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Letter from the Guest Editor

Dear colleagues,

On behalf of the organizing committee of the 6th Meeting of the Greek Society of Pharmacology, I welcome you to the Faculty of Medicine of the University of Crete in Heraklion. The Greek Society of Pharmacology was inaugurated in 1984 by a few members of Academia in Greece. We are still a small society, yet we have an active voice in EPHAR and IUPHAR. Our members, present in academia, research institutes and hospitals all over Greece, are actively involved in basic and clinical research.

The goal of our Society is to promote a forum for discussion on state of the art issues in Pharmacology and to provide the opportunity especially to our young colleagues, to meet, interact and discuss issues with prominent scientists from all over the world. Innovations in Pharmacology teaching is also of primary interest to the Society, since we want to guarantee the best training possible that will assist the medical doctor, pharmacist or doctor-scientist in the decades to follow.

I welcome you again, and I wish you a fruitful meeting and a pleasant stay in Heraklion.

The Guest Editor

Kiki Thermos

Professor of Pharmacology
Chair of the Organizing Committee

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Zebrafish Models of Cardiac Valve Development and Disease

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SUMMARY

Cardiac valves derive from endocardial cells and function throughout the life of vertebrates to prevent retrograde blood flow. We have identified a number of mutants in different stages of valve development and work on characterizing and cloning the corresponding genes. These mutations disrupt atrioventricular (AV) valve development at discrete stages, indicating that such a complex morphogenetic process can be broken down into genetically separable steps.

We have identified a mutation in the glycyl t-RNA synthetase (GARS) where valve development comes to a halt, at a stage following atrioventricular canal specification and squamous to cuboidal epithelium transition. We have also identified two novel alleles of weak atrium that carry premature stop codons in myh6 and develop pericardial edema and AV valve stenosis by 60 hpf. However, when the pericardial edema is surgically released, adult animals can be raised indicating that the sole beating of the ventricle in the zebrafish heart can support circulation throughout development and adulthood. Nevertheless, the changes in atrial contractility and hemodynamics interfere with the wild-type remodelling/maturation of the AV valves (from two to four cusps) and result in a hypertrophic ventricle. The 3rd line we isolated has an outflow tract stenosis by 72hpf and the bi-directional blood flow causes dilation of the heart chambers and single-layer endocardial cells at the AV canal. Finally, we also isolated an adult viable mutant line where the heart does not loop properly resulting in delayed AV valve development.

The long-term aim of our efforts is to find out how heart valves form and function throughout the life of vertebrates.

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Anti-inflammatory Actions of hsp90 Inhibitors

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SUMMARY

Although inflammation has been implicated in the pathogenesis of most cardiovascular disease, appropriate anti-inflammatory drugs are sparse. COX inhibitors are single-target agents that lack wide-spread efficacy, whereas glucocorticosteroids exert widespread anti-inflammatory actions, but also severe side effects. Heat shock protein 90 (hsp90) inhibitors may fill this void. Hsp90 exists in complexes with numerous pro-inflammatory ("client") proteins; these complexes promote client protein survival and, in the case of many enzymes, also optimize their activity. Accordingly, hsp90 inhibitors produce anti-inflammatory effects by preventing the association of hsp90 with its pro-inflammatory client proteins resulting in their deactivation and/or degradation.

We have recently reported that the hsp90 inhibitor, 17-AAG (tanespimycin), prevents, as well as repairs endothelial cell hyper-permeability, *in vitro* and *in vivo* (*Am J Respir Crit Care Med 176:667-75, 2007; Am J Resp Cell Mol Biol 39: 551-59, 2008; Amer J Physiol Lung Cell Mol Physiol. 294(*4*):L755-63, 2008*). More recent work suggests that 17-AAG also inhibits the release of a wide spectrum of pro-inflammatory cytokines from human peripheral blood monocytes, attenuates the right ventricular hypertrophy, vascular remodeling and inflammation associated with pulmonary arterial hypertension, and reduces the inflammation and airway hyperreactivity in a mouse model of allergic asthma. The specific targets of 17-AAG in these studies are under investigation; initial findings point towards $pp60^{src}$, hsp27, IκK and GSK-3β as key mediators.

17-AAG has recently finished phase II clinical trials as an anti-neoplastic agent, exhibiting a favorable profile of generally mild side effects. It remains to be determined whether a similar profile will characterize the anti-inflammatory use of 17-AAG and other hsp90 inhibitors.

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Basic needs, essential drugs and dispassionate medicine

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SUMMARY

The processes of economic globalization are shaping people's health across the world – and not for the better. The number of people in poverty has been increasing in some parts of the world, as has inequality between richer and poorer both within and between countries.

The deeply unjust mismatch between expenditure on medicines and health need mirrors global socio-economic disparities. 42% of global expenditure on medicines is spent on 5% of the world's population living in North America, while only 20% is spent on the majority of the world's population with the highest burdens of disease in Africa, Asia, the Middle East and Latin America.

The combined worth of the world's top five drug companies (Big Pharma) is twice the combined Growth National Product of all Sub-Saharan Africa, and their influence on the rules of world trade is many times stronger because they bring their wealth to bear directly on the levers of western power.

New ways to fund and stimulate pharmaceutical Research & Development are needed to achieve the goal of universal access to essential medicines and avoid the huge inefficiencies and corruption of the current system.

Profit-motivated pharmaceutical companies, whether Big Pharma or generic manufacturers, cannot be left to operate without a strong regulatory framework to promote rational medicine use and patient safety. The erosion of independent national and international regulatory structures and powers must be reversed. Civil and especially scientific society must play a further watchdog role that holds pharmaceutical companies and government regulators accountable to high standards of ethical practice.

In parallel, the medicine practiced both in less developed countries and western societies must be impartial and focused in basic needs first.

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Innovations in Pharmacology Teaching

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Key words: e-learning, pharmacology teaching, innovative teaching and learning

S u m m a r y. Teaching of medicine and in particular the basic sciences such as pharmacology is renowned for being innovative and rising to the challenge of using technology as an enhancement to traditional methods. This presentation will use illustrative examples from Edinburgh of how e-learning has been used to enhance the student learning experience and provide support for teachers particularly in pharmacology.

INTRODUCTION

Pharmacology is an important subject both in its own right and as a major component of medicine, pharmacy, dentistry, veterinary medicine and the healthcare professions. Many people have recognized for many years the potential for e-learning in higher education, particularly in those professions such as medicine and healthcare which are highly visual and where real-life scenarios can be simulated virtually and learning can be contextualized. The mantra has always been that e-learning would transform the way we teach and the way that students learn and the potential for cost-savings and efficiency gains has been a major driver.

Pharmacology teaching has been at the forefront of innovation and in particular, over the last twenty years or so the use of Information and Communication Technology (ICT) to support teaching and learning in pharmacology has become widespread (Hughes, 2003). This will be the focus of this presentation but innovations, not reliant on technology, should not overlooked. For example, didactic, teacher-led teaching has been supplemented or even replaced by problem-based learning which, in the opinion of advocates, better engages students in managing their own learning and prepares them for lifelong learning. Lectures have developed from the typical one-way (expert to novice) transfer of information into more interactive events which may or may not be facilitated through the use of technologies such as audience/personal response systems (clickers). Peer marking of student assignments or practical class write-ups (Hughes, 2002) has been developed as a learning method, and there are interesting examples of bringing fun into learning such as the use of topical debates, the use of games, e.g. crosswords (Rangachari, *Writing Problems,* 1998-2007), a pharmacological version of *Who wants to be a millionaire* and board games such as *Autonomic Monopoly* and *Cardiovascular Pursuit* (MacDonald cited in Atkinson & Hughes, 2001)) and even using music to enhance lectures (MacDonald cited in Atkinson & Hughes, 2001).

Technological innovations

The use of technology to support teaching and learning has a long history but, in the UK in particular, real impact at a national level was seen during the 1990s when there was significant development of interactive computer programs, usually delivered on CDROM, and designed to either cover discrete lecture topics or support laboratory practical classes. In the UK there were a number of Government-backed educational initiatives often involving consortia of universities working on specific disciplines. For example, in pharmacology there were: the *pharma-CAL-ogy* Project (http://www.pharmacalogy.com) which was responsible for creating a suite of around 40 interactive computer-assisted learning programs which were distributed through the British Pharmacological Society.

The Pharmacy Consortium for Computer-aided Learning (PCCAL: http://www.coacs.com/PCCAL/) was a consortium of UK university pharmacy departments established with Government funding to create computer-assisted learning programs to

support pharmacy teachers; individuals developing resources in their own time and making these available through commercial publishers e.g. Sheffield BioScience Programs (www.sheffbp.co.uk); Thieme Medical Publishers (http://www.thieme.com/); Biosoft England (http://www.biosoft.com);

The educational effectiveness of computer programs of this sort in supporting or even replacing traditional teaching methods has been studied and in the main effective learning has been demonstrated (Dewhurst, et al 1994; Leathard & Dewhurst 1995; Brain, et al 1999; Dewhurst, et al 2000; Hughes (2001).

In the mid-1990s the Internet was developed and it has increasingly become a feature of education during the last decade. The World Wide Web and search engines such as Google have rapidly transformed the way that students and teachers find information with sources such as Wikipaedia now accepted as providing quality up-to-date information. Virtual or managed learning environments (VLEs or MLEs) are now the norm in many universities. They enable whole courses to be managed online and, when used effectively, they integrate into a single website the provision of high-quality content with access to tools to support educational processes. Initially course content was still developed by the experts and delivered online to support student learning enabling students to study flexibly either on- or off-campus. Some distance-learning courses are delivered wholly online and there is no face-to-face contact between tutors and students. Typically a VLE will provide students with: (i) personalized information, an online timetable and notice board, access to a personal portfolio and to their assessments and course evaluations; (ii) a discussion board through which students and teachers can initiate discussions and students can learn from each other and from their tutors. Sometimes these discussions can be archived and used to enhance student feedback; (iii) self-assessments covering discrete areas of the curriculum allow students to assess their own understanding and provide excellent revision aids; (iv) interactive content including resources to supplement lectures, animations and video to enhance the learning of dynamic events such as drug-receptor interaction, scenario-based learning activities such as virtual patients which contextualise learning and which can also be used to teach clinical decision-making skills, simulations of laboratory exercises, resources to better prepare medical students to be safe prescribers etc.

Lecture support materials, such as PowerPoint presentations, enhanced by audio commentary, lecture notes, reading lists etc

More recently, the advent of Web 2.0 applications has enabled learners to publish their own content (wikis, weblogs, photos, videos) more easily and posed challenges to educational institutions in how to manage this new content and use the myriad of social communication tools effectively for education.

This presentation will focus on ways in which technology has impacted on teaching and learning medicine and veterinary medicine in Edinburgh and in the wider national and international context over the last two decades and how the current financial climate, the speed of technological advances and the move towards open content present challenges and opportunities to the medical and healthcare e-learning communities.

REFERENCES

1. Atkinson J., Hughes I.: Teaching pharmacology together in Europe. *Fund. Clin. Pharmacol. 15*: 365–372 (2001)
2. Brain S., Dewhurst D.G., Williams A.D.: Evaluation of the usefulness of a computer-based learning program to support student learning in pharmacology. *Ass. Lear. Technol. J. (ALT-J) 7(2)*: 37-45 (1999)
3. Dewhurst D.G., Hardcastle J., Hardcastle P.T., Stuart E.: Comparison of a computer simulation program with a traditional laboratory practical class for teaching the principles of intestinal absorption. *Am. J. Physiol. 267 (Advances in Physiology Education 12(1)*: S95-S103 (1994)
4. Hughes I.E.: Do computer simulations of laboratory practicals meet learning needs? *Trends Pharmacol. Sci. 22*: 71-74 (2001)
5. Hughes I.E.: Peer marking of simulated pharmacological experiments. Teaching Pharmacology Tomorrow: Tools and Techniques, 2002
6. Hughes I.E.: Teaching pharmacology in 2010 - new knowledge, new tools, new attitudes. *Folia Pharmacologica Japonica 122*: 411-418 (2003)
7. Leathard H.L., Dewhurst D.G.: Comparison of the cost-effectiveness of a computer assisted learning program with a tutored demonstration to teach intestinal motility to medical students. *Ass. Learn. Technol. J. 3*: 118-125 (1995)
8. Rangachari: Writing Problems (1998-2007) http://fhs.mcmaster.ca/pbls/writing/

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Appication of Classic Pharmacological Principles to Modern Drug Didcovery

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SUMMARY

Various pharmacological, biochemical and structural methodologies have defined specific mechanisms that have enabled the discovery of novel therapeutics in a variety of disease states. The superfamily of GPCRs (G-Protein Coupled Receptors) is the largest cell membrane-bound protein family encompassing more than 1000 different and distinct proteins. These membrane-bound proteins are responsible for translating extracellular stimuli into intracellular signals. Many decades of research have been focused on the identification of small molecules that interact with these proteins to be used as potential therapeutics in disease. While the experimental methodologies used in modern drug discovery have greatly evolved, the basic underlying pharmacological principles that these are based upon are still being utilized in our understanding of these complex cellular systems. The superfamily of GPCRs has been subdivided into 5 distinct classes. This presentation will use examples from two of these classes (Class A; GnRH and Class B; CRF_1 and CRF_2) to describe our current understanding of the discrete molecular interactions of the endogenous ligands and novel small molecules. These differences have been elucidated through a detailed examination of the binding, activation and regulation characteristics of peptide and non-peptide antagonists and will demonstrate key features of the signaling properties of these ligands that have been exploited for the discovery of unique chemical compounds. The results of these efforts over the past decade has enabled us to discover and identify small molecule drug candidates that are currently undergoing Phase 2 Clinical trials in diseases such as endometriosis (GnRH receptor antagonists), major depressive disorder (CRF_1 receptor antagonists) and acute decompensated heart failure (CRF_2 receptor agonists). These molecules all represent unique approaches in their interaction with their respective GPCRs and hold tremendous potential as novel therapeutics in the treatment of these diseases and disorders.

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Adenoviral Vectors in the Treatment of HDL Deficiencies

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SUMMARY

Epidemiological studies and clinical intervention trials have established that levels of high density lipoproteins (HDL) in plasma are reversely associated with the incidence of coronary artery disease. We are using adenovirus-mediated gene transfer in appropriate mouse models in order to dissect the pathways of biogenesis and maturation of HDL *in vivo*. This technology involves the generation of recombinant adenoviruses carrying the gene of interest, amplification of the adenoviruses in appropriate mammalian cell lines, purification, titration and injection into the tail vein of mice. The resulting phenotype is monitored 1 to 5 days post-injection. Using this technology, we have functionally characterized natural human mutations in apolipoprotein A-I (apoA-I), the main protein component of HDL, such as apoA-I(R151C)$_{Paris}$, apoA-I(R160L)$_{Oslo}$, apoA-I(Leu141Arg)$_{Pisa}$ and apoA-I(Leu159Arg)$_{FIN}$ and have shown that these mutations disrupt HDL biogenesis and maturation. These defects in HDL caused by the natural apoA-I mutations can be corrected by administration of recombinant adenoviruses expressing lecithin cholesterol acetyl transferase (LCAT). Using this approach we have also studied the role of apoA-IV in the biogenesis of HDL. We have found that gene transfer of apoA-IV in apoA-I$^{-/-}$ x apoE$^{-/-}$ mice increased 1.5-fold plasma cholesterol levels and induced mild hypertriglyceridemia. The plasma cholesterol and apoA-IV were distributed mainly in the VLDL/IDL/LDL region and to a lesser extent in HDL. Electron microscopy analysis of the HDL fractions obtained by density gradient ultracentrifugation showed the presence of lipoprotein particles along with lipoprotein aggregates. Two-dimensional gel electrophoresis showed formation of distinct HDL subpopulations. Currently, we are also employing recombinant adenoviruses to investigate the effect of naturally occurring human LCAT mutations as well as the role of new genes, identified recently by genome-wide association studies, in the biogenesis and/or catabolism of HDL in mice.

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Peptidomimetic Inhibitors of Cyclin A and PCNA: Discovery and Design

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SUMMARY

Structure based design is one of the most common used approaches for discovery and development for new drugs. Applications of those methods have been used in the discovery of peptide inhibitors of Cyclin A and PCNA. Additional a new computational method allowing non-peptide fragments to be identified have been employed successfully in the discovery of Cyclin A inhibitors. The latest approach should be generally applicable in replacing amino acids as individual residues or groups in peptide inhibitors to generate pharmaceutically acceptable lead molecules.

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Emerging Therapies in Multiple Sclerosis: Current Concepts and Future Perspectives

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SUMMARY

Multiple Sclerosis (MS) represents the main cause of neurological disability in young adults of Caucasian ancestry. However for more than a decade the only therapies available were two immunomodulatory drugs, interferon beta and glatiramer acetate, and the immunosuppressant mitoxantrone. Although the etiology of MS remains obscure, the immunopathogenesis appears to involve both the cell- and humorally-mediated arms of the immune system acting in concert with genetic and environmental factors. In that respect, natalizumab, a humanized monoclonal antibody (mAb) raised against α4-integrin, showed increased efficacy compared to existing therapies, which led to its recent approval in Europe and the USA as second-tier treatment of Relapsing Remitting MS. Natalizumab acts by blocking lymphocyte adhesion to endothelium and thus prevents the transmigration of autoreactive T cells across the blood brain barrier into the CNS. However treatment with natalizumab was associated with JC virus reactivation and development of progressive multifocal leucoencephalopathy (PML). In addition, 2 recently completed phase III trials showed significant reduction in both MRI and clinical markers of disease activity with the orally administered fingolimod, a sphingosine-1-phosphate (S1P) modulator that prevents lymphocyte egress from lymph nodes. Cladribine is an adenosine deaminase-resistant purine nucleoside analogue that causes longlasting lymphocyte depletion preferentially affectingCD4$^+$ T cells.A short treatment course (8-20 days per year) with cladribine tablets provided a significant benefit for patients withRRMS. Very promising results were obtained with alemtuzumab, a humanised mAb that targets CD52, a cell-surface glycoprotein abundantly expressed on T and B lymphocytes, monocytes, and eosinophils, thus resulting in depletion of both T and B cells. In a phase II trial, alemtuzumab administered yearly demonstrated superior to interferon efficacy, with respect to the relapse rate, disability progression and MRI activity, albeit with an excess risk of developing an antibody-mediated autoimmune disease (Graves disease, idiopathic thrombocytopenia). The growing evidence that humoral immunity is operative in MS pathogenesis prompted the use of Rituximab, a chimeric murine/ human mAb directed against CD20, a surface antigen expressed on pre–B cells and mature B cells. A phase II trial confirmed the efficacy of rituximab in RRMS without significant adverse effects or opportunistic infections. However recent reports of PML occuring in patients with lymphoproliferative and other autoimmune disorders treated with rituximab cause an increasing concern over its safety. Recently daclizumab, a humanized mAb directed against IL-2Rα (CD25) was tested either as monotherapy or in combination with interferon-β in RRMS patients, showing benefit in both clinical and MRI parameters. Although the exact mechanism of action has not fully been elucidated, an expansion of the immunoregulatory CD56^{bright} natural killer cells is postulated. Finally clinical trials are under way with three oral immuno-modulatory drugs: Teriflunomide, Laquinimod and Dimethyl Fumarate (BG00012).

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From Peptides to Non Peptide Mimetics - A New Generation of Drugs: The Examples of Angiotensin II and Myelin

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SUMMARY

The discovery of Losartan a non peptide Angiotensin II Receptor antagonist was announced in 1989 during the Gordon Research Conference on Angiotensin and the Renin-Angiotensin-System (RAS). The drug was discovered in the Laboratories of Dupont and the announcement at the Conference was the approval for Clinical trials which led to the first Angiotensin II nonpeptide Receptor antagonist. Previous Angiotensin II peptide antagonists such as Sarilesin and Saralasin failed to become drugs due to its peptide nature rendering them susceptible to proteolytic enzymes which hydrolyze them. The announcement was the result of many years work on Angiotensin and the RAS System, since it was discovered 80 years ago. Breakthroughs in this evolution was the discovery of Captopril by Miguel Ondetti in 1975 and Losartan by Timmermans in 1989. In this lecture the main steps followed in our laboratories in Patras are mentioned which led to our Sartan, named Elsartan. Briefly the main steps are: 1. Peptide (The tool), 2. Peptide Model (The ligand – receptor interaction), 3. Cyclic Peptide (The drug lead), 4. Non-peptide mimetic (The Drug).

Immunodominant Epitopes MBP 83-99, PLP 139-151, MOG35-55 of human proteins MBP, PLP, MOG of myelin sheath are implicated in Multiple Sclerosis. These epitopes have been the tools in our laboratories for the Design Synthesis and Preclinical Evaluation in a large number of rationally designed linear and cyclic analogues conjugated to reduced or oxidized mannan via [Lys-Gly] bridge. Specific Analogues have been found to immune rats rendering them potential therapeutics vaccine drugs in the Immunotherapy of Multiple Sclerosis. Furthermore, our cyclic MBP 83-99 peptides, for the first time to be reported as HLA and MHC binders and more stable compared to linear counterparts, possess a series of important immunomodulatory properties rendering them as putative drugs for treating multiple sclerosis and potentially other Th1 – mediated autoimmune diseases. In the light of the results and findings in our research, the main immunodominant peptides MOG35-55, PLP139-151 and MBP83-99 and their head to tail cyclic counterparts conjugated to reduced mannan have been selected to constitute a mixture cocktail drug for preclinical investigation in preparation of New Drug Application (NDA) for Clinical Phase I and II studies in the Immunotherapy of Multiple Sclerosis.

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Current Advances in Gene Therapy for Parkinson's Disease

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SUMMARY

Parkinson's disease (PD) is a neurodegenerative disorder characterized by severe motor symptoms (including resting tremor, cogwheel rigidity and bradykinesia) caused by the progressive loss of the dopaminergic nigrostriatal projection neurones by apoptosis (1). This leads to gradual depletion of the neurotransmitter dopamine from the striatum and consequent imbalance between the striatopallidal and striatonigral (basal ganglia) output pathways controlling movement (2). The main treatment for PD in its early stages is to provide dopamine via oral uptake of L-3, 4-dihydroxyphenylalanine (L-Dopa). However, chronic administration of L-Dopa eventually leads to loss of drug efficacy (*wearing off*) and the onset of disabling dyskinesias and motor fluctuations (*on-off*) (2).

We have used an equine infectious anemia virus (EIAV) vector (ProSavin®) to express three dopamine biosynthetic enzymes, namely tyrosine hydroxylase, aromatic L-amino acid decarboxylase, and GTP cyclohydrolase-1, essential for metabolising tyrosine to dopamine. This vector mediates dopamine production *in vitro* and *in vivo* and was previously demonstrated to correct the 6-OHDA lesion rat model of PD after stereotactic delivery to the striatum (3). We have further improved the vector and evaluated its therapeutic potential in a bilateral non-human primate (NHP) model of PD. ProSavin® was injected bilaterally into the striatum of MPTP-lesioned macaques using MRI-guided stereotactic surgery, and resulted in an increase in striatal dopamine and significant long-term reversal of motor deficits compared to control MPTP animals. Furthermore, gene transfer normalised internal globus pallidus activity and subthalamic nucleus metabolism, indicating restoration of normal basal ganglia functioning. L-Dopa administration to ProSavin® treated macaques led to increased levels of striatal dopamine but did not result in any runaway dyskinesias (4). This study has led to the initiation of a Phase I/II dose escalation-safety/efficacy clinical trial in France aiming at assessing the utility of this approach in treating late stage Parkinson's disease patients. This study will be discussed in relation to other gene therapy approaches also in clinical trials for PD.

1. Jenner P., Olanow W.C.: *Neurology 66*: S24-S36 (2006)
2. Thanvi B., Lo N., Robinson T.: *Postgrad. Med. J. 83*: 384-388 (2007)
3. Azzouz et al.: *J. Neurosci. 22*: 10302-10312 (2002)
4. Jarraya et al.: *Sci. Transl. Med. 1(2)*: 2ra4 (2009)

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Hydrogen Sulfide is an Inducer of Angiogenesis

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SUMMARY

Hydrogen sulphide (H_2S) is emerging as an important signalling molecule in the cardiovascular system. H_2S can be produced by endothelial cells and has been shown to relax vascular smooth muscle leading to reduced mean arterial blood pressure. H_2S is endogenously synthesized in a range of mammalian tissues through the activity of at least two enzymes, cystathionine β synthetase (CBS) and cystathionine γ lyase (CSE), the latter being mainly expressed in the vasculature. Herein, we studied the role of this gasotransmitter in angiogenesis.

In vitro, H_2S triggered endothelial cell (EC) growth and motility and induced the assembly of EC into tube-like networks. H_2S induced p38 MAPK phosphorylation; inhibition of p38 activity with SB203580 led to a reduction in the migratory rate of EC in response to H_2S. Activation of p38 and migration of cells treated with H_2S was inhibited by the ATP-sensitive (K^+_{ATP}) potassium channels channel blocker, glibenclamide. On the other hand, the K^+_{ATP} channel opener SG209 promoted EC migration. The results described above reveal the importance of K+ currents and MAPK pathways in the angiogenic actions of H_2S in vitro. To determine if H_2S also enhances angiogenesis in vivo we treated chicken chorioallantoic membranes (CAM) with H_2S; such treatment induced new vessel growth while topical administration of H_2S in a rat burn wound assay promoted re-epithilization and wound-healing.

To test whether endogenously produced H_2S affects angiogenesis, we used inhibitors of H_2S synthesis. Treatment of CAM with PAG or BCA reduced H_2S production and neovascularization, while exogenous addition of H_2S reversed the inhibitory actions of PAG or BCA in the CAM. To further investigate a possible role of endogenous H_2S to the actions of the well-known EC mitogen vascular endothelial growth factor (VEGF), we treated EC with VEGF. This induced H_2S release from EC, although concomitant application of H_2S and VEGF did not result in an additive effect in motility. Furthermore, pre-treatment of EC with PAG, BCA or K^+_{ATP} channel inhibitors reduced or even abolished VEGF-stimulated EC migration. siRNA knock-down of CSE attenuated VEGF-signalling and EC migration triggered by VEGF. In an aortic ring in vitro angiogenesis assay, addition of VEGF resulted in the growth of fewer vessels in rings from $CSE^{-/-}$ compared to wild-type rings. Moreover, in $CSE^{-/-}$ mice wound healing was delayed. Taken together the above mentioned results support a pro-angiogenic role for endogenous H_2S.

In conclusion, our findings indicate that H_2S enhances new blood vessel formation through a K^+_{ATP} channel/p38 pathway and that anti-diabetic agents that inhibit K^+_{ATP} channels may be useful tools in diseases where inhibition of neovascularization is desirable.

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A Visit to Vergina

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SUMMARY

Recent archaeological investigation in Northern Greece has contributed largely to our knowledge of ancient Macedonians and their culture.

The Aristotle University Excavation at Vergina, on the northern slopes of the Pierian Mountains, has contributed largely to this new knowledge, due to its identification with the ancient city of *Aegae*, the old capital and royal necropolis of the ancient Macedonian kingdom.

Various speculations about the idiom, the religion and the burial customs of its people, as well as gaps in its history have already been reconsidered, in the light of the abundant material evidence from their cultural cradle

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Biologic Agents in Rheumatic Diseases: Lessons Learned and Perspective

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SUMMARY

Autoimmune rheumatic diseases are chronic, systemic inflammatory disorders of complex etiology and pathophysiology. Uncontrolled inflammation results in organ damage and functional impairment.

During the past 15 years we have enhanced the understanding of molecular pathogenesis of autoimmune and autoinflammatory rheumatic diseases. This has enabled the development of innovative biological agents that target specific parts of the immune system. Biologic therapies such as monoclonal antibodies and fusion proteins have revolutionized the management of rheumatic disease. By targeting key cytokines (TNFα, IL-1, IL-6) and immune cells (T-cells, B-cells), biologics have provided more specific therapeutic interventions with less immunosuppression. These treatments have changed the course and face of inflammatory arthritides and outcomes for patients and society. Clinical use, however, has revealed that their theoretical simplicity hides a more complex reality. Residual disease activity, immunogenicity, specific infections, are issues which have been emerged in clinical application of novel therapies.

Concerning pathophysiology of rheumatoid arthritis, new knowledge has emerged of how environmental factors interact with susceptibility genes and the immune system in the pathogenesis of the disease. Research undertaken on the longitudinal disease process and molecular pathology of joint inflammation has led to new therapeutic strategies that promote early use of disease-modifying drugs with tight disease control and distinct and quantifiable treatment goals. Today, such approaches can halt most cases of joint destruction but not all instances of joint inflammation and comorbidity. Understanding the cause and pathogenesis of different rheumatoid arthritis and other autoimmune and autoinflammatory diseases, will lead not only to individualized treatments during early phases of the illness but also, possibly, to disease prevention

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Advances in Medical Treatment of Diabetic Retinopathy

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SUMMARY

Despite improved control of blood glucose levels and hypertension, diabetic retinopathy remains the leading cause of blindness in working-age individuals of developed countries. When proliferative diabetic retinopathy or clinically significant diabetic macular oedema appears, argon-laser photocoagulation is presently indicated and its efficacy has been widely demonstrated. However, this is an aggressive treatment that destroys healthy parts of the retina and plays no role in retinopathy prevention. Intravitreal corticosteroids have been successfully used in eyes with persistent DME and loss of vision following the failure of conventional treatment. However, reinjections are commonly needed, and there are substantial adverse effects such as infection, glaucoma and cataract formation. In advanced stages of DR intravitreous anti-VEGF agents have emerged as new treatments but they are yet to be approved for DR. Vitreo-retinal surgery is an expensive and complicated treatment that should be carried out only by vitreoretinal specialists experienced in this procedure and it is normally reserved for the ultimate blinding complications of PDR such as severe vitreous hemorrhage and secondary retinal detachment. For all these reasons, new pharmacological treatments based on the understanding of the pathophysiological mechanisms of diabetic retinopathy are needed.

The beneficial effects of fenofibrate and candesartan on ophthalmological outcomes have been reported in clinical trials. It should be noted that their positive effects on diabetic retinopathy were unrelated to the primary actions of these drugs (ie. reducing serum lipids and blood pressure, respectively), and that the mechanisms involved in their action at retinal level are currently under investigation. The results from our lab on the mechanisms by which fenofibrate exerts its beneficial effects will be presented.

Finally, it should be emphasized that before any microcirculatory abnormalities can be detected in ophthalmoscopic examination, retinal neurodegeneration is already present. Therefore, new strategies based on either the delivery of neuroprotective agents or the blockade of neurotoxic factors are currently being tested in experimental studies and in clinical pilot studies. The results of our group concerning new targets for neuroprotection will be discussed.

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Omega 3 fatty acids suppress retinopathy

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SUMMARY

Many sight-threatening diseases have two critical phases, vessel loss followed by hypoxia-driven destructive neovascularization. These diseases include retinopathy of prematurity and diabetic retinopathy, leading causes of blindness in childhood and middle age. We studied the influence of omega-3- and omega-6-polyunsaturated fatty acids (PUFAs) on vascular loss, vascular regrowth after injury, and hypoxia-induced pathological neovascularization in a mouse model of oxygen-induced retinopathy. We show that increasing omega-3-PUFA tissue levels by dietary or genetic means decreases the avascular area of the retina by increasing vessel regrowth after injury, thereby reducing the hypoxic stimulus for neovascularization. The bioactive omega-3-PUFA-derived mediators neuroprotectinD1, resolvinD1 and resolvinE1 also potently protected against neovascularization. The protective effect of omega-3-PUFAs and their bioactive metabolites is mediated, in part, through suppression of tumor necrosis factor-a. This inflammatory cytokine is found in a subset of microglia that is closely associated with retinal vessels. These findings indicate that increasing the sources of omega-3-PUFA or their bioactive products reduces pathological angiogenesis. Western diets are often deficient in omega-3-PUFA, and premature infants lack the important transfer from the mother to the infant of omega-3-PUFA that normally occurs in the third trimester of pregnancy. Supplementing omega-3-PUFA intake may be of benefit in preventing retinopathy.

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New Therapeutic Targets in Retinal Disease

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SUMMARY

Retinal diseases such as Diabetic Retinopathy (DR) and Age-Related Macular Degeneration (ARMD) lead to vision impairment and blindness. Their global prevalence is increasing due to the rapidly increasing numbers of people with diabetes and older people worldwide, respectively. These diseases are defined as microvascular diseases and characterized by neovascularization. However, neural retinal defects, such as an increase in apoptosis and retinal cell loss, have been detected. These processes are reminiscent of ischemia, glutamate excitotoxicity and neurodegeneration. New pharmacological targets are essential in order to treat both vascular and neural elements of the retina and to provide more efficacious therapeutics for retinal disease.

The neuropeptide somatostatin (somatotropin release inhibitory factor, SRIF) has been shown to have antineovascular actions due to its ability to inhibit the actions of growth hormone, and a variety of other growth factors. We have focused our studies on the elucidation of somatostatin's neuroprotective (anti-ischemic) effects in different models of ischemia induced retinopathies, namely an ex vivo model of chemical ischemia and an in vivo model of AMPA excitotoxicity. Our results support that somatostatin and its $sst_{2/5}$ analogues protect the retina from ischemic insults. The protective effect of the sst_2 ligands was shown to be mediated via a NO/cGMP mechanism. These results, in combination with the increasing literature data on the antineovascular effects of the $sst_{2/5}$ analogues, support the use of these agents as therapeutics in ischemia induced retinal diseases.

Recently, neurosteroids such as dehydroepiandrosterone (DHEA) were shown to have neuroprotective antiapoptotic properties in different paradigms. In addition, these actions were shown to be mediated via a mechanism that involved the NGF pro-survival receptor, TrkA. This evidence provided the impetus for the investigation of the putative neuroprotective actions of DHEA, and synthetic analogues, in retinal models of retinopathies. The endogenous (DHEA) and synthetic androstene neurosteroids employed protected the rat retina from chemical ischemia. In this model, the data suggested that the neuroprotective effects of the neurosteroids are mediated via the NGF receptor and its signaling cascades. In addition, DHEA was shown to protect the retina from AMPA induced excitotoxicity *in vivo*, an effect that was blocked by the TrkA inhibitor and mimicked by NGF itself. These studies are still in preliminary phase, but the present data suggest that neurosteroids may be a new target for retinal therapeutics.

In conclusion, somatostatin analogues and neurosteroids target excitotoxicity and apoptosis in *ex vivo* situations, but most importantly when delivered intravitreally. These properties lend these agents a therapeutic potential in retinal disease, whose pathophysiology involves ischemia induced neurodegeneration. The inclusion of these molecules in a multi drug treatment could ensure a more efficacious therapy.

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Parstatin: a Novel Endogenous Anti-angiogenic Peptide with Potential Therapeutic Applications

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SUMMARY

The proteolytic activation of proteinase-activated receptor 1 (PAR1) unveils the tethered peptide ligand and cleaves a 41-amino acid peptide. We have recently shown that this peptide, which we have designated as *parstatin* is a potent inhibitor of angiogenesis. Synthesized parstatin suppressed both basic angiogenesis and that stimulated by bFGF and VEGF in chick chorioallantoic membrane and in the rat aortic ring model of angiogenesis. Parstatin also abrogated endothelial cell proliferation, migration and capillary-like network formation in vitro, by promoting cell cycle arrest and apoptosis. We have also evaluated parstatin in three animal models of ocular neovascularization. Intravitreous injections of parstatin significantly suppressed retinal neovascularization in mice with oxygen-induced ischemic retinopathy. In mice with laser-induced rupture sites in Bruch's membrane, intraocular injection of parstatin strongly reduced the area of choroidal neovascularization. Similarly, rats with chemical burn-induced corneal neovascularization, that received subconjunctival injections of parstatin had areas of corneal neovascularization that were significantly smaller than those seen in control subjects treated with vehicle. These results support the notion that parstatin represents an important negative regulator of angiogenesis and indicate that it may provide a new agent for consideration for treatment of patients with corneal, retinal, or choriodal neovascularization.

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Functional Mechanisms of GPCRs and Transporters Offer Insights for Targeted Drug Design

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The focus of the presentation is the molecular machinery involved in cell-cell communication and intracellular signal transduction, specifically GPCRs and Na^+-coupled neurotransmitter transporters (NSS). These molecular systems are modulated by the binding of endogenous ligands and pharmaceutical agents, and the presentation will illustrate the challenges encountered in the acquisition of the structure-based information that is required to understand these processes and to design new drugs that target them. The results from our studies are obtained from a combined experimental and computational approach that includes functional analysis, data-driven simulation and experimental validation that reveals how physiological function emerges from the activity of the membrane-associated proteins. Atomistic level formalisms in structural and computational molecular biophysics are employed to understand functional mechanisms triggered by the GPCRs and NSS transporters, and their allosteric modulation by ligands and by protein-protein interactions (e.g., with PDZ and BAR domains). The computational methods used to determine the mechanistic details, to design constructs with predetermined properties, and to guide novel experimental approaches, include (i) various forms of targeted molecular dynamics, (ii) mesoscale representations of protein and membrane dynamics and of lipid reorganization driven by interactions with proteins, based on free energy minimization methods and Monte Carlo sampling; and (iii) simulation of cell signaling pathways and networks.

ILLUSTRATIVE PUBLICATIONS

1. Shi L., et al., - The mechanism of sodium-coupled symport by a homolog of neurotransmitter transporters: Two substrates are required. *Mol. Cell. 30*: 667-677 (2008) PMID: 18570870
2. Beuming T., et al., - The binding sites for cocaine and dopamine in the dopamine transporter are overlapping. *Nature Neurosci. 11*: 780-789 (2008) PMID: 18568020.
3. Ericksen S.S., et al.: Ligand selectivity of D2 dopamine receptors is modulated by changes in local dynamics produced by sodium binding. *J. Pharmacol. Exp. Ther. 328*: 40-54 (2009) PMID:18849360 .
4. Chang C.W., et al.: Towards a quantitative representation of the cell signaling mechanisms of hallucinogens: Measurement and mathematical modeling of 5-HT1A and 5-HT2A receptor-mediated ERK1/2 activation. *Neuropharmacology 56*: 213–225 (2009) PMID: 18762202
5. Han Y., et al. - Allosteric communication between protomers of dopamine class A GPCR dimers modulates activation. *Nature Chem. Biol. 5*: 688-695 (2009) PMID: 19648932
6. Khelashvili G., Harries D., Weinstein H.: Modeling membrane deformations and lipid demixing upon protein-membrane interaction: the BAR dimer adsorption. *Biophys. J. 97*: 1626-1635 (2009) PMID: 19751667
7. Thorsen T.S., et al. : Identification of a small-molecule inhibitor of the PICK1 PDZ domain that inhibits hippocampal LTP and LTD. *Proc. Natl. Acad. Sci. USA 107*: 413-418 (2010) PMID: 20018661
8. Zhao Y., et al.: Substrate-dependent proton antiport in neurotransmitter:sodium symporters. *Nature Chem. Biol. 6*: 109-116 (2010) PMID: 20081826
9. Zhao Y., et al.: Single molecule studies of the allosteric modulation of intracellular gating in a neurotransmitter transporter homolog. *Nature* 2010 – (May 13 issue).
10. Claxton D.P., et al.: Ion/substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter:sodium symporters. Nature Struct. Mol. Bio 2010 – accepted April 2010.

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Adeno-associated Vectors and Neuropsychiatric Disorders

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SUMMARY

Most of the medications used for neuropsychiatric disorders target G protein coupled receptors (GPCR). Over the last ten years our work evolves towards the understanding of mechanisms controlling GPCR responsiveness and desensitization. Among the most important players in GPCR function, RGS proteins (Regulators of G protein signaling) and their interactive partners, act in a highly selective manner to control various neuronal responses. A large amount of clinical and preclinical studies implicate members of the RGS family in the pathophysiology of disorders such as addiction, depression and schizophrenia. Moreover, complexes between RGS proteins, G alpha subunits and other scaffolding molecules appear to dynamically regulate the actions of several drugs used for the treatment of CNS diseases, including L-dopa, antidepressant agents and opiate analgesics. Our group combines genetic mouse models with biochemical and molecular biology approaches in order to understand the brain region/receptor selective actions of RGS family members, such as RGS9-2, RGS4, RGS2 and RGSz. Adeno-associated viral constructs (AAV) provided a very important tool for brain region selective overexpression or deletion of a protein, as they are nontoxic, they have a long half life and they selectively infect neurons. This presentation provides some examples of tissue specific interventions in RGS protein activity in animal models of addiction, neuropathic pain and depression.

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Investigating the Signal Transduction Pathways Underlying Remote Ischaemic Preconditioning in Pigs

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Key words: Remote ischaemic preconditioning, myocardium, infarct size, adenosine receptor, PI3K-Akt pathway

INTRODUCTION

Applying brief ischaemia/reperfusion to a limb either prior to myocardial ischaemia (remote ischaemic preconditioning, RIPC) can reduce myocardial infarct size. We hypothesised that RIPC limits infarct size by activating the adenosine receptor and the PI3K-Akt pathway at the onset of myocardial reperfusion.

METHODS

Under general anaesthesia mini swines (25-30kg) were randomly divided into four groups and were subjected to 60min regional ischemia of the heart after ligation of a prominent coronary artery with the following additional interventions: *Control group* with no additional interventions, *RIPC group* subjected to four cycles of 5min ischemia/5min reperfusion of a lower limb prior to the onset of myocardial ischaemia, *RIPC+Wort group* and *RIPC+8-SPT groups* were subjected to the same intervention were treated with 20 μg/kg Wortmannin or 10 mg/kg 8-SPT respectively that were intravenously given 30 seconds before the end of prolonged ischemia. After the end of the long reperfusion period, the infarct size (I) was delineated by TTC staining, the area at risk (R) by fluorescent particles and the percent (%) I/R ratio was calculated. In a second series of experiments, three respective to the first series groups were subjected to the same protocol. Myocardial biopsies were taken from all studied groups, at three different time points: (A) baseline – healthy myocardial tissue – up and away from the ischemic region and the apex of the heart, (B) 5 min and (C) 15 min respectively after the onset of reperfusion from area at risk. PI3, phospho-PI3, Akt, phospho-Akt and total GSK-3β were assessed by Western blot.

RESULTS

RIPC significantly reduced myocardial infarct size (13.3±2.2% versus 48.8±4.2% in control: P<0.05:N>5/group). Wortmannin, the PI3K-Akt inhibitor, partially abolished the infarct-limiting effects of RIPC (33.2±6% with RIPC+Wort versus 13.3±2.2% with RIPC:P<0.05:N>5/group). 8-SPT, the adenosine receptor inhibitor, did not influence the infarct-limiting effects of RIPC (10.4±2.0% with RIPC+8-SPT versus 13.3±2.2% with RIPC: P>0.05:N>5/group). The phosphorylation of PI3 and Akt was significantly higher in the ischaemic regions of the heart, in the protected *RIPC group* compared to the *Control*, *RIPC+Wort* and *RIPC+8-SPT* groups. GSK-3β was inhibited only in the protected *RIPC* and *RIPC+SPT* groups and not in the *Control* and the non protected *RIPC+Wort* groups.

CONCLUSION

RIPC reduces myocardial infarct size by activating the PI3K-Akt pathway at reperfusion. RIPC

does not require activation of the adenosine receptor at the time of reperfusion for its infarct-limiting effects. The complete absence of PI3 and Akt does not abrogate the protective effect of RIPC if it is combined with simultaneous inhibition of GSK-3β. Total GSK-3β prevents the protection afforded by RIPC.

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STAT5B Forms Dynamic Complexes with the δ-Opioid Receptor and Selective G Protein Subunits

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Key words: Opioid receptor, Signal Transducer and Activator of Transcription, G protein, signaling complex, tyrosine phosphorylation

Summary. Recent observations from our laboratory have shown that the Signal Transducer and Activator of Transcription 5A (STAT5A) interacts with the μ-opioid receptor (μ-OR) and is phosphorylated upon μ-OR stimulation. In the present study we demonstrate that another member of the STAT family, STAT5B, interacts directly with the conserved juxtamembrane region of the C-terminal tail of the δ-opioid receptor (δ-CT). Agonist exposure of HEK293 cells, stably expressing the flag-δ-opioid receptor (δ-OR), leads to a G protein-dependent STAT5B phosphorylation and transcriptional activation mediated by c-Src kinase. Co-immunoprecipitation studies demonstrate that δ-OR serves as a platform for the formation of a multi-component signaling complex signalosome, consisting of STAT5B, c-Src, Gβγ and selective Gα protein subunits. Collectively, our results uncover a novel signaling pathway through which δ-OR might be involved in the transcriptional regulation of STAT5B-dependent genes.

INTRODUCTION

STATs comprise a family of seven structurally and functionally related transcription factors (2). Although STATs are considered to be primarily regulated by cytokines, their activities can also be modulated by G protein-coupled receptors (GPCRs) and distinct members of the STAT family have been shown to interact directly with several GPCRs (3,4).

Opioid receptors belong to the superfamily of GPCRs and are prototypical Gi/Go-coupled receptors. Like many receptors that utilize Gi subfamily members for signal transduction, the opioid receptors have long been known to inhibit adenylyl cyclases (5,6), while ample experimental evidence has revealed that opioid receptor activation leads to alterations in the expression of target genes, involved in the development of opiate tolerance and dependence. Opioid administration causes activation of several transcription factors, including cyclic AMP-response element DNA-binding protein (CREB), Activator protein AP-1, members of the MAPK cascade and the NF-kB (7). Upon activation, opioid receptors are also capable of inducing phosphorylation and activation of STAT family members (1).

We have previously shown that the conserved YXXL motif within the C-terminal tail of μ-OR serves as a docking site for STAT5A binding (1). This YXXL motif found frequently in GPCRs, is part of the helix VIII of opioid receptors and serves as a docking site for various opioid receptor interacting partners (8-10). Given that the δ-CT contains the same structural motif, we wondered whether STAT5A/B interact in a similar manner with δ-OR and whether δ-OR could alter transcription by forming a multi-component signaling complex with STAT5 and/or other signaling intermediates.

METHODS

Cell cultures and transient transfections: HEK293 cells stably transfected with the human flag-δ-OR (δ-HEK293) and Neuro-2A mouse neuroblastoma cells were used. Transient transfections were

performed using Lipofectamine 2000 reagent, according to the manufacturer's instructions.

Immunoprecipitation assays: δ-HEK293 or SH-SY5Y cells, endogenously expressing STAT5B were treated or not, with 1 μM of opioid agonists and lysed in buffer containing 1% Igepal and protease/phosphatase inhibitors. Cell lysates were incubated with the appropriate antibodies and immunoblotted as described (11,12).

Luciferase measurements: δ-HEK293 cells were transfected with an expression vector containing a 4xGAS STAT-activation dependent firefly-luciferase reporter construct. 48 h after transfection cells were treated with 1 μM DSLET for 15 min and luciferase expression was measured 6 h after agonist challenge (1).

Isolation of cytoplasmic and nuclear extracts: δ-HEK293 cells treated or not with DSLET were lysed in buffer containing DTT and were incubated with cytochalasin B. Cell lysates were homogenized and the nuclei were collected by centrifugation at 600xg. Cytoplasmic extracts were obtained with further centrifugation at 10 000 x g. Nuclear fractions were isolated after ice incubation in a Triton buffer and centrifugation at 10 000 x g.

Preparation of GST fusion constructs and GST pull-down assays: GST fusion peptides generation and pull-down experiments were performed as described (13,14).

RESULTS AND DISCUSSION

In an attempt to examine whether STAT5B interacts with the δ-OR, pull-down assays were performed, encompassing parts of the δ-CT. As shown in Figure 1, these studies revealed the direct interaction of STAT5 with the C-terminal tail of δ-OR. Mapping the sites of interaction demonstrated that the conserved YAFL region at the δ-CT is the structural determinant responsible for STAT5B binding. Co-immunoprecipitation assays confirmed the binding of STAT5B to δ-OR and demonstrated that STAT5B interacts constitutively with the receptor and dissociates upon DSLET administration. These results suggest that the DSLET-activated conformation of δ-OR destabilizes the interaction between STAT5B and the receptor, possibly due to tyrosine phosphorylation of δ-OR and/or STAT5B activation.

Given the interaction of STAT5B within the δ-CT and in view of the STAT5A tyrosine-phosphorylation upon μ-OR activation (1), we explored whether similar effects occur upon activation of δ-OR with various specific agonists. Our results have shown that STAT5B is phosphorylated upon δ-OR activation in HEK293 and SH-SY5Y cells by both DSLET and morphine. This phosphorylation occurs in a G protein-dependent manner and is carried out by a c-Src kinase. Additional experimental studies indicated the transcriptional activation of a STAT5-directed Luciferase reporter gene upon δ-OR stimulation together with an increase of STAT5B in the cell nucleus. This observation suggests that activation of flag-δ-OR induces STAT5B translocation to the nucleus and STAT5-mediated transcriptional responses.

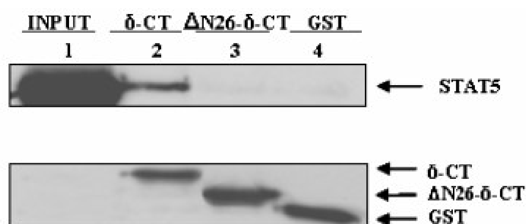

Figure. 1 STAT5B interacts with the δ-CT

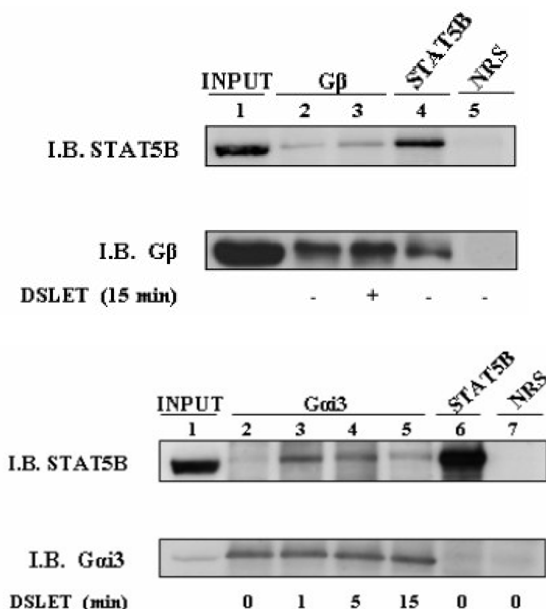

Figure 2. STAT5B interacts with Gαi/Gαo and Gβγ subunits in HEK293 cells

Src kinases were previously shown to associate with and phosphorylate members of the STAT

family (15). In view of this consideration, we examined whether c-Src could also associate directly with STAT5B. Our co-immuno-precipitation studies showed that STAT5B interacts with c-Src only upon DSLET-activation of δ-OR in HEK293 cells. Knowing that Gαi and Gβγ subunits interact *in vitro* with the δ-CT [14] and that δ-OR-induced STAT5B phosphorylation occurs in a G protein-dependent manner, we wondered whether STAT5B could directly associate with Gα and/or Gβγ subunits. Our experiments indicated that, in HEK293 cells, Gβγ complex interacts spontaneously with STAT5B, an interaction that is enhanced upon δ-OR activation. Apart from Gβγ, STAT5B interacts also with Gαi3 and Gαo, but not Gαi2, in the presence or absence of opioid agonists (Fig. 2).

To detect whether a multi-component signaling complex is initiated upon activation of δ-OR, the spatial organization between the receptor, G proteins, Src kinase and STAT5B was investigated. Gβγ associates with δ-OR independently of DSLET-stimulation, whereas c-Src fails to interact with δ-OR at any receptor conformation. Given that c-Src phosphorylates STAT5B but does not interact with the receptor we explored whether the Gβγ complex serves as a scaffold to recruit c-Src to the δ-OR. Indeed, c-Src, in the resting state of the receptor, associates with Gβγ subunits, whereas upon δ-OR stimulation interacts with Gαi3.

Collectively, our results propose a novel signaling pathway initiated at the δ-CT, which serves as a protein platform. As demonstrated in Figure 3, δ-OR interacts constitutively with STAT5B, Gα and Gβγ subunits and indirectly with c-Src via Gβγ dimer, giving rise to a signaling complex *signalosome*. In the DSLET-activated state of δ-OR, Gβγ remains bound to δ-OR; STAT5B dissociates from the receptor, forming a new complex, consisting of Gβγ, active Gαi/o subunits and activated c-Src. Subsequently, phosphorylated STAT5B dimerizes and translocates to the nucleus where it binds to specific DNA target sequences, altering gene transcription.

The formation of dynamic complexes between δ-OR, G protein subunits and STAT5B uncovers a novel signaling pathway through which δ-opioid receptor might regulate gene transcription in the nervous system and alter synaptosomal plasticity.

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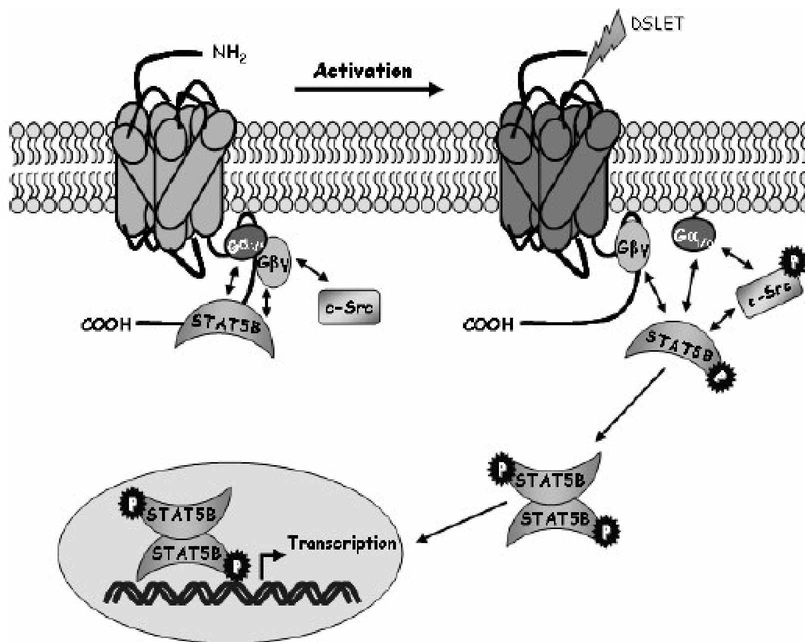

Figure. 3 A putative signaling pathway of the δ-OR-STAT5B-G protein complex

REFERENCES

1. Mazarakou G., Georgoussi Z.: STAT5A interacts with and is phosphorylated upon activation of the μ-opioid receptor. *J. Neurochem. 93*: 918-931 (2005)

2. Levy D.E., Darnell J.E., Jr.: 2002. Stats: transcriptional control and biological impact. Nat. Rev. Mol. Cell. Biol. 3, 651-662.

3. McWhinney, C. D., Dostal, D., Baker, K., 1998. Angiotensin II activates Stat5 through Jak2 kinase in cardiac myocytes. J. Mol. Cell. Cardiol. 30, 751-761.

4. Wu, E. H., Lo, R. K., Wong, Y. H., 2003. Regulation of STAT3 activity by G16- coupled receptors. Biochem. Biophys. Res. Comun. 303, 920-925.

5. Georgoussi, Z., Zioudrou, C., 1993. Effect of delta-opioid antagonists on the functional coupling between opioid receptors and G-proteins in rat brain membranes. Biochem Pharmacol. 45, 2405-2410.

6. Megaritis, G., Merkouris, M., Georgoussi, Z., 2000. Functional domains of delta- and mu-opioid receptors responsible for adenylyl cyclase inhibition. Recept. Channels 7, 199-212.

7. Tso, P. H., Wong, Y. H., 2003. Molecular basis of opioid dependence: role of signal regulation by G-proteins. Clin. Exp. Pharmacol. Physiol. 30, 307-316.

8. Georgoussi, Z., 2008. Novel interacting partners regulating opioid receptor signaling. In: Ciruela, F., Lujan, R., (Eds), Molecular Aspects of G Protein-Coupled Receptors. Nova Science Publishers Inc, pp. 169-206.

9. Leontiadis, L. J., Papakonstantinou, M. P., Georgoussi, Z., 2009. Regulator of G protein signaling 4 confers selectivity to specific G proteins to modulate μ- and δ-opioid receptor signaling. Cell. Signal. 21, 1218-1228.

10. Georgoussi, Z., Georganta, E-M., Milligan, G., 2010. The other side of opioid receptor signalling: Regulation by protein-protein interaction. Curr. Drug Targets. In press.

11. Morou, E., Georgoussi Z., 2005. Expression of the third intracellular loop of the δ-opioid receptor inhibits signaling by opioid receptors and other G protein coupled receptors. J. Pharmacol. Exp. Ther. 315, 1368-1379.

12. Swevers, L., Morou, E., Balatsos, N., Iatrou, K., Georgoussi, Z., 2005. Functional expression of mammalian opioid receptors in insect cells and high-throughput screening platforms for receptor ligand mimetics. Cell. Mol. Life Sci. 62, 919-930.

13. Douris, V., Swevers, L., Labropoulou, V., Andronopoulou, E., Georgoussi, Z., Iatrou, K., 2006. Stably transformed insect cell lines: tools for expression of secreted and membrane-anchored proteins and high-throughput screening platforms for drug and insecticide discovery. Adv. Virus Res. 68, 113-156.

14. Georgoussi, Z., Leontiadis, L., Mazarakou, G., Merkouris, M., Hyde, K., Hamm, H., 2006. Selective interactions between G protein subunits and RGS4 with the C-terminal domains of the μ- and δ-opioid receptors regulate opioid receptor signalling. Cell. Signal. 18, 771-782.

15. Cao, X., Tay, A., Guy, G. R., Tan, Y. H., 1996. Activation and association of Stat3 with Src in v-Src-transformed cell lines. Mol. Cell. Biol. 16, 1595-1603.

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Patterns of Human Interleukin-8 (IL-8) Gene Expression during Growth Related Changes and Hemin-induced Differentiation in K562 CML Cells

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Key words: Interleukin-8, K562 cells, hemin, mRNA levels

S u m m a r y. *IL-8, a pro-inflammatory cytokine, is produced from a variety of cancer cells and it was found to contribute to a more aggressive attitude of cancer cells. In the present study, we have found that K562 cells produce some basal levels of IL-8 mRNA, which are increased by several folds by hemin. The mechanism that contributes to this increase is found to be highly and rapidly-responsive. The biological significance of IL-8 production for the progression of leukeamia is being proposed.*

INTRODUCTION

Human Interleukin-8 (IL-8) belongs to the family of CXC chemokines and it was first found to be produced by macrophages and to act as a chemoattractant for neutrophils (1). Moreover, IL-8 was also found to be produced by nonimmune cells and especially by a large variety of cancer cells (ovarian cancer, breast cancer, lung cancer, pancreatic adenocarcinoma and by leukemic cells) (2). Interleukin-8 is a secreted protein of 79 amino acids, which is processed extracellularly to yield the signaling protein of either 77 amino acids in nonimmune or 72 amino acids in immune cells.

IL-8 has been shown to contribute to human cancer progression through its potential functions as a mitogenic, motogenic and angiogenic factor (2,3). IL-8 acts in an autocrine loop on cancer cells by increasing their proliferation and migration capacity and in a paracrine loop by influencing endothelial cells and promoting angiogenesis (4). Finally, IL-8 derived from cancer cells can attract neutrophils in the site of tumor, which can either produce growth factors in a way to enhance the growth of cancer cells or attack them and reducing their proliferative capacity. IL-8 exerts these actions via interactions with the CXCR1 and CXCR2 G-coupled receptors.

Apart from the constitutive production of IL-8 protein from a variety of cancer cells, IL-8 gene expression is activated by environmental stimuli, such as acidosis (5), hypoxia, NO and other interleukins like TNF and INF-γ (6). Mechanistically, the increase in IL-8 mRNA levels by the above mentioned stimuli has been attributed either to an increase in the rate of transcription, or to stabilization at the mRNA level or both. The activation of transcription of IL-8 gene requires the activation of either the combination of NF-κB and AP-1 transcription factors or that of NF-κB and NF-IL6, depending on the type of cells (7). On the other hand, in the 3' UTR of the mRNA of IL-8 (NM_000584.2) there are ARE (AU-Rich-Elements). These elements were found in many labile mRNAs to act as destabilizing determinants by promoting the deadenylation of mRNA, which results in the subsequent mRNA decay (8).

In our study we have observed that K562 CML cells express some basal levels of mRNA of IL-8, which are increased proportionally with the density of the cells in the culture. Furthermore, we have noticed that hemin, the oxidised form of heme and a key microenvironmental regulator of erythropoiesis[9], increases the levels of mRNA of IL-8 by several folds. The initial characterization was performed as function of the optimum concentration of hemin used and the time

that has to lapse in order the increase in the mRNA of IL-8 to be observed.

MATERIALS AND METHODS

Human K562 chronic myelogenous leukemia cells were used throughout this study. K562 cells were grown in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37^{0}C with 5% CO_2 and humidified atmosphere (~95%). Hemin (Fluka) was prepared as stock solution at 4 mM concentration in slightly alkaline solution. Throughout these experiments K562 cells were seeded at 1 x 10^5 cells/ml, after determination of the concentration of a stock culture using Neubaeur hematocytometer. Cells were cultured with hemin at concentrations varying from 5-90 μM or in absence of hemin for time periods between 12-96 h. Total cytoplasmic RNA was isolated from the cells with acid phenol-guanidium thiocyanate-chloroform extraction protocol as described (10). The concentration of RNA in the aqueous solution was estimated by measuring the absorbance at 260 nm. One step RT-PCR was performed with the RobusT-I RT-PCR kit (Finnzymes) in 50 μl reactions using as template 1 μg RNA/ reaction from K562 cells treated at different conditions, as indicated at the text. For RNA loading control a housekeeping gene, GAPDH, was used, whose mRNA levels are steady under different conditions. The RT-PCR running conditions were the following: 48 ^{0}C for 30 min, 94 ^{0}C for 2 min, 94 ^{0}C for 30 sec, x ^{0}C for 1 min, 72 ^{0}C for 50 sec, 35 cycles and 72 ^{0}C for 10 min. The annealing temperatures (x ^{0}C) for the human genes used were 63^{0}C (IL-8) and 57^{0}C (GAPDH). The following primer sequences were used: IL-8 5'-GGCACAAACTTTCAGAGACAGCAGA-3' (forward) and 5'-CCCTCTGCACCCAGTTTTCCTT-3' (reverse), GAPDH 5'-GAAGGTGAAGGTCGGAGT-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse). The PCR products whose sizes were 355bp (IL-8) and 225bp (GAPDH) were analyzed in 1% agarose gel.

RESULTS AND DISCUSSION

In the current study, we have observed the levels of mRNA of IL-8 to increase in K562 cultures treated with hemin. In particular, we have found that the increase in mRNAs is dose-dependent to hemin in concentrations of 5 to 25 μM. The last concentration seems to be the optimum of inducing IL-8 by hemin in K562 cells (Figure 1). The levels of mRNA of IL-8 were found to remain constant in higher concentrations of hemin (30 to 90 μM).

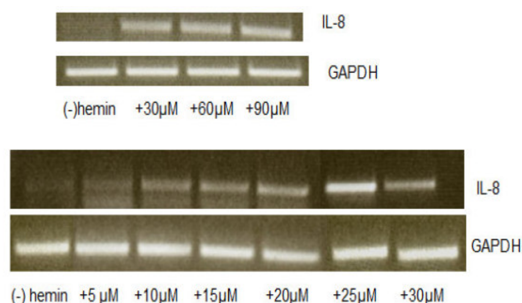

Figure 1: Assessment of IL-8 gene expression transcripts (mRNA) by RT-PCR in RNA derived from 48 h cultures of hemin-treated (at concentrations varying from 5 to 90 μM) K562 cells. Results are representative of two independent experiments

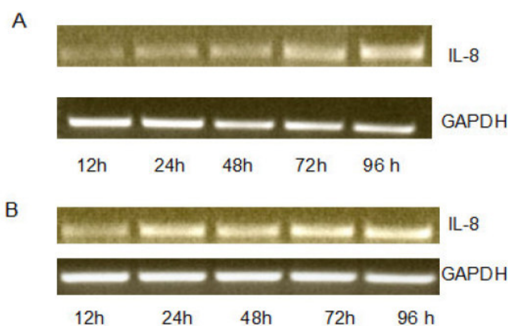

Figure 2: Assessment of IL-8 gene expression by RT-PCR in RNA derived from untreated (A) and treated with 25 μM hemin (B) and at various time points (ranging from 12 to 96 h) K562 cell cultures. Results are representative of two independent experiments.

Moreover, we have found that K562 cells were able to produce some basal levels of mRNA of IL-8, which are increased proportionally with the density of the cells in the culture (Figure 2A). This observation may reflects that the constitutive production of mRNA of IL-8 from K562 cells depends on cell cycle phase, since the majority of cells at 96 h are in G1 phase. In the presence of hemin, we have observed that the increase in mRNA of IL-8 had already begun in 12h (Figure 2B in comparison to Figure 2A). From the current observations we propose that hemin augments the levels of mRNA of IL-8 by a mechanism that seems to be highly and rapidly-responsive, since it is accomplished in surprisingly low concentrations of

hemin and it had already begun at 12h, respectively.

In the immediate future, we are going to investigate the time that has to lapse in order the mechanism of increase of IL-8 mRNAs stimulated by hemin to begin, by analysing with RT-PCR RNAs isolated at time points varying from 2 to 10 h. Our next goal is to investigate in which level (transcriptional or post-transcriptional) the mechanism of increase of mRNA of IL-8 induced by hemin functions. In this frame, we are going to block transcription with actinomycin D, an RNA polymerase inhibitor (11), and estimate the $t_{1/2}$ of mRNA of IL-8 with Northern analysis in untreated and cells treated with hemin. Furthermore, it would be useful to see if the increase in mRNA levels is accompanied with an increase in the protein level by performing ELISA in the cell culture medium.

In conclusion, we tend to suggest that as leukeamia cells are exposed to hemin in the microenvironment of bone marrow, the specific mechanism of increase of IL-8 mRNA levels is activated in a way to influence the proliferation capacity of cancer cells and enhance the angiogenesis in the bone marrow. So, understanding the mechanisms of both constitutive and inducible Il-8 expression will be helpful in designing potential therapeutic strategies of targeting Il-8 to control tumor growth and metastasis.

REFERENCES

1. Baggiolini M., Clark-Lewis I.: Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS 307*: 97-101 (1992)
2. Xie Keping: Interleukin-8 and human cancer biology. *Cytokine and Growth Factors Review 12*: 375-391 (2001)
3. Waugh D., Wilson C.: The Interleukin-8 Pathway in Cancer: *Clin. Cancer Res. 21*: 6735-6741 (2008)
4. Heidemann J., Ogawa H., Dwinell M., Raffie P., Maaser C., Gockel H., Otterson M., Ota D., Lugering N. Domschke W., Binion D.: Angiogenic effects of Interleukin-8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. *J. Biol. Chem. 278*: 8508-8515 (2003)
5. Xu L., Fidler I.: (2001) Acidic pH-induced elevation in interleukin-8 expression by human ovarian carcinoma cells. *Cancer Res. 60*: 4610-4616 (2001)
6. Yasumoto K., Okamoto S., Mukaida N., Murakami S., Mai M., Matsushima K.: Tumor Necrosis Factor and Interferon-γ synergistically induce interleukin-8 production in a human gastric cancer cell line through acting on AP-1 and NF-κB-like binding sites of the interleukin gene. *J. Biol. Chem. 267*: 22506-22511 (1992)
7. Mukaida N., Okamoto S., Ishikawa Y., Matsushima K.: Molecular mechanism of interleukin-8 gene expression. *J. Leukocyte Biol.*: 554-558 (1994)
8. Zublaga A., Belasco J., Greenberg M.: The nonamer UUAUUUAUU is the key AU-Rich sequence motif that mediates mRNA degradation. *Mol. Cell. Biol. 15*: 2219-2230 (1995)
9. Tsiftsoglou A., Tsamadou A. Papadopoulou L.: Heme as a key regulator of major mammalian cell functions: molecular, cellular and pharmacological aspects. *Pharmol. Ther. 111*: 327-345 (2006)
10. Sambrook J. Russel DW, Laboratory. CSH: Molecular cloning: A laboratory manual. Cold Spring Harbor, NY, 2001
11. Hyman R., Davinson N.: Kinetics of the in vitro inhibition of transcription by Actinomycin D.: *J. Mol. Biol. 50*: 421-438 (1970)

REVIEW OF CLINICAL PHARMACOLOGY AND
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Role of Adrenoceptor Signaling in PPARα Regulation

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SUMMARY

PPARα holds a fundamental role in lipid homeostasis by directly regulating genes involved in fatty acid uptake, β- and ω-oxidation. It is worthy of note that PPARα agonists are effective in raising HDL-cholesterol and reducing triglycerides, properties that prevent atherosclerosis and reduce the risk for cardiovascular diseases. This study investigated the role of adrenoceptor signaling in PPARα regulation using wild type and humanized PPARα mice treated with either phenylephrine hydrochloride (2 mg/kg i.p., α_1-agonist) or isoprenaline hydrochloride (2 mg/kg, i.p., β1/2-agonist). Dexmedetomidine hydrochloride (5 μg/kg, s.c.) was used for α2-adrenergic receptor stimulation. The data of this study showed that adrenergic receptors (ARs), major components of the stress system and targets of various drugs, used in the treatment of cardiovascular diseases hold key roles in PPARα regulation. In particular, stimulation of α1-ARs with phenylephrine and beta-ARs with isoprenaline was followed by a significant up-regulation of PPARα and target genes, including ACOX, ACOT-1, ACOT-4, cyp4α10 and cyp4α14 that regulate the metabolism of fatty acids. *In vitro* studies using primary hepatocyte cultures treated with AR-agonists confirmed the involvement of hepatic AR-signaling in PPARα regulation. Overall, the data of this study set the basis of a better understanding the complex physiopathological states related to lipid disturbances and potentially introduce innovative therapeutic approaches.

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Possible Protective Effect of 2,3-dihydro-2H-1,4-Benzoxazine Derivatives in a Prion Disease Model

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Key words: Transmissible spongiform encephalopathies, prion protein, benzoxazines, drug screening

S u m m a r y. Prion diseases are fatal neurodegenerative disorders characterized by the structural conversion of a normal, cellular protein, PrP^C, into an aberrant isoform, termed PrP^{Sc}. The physiological function of PrP^C has remained enigmatic, although there is evidence that PrP^C may play a role in protecting cells from oxidative stress, which, per se, has been implicated in prion pathogenesis. Benzoxazines are bicyclic heterocyclic compounds with several pharma-ceutically important properties, including neuropro-tection and antioxidation. In this study, a series of novel benzoxazine derivatives was evaluated in vitro regarding their effect on the levels of both the physiological PrP^C and the abnormal PrP^{Sc}. A possible involvement in the inhibition of neuronal cell death, would render these compounds valuable neuroprotective agents. Moreover, our in vitro system could serve as a particularly useful model in further screening assays.

INTRODUCTION

Transmissible Spongiform Encephalopathies (TSEs), commonly known as prion diseases are fatal neurodegenerative disorders affecting a wide range of species, varying from humans to rodents. The most notorious representatives include Bovine Spongiform Encephalopathy (BSE) in cattle, scrapie in sheep and goats and Creutzfeldt Jakob disease (CJD) in humans. Prion diseases are invariably fatal, involving the structural conversion of a normal, endogenous protein, called PrP^C into its conformational, proteinase K (PK) - resistant, possibly pathogenic isoform, termed PrP^{Sc}. Although substantial research has been performed during the last 30 years, in order to better comprehend prion pathogenesis, principal issues, including PrP^C physiological role, prion pathogenic mechanism and transmissibility, still remain elusive.

Several compounds have been considered to display anti-prion activity both *in vitro* and *in vivo* (1). The most effective ones include inhibitors of PrP^{Sc} accumulation in infected cell lines, whereas strategies for both eliminating the physiological PrP^C as the substrate for prion conversion and for enhancing PrP^{Sc} degradation have also been employed (2).

On an attempt to identify novel neuroprotective compounds, a series of new 2,3-dihydro-2H-1,4-benzoxazine derivatives modified at positions 2-, 3-, 4- and 6-, was examined regarding their putative protective effect on the levels of both PrP^C and PrP^{Sc} *in vitro*. Here we report our findings on three out of seventeen compounds.

METHODS

Cell culture: Mouse neuroblastoma-2A cells (N2a) (3-5), permanently infected with the 22L mouse-adapted scrapie strain were used. 22L-ScN2a were kindly provided by Dr. H. Schätzl, Institute of Virology, Technical University of Mu-

nich, Germany, and cells were cultivated as previously described (6).

Compound Preparation and Incubation: Compounds were diluted in DMSO, in order to reach the proposed EC50 (as indicated by studies in hippocampal HT22 cells) in the cell culture [EC50 value: TC130, 495 μM; TC177, 142 nM; TC178, 190 nM] per treatment. Control cell cultures received only DMSO. For each treatment duplicate cultures were used. The dosage scheme for each compound included administration of the compound on day 1 post subpassaging for TC130; days 1, 2 and 3 for TC177 and TC178 (medium was exchanged before the second administration). Cells were lysed on the fourth day post subpassaging.

Trypsinization, Lysis and Proteinase K treatment: Trypsin treatment and lysis were performed as described elsewhere (7). Following lysis, the lysates were centrifuged for 1 min at 14000 rpm. For PK-treatment, cell lysates were incubated with 1μg/ml PK (Merck) for 1 h at 37 ^{o}C. The digestion was stopped by the addition of PMSF 5mM. Finally, sarcosyl 1% was added and the proteins were methanol-precipitated. The untreated control samples were methanol-precipitated after the addition of PMSF 5 mM. Following centrifugation at 4000 rpm for 40 min, the protein pellets were solubilized in 2.5X O'Farrell loading buffer, heated for 10 min at 100 ^{o}C and then analyzed by SDS-PAGE and western blotting.

Western blot analysis: Western blotting was performed as previously described [8]. The antibodies used were 6H4, 1:5000 (Prionics) for endogenous PrP levels and goat-anti-mouse HRP-conjugated as secondary antibody, 1:10000 (Pierce) in blocking buffer (5% powder milk in PBST 0.1%). Monoclonal anti-actin antibody, 1:2000 (Santa Cruz Biotechnology) was used for estimation of endogenous actin levels.

Densitometry: Normalization of protein levels was performed based on actin and using Gel-Pro Analyzer (Version 3.0.00.00, Serial Nr. 50M00000-00001, Copyright © Media Cybernetics, 1993-97).

RESULTS

Initially, we tested the endogenous ability of the cells to continuously convert PrP^{C} into PrP^{Sc} and compared the PrP-immunostaining pattern with the one obtained following PK-treatment of TSE-infected rodent brain homogenates (Figure 1). As indicated by the prion-characteristic three band-pattern following treatment with PK, the cells were able to accumulate adequate amounts of PrP^{Sc}.

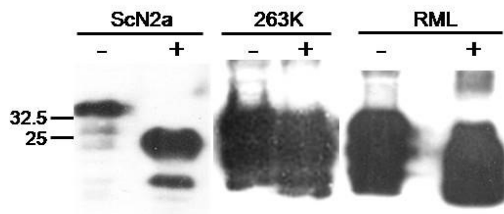

Figure 1. PK treatment of prion-infected cells and brain homogenates. Samples were either treated (+) or non treated (-) with PK. ScN2a, 22LScN2a cells [100.000 cells/load for (-) and 400.000 cells/load for (+)]; 263K, scrapie hamster brain homogenate (2mg brain equivalents/load); RML, scrapie mouse brain homogenate (2 mg brain equivalents/load). Western blotting using 6H4 monoclonal antibody (1:5000). The molecular weight protein markers are indicated in kDa.

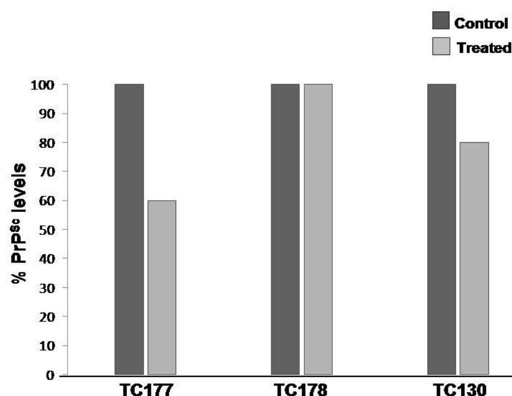

Figure 3. Graphic illustration of % reduction of PrP^{Sc} levels. Bars indicate the % reduction of PrP^{Sc} levels in treated cells in comparison to control cells that did not receive any compound. Normalization was performed based on actin levels in treated and non treated cell lysates.

In order to test the possible effect of certain benzoxazine derivatives on the levels of PrP^{C} and/or PrP^{Sc}, we incubated cells with the compounds as indicated in the Methods section. Consequently, cells were either treated or not treated with PK and PrP-immunolabeling was compared to the one of control cells (Figs 2 and 3).

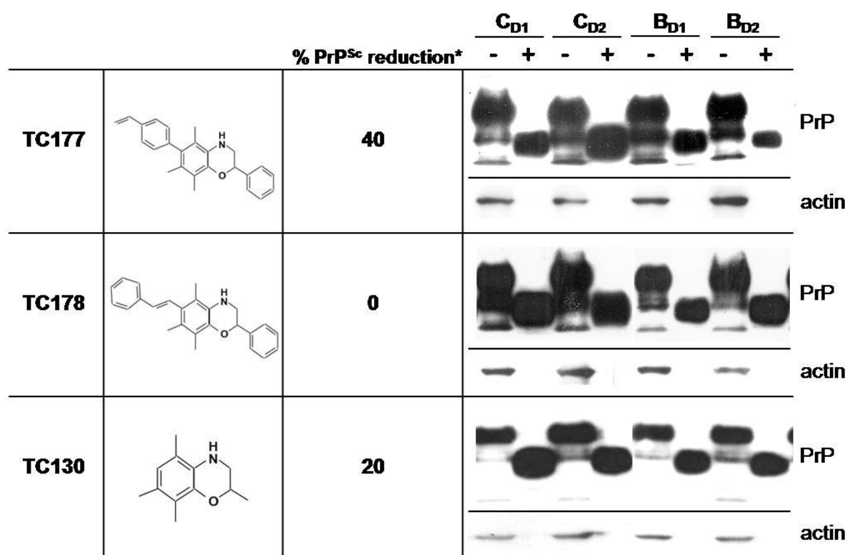

*Figure 2. Effect of benzoxazine derivatives on PrP levels. Following incubation with the compound, the cells were treated (+) or non treated (-) with PK. All treatments were performed in duplicates (C_{D1} & C_{D2} for the controls and B_{D1} & B_{D2} for the benzoxazine-treated cells). For PrP-immunostaining, 6H4 (1:5000) was used, whereas for protein content normalization, blots were probed with anti-actin monoclonal antibody (1:2000). *, Calculations were performed using densitometry based on normalization of protein amounts according to actin levels*

DISCUSSION

We have employed a well established *in vitro* prion disease model, namely the scrapie-infected mouse neuroblastoma (22L-ScN2a) cells, in order to screen a series of benzoxazine derivatives for a putative effect on PrP^{C} and/or PrP^{Sc} levels. Preliminary data indicate that certain benzoxazines may exert a diminishing effect on the levels of PrP^{Sc}. The mechanism, by which these compounds inhibit PrP^{Sc} formation and/or enhance its clearance, is still unknown. Further *in vitro* studies are required, in order to define the structure-activity relationships and also assess issues concerning dose dependence and cytotoxicity. Ultimately, *in vivo* assays should be employed, in order to evaluate the therapeutic potential of these benzoxazine derivatives.

REFERENCES

1. Trevitt C.R., Collinge J.: A systematic review of prion therapeutics in experimental models. *Brain 129*: 2241-2265 (2006)
2. Gilch S., et al.: Strategies for eliminating PrP(c) as substrate for prion conversion and for enhancing PrP(Sc) degradation. *Vet. Microbiol. 123*: 377-386 (2007)
3. Race R.E., Fadness L.H., Chesebro B.: Characterization of scrapie infection in mouse neuroblastoma cells. *J. Gen. Virol. 68*: 1391-1399 (1987)
4. Caughey B., et al.: Prion protein biosynthesis in scrapie-infected and uninfected neuroblastoma cells. *J. Virol. 63*: 175-181 (1989)
5. Bosque P.J., Prusiner S.B.: Cultured cell sublines highly susceptible to prion infection. *J. Virol. 74*: 4377-4386 (2000)
6. Nishida N., et al.: Successful transmission of three mouse-adapted scrapie strains to murine neuroblastoma cell lines overexpressing wild-type mouse prion protein. *J. Virol. 74*: 320-325 (2000)
7. Greenwood A.D., et al.: Cell line dependent RNA expression profiles of prion-infected mouse neuronal cells. *J. Mol. Biol. 349*: 487-500 (2005)
8. Salta E., et al.: Evaluation of the possible transmission of BSE and scrapie to gilthead sea bream (Sparus aurata). *PLoS One 4*: e6175 (2009)

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Nerve Growth Factor Receptors Mediate the Neuroprotective Effects of Neurosteroid Dehydroepiandrosterone

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Key words: Neurosteroids, neurotrophins, apoptosis, membrane receptors, cell signaling

SUMMARY

Neurosteroid DHEA is biosynthesized in neurons and glia, regulating neuronal survival and neurogenesis during development and in aging. We provide evidence that DHEA acts as a neurotrophic factor, protecting neuronal cells against apoptosis via activation of TrkA and $p75^{NTR}$, membrane receptors of neurotrophin NGF. Specifically, we have shown that siRNA against prosurvival TrkA receptors blocked the anti-apoptotic effect of DHEA and reversed its stimulatory action on anti-apoptotic Bcl-2 proteins. Radiolabeled [^{3}H]DHEA bound with high affinity to membranes isolated from HEK293 cells transfected with the cDNAs of TrkA and $p75^{NTR}$ receptors (Kds 0,9 and 5.6 nM respectively). Membrane binding of DHEA on $HEK293^{TrkA}$ and $HEK293^{p75NTR}$ transfectants was also shown with flow cytometry and immunofluoresence microscopy, using the membrane impermeable DHEA-BSA-FITC conjugate. DHEA-polyethylene-glycol beads effectively pulled down recombinant TrkA and $p75^{NTR}$ proteins, and precipitated both proteins from extracts prepared from cells expressing both receptors. DHEA was mimicking NGF in stimulating the phosphorylation of TrkA and in controlling TrkA and $p75^{NTR}$ protein levels. Furthermore, DHEA effectively activated NGF receptor-mediated signaling; Shc, Akt, and ERK1/2 kinases down-stream to TrkA receptors and TRFA6, RIP2 and RhoGDI effectors of $p75^{NTR}$ receptors. Finally, DHEA rescued from apoptosis sensory neurons of dorsal root ganglia in NGF null embryos and compensated NGF in rescuing sympathetic neurons of embryonic superior cervical ganglia. Our findings suggest that DHEA and NGF cross-talk via their activation of NGF receptors to afford brain shaping and maintenance. Phylogenetic findings on the evolution of neurotrophins, their receptors and CYP17, the enzyme responsible for DHEA biosynthesis, combined with our data support the hypothesis that DHEA served as a phylogenetically ancient neurotrophic factor.

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Interactions between Carbon Monoxide Releasing Molecules and Nitric Oxide Donors in Vascular Tissue

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Key words: Carbon monoxide-releasing molecule (CORM), guanylate cyclase (GC), cyclic guanosine monophosphate (cGMP), rat aortic smooth muscle cell (RASMC), vasorelaxation; nitric oxide (NO), Reactive oxygen species (ROS)

SUMMARY

Carbon monoxide (CO) is a weak sGC stimulator, leading to transient increases in cGMP and vasodilation. Transition metal carbonyls liberate CO in a controlled fashion and function as CO-releasing molecules (CORMs). The aim of the present work was to test the ability of a number of CORMs to modulate basal and nitric oxide (NO)-induced cGMP formation and vasorelaxation. cGMP accumulation was measured in rat aortic smooth muscle cells in the presence of CORMs and/or nitric oxide (NO) donors using ELISA. Basal and nitric oxide-stimulated sGC activity was determined using purified rat recombinant sGC. Vasodilation was determined using pre-contracted rat aortic rings after incubation with a CORM, in the presence or absence of S-nitroso N-acetylpenicillamine. Incubation of cells with some, but not all of the CORMs caused a minor increase in cGMP levels. Concentration-response curves were bell-shaped for most of the CORMs studied. Although exposure of cells to CORM-2 or ALF157 enhanced cGMP formation we observed that both compounds inhibited NO-stimulated cGMP accumulation in cells and NO-stimulated sGC activity that could be reversed by superoxide anion scavengers. Superoxide anion generation from both CORM-2 and ALF157 was confirmed using luminol-induced chemiluminescence. Furthermore, we observed that NO is scavenged by CORM-2. When used alone CORM-2 relaxed vessels through a cGMP-mediated pathway, but attenuates NO donor-stimulated vasorelaxation. We conclude that CORMs have variable, context-dependent effects on vessel tone as they can directly dilate blood vessels and also block NO-induced vasorelaxation.

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Biologic agents in the treatment of Non-Small-Cell Lung Cancer (NSCLC)

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INTRODUCTION

Non-Small Cell Lung Cancer (NSCLC) accounts for approximately 80% of the 170.000 new cases of lung carcinoma diagnosed each year in the United States and remains the leading cause of cancer-related death in both men and women in Western countries. The majority of these patients present with advanced, unresectable or metastatic disease, and approximately 15% of unselected patients are expected to be alive at five years (1).

Although, chemotherapy remains the cornerstone of treatment in advanced/metastatic NSCLC. Its use is not associated with a substantial survival improvement for most lung cancer patients, while it is associated with significant toxicity and it is clear that chemotherapy has reached a plateau of activity in the treatment of NSCLC (2). Thus, there is a clear need for new more active and more tolerant agents. Advances in our understanding of molecular biology of cancer and mechanisms of tumorigenesis, have enabled the discovery of several potential molecular targets and development of novel *targeted therapies*. These therapies inhibit signaling pathways involved in the development and progression of cancer. As these pathways are preferentially activated in cancer cells as contrasted to normal cells, targeted therapies are presumably better tolerated compared to classical cytotoxic agents.

EPIDERMAL GROWTH FACTOR RECEPTOR ANTAGONISTS

The EGFR family consists of four structurally similar tyrosine kinase proteins, including Erb-1 (EGFR), Erb-2 (HER2/neu), Erb-3 and Erb-4. The receptors exist as inactive monomers that homo- or heterodimerize (between EGFR and another member of the Erb family) after ligand binding. EGFR can be activated by several ligands, including Epidermal Growth Factor (EGF), Tumor Growth Factor-α (TGF-a), betacellulin, epiregulin and amphiregullin (3). Binding of these specific ligands to EGFR, results in activation of this receptor via autophosphorylation of the associated tyrosine kinase, which initiates an intracellular signal transduction cascade that affects DNA synthesis, cell growth, and survival (4). EGFR is over-expressed in the majority of NSCLC and therefore inhibition of EGFR signaling represents an ideal therapeutic target in NSCLC (5).

Strategies to block EGFR include tyrosine kinase inhibitors, monoclonal antibodies, antisense approaches and ligand-linked toxins. Among these approaches only tyrosine kinase inhibitors and monoclonal antibodies have reached clinical development.

Tyrosine Kinase Inhibitors

Four placebo controlled phase III trials have evaluated anti-EGFR TKIs as maintenance (6,7) (SATURN and WJTOG0203 trials), second (8) or third line (9) (ISEL and BR.21 trials) treatment in patients with NSCLC (Table 1). BR.21 was the first published study that compared erlotinib as second or third line treatment versus placebo in 731 NSCLC patients and demonstrated a survival benefit of about 2 months in favor of erlotinib (9). However, a similarly designed bigger phase III trial that randomly allocated 1692 NSCLC patients to gefitinib or placebo as second line treatment (ISEL trial) failed to demonstrate a benefit in terms of survival in favor of gefitinib. It is not clear why these conflicting results were observed between these two trials. These differences may be attributed to differences in the population included in the trial, differences regarding response

to previous therapy (more refractory patients were included in the ISEL study) or to the fact that gefitinib was administered in the ISEL trial at a dose significantly lower than maximum tolerated dose (MTD), while erlotinib in the BR.21 trial was administered at its MTD. Two other trials evaluated erlotinib (SATURN trial) (6) or gefitinib (WJTOG0203 trial) (7) respectively as maintenance therapy after standard platinum-based first line treatment. Both maintenance trials demonstrated a statistically significant benefit in terms of progression free survival (PFS) (SATURN: HR 0.71 [95% CI 0.62-0.82]; p<0.0001; WJTOG0203: HR 0.68; [95% CI, 0.57 to 0.80]; P <0.001). Survival data are not yet available for SATURN, while WJTOG0203 failed to yield a benefit; however it should be noted that this was a small underpowered study for overall survival (OS) differences.

Three phase III trials compared TKIs with chemotherapy either in first-line setting (10,11) or in second-line setting (12). IPASS study randomly assigned chemo-naïve NSCLC patients (never-smokers or former light smokers) to gefitinib or to chemotherapy doublet with paclitaxel/carboplatin (10). This trial met its primary end-point of showing non-inferiority of gefitinib, but furthermore demonstrated its superiority compared to chemotherapy (HR: 0.74, 95% CI 0.65 to 0.85, p<0.001). WJTOG3405 randomized 177 NSCLC patients harboring EGFR activating mutations to chemotherapy (docetaxel-cisplatin) or gefitinib and yielded a significant prolongation of PFS in favor of gefitinib with a median progression-free survival time of 9·2 months (95% CI 8·0–13·9) versus 6·3 months (5.8-7.8; HR 0·489, 95% CI 0·336-0.710, log-rank p<0.0001) for chemotherapy. INTEREST trial was a non-inferiority phase III trial that compared gefitinib with docetaxel as second line treatment in NSCLC patients (12). This was also a positive trial and non-inferiority of gefitinib compared to docetaxel was confirmed for overall survival (HR 1.020, 96% CI 0.905 to 1.150) (Table 2).

Monoclonal antibodies

Two prospective, randomized phase III trials of the combination of cetuximab with cytotoxic drugs are published. The first compared a cisplatin/vinorelbine doublet versus the same chemotherapy plus cetuximab in 1125 chemo-naïve NSCLC patients with EGFR immunohistochemistry (IHC) positive tumors. Although, PFS was identical between the two arms (4.8 vs 4.8 months), the cetuximab arm had significantly longer survival (11.3 vs. 10.1 months; P=0.044) (FLEX study) (13). Furthermore, a new analysis demonstrated that OS for patients receiving cetuximab, who experienced any grade of rash (acne-like rash) within three weeks of treatment initiation was 15.0 months compared to 8.8 months in those patients who developed no rash (HR=0.63; p<0.001) (14). The magnitude of benefit while statistically significant was modest (1.2-month improvement in median survival) and noteworthy, this trial has been criticized as being *over-powered* (15). A second phase III trial investigating carboplatin plus a taxane (either paclitaxel or docetaxel) with or without cetuximab as first line treatment for patients with metastatic NSCLC failed to show any improvement in progression free survival, the study's primary end point (BMS099 study) (16). A possible explanation for the discrepancy between the FLEX and the BMS099 study might be related to the relatively low number of patients participating in the latter trial. However, given that survival benefit observed in the FLEX study was modest and that BMS099 failed to demonstrate an OS benefit (although not adequately powered for OS), further studies are needed to elucidate the role of cetuximab in the treatment of NSCLC.

Molecular predictors of response

EGFR expression as determined by immunohistochemistry and its correlation with sensitivity to EGFR TKIs has been extensively studied in NSCLC. Published results are conflicting and both positive and negative correlations have been reported (17). However, it should be underlined that these differences could be attributed to differences regarding the methodologies used in all these studies (17). EGFR gene copy number, as assessed by fluorescence in situ hybridization (FISH) has also been tested as predictive factor for response with TKIs treatment and has been associated with significantly higher response rate and overall survival in randomized phase III trials of TKIs versus placebo while no predictive value of FISH EGFR analysis for survival was observed in randomized trials comparing TKI treatment with chemotherapy. Increased EGFR gene copy number has also been correlated with clinical outcome in NSCLC patients treated with monoclonal antibodies against EGFR. Most of the somatic mutations of EGFR observed in NSCLC, affect the tyrosine kinase coding domain (exons 18-21). Most common of these are in-frame deletions in exon 19 (codons 746-750) and a missense mutation leading to a substitution of arginine for leucine at codon 858 (L858R). There is strong correlation between the presence of these mutations and EGFR TKIs sensitivity in vitro and in vivo and

is considered the most valid predictive biomarker for benefit from TKIs treatment (17).

Table 1
Phase III placebo-controlled trials of anti-EGFR TKIs vs placebo

Trial	Treatment	n	ORR (%)	Median survival (mo)	P-value
BR.21 (9)	Erlotinib Placebo	427 211	8.9 <1	6.7 4.7	<0.001
ISEL (8)	Gefitinib Placebo	1129 563	8 <1	5.6 5.1	0.11
SATURN (6)	Erlotinib Placebo	438 451	12 5	PFS HR 0.71	0.001
WJTOG0203 (7)	Gefitinib Placebo			HR 0.86	0.11

HR=hazard ratio

Table 2
Phase III trials of TKIs versus chemotherapy

Trial	Treatment	N	ORR (%)	Median survival (mo)	P-value
INTEREST (12) *(non-inferiority)*	Gefitinib Docetaxel	733 733	9.1 7.6	7.6 7.6	-
WJTOG3405 (11)	Gefitinib TXT/CDDP	88 89	22.5 12.8	PFS HR 0.489	<0.001
IPASS (10)	Gefitinib PCL/Carbo	609 608	43 32.2	PFS HR 0.74	0.0001

TXT=docetaxel, CDDP=cisplatin, PCL=paclitaxel, Carbo=carboplatin, PFS=progression free survival, HR=hazard ratio

Antiangiogenic therapies

Bevacizumab is a recombinant, humanized, monoclonal antibody against VEGF, and is the most extensively studied antiangiogenic agent. One phase III trial demonstrated a survival benefit (prolongation of median survival by two months) for the combination of bevacizumab and paclitaxel/carboplatin followed by bevacizumab until disease progression (ECOG 4599) (18), while in the European AVAiL phase III trial, two doses of bevacizumab were compared to gemcitabine/cisplatin followed by bevacizumab. This study failed to show survival improvement, although time-to-progression was significantly longer for either dose of the drug (19). Squamous histology, metastases to central nervous system, history of hemoptysis, and history of documented hemorrhagic diathesis were reported as exclusion criteria in both studies, limiting the potential use of bevacizumab to selected NSCLC patients. The ECOG 4599 study reported significantly higher toxicity for patients receiving bevacizumab. Importantly, there were only two treatment related deaths in the chemotherapy arm and 15 in the bevacizumab arm (18). Similarly, in the AVAiL study, treatment related deaths were higher in the 15 mg/kg bevacizumab arm (1% higher death rate due to serious adverse events) while it was similar in the 7.5 mg/kg bevacizumab arm and chemotherapy alone arm.

REFERENCES

1. Jemal A., Siegel R., Ward E., Murray T., Xu J., Smigal C., et al.: Cancer Statistics. Cancer J. Clin. 56: 106-130 (2006)
2. Carney D.: Lung Cancer-Time. To Move On From Chemotherapy. N. Engl. J. Med. 346: 126-128 (2002)
3. Franklin W., Veve R., Hirsch F., Helfrich B., Bunn P., Jr.: Epidermal growth factor receptor family in lung cancer and premalignancy. Semin. Oncol. 29(1 Suppl 4): 3-14 (2002)
4. Sedlacek H.: Kinase Inhibitors In Cancer Therapy: A Look Ahead. Drugs 59: 435-76 (2000)
5. Ciardiello F., Tortora G.: Egfr Antagonists In Cancer Treatment. N. Engl. J. Med. 358: 1160-1174 (2008)
6. Cappuzzo F., Ciuleanu T., Stelmakh L., Cicenas S., Szczesna A., Juhasz E. et al.: Saturn: A double-blind, randomized, phase iii study of maintenance erlotinib versus placebo following nonprogression with first-line platinum-based chemotherapy in patients with advanced Nsclc. J. Clin. Oncol. 27(15s): Abstr 8001 (2009)
7. Takeda K., Hida T., Sato T., Ando M., Seto T., Satouchi M., et al.: Randomized Phase Iii Trial Of Platinum-Doublet Chemotherapy Followed By Gefitinib Compared With Continued Platinum-Doublet Chemotherapy In Japanese Patients With Advanced Non-Small-Cell Lung Cancer: Results Of A West Japan Thoracic Oncology Group Trial (Wjtog0203). J. Clin. Oncol. 28: 753-760 (2010)
8. Thatcher N., Chang A., Parikh P., Rodrigues Pj, Ciuleanu T., Von P., et al.: Gefitinib Plus Best Supportive Care In Previously Treated Patients With Refractory Advanced Non-Small-Cell Lung Cancer: Results From A Randomised, Placebo-Controlled, Multicentre Study (Iressa Survival Evaluation In Lung Cancer). Lancet 366: 1527-1537 (2005)
9. Shepherd F., Rodrigues P., Ciuleanu T., Tan E., Hirsh V., Thongprasert S.. et al.: Erlotinib In Previously Treated Non-Small-Cell Lung Cancer. N. Engl. J. Med. 353: 123-132 (2005)
10. Mok T., Wu Yl, Thongprasert S., Yang Ch., Chu Dt., Saijo N., et al.: Gefitinib Or Carboplatin-Paclitaxel In Pulmonary Adenocarcinoma. N. Engl J. Med. 361: 947-957 (2009)
11. Mitsudomi T., Morita S., Yatabe Y., Negoro S., Okamoto I., Tsurutani J., et al.: Gefitinib Versus Cisplatin Plus Docetaxel In Patients With Non-Small-Cell Lung Cancer Harbouring Mutations Of The Epidermal Growth Factor Receptor (Wjtog3405): An Open Label, Randomised Phase 3 Trial. Lancet Oncol. 11: 121-128 (2010)
12. Kim E., Hirsh V., Mok T., Socinski M., Gervais R., Wu Yl, et al.: Gefitinib Versus Docetaxel In Previously Treated Non-Small-Cell Lung Cancer (Interest): A Randomised Phase Iii Trial. Lancet 372(9652): 1809-1818 (2008)
13. Pirker R., Szczesna A., Von Pawel J., Krzakowski M., Ramlau R., Park K., et al.: Flex: A Randomized, Multicenter, Phase Iii Study Of Cetuximab In Combination With Cisplatin/Vinorelbine (Cv) Versus Cv Alone In The First-Line Treatment Of Patients With Advanced Non-Small Cell Lung Cancer (Nsclc). Proc. Am. Soc. Clin. Oncol. 26(20): Abstr 3 (2008)
14. Gatzemeier U., Vonpawel J., Vynnchenko I., Zatloukal P., Demarinis F., O'byrne K., et al.: Cetuximab In Combination With Platinum-Based Chemotherapy Improves Survival Versus Chemotherapy Alone In The 1st-Line Treatment Of Patients With Advanced Non-Small Cell Lung Cancer

(Nsclc). Multidiciplinary Symposium In Thoracic Oncology Chicago, November 2008 2008
15. Langer C.: Will Flex Allow Us Flexibility In The Therapy Of Advanced Non-Small-Cell Lung Cancer? Insights From The 2008 American Society Of Clinical Oncology Meeting. Clin. Lung Cancer 9: 249-251 (2008)
16. Lynch T., Patel R., Dreisbach L., Mcleod M., Heim W., Hermann R., et al.: A Randomized Multicenter Phase Iii Study Of Cetuximab (Erbitux(R)) In Combination With Taxane/Carboplatin Versus Taxane/Carboplatin Alone As First-Line Treatment For Patients With Advanced/Metastatic Non-Small Cell Lung Cancer (Nsclc): B3-03. J. Thorac. Oncol. 2(8): S340-S341 (2007)
17. Pallis A., Serfass L., Dziadziusko R., Van Meerbeeck J., Fennell D., Lacombe D., et al.: Targeted Therapies In The Treatment Of Advanced/Metastatic Nsclc. *Eur. J. Cancer 45*: 2473-2487 (2009)
18. Sandler A., Gray R., Perry M., Brahmer J., Schiller J., Dowlati A., et al.: Paclitaxel-Carboplatin Alone Or With Bevacizumab For Non-Small-Cell Lung Cancer. *N. Engl. J. Med. 355*: 2542-2550 (2006)
19. Reck M., Von P., Zatloukal P., Ramlau R., Gorbounova V., Hirsh V., et al.: Phase Iii Trial Of Cisplatin Plus Gemcitabine With Either Placebo Or Bevacizumab As First-Line Therapy For Nonsquamous Non-Small-Cell Lung Cancer: Avail. *J. Clin. Oncol. 27*: 1227-1234 (2009)

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Neurobiological Insights into the Chronic Mild Stress (CMS) Model of Depression: Sex Differences in Serotonergic Neurotransmission

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Key words: *In situ* hybridization; antidepressant, serotonin (5-HT), major depression, psychopharmacology

Summary: Chronic mild stress (CMS) was developed in the late 1980s and is one of the most extensively investigated animal models of depression to-date. Research from our lab has underlined the importance of serotonin in the manifestation of sexually dimorphic neurochemical, behavioural and immune responses. Novel data reveal that both CMS application and chronic antidepressant treatment modulate hippocampal serotonergic 1A (5-HT_{1A}) receptor mRNA levels in a sexually dimorphic manner.

INTRODUCTION

Major depression affects both sexes, but more women than men are likely to be diagnosed with depression in any given year. This sex-dependent differentiation has been largely attributed to the pronounced sex differences that predominate in both the anatomy and function of the human brain, as well as to the sexually dimorphic hormonal *milieu.* Chronic mild stress (CMS) was developed in the late 1980s and is one of the most extensively investigated animal models of depression to-date. In our initial studies in male rats exposed to a CMS regimen, we observed a decrease in the prefrontocortical serotonergic activity accompanied by an increase in hippocampal serotonergic activity; all alterations were reversed by chronic *imipramine* treatment [1]. In further comparative studies between male and female rats, we used a milder CMS protocol, which did not induce neurochemical alterations in male rats, but resulted in a significant decrease in hippocampal serotonergic activity in females [2]. Accordingly, exposure to CMS induces a wide spectrum of relevant neurobiological alterations in specific brain regions implicated in the pathophysiology of major depression. For instance, it has previously been reported that hippocampal serotonergic 1A (5-HT_{1A}) receptors are increased by CMS in male rats [3]. Given the prominent role of the hippocampus in the sex-dependent processing of stressful stimuli, in the present study we investigated whether sex differences exist in the expression of hippocampal 5-HT_{1A} receptor upon CMS application and chronic antidepressant treatment.

METHODS

Male and female *Sprague–Dawley* rats, weighing 200-300 g, were used throughout this study and were either subjected to CMS and chronic antidepressant regimens or served as controls, as previously described [4]. At 24 h following the cessation of stressors/drug administrations, CMS-treated and control rats of both sexes were decapitated and their brains were processed accordingly for the immunohistochemical detection of digoxygenin (DIG)-labelled 5-HT_{1A} riboprobes in the region of the hippocampus.

RESULTS

CMS application resulted in increased 5-HT_{1A} mRNA levels in the CA1 and CA3 regions of the hippocampus in males but not in female rats. On

the other hand, $5\text{-}HT_{1A}$ mRNA levels in the CA1 region were induced in male but decreased in female controls upon chronic antidepressant treatment.

DISCUSSION

These novel data reveal that both chronic stress and antidepressant treatment induce sexually dimorphic effects on 5-HT1A receptor mRNA expression in the CA1 and CA3 regions of the hippocampus and lend further support to the prominent role of the hippocampal serotonergic system in the manifestation of sexually dimorphic responses in the CMS model of depression.

REFERENCES

1. Bekris S., Antoniou K., Daskas S., Papadopoulou-Daifoti Z.: Behavioural and neurochemical effects induced by chronic mild stress applied to two different rat strains. *Behav. Brain Res. 161*: 45-59 (2005)
2. Dalla C., Antoniou K., Drossopoulou G., *et al.*: Chronic mild stress impact: Are females more vulnerable? *Neuroscience 135:* 703-714 (2005)
3. Papp M., Klimek V., Willner P.: Effects of imipramine on serotonergic and beta-adrenergic receptor binding in a realistic animal model of depression. *Psychopharmacology (Berl) 114*: 309-314 (1994)
4. Pitychoutis P.M., Griva E., Ioannou K., Tsitsilonis O.E., Papadopoulou-Daifoti Z.: Chronic antidepressant treatment exerts sexually dimorphic immunomodulatory effects in an experimental model of major depression: Do females lack an advantage? *Int. J. Neuropsychopharmacol 12*: 1157-1163 (2009)

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Antagonism of Cannabinoid CB1 Receptors Modulates the Effects of Amphetamine on Locomotor Activity and Dopamine and Glutamate in the Nucleus Accumbens *in vivo*

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Key words: Dopamine, glutamate, amphetamine, cannabinoids, motor activity

S u m m a r y. The endocannabinoid system (ECS) modulates many neurotransmitter systems and is implicated in reward, addiction and the effects of psychostimulants. The psychostimulant d-amphetamine (d-amp) is well known to modulate locomotor activity as well as dopamine and glutamate function; however, it is yet unclear how the ECS is able to intervene in these effects of d-amp. Thus, the aim of the present study was to investigate the effects of CB1 antagonism on d-amp-induced behavioural and neurochemical effects. Sprague- Dawley rats were either observed for locomotor activity after administration of vehicle, d-amp, SR or SR and d-amp or underwent surgery for probe implantation and administered the same treatments in microdialysis experiments measuring dopamine and glutamate in the nucleus accumbens. Our results showed that d-amp on its own induced hyperlocomotion and increased dopamine and glutamate. When coadministered with SR, these effects on locomotor activity and neurotransmitter levels were modulated. This study provides further evidence for the role of the ECS in d-amp- induced behavioural and neurochemical effects in vivo, and furthermore, emphasizes the importance of this modulatory neurotransmitter system in psychostimulant addiction.

INTRODUCTION

The ECS modulates many neurotransmitter systems and thus it is implicated in many physiological processes including reward and cognition. This interaction can also be extended to pathological states such as drug dependence and addiction. Interestingly, the ECS has been implicated in psychostimulant addiction and has even been considered a potential target for treatment, however, at present, this phenomenon is not completely understood. The effects of drugs of abuse such as cannabinoids and the psychostimulant d-amp on the brain and on behaviour are not only mediated by the mesolimbic dopaminergic system but also by the excitatory glutamatergic neurotransmitter system. Amphetamine, an indirect dopamine agonist, is universally known to modulate locomotor activity and extracellular dopamine and glutamate. Evidence demonstrates that cannabinoids also modify locomotor activity as well as the neurotransmitters dopamine and glutamate, mainly via their action on the CB1 receptor in the brain. In the present study, we chose to investigate the effects of SR, a CB_1 receptor antagonist, coadministered with the psychostimulant, d-amp on locomotor activity, in vivo extracellular dopamine levels and glutamate levels in the nucleus acccumbens, the epicentre of reward.

METHODS

Male Sprague-Dawley rats were divided into two groups; the first group was intraperitoneally (i.p.) administered one of four treatments and locomotor activity was measured. The second group of rats underwent surgery and a microdialysis probe was implanted in the nucleus ac-

cumbens. Sample collection began 24 h later. Once baseline samples were collected, the rats were injected i.p. with one of four treatments and sample collection resumed. All samples were then measured for dopamine and glutamate using High Performance Liquid Chromatography with electrochemical detection. The four treatment groups were as follows: vehicle, d-amp, SR or SR + d-amp.

RESULTS

Our results show that d-amp- administered rats exhibited increased motor activity and this effect was modulated by coadministration with SR. Likewise, increased extracellular dopamine and glutamate observed after d-amp administration in the nucleus accumbens, were also modified by SR.

DISCUSSION

This study addresses the effects of the CB1 antagonist, SR, on the universally known stimulatory effects of d-amp on locomotor activity as well as dopamine and glutamate release in the nucleus accumbens. Our results show that SR was indeed able to modulate both locomotor activity and neurotransmitter release induced by d-amp, thus providing us with further evidence for a role of the ECS in the effects of d-amp. On a larger scale, these results contribute to the expansively growing literature on the significance of the ECS in reward and addiction.

REFERENCES

1. Corbille A.G., Valjent E., Marsicano G., Ledent C., Lutz B., Herve D., Girault J.A.: Role of cannabinoid type 1 receptors in locomotor activity and striatal signaling in response to psychostimulants. *J Neurosci 27*: 6937-6947 (2007)

2. Gardner E.L.: (2005) Endocannabinoid signaling system and brain reward: emphasis on dopamine. *Pharmacol. Biochem. Behav. 81*: 263-284 (2005)

3. Parolaro D., Rubino T.: The role of the endogenous cannabinoid system in drug addiction. *Drug News Perspect. 21*: 149-57 (2008)

4. Polissidis A., Chouliara O., Galanopoulos A., Rentesi G., Dosi M., Hyphantis T., Marselos M., Papadopoulou-Daifoti Z., Nomikos G.G., Spyraki C., Tzavara E.T., Antoniou K.: Individual differences in the effects of cannabinoids on motor activity, dopaminergic activity and DARPP-32 phosphorylation in distinct regions of the brain. *Int. J. Neuropsychopharmacol*: 1-17 (2009)

5. Wiskerke J., Pattij T., Schoffelmeer A.N., De Vries T.J.: The role of CB1 receptors in psychostimulant addiction. *Addict. Biol. 13*: 225-38 (2008)

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A Novel Role of Endogenous Corticotropin-releasing Hormone (*Crh*) on Dermal Fibroblast Function

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Key words: Fibroblasts, CRH, interleukin-6, proliferation, migration, skin

INTRODUCTION

Hypothalamic CRH, a major mediator of the stress response, is involved in the inflammatory response by exerting indirect anti-inflammatory effects via stimulation of glucocorticoid release as well as potent direct proinflammatory effects in a plethora of tissues, including skin. The latter is further supported by that, CRH and its receptors are expressed in human and murine skin. In the process of wounded skin repair, fibroblasts from the wound edges migrate into the wound and proliferate in order to fill the site of the wound. This process is highly coordinated and mediated by locally released growth factors and cytokines which likely act in an autocrine/paracrine manner. IL-6 is a pro-inflammatory cytokine critically involved in cutaneous wound healing. We have previously shown that CRH regulates IL-6 expression during inflammation and that *Crh* deficient mice have accelerated wound healing in vivo and suppressed tissue IL-6 expression. Based on the above, *the aim of our study* was to clarify the role of endogenous CRH on the function of primary dermal fibroblasts, *in vitro*.

METHODS

Primary fibroblasts were isolated from the skin of wild type (*Crh+/+)* and *Crh* deficient (*Crh-/-*) neonatal mice as well as from human foreskin. Evaluation of the proliferation rate of all cells was performed using the MTT and the thymidine incorporation methods. Apoptotic cell death was measured with FACS analysis. Cytokine levels were measured with ELISA (murine cells) or with Chemiluminescence (human cells). Evaluation of the migration rate was performed using the scratch assay. The presence and the functional role of CRF receptors in dermal fibroblasts was tested with RTPCR and cAMP accumulation assays, respectively.

RESULTS

Our results showed that *Crh-/-* fibroblasts have significantly compromised IL-6 and TGF-β secretion compared to that of *Crh*+/+ mice. Treatment of cells with CRH (10 nM) did not affect the secretion of either IL-6 or TGF-β in both genotypes. Furthermore, *Crh-/-* fibroblasts had significantly higher basal proliferation rate compared to *Crh+/+* fibroblasts. Treatment with CRH (10 nM) had no effect on the proliferation rate of cells of either genotype. No difference was observed in apoptosis between the two genotypes. Furthermore, the number of *Crh-/-* cells migrated into the wounded area was significantly higher than that of *Crh+/+* cells in the in vitro wound assay.

Treatment of cells of either genotype with CRH did not alter their migration rate. Finally, in order to examine the effect of CRH on human fibroblasts, experiments using the CRF receptor antagonists, antalarmin and a-helical CRF(9-41) were performed. These experiments revealed increased proliferation and migration rate and suppressed IL-6 secretion of CRF_1 antagonist-treated fibroblasts.

DISCUSSION

In summary, our *in vitro* and *in vivo* findings on the role of CRH in–cutaneous wound healing demonstrate accelerated repair in the *Crh-/-* mice, possibly due to the increased migration and proliferation rate of the *Crh-/-* dermal fibroblasts. Furthermore, the reduced secretion of IL-6 by *Crh-/-* fibroblasts, in conjunction with the interrelated roles of IL-6 and CRH and the presence of CRH and its receptors in the skin, underlines the significance of locally acting CRH in the biology of these cells. Our findings support the direct effects of CRH in skin biology and provide interesting insights for the potential usefulness of the developing specific agonists and antagonists of the CRH family in dermal injury or other skin diseases.

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Role of Pleiotrophin and its Receptor Protein Tyrosine Phosphatase β/ζ in Vascular Endothelial Growth Factor-induced Endothelial Cell Migration

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Key words: Heparin affin regulatory peptide, heparin-binding growth associated molecule, angiogenesis, tumour, KDR, $\alpha_v\beta_3$ integrin

SUMMARY

A considerable progress has been made during the past years in elucidating the molecular actors of angiogenesis, with vascular endothelial growth factor (VEGF) representing the major inducer of angiogenesis up to date. VEGF induces several angiogenic functions of endothelial cells through its receptors VEGFR1 and VEGFR2 or KDR. VEGF-induced endothelial cell migration seems to be mediated by KDR, possibly via engagement of integrin $\alpha_v\beta_3$. Pleiotrophin (PTN) is a secreted heparin-binding growth factor that through its receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ) and $\alpha_v\beta_3$ integrin induces human endothelial cell migration. We have previously shown that exogenous PTN inhibits VEGF-induced endothelial cell migration. In the present work we studied the effect of endogenous PTN and its receptor RPTPβ/ζ in VEGF-induced endothelial cell migration. We found that endogenous PTN is not involved, while RPTPβ/ζ is required for VEGF-induced endothelial cell migration. Although VEGF may directly interact with RPTPβ/ζ, down-regulation of the latter by siRNA does not affect VEGF-induced ERK1/2 activation, but seems to affect the interaction of KDR with $\alpha_v\beta_3$, which is important for VEGF-induced cell migration. Collectively, RPTPβ/ζ seems to be required for VEGF-induced endothelial cell migration through its receptor KDR.

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Differential expression of preoteoglycans in intrinsic and extrinsic skin ageing

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Key words: Skin ageing, proteoglycans, aggrecan, versican, perlecan

INTRODUCTION

Skin ageing is a multifactorial process consisting of two distinct and independent components: intrinsic ageing which affects the skin in the same pattern as it affects all internal organs and extrinsic ageing. Extracellular matrix molecules, like hyaluronan, are highly implicated in both processes (1). Proteoglycans are major components of the extracellular matrix of the skin. However, they have been poorly studied in intrinsic and extrinsic skin ageing. In the present study we have tried to elucidate the involvement of certain important proteoglycans in intrinsic and extrinsic skin ageing in humans.

METHODS

Facial photo-protected and photo-exposed skin tissue specimens were collected from 10 male (mean age 73.3) and 6 postmenopausal female (mean age 67) patients. Photo-protected skin tissue specimens were also obtained from juvenile (mean age 5 years) patients. Gene expression of versican 1, versican 0, biglycan, decorin, aggrecan, perlecan and syndecan-3 was analyzed using RT-PCR.

RESULTS

Gene expression of versican 1, versican 0, biglycan, decorin, perlecan and syndecan-3 was down regulated in intrinsic skin ageing, whereas the exception of aggrecan, was up regulated. Extrinsic skin aging was associated with decreased expression of perlecan and decorin and increased expression of versican 0, versican 1 and aggrecan. Biglycan and syndecan-3 remained practically unaltered.

CONCLUSIONS

The differential expression of proteoglycans in extrinsic and intrinsic skin ageing indicate that these extracellular matrix molecules are involved in skin ageing and may offer novel pharmacological targets to confront skin ageing.

REFERENCES

1. Tzellos T.G., Klagas I., Vahtsevanos K., Triaridis S., Printza A., Kyrgidis A., Karakiulakis G., Zouboulis C.C., Papakonstantinou E.: Extrinsic ageing in the human skin is associated with alterations in the expression of hyaluronic acid and its metabolizing enzymes. *Exp. Dermatol. 18*: 1028-1035 (2009)

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Bioequivalence of two Formulations of Gabapentin 400 mg Capsules: Single-Dose, Open-Label, Randomized, Two-Period Crossover Comparison in Healthy Pakistani Adult Subjects

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Key words: Gabapentin, capsules, bioequivalence, pharmacokinetics, HPLC

S u m m a r y. A randomized, two-way, crossover, bioequivalence study was conducted in 20 fasting, healthy, male volunteers to compare two brands of Gabapentin 400 mg capsules, NEOGAB® (manufactured by Hilton Pharma (Pvt) Limited, Progressive Plaza, Beaumont Road, Karachi, Pakistan) as a test and NEURONTIN® (manufactured by Godecke AG/ Germany under license of Parke-Davis) as a reference product. One capsule of either formulation was administered with low-carbonate water after 10 h of overnight fasting. After dosing, serial blood samples were collected during a period of 48 hours. Plasma samples were analysed for Gabapentin by a validated HPLC method. The pharmacokinetic parameters AUC_{0-24}, $AUC_{0-\alpha}$, C_{max}, T_{max}, K_{el}, $T_{1/2}$, and CL were determined from plasma concentration-time profiles for both formulations and were compared statistically to evaluate bioequivalence between the two brands of Gabapentin, using the statistical modules recommended by FDA. The analysis of variance (ANOVA) did not show any significant difference between the two formulations and 90% confidence intervals (CI) fell within the acceptable range for bioequivalence. Based on these statistical inferences it was concluded that the two brands exhibited comparable pharmacokinetics profiles.

INTRODUCTION

Gabapentin (1-[aminomethyl] cyclohexaneacetic acid) is an analogue of γ-aminobutyric acid (GABA), a substance approved by the US Food and Drug Administration (FDA) for add-on therapy in treating seizures thought to be associated with interference in GABA-ergic pathways or provoked by excitory amino acids (1-3). Gabapentin has been shown to increase γ-aminobutyric acid concentrations in the brain of epilepsy patients (4). Gabapentin is well established in the treatment of seizures and also has a demonstrated analgesic effect in patients with chronic neuropathic pain (5). The absorption of gabapentin is saturable in a dose dependent manner involving an active transport process mechanism by an L-amino acid transporter (6). In humans, GBP is eliminated exclusively by renal excretion of unchanged drug, and plasma protein binding is negligible. Renal clearance (CL) is similar to glomerular filtration rate indicating that no net renal secretion or reabsorption takes place (7).

The objective of this study was to evaluate, in healthy volunteers, the bioequivalence of a test formulation of the 400 mg (capsule) of Neogab® elaborated by Hilton Pharma (Pvt) Limited, Progressive Plaza, Beaumont Road, Karachi, Pakistan and 400 mg (capsule) of Neurontin® made by Godecke AG/ Germany under license of Parke-Davis, used as a reference formulation.

The study was conducted in a randomized, single-dose, two-way, cross-over design with a 10 days washout period between two doses. During each period, the volunteers were admitted to hospital and after an overnight fasting they received a single reference or tested 400 mg Gabapentin capsule. Low-carbonate water (240 mL) was given immediately after drug administration. All volunteers fasted 2 h after the drug administration. The standardized meals (break-

fast, lunch and afternoon snack) were provided to volunteers. No other food was permitted during the first 24 h after drug administration. The study was performed in accordance with the guidelines of the revised Declaration of Helsinki on biomedical research involving subjects and the requirements of Good Clinical Practice.

METHODOLOGY

Formulations and sample collection

The following formulations were employed: Neogab® 400 mg Capsule (Batch # 5462, expiration date 04/2008) as test formulation, and Neurontin® 400 mg tablets (Batch # 0120094, expiration date 08/2006) as reference formulation. Blood samples were drawn at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12, 24, 36 and 48 hours after drugs administration. The blood samples were centrifuged at 4000 rpm for 15 min and the separated plasma was collected and stored at -20 °C until drug analysis. After a wash-out period of 10 days, the study was repeated in the same manner to complete the cross-over design.

Sample preparation for HLPC injection

To a microcentrifuge tube (2.0 ml, Eppendorf), 50 µl plasma sample, 25 µl of the internal standard (200 µg/ml), 250 µl of acetonitrile were added. The mixture was vortex-mixed on a vortex mixer (Scientific Industries, Inc., NY, USA) for 2 min. The mixture was then centrifuged at 17,000 g for 5.0 min (Jouan, GR 412, Saint Mazaire, Cedex, France). Two hundred fifty microliters of the supernatant was transferred into a 2.0 mL Eppendorf vial and mixed in darkness with 25 µL dansyl chloride (2.5 mg/mL in acetone) and 10 µL 0.5 mol/L Na_2HPO_4, pH 9.2. After centrifugation, at 17,000 g for 2 minutes the supernatant was transferred to glass tubes with caps (Millipore, Bedford, MA, USA) and 20 µl was directly injected into the HPLC system.

Chromatographic conditions

Chromatographic analysis was carried out at 30 °C. Separation was carried out with a µBondapak C-18, 10 µm, 300 × 3.9 mm column maintained at 30 °C. The mobile phase was 50 mmol/L NaH_2PO_4 containing 40% acetonitrile, vacuum-degassed before use, at a flow rate of 2.5 mL/min. Detection was carried out at excitation and emission wavelengths of 318 and 510 nm, respectively. The mobile phase was filtered by passing through a 0.22 µm membrane filter (Millipore, Bedford, MA, USA).

HPLC analysis

The mobile phase consisting of 50 mM sodium dihydrogen phosphate buffer and acetonitrile in the ratio of 60:40 (%, v/v) was found to be an appropriate mobile phase allowing adequate separation of drug and the internal standard using a C18 column at a flow rate of 2.5 ml/min.

The chromatographic separations and quantitative determination were performed using a high-performance liquid chromatograph from Agilent Technologies, Agilent 1100 consisting of a quaternary HPLC pump (Model 1100 Agilent), Micro vacuum degasser (1100 Agilent), a Fluorescence detector (light source xenon flash lamp, excitation monochromator 200-700 nm and zero order, emission monochromator 280-900 nm and zero order, flow cell: 8 µl volume, 20 bar pressure maximum, Model 1100 Agilent), Autosampler, containing 100 vials capacity and volume range 0.1 to 100 µL (Model 1100 Agilent), Thermostated column compartment (Model 1100 Agilent), and a software on 2d-chem station (Rev a.10.02 1757 Agilent Technology). The analytical column used to achieve chromatographic separation was a µBondapak C-18, 10 µm, 300 × 3.9 mm column protected by a guard column of the same material.

The plasma standard curves were prepared over the range of 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 µg/ml. Standard curves were analyzed and for each standard curve; determined the variability of the slopes and intercepts. The LOD of the drug in plasma samples was 25 ng/ml. The calibration curves of Gabapentin were typically described by the following regression analysis equations: $y = 1.1627x \pm 0.1693 + 0.0617 \pm 0.0243$, $r^2 = 0.9991$ (for plasma).

Pharmacokinetic and statistical analysis

For computation and analysis of the drugs plasma concentration versus time data and the graphics, the computer software Microsoft Excel 7.0 was used. The plasma concentration versus time data was used to calculate pharmacokinetics, absorption kinetics, and bioavailability and bioequivalence parameters by using PC-Computer Program, APO, MWPHARM version 3.02 a MEDIWARE product Holland. The area under the plasma concentration-time curve AUC_{0-24} and the area to the infinity $AUC_{0-\alpha}$ were calculated by using the linear trapezoidal method. For the purpose of bioequivalence analysis, two-way analysis of variance (ANOVA, GLM procedure) was used to assess the effect of formulations, periods, sequences, and subjects on AUC, T_{max} and C_{max}. The difference of two related parameters was

considered statistically significant for p-values equal or less than 0.05.

RESULTS

After a single oral administration of Gabapentin 400 mg capsule of test and reference in 20 healthy male subjects, the pharmacokinetic parameters showed in Table 1. And the mean plasma concentration-time curve of Gabapentin is shown in Figure 1.

Table 1
Pharmacokinetic parameters of Gabapentin after a single oral administration of test or reference tablet containing 400 mg Gabapentin in 20 healthy subjects

Kinetic Parameters	*Test capsule*	*Reference capsule*
C_{max} *(ng/L)*	2.27 ± 0.05	2.38 ± 0.10
T_{max} *(hr)*	1.70 ± 0.06	1.68 ± 0.04
$T_{1/2}$ *(hr)*	0.54 ± 0.04	0.62 ± 0.03
AUC_{0-t} *(μg. hr. L-1)*	17.05 ± 0.84	18.18 ± 0.74

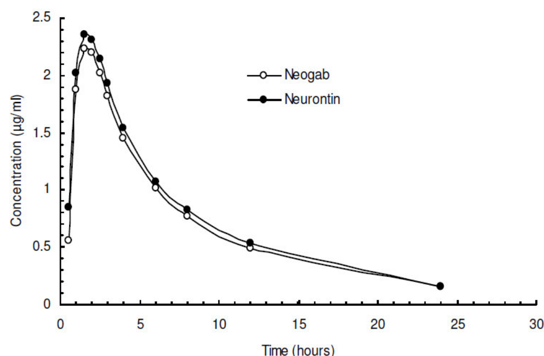

Figure 1. Mean plasma concentration of Gabapentin on ordinary scale after oral dose of 400 mg Capsule each Neogab® (Hilton) and NEURONTIN® (PARKE DAVIS) in 20 healthy male subjects

Bioequivalence evaluation

The analysis of variance on AUC and C_{max} indicated that there were no significant difference between the test and reference. Bioequivalence of Gabapentin after oral dose of 400 mg in NEOGAB® (Hilton) and NEURONTIN® (PARKE DAVIS) Capsules at 90% Confidence Interval (CI) lower and upper limits for AUC were 85.5 and 101.6 respectively and for C_{max} were 90.5 and 103.9, respectively. The lower and upper limits are within the acceptable limits of 80-125%.

DISCUSSION

Peak plasama levels (C_{max}) of gabapentin of 2.7-2.99 mg.l^{-1} are achieved 3-3.2 hr after ingestion of a single 300 mg capsule (7,8). Plasma GBP concentrations peaked at 2-6 h after the dose of 400 mg capsule (mean, 3.4 h) with C_{max} values ranging from 2.15 to 7.02 µg/ml (mean, 4.2 µg/ml), and then declined in apparent monoexponential fashion with $t_{1/2}$ values of 3.8-1 1.4 h (10). And the peak plasma levels (C_{max}) of gabapentin of 2.27/2.38 ng.l^{-1} are achieved 1.70/1.68 hr after ingestion of a single 400 mg dose of Neogab®/Neurontin® capsule. The elimination half-life of gabapentin is between 4.8 and 8.7 hr reported in the literature (7,8) whereas the elimination half life of gabapentin is between 7.25/ 6.77 hr after ingestion of a single 400 mg dose of Neogab®/Neurontin® capsule. The obtained values were in good agreement with reported studies.

The main objective of bioequivalence studies is to assure the safety and efficacy of generic formulations. Two formulations of the same drug are considered to be bioequivalent and therapeutically equivalent if they exhibit a comparable extent and rate of absorption, when they are administered in the same molar dose and under similar experimental conditions (9).

Statistical comparison of the main pharmacokinetic paramenters, AUC and C_{max}, clearly indicated no significant difference between Neogab® and Neurontin® 400 mg capsules, in any of the calculated pharmacokinetic parameters. Since the 90 % CI for AUC and C_{max} mean ratios are within the 80%-125% interval, it was concluded that the tested formulation Neogab® is bioequivalent to Neurontin®.

REFERENCES

1. Hengy H., K. Ernst-Ulrich.: Determination of Gabapentin in plasma and urine by high-performance liquid chromatography and pre-column labeling for ultraviolet detection. *J. Chrom. 341*: 473-478 (1985)
2. Goa K.D., E.M. Sorkin.: Gabapentin: A Review of Its Pharmacological Properties and Clinical Potential in Epilepsy. *Drugs 46*: 409-427 (1993)
3. Upton N.: Mechanisms of action of new antiepileptic drugs: Rational Design and serendipitous findings. *Trends Pharmacol. Sci. 15*: 456-463 (1994)
4. Patsalos P.N.: New antiepileptic drugs. *Ann. Clin. Biochem. 36*: 10-19 (1999)
5. Rowbotham M., Harden N., Stacey B., et al.: Gabapentin for the treatment of postherpetic neuralgia: a randomized controlled trial. *JAMA 280*: 1837-1842 (1998)
6. Stewart B.H., Kugler A.R., Thompson P.R., et al.: A saturable transport mechanism in the intestinal absorption of gabapentin is the underlying cause of the lack of proportionality between increasing dose and drug levels in plasma. *Pharmac. Res. 10*: 276-281 (1993)
7. Vollmer K.O., Van Hodenberg A.V., Kolle E.U.: Pharmacokinetics and metabolism of gabapentin in rat, dog and man. Arzneimittel-Forschung/Drug Res. 36: 830-839 (1986)

8. Vollmer KO, Anhut H, Thomann P, Wagner F, Jahncken D. pharmacokinetic model and absolute bioavailability of the new anticonvulsant gabapentin. *Adv. Epileptol. 17*: 207-211 (1989)
9. Chow S.C., Liu J.P.: Design and Analysis of Bioavailability and Bioequivalence Studies. Marcel Dekker, New York, 1992
10. Boyd R.A, Turck D., Abel R.B., Sedman A.J., Bockbrader H.N.: Effects of age and gender on single-dose pharmacokinetics of gabapentin. *Epilepsia 40*: 474-479 1999

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Inhibition of the Inflammatory Response of Activated Microglia by a Novel 17 Spiro Analog of Neurosteroid Dehydroepiandrosterone

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Key words: Microglia, BV2 cells, inflammation, DHEA, BNN27

INTRODUCTION

Activated microglia plays an important role in the pathogenesis of the majority of neurodegenerative disorders, such as Alzheimer's, Parkinson's disease and Multiple Sclerosis. Upon activation, microglia, which possesses the role of the CNS macrophages, produces and secretes most of the pro-inflammatory cytokines including Tumor Necrosis Factor α (TNFα), Interleukin 6 (IL-6), and Interleukin 1α and β (IL-1α and IL-β). Recent studies have shown that microglia also expresses the necessary biosynthetic enzymes for the production of neurosteroids, such as Dehy-droepiandrosterone (DHEA), Pregnanolone and Allopregnanolone (Allo). DHEA is a multi-functional neurohormone exerting both neuroprotective effects as well as possible modulatory actions on the immune system. Although there are several studies on the role of DHEA on peripheral macrophages, the impact of neurosteroids on the production of inflammatory mediators from microglia cells has not been yet elucidated. Based on the above, the aim of our study was to characterize in details the role of DHEA and its synthetic analog BNN27 (a 17 spironeurosteroid which is recently developed in our laboratory, deprived of endocrine properties), on the inflammatory response of activated microglia cells.

METHODS

For our studies we used the murine microglia BV2 cell line. BV2 cells have been shown to maintain many microglia responses. The experimental procedure was as follows: BV2 cells were stimulated with lipopolysaccharide (LPS) in the absence or presence of DHEA or BNN27 (100 nM) for several time points (3, 6, 12, 24, and 48 hr following stimulation with LPS). The levels of TNF-α and IL-6 were determined by ELISA and proliferation rate was measured using the MTT assay. Moreover, the possible effect of DHEA and BNN27 on apoptotic cell death of BV2 cells was examined by Flow Cytometry Analysis (FACS).

RESULTS AND DISCUSSION

Our results showed that BNN27 significantly reduced the secretion of TNF-α following activation with LPS in every time point tested. However, DHEA did not have a similar effect, due to its possible metabolic conversion, by specific steroid converting enzymes that BV2 cells produce. Neither DHEA nor BNN27 affected the levels of IL-6 in the time points tested. In addition, the synthetic analog also reduced the proliferation rate of BV2 cells after LPS stimulation, thus further indicating its anti-inflammatory role, since increased rates of proliferation characterize activated microglia. Studies in progress aim to elucidate the role of DHEA and BNN93 on apoptosis. In summary, our findings provide evidence for the possible pharmacological use of the synthetic analog BNN27 in the inhibition of inflammatory responses of activated microglia and thus ultimately, in the combined treatment of various neurodegenerative disorders.

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Methylation of the *Adenomatous Polyposis Coli* (*APC) Gene* in Cell-free DNA Circulating in Serum of Colorectal Cancer Patients

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Key words: Colorectal cancer, Molecular biomarkers, circulating DNA, DNA methylation, *adenomatous polyposis coli (APC).*

SUMMARY. The serum of cancer patients often contains increased free DNA levels, which could potentially offer material for early cancer detection or even information for the development of new therapeutic interventions. Genetic and epigenetic alterations of the *adenomatous polyposis coli* (*APC*) gene are common events in gastrointestinal tumor development. In the present study, we investigated the frequency of aberrant *APC* promoter methylation in circulating cell-free DNA isolated by colorectal cancer patients (CRC). We detected methylation of *APC* in 26 of 72 (38%) cases. Methylation was strongly correlated with metastasis. *APC* methylation analysis appears to be promising as a noninvasive tumor marker in serum DNA of CRC patients.

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Effect of JNK Inhibition on Post-ischemic Inflammation

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Key words: Neuroinflammation, neuroprotection, cerebral ischemia, JNK, microglia

INTRODUCTION

The c-Jun-N-terminal kinase signaling pathway (JNK) is activated during ischemia and plays an important role in apoptosis and inflammation. We have previously demonstrated that D-JNKI1, a specific JNK inhibitor, is strongly neuroprotective in animal models of stroke. We presently evaluated if D-JNKI1 modulates post-ischemic inflammation.

METHODS AND RESULTS

D-JNKI1 (0.1 mg/kg) or vehicle (saline) was administered intravenously 3 h after 45 min middle cerebral artery occlusion (MCAo) in outbred CD1 mice. Lesion size at 48 h was significantly reduced in the treated group. Activation of JNK (phosphorylation of c-Jun) was observed in neurons as well as in Isolectin B4 positive microglia 48 h after MCAo. We quantified microglial cells (CD11b) by measuring the average intensity of CD11b labelling (infra-red emission) within the ischemic tissue. No significant difference was found between groups. Cerebral ischemia was modelled *in vitro* by subjecting rat organotypic hippocampal slice cultures to oxygen (5%) and glucose deprivation for 30 min. In *vitro*, D-JNKI1 was found predominantly in NeuN positive neurons of the CA1 region and in few Isolectin B4 positive microglia. 48 h after OGD microglia were activated whereas resting microglia were found in controls and in D-JNKI1-treated slices. The secretion of inflammatory mediators was analysed *in vivo* (immunohistochemistry) and *in vitro* (Luminex technology) 48 h after induction of ischemia. Preliminary results suggest a decrease in interleukin-1-beta (IL-1-beta) and monocyte chemoattractant protein-1 (MCP-1) after treatment, respectively.

CONCLUSION

Our *in vivo* study shows that D-JNKI1 reduces the infarct volume 48 h after transient MCAo and does not act on the activation and accumulation of microglia. In contrast, *in vitro* data show a modulation of microglial activation after treatment.

All together, our data suggest that D-JNKI1 does not act directly on microglia. Further experiments will show whether D-JNKI1 reduces inflammatory mediators in the brain and in peripheral tissue or systemic circulation.

REFERENCES

1. Borsello et al.: *Nat. Med. 9*: 1180-1186 (2003)
2. Hirt et al.: *Stroke 35*: 1738-1743 (2004)
3. Wiegler et al.: *Cerebrovasc. Dis. 26*: 360-366 (2008)

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Assessing the Effect of Imatinib on the Expression of Genes Involved in Mitochondrial Cell Respiration in Human K-562 CML Cells

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Key words: Imatinib; COX deficiency; Mitochondrion, K-562 cells

Summary: Imatinib (IM) is a selective targeted inhibitor of chimeric Bcr-Abl tyrosine kinase, developed as a highly effective first line therapeutic agent to treat human chronic myelogenous leukemia and acute lymphocytic leukemia. Although IM is a designed molecular inhibitor of Bcr-Abl tyrosine kinase, additional mechanisms perhaps are involved leading to IM-induced drug resistance and cardiovascular toxicity. Our most recent observations that hemin (the oxidized form of heme) can counteract the cytotoxic effect of IM, prompted us to investigate the question whether IM dismantles the mitochondrial cell respiration pathway by blocking the expression of several genes encoding vital hemoproteins and other related proteins involved in the biosynthesis of cytochrome c oxidase. RT-PCR analysis revealed that IM repressed the expression of SCO2, COX17, Frataxin and Ferrochelatase genes, among others involved in mitochondrial respiratory chain pathway. These results indicate that IM selectively represses the expression of key-genes and provide novel mechanism(s) of IM that may explain its cardiovascular toxicity.

INTRODUCTION

Imatinib (IM/Gleevec/Glivec/STI-571/CGP57148B), a selective targeted inhibitor of Bcr-Abl chimeric tyrosine kinase, has been developed and used as a highly effective first line drug to treat human Bcr-Abl$^+$ Chronic Myelogenous Leukemia (CML) and Acute Lymphocytic Leukemia (ALL) (1). Unfortunately, a substantial portion of CML/ALL patients acquire resistance to IM (2) and/or suffer from cardiovascular toxicity (3,4). Whether those two adverse reactions of IM are attributed to common or disparate molecular mechanism(s) of action is not known yet. Furthermore, it is also known that mutations in *SCO2* gene, encoding a cytochrome *c* oxidase (COX) assembly protein, lead to fatal infantile cardioencephalomyopathy and COX deficiency (5).

Our earlier observations indicated that: a) heme [an iron Protoporhyrin-IX (Pp-IX), playing a key role in hematopoiesis] (6) counteracts directly or indirectly the Bcr-Abl$^+$-induced cell killing of IM (7), either via *HO-1* (*Hsp32*) induction (8,9) or via activation of antiapoptotic *Bcl-2* and Nuclear Factor E2-Related factor 2 (*Nrf2*) genes (10) and b) IM represses the mitochondrial COX activity through suppression of *SCO2* gene expression (11). In this study, we further explored the question whether IM represses the transcription of various genes involved in the biosynthesis of heme, hemoproteins and COX assembly genes.

METHODS

Human Bcr-Abl$^+$ K-562 CML cells were used throughout this study (12). These cells were seeded at $1x10^5$ cells/ml and grown in RPMI-1640 medium supplemented with 10% v/v FBS (Fetal Bovine Serum) and 1% v/v PS (Penicillin-Streptomycin), at 37°C in 5% CO_2 humidified atmosphere.

Total RNA isolated from human K-562 CML cells according to acid guanidinium thiocyanate-phenol-chloroform extraction method (13).

One step RT-PCR was performed with the RobusT-I RT-PCR kit (Finnzymes).

RESULTS

Human K-562 CML cells were grown with and/or without IM and assessed for cell growth at various time intervals. IM at concentrations as low as 1μM inhibited cell growth and caused cell cycle arrest after 24 to 96h.

To further examine whether IM treatment affects the transcription of genes encoding proteins of different classes that facilitate COX biosynthesis, RT-PCR assessment of total RNA derived from control and IM-treated human K-562 CML cells was performed. This analysis revealed that IM: a) altered to a different extent the expression of *SCO2*, *COX17*, *Frataxin* (*FXN*) and *Ferrochelatase* (*FECH*) genes; b) had no effect on the expression level of *SCO1*, *MTCOXI*, *MTCOXII*, *MTCOXIII*, *COXIV-1*, *COXVa*, *COXVIa-1*, *COX-VIc* and *COX10* genes.

DISCUSSION

IM induces apoptosis by blocking Bcr-Abl tyrosine kinase activity. Moreover, IM was found to repress the expression of *Bcl-2a*, *Bcl-2b* as well as *Nrf2* genes, which are involved in cell survival (10). However, despite these beneficial antineoplastic effects, a portion of patients undergoing IM treatment relapse by exhibiting acquired resistance to IM (2), while some of them exhibit cardiovascular toxicity (3, 4). Our results provide evidence that IM dismantles the mitochondrial cell respiration pathway and causes COX deficiency by mainly blocking the *SCO2* gene. This is an interesting, although unexplored, aspect of IM action. Possibly, *SCO2* gene suppression and COX deficiency induced by IM may explain why this agent may lead to cardiovascular toxicity in certain cases.

REFERENCES

1. Capdeville R., Buchdunger E., Zimmermann J., Matter A.: Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. *Nat. Rev. Drug Discov. 1*: 493 (2002)
2. Melo J.V., Chuah C.: Resistance to imatinib mesylate in chronic myeloid leukaemia. *Cancer Lett. 249*: 121 (2007)
3. Kerkela R., Grazette L., Yacobi R., Iliescu C., Patten R., Beahm C., Walters B., Shevtsov S., Pesant S., Clubb F.J., Rosenzweig A., Salomon R.N., Van Etten R.A., Alroy J., Durand J.B., Force T.: Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. *Nat. Med. 12*: 908 (2006)
4. Will Y., Dykens J.A., Nadanaciva S., Hirakawa B., Jamieson J., Marroquin L.D., Hynes J., Patyna S., Jessen B.A.: Effect of the multitargeted tyrosine kinase inhibitors imatinib, dasatinib, sunitinib, and sorafenib on mitochondrial function in isolated rat heart mitochondria and H9c2 cells: *Toxicol. Sci. 106*: 153 (2008)
5. Papadopoulou L.C., Sue C.M., Davidson M.M., Tanji K., Nishino I., Sadlock J.E., Krishna S., Walker W., Selby J., Glerum D.M., Coster R.V., Lyon G., Scalais E., Lebel R., Kaplan P., Shanske S., De Vivo D.C., Bonilla E., Hirano M., DiMauro S., Schon E.A.: Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in SCO2, a COX assembly gene, *Nat. Genet. 23*: 333 (1999)
6. Tsiftsoglou A.S., Tsamadou A.I., Papadopoulou L.C.: Heme as key regulator of major mammalian cellular functions: molecular, cellular, and pharmacological aspects. *Pharmacol. Ther. 111*: 327 (2006)
7. Bonovolias I.D., Manley P.W., Tsiftsoglou A.S.: Hemin confers resistance to Imatinib (Gleevec)-induced cell death but enhances Nilotinib (AMN107) cytotoxicity in human K-562 CML leukemia cells. *Blood 108*: 4769 (2006)
8. Mayerhofer M., Florian S., Krauth M.T., Aichberger K.J., Bilban M., Marculescu R., Printz D., Fritsch G., Wagner O., Selzer E., Sperr W.R., Valent P., Sillaber C.: Identification of heme oxygenase-1 as a novel BCR/ABL-dependent survival factor in chronic myeloid leukemia. *Cancer Res. 64*: 3148 (2004)
9. Mayerhofer M., Gleixner K.V., Mayerhofer J., Hoermann G., Jaeger E., Aichberger K.J., Ott R.G., Greish K., Nakamura H., Derdak S., Samorapoompichit P., Pickl W.F., Sexl V., Esterbauer H., Schwarzinger I., Sillaber C., Maeda H., Valent P.: Targeting of heat shock protein 32 (Hsp32)/heme oxygenase-1 (HO-1) in leukemic cells in chronic myeloid leukemia: a novel approach to overcome resistance against imatinib. *Blood 111*: 2200 (2008).
10. Bonovolias, I. D., and Tsiftsoglou, A. S., Hemin counteracts the repression of Bcl-2 and NrF2 genes and the cell killing induced by imatinib in human Bcr-Abl(+) CML cells, *Oncol Res, 17*, 535 (2009)
11. Foltopoulou P.F., Tsiftsoglou A.S., Bonovolias I.D., Ingendoh A.T., Papadopoulou L.C.: Intracellular delivery of full length recombinant human mitochondrial L-Sco2 protein into the mitochondria of permanent cell lines and SCO2 deficient patient's primary cells. *Biochim. Biophys. Acta*: (2010)
12. Lozzio B.B., Lozzio C.B.: Properties and usefulness of the original K-562 human myelogenous leukemia cell line. *Leuk Res. 3*: 363 (1979)
13. Chomczynski P., Sacchi N.: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem. 162*: 156 (1987)

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Effects of low doses of the nitric oxide synthase inhibitor L-NAME on recognition and spatial memory deficits produced by NMDA receptor antagonists in rats

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SUMMARY

There is experimental evidence that the nitric oxide synthase (NOS) inhibitor L-NAME is involved in learning and memory processes, although its exact role is still matter of investigations. The aim of the present study was to clarify the exact role of L-NAME on memory using different testing procedures. In a first study, the effects of a single injection of L-NAME (1, 3 and 10 mg/kg, i.p.) on recognition memory and its ability in counteracting MK-801-induced memory deficits were evaluated in the novel object recognition test. Subsequently, the effects of L-NAME on rats' spatial reference and spatial working memory and its ability to counteract ketamine-induced spatial memory deficits were assessed in the radial water maze task. L-NAME alone did not affect rats' performance and at 1 and 3 mg/kg, antagonized MK-801 (0.1 mg/kg)-induced performance deficits in the novel object recognition task. L-NAME (3 mg/kg) disrupted rodents' performance in the radial water maze test, whereas at 10 mg/kg, it attenuated ketamine (15 mg/kg)-induced spatial working, but not spatial reference memory deficits. In a last experiment aiming to evaluate the effects of the treatment conditions (acute vs. sub-chronic) on rats' performance, L-NAME given sub-chronically antagonized delay-dependent deficits in the novel object recognition task (1 and 10 mg/kg), while at 3 mg/kg it was unable to do so. The present results indicate that the L-NAME displays a dual effect on rats' memory and this effect might be influenced by the treatment regimen.

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Anesthetic ketamine impairs rats' retrograde memory. Reversal of its behavioural effects by the nitric oxide synthase inhibitor L-NAME

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SUMMARY

There is poor experimental evidence concerning the effects of anesthetic doses of the non-competitive NMDA receptor antagonist ketamine on rodents' memory abilities. The present study was designed to investigate a) the consequences of post-training administration of anesthetic ketamine on rats' recognition memory and b) to evaluate whether or not the nitric oxide synthase inhibitor L-NAME (1, 3, 10 mg/kg, i.p.) was able to counteract the expected behavioural deficits produced by anesthetic ketamine. For this purpose, the novel object recognition task was selected. Post-training administration of ketamine (100 mg/kg; i.p.) disrupted animals' performance in the novel object recognition paradigm indicating that anesthetic ketamine impaired recognition memory. L-NAME (1-3, but not 10 mg/kg) antagonized this deficit on cognition produced by anesthetic ketamine. The current results indicate that anesthetic ketamine impaired rats' retrograde memory and that an NO component modulates these effects.

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Activation of the p75 Neurotrophin Receptor through Conformational Rearrangement of Disulphide-linked Receptor Dimers

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Ligand-mediated receptor dimerization has emerged as a universal mechanism of growth factor receptor activation. Neurotrophins interact with dimers of the p75 neurotrophin receptor ($p75^{NTR}$), but the mechanism of receptor activation has remained elusive. Here, we show that $p75^{NTR}$ forms disulphide-linked dimers independently of neurotrophin binding through the highly conserved Cys^{257} in its transmembrane domain. Mutation of Cys^{257} abolished neurotrophin-dependent receptor activity but did not affect downstream signaling by the $p75^{NTR}$/NgR/Lingo-1 complex in response to MAG, indicating the existence of distinct, ligand-specific activation mechanisms for $p75^{NTR}$. FRET experiments revealed a close association of $p75^{NTR}$ intracellular domains that was transiently disrupted by conformational changes induced upon NGF binding. Although mutation of Cys^{257} did not alter the oligomeric state of $p75^{NTR}$, the mutant receptor was no longer able to propagate conformational changes to the cytoplasmic domain upon ligand binding.

In addition, we also show that cross-linking of $p75^{NTR}$ dimers by cysteine-scanning mutagenesis results in constitutive, ligand-independent activity in several pathways that are normally engaged upon neurotrophin stimulation of native receptors. The activity profiles of different disulfide-crosslinked $p75^{NTR}$ mutants were similar but not identical, suggesting that different configurations of $p75^{NTR}$ dimers might be endowed with different functions.

Together, these results indicate that dimeric mutants of $p75^{NTR}$ functionally resemble neurotrophin bound, not neurotrophin-free, receptors, support formation or stabilization of receptor dimers and oligomers as the mechanism by which neurotrophins activate $p75^{NTR}$, and reveal a genetic approach to generate gain-of-function receptor variants with distinct functional profiles.

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Possible Interactions with Drugs of Herbals Used for Weight Loss Enhancement

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Key words: Herbals, weight loss, adverse effects, drug interactions

S u m m a r y. The use of dietary supplements, including herbals, vitamins and minerals, is extensive. The majority of patients have little or no information about the possible risks and adverse effects from interactions with prescribed medicines. Concerns about herbal medications include their actual pharmacological properties. Among the more common herbs used for weight loss are Ephedra sinica (ephedra), Hoodia gordonii (hoodia), Citrus aurantium (bitter orange) and Larrea tridentate (chaparral), and are mostly used in an effort to enhance metabolism. Receiving ephedra with various medications may lead to interactions, (i.e. dysrythmias, hypertension, anxiety). Hoodia has been found to suppress appetite, however it has a potential hepatoxicity and therefore safety and efficacy have to be validated. Bitter orange acts similarly to ephedra and when received together with MAOIs can lead to tachycardia and hypertension. Chaparral acts similarly to caffeine and also has a mild diuretic effect. it's use may be complicated with renal, hepatic and neurological toxicity, and is also considered carcinogenic. Patients should be extremely cautious when receiving herbal medicines especially when are on any prescribed medication.

INTRODUCTION

Herbal-drug interactions are not less frequent and less important than drug-drug interactions. In general, herbals are less potent than drugs and thus pose a lesser threat to the person taking them. Herbal-drug interactions depend on the active(s) ingredient(s) of the herb or herbs in case of a multi-herbal mixture. Both herbals and drugs are metabolized by the cytochrome P-450 enzyme system of the liver, thus increasing the potential for interactions. Different herbs are used for weight loss and these include ephedra, hoodia, bitter orange and chaparral, which are mostly used in an effort to enhance metabolism. They are thought to act as thermogenics and mild diuretics. Most herbal products have not been scientifically evaluated, and therefore information regarding pharmacokinetics, pharmacodynamics, efficacy and safety is in most cases limited.

RESULTS

Ephedra sinica (ephedra)

It is a synonym for *Ephedra distachya,* and is also known as ma-huang or epitonin. It can be found in traditional Chinese herbal medications, herbal teas and also other products available via the web Ephedra contains among others the alkaloids ephedrine, pseudoephedrine (isoephedrine), and norpseudoephedrine (cathine) and other constituents, including quinoline. The stem contains approximately 0.5-2.5% alkaloids, with ephedrine accounting for 30- 90% of the total alkaloid content. Ephedrine and pseudoephedrine are found in the leaves and stems of ephedra and are structurally related to amphetamines and therefore ephedra is considered a herbal amphetamine (1). It has been shown to increase the availability and action of the endogenous neurotransmitters norepinephrine and epinephrine and stimulates catecholamine receptors in the brain, heart, and blood vessels both directly and indirectly (2). There is strong evidence that ephedra is associated with an increased risk of side effects, possibly even fatal ones, and therefore it was banned by the US FDA in 2004 (3). Ephedra or ephedrine alkaloids are considered toxic to the cardiovascular system, and potentially toxic to the neurological, gastrointestinal and endocrine systems. Taking ephedra with medication may lead to various interactions, which may include dysrythmias (antiarrythmics, theophylline, caffeine, halothane, cardiac glycosides), hypertension (caf-

feine, oxytocin, MAOIs), psychosis (antidepressants), anxiety (antidepressants) (1). Another possible effect worth mentioning is the reduced effect of certain drugs (antihypertensives, antiseizures, benzodiazepines, antiglycemics) when received together with ephedra (4).

Hoodia gordonii (hoodia)
Also known as Kalahari cactus and xhoba. Hoodia gordonii is widely distributed through the arid areas of South Africa and Namibia. The active appetite suppressant component of *Hoodia gordonii* was identified as a triglycoside of 12β-tigloyloxy-14β-hydroxypregn-5-en-20-one, a minor component in the plant extract (5). This has been found to suppress appetite for several hours, by signaling satiety on hypothalamic cells far more intensely than glucose (6). There are not any known herb-drug interactions of hoodia; however it has a potential hepatoxicity and therefore its safety and efficacy have to be validated (6).

Citrus aurantium (bitter orange)
Other common names include Seville orange, zhi zhi or sour orange. Bitter orange contains several compounds including synephrine alkaloids (7). It is commonly used as a substitute for ephedra in dietary supplements (8) and has a mechanism of action which is similar to ephedra and may therefore also lead to cardiovascular and neurologic toxicity when received together with other stimulants (e.g. caffeine). When received together with MAOIs can lead to tachycardia and hypertension (9). Larger clinical trials are necessary to draw adequate conclusions regarding the safety and efficacy of Citrus aurantium and synephrine alkaloids for promoting weight loss (7).

Larrea divaricata (chaparral)
It is synonymous with for *Larrea tridentate* and can also be found as creosote bush, hediondilla and greasewood. Chaparral grows in deserts and is used as a herbal remedy among Native Americans in the Southwestern United States and Northern Mexico (10). It is thought to act as an energy booster and a possible appetite suppressant, having a mechanism of action similar to caffeine and also a mild diuretic effect (11). Chaparral use has been associated with severe hepatotoxicity (10) and is also considered to be a carcinogenic substance (9). There are not any known herb-drug interactions of chaparral.

DISCUSSION

Unethical marketing techniques lead to false expectations about the safety and efficacy of herbal products (4). Many patients think that receiving a dietary supplement (herbals are thought to be dietary supplements and not medicines, and therefore their purity, efficacy and safety do not have to be tested or proven) is not going to lead to any adverse effects or interfere with prescribed medication (12). Physicians should always ask their patients about possible use of any dietary supplement or herbal product in order to counsel them in the most proper way. Since there are numerous possible adverse effects and herb-drug interactions of herbs used for weight loss, patients should be able to receive all the needed information from their physician or pharmacist in order to avoid them.

REFERENCES

1. National Center for Complementary and Alternative Medicine: Ephedra. Herbs at a Glance 2006. http://nccam.nih.gov/health/ephedra/ataglance.htm
2. Kalix P.: The pharmacology of psychoactive alkaloids from ephedra and catha. *J. Ethnopharmacol. 32*: 201-208 (1991)
3. National Center for Complementary and Alternative Medicine: Ephedra. Consumer Advisory 2004 http://nccam.nih.gov/news/alerts/ephedra/consume-radvisory.htm
3. Tachjian A., Maria V., Jahangir A.: Use of herbal products and potential interactions in patients with cardiovascular diseases. *J. Am. Coll. Cardiol. 55*: 515-25.
5. van Heerden F.R., Marthinus Horak R., Maharaj V.J., Vleggaar R., Senabe J.V., Gunning P.J.: An appetite suppressant from Hoodia species. *Phytochemistry 68*: 2545-2553 (2007)
6. National Center for Complementary and Alternative Medicine: Herbs at a Glance. Hoodia. http://nccam.nih.gov/health/hoodia/
7. Bent S., Padula A., Neuhaus J.: Safety and efficacy of citrus aurantium for weight loss. *Am. J. Cardiol. 94*: 1359-1361 (2004)
8. Haaz S., Fontaine K.R., Cutter G., Limdi N., Perumean-Chaney S., Allison D.B.: Citrus aurantium and synephrine alkaloids in the treatment of overweight and obesity: an update. *Obes. Rev. 7*: 79-88 (2006)
9. Kuhn M.A., Winston D.: Herbal Therapy and Supplements. Philadelphia, PA: Lippincott Williams & Wilkins, 2007
10. Rodriguez-Fragoso L., Reyes-Esparza J., Burchiel S.W., Herrera-Ruiz D., Torres E.: Risks and benefits of commonly used herbal medicines in Mexico. *Toxicol. Appl. Pharmacol. 227*: 125-135 (2008)
11. Holcomb S.S.: Common herb-drug interactions: what you should know. *Nurse Pract. 34(5)*: 21-29 (2009)
12. Bishop F.L., Yardley L., Lewith G.T.: Treat or treatment: a qualitative study analyzing patients' use of complementary and alternative medicine. *Am. J. Public. Health 98*: 1700-1705 (2008)

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Relationship between a Polymorphism of the eNOS Gene and Myocardial Infarction in a Subgroup of Greek MI Patients

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Key words: eNOS gene, myocardial infarction, nitric oxide synthase, nitric oxide

S u m m a r y. Nitric oxide (NO), produced by endothelial nitric oxide synthase (eNOS), plays a key role in the regulation of vascular tone. The E298D polymorphic variant of eNOS has been associated with myocardial infarction (MI), but data relating to this variant are divergent in Greece. Accordingly, we examined a possible association between the E298D polymorphism of the eNOS gene and MI in a subgroup of the Greek population. The study population consisted of 204 patients with a history of MI and 218 control subjects. All subjects were of Greek origin. According to the univariate findings, the risk for MI in E298D TT was 2.06 (95%CI: 1.06-4.00, p=0.032) versus GG+GT and 2.34 (95%CI: 1.17-4.68, p=0.016) versus GG. The risk for the T allele was estimated at 1.42 (95%CI, 1.06-1.89, p=0.022) as compared to G allele. The positive association of TT versus GG+GT with MI risk remained even after adjusting for the main study covariates. Moreover, strong evidence was found for an increased risk for MI among carriers of the TT genotype who were smokers, hypertensive and had a family history of CAD. This study indicates that E298D polymorphism of the eNOS gene seems to be associated with MI occurrence in the Greek population. It is possible that TT genotype is closely linked to the etiology of MI even after adjusting for known MI risk factors.

INTRODUCTION

Nitric oxide (NO), the endothelium-derived relaxing factor, is synthesized from L-arginine by at least three isoforms of the Nitric Oxide Synthase (NOS) (inducible NOS, iNOS or NOS2, constitutive neuronal NOS, nNOS or NOS1 and constitutive endothelial NOS, eNOS or NOS3). Nitric oxide, produced by eNOS, diffuses from the endothelium to vascular smooth muscle cells, where it increases the concentration of cyclic guanosine monophosphate (cGMP) by stimulating soluble guanylate cyclase, leading to vascular relaxation (1). The gene encoding eNOS is located on chromosome 7q35-36 and contains 26 exons that span 21 kb (2). The common polymorphism G894T in exon 7 of the eNOS gene results in the substitution of glutamic acid (E) at codon 298 by aspartic acid (D) (E298D). This is the only known polymorphism changing the eNOS protein sequence, leading to speculation that genetic variation at this site may alter e-NOS activity or regulation and possibly leads to endothelial dysfunction and to pathogenesis of several cardiovascular diseases (3). The 298D variant has been associated with myocardial infarction (MI) in Japanese (4), English (5), German (6) and American (7) populations, whereas other studies do not support these findings in Koreans (8) and in Caucasians, i.e. Austrian (9), French-Nothern Irish (10) and Dutch (11) populations. In Greece studies of the E298D polymorphism had controversial results (12-14). Thus, it was the aim of the present study to investigate in a subgroup of the Greek population whether the E298D gene variation was related to the risk of myocardial infarction in the total population and among subjects who were at lower or higher risk to this disease.

METHODOLOGY

A total of 422 subjects of Greek origin were prospectively enrolled in the study. 204 patients, diagnosed with an Acute Myocardial Infarction (AMI) or Non ST Segment Elevation Myocardial Infarction (NSTEMI) were compared to 218 control subjects. The 204 patients with MI admitted to the 1st Department of Cardiology, Onassis Cardiac Surgery Center Athens, Greece from October 2007 to April 2008. Diagnosis of AMI and NSTEMI was made by chest symptoms, electrocardiographic

changes and serum creatinine kinase-MB isoenzyme (CKMB) elevations according to guidelines [15]. The control group consisted of 218 volunteers who were selected from the general population of the greater Athens area. Controls were not eligible to participate if they had a history of MI or angina, clinical evidence of coronary artery disease (CAD), stroke, or any atherosclerotic disease in the past, based on a detailed medical history and a physical examination followed by a normal electrocardiogram. From each participant genomic DNA was extracted from peripheral blood leukocytes. The e-NOS E298D polymorphism was determined by Real-Time Polymerase Chain Reaction (RT-PCR) with melting curve analysis of PCR products from acceptor (5´end-labeled) and donor probes (3´end-labeled with fluorescein) specific for the polymorphism.

RESULTS

Table 1 shows the distribution of 204 cases with MI and 218 controls by sociodemographic and clinical characteristics.

Table 1

Variables	MI Patients (n = 204)	Controls (n = 218)	P-value
Age (years)			0.580
<60	104 (51.0)	117 (53.7)	
60+	100 (49.0)	101 (46.3)	
Gender			0.658
Male	178 (87.2)	187 (85.8)	
Female	26 (12.8)	31 (14.2)	
BMI (kg/m^2)	26.88±3.47	26.19±2.92	0.028
Smoking			<0.0001
No	37 (18.1)	99 (45.4)	
Yes	167 (81.9)	119 (54.6)	
Family history of CAD			<0.0001
No	119 (58.3)	195 (89.5)	
Yes	85 (41.7)	23 (10.5)	
Hypertension			<0.0001
No	59 (28.9)	167 (76.6)	
Yes	145 (71.1)	51 (23.4)	
Diabetes mellitus			<0.0001
No	131 (64.2)	208 (95.4)	
Yes	73 (35.8)	10 (4.6)	
Hypercholesterolemia			<0.0001
No	28 (13.7)	198 (90.8)	
Yes	176 (86.3)	20 (9.2)	

Data are presented as mean ± SD or N (%)

Regarding age and gender, no significant differences were identified between cases and controls (p=0.580 and p=0.658 respectively). The mean BMI was significantly higher in cases as compared to controls (p=0.028). The prevalence of atherogenic risk factors such as smoking, family history of CAD, hypertension, diabetes mellitus and hypercholesterolemia was significantly higher in the patient group.

Table 2 presents the distribution of genotypes GG, GT and TT in the two groups of participants.

Table 2

eNOS E298D Polymorphism	MI Patients N (%)	Controls N (%)	P-value
eNOS			0.044
GG	83 (40.7)	108 (49.5)	
GT	94 (46.1)	95 (43.6)	
TT	27 (13.2)	15 (6.9)	
allele			0.018
G	260 (63.7)	311 (71.3)	
T	148 (36.3)	125 (28.7)	

The frequencies of the eNOS GG genotype were 40.7% and 49.5%, of heterozygous GT 46.1% and 43.6% and of homozygous TT 13.2% and 6.9% among cases and controls respectively. This difference was statistically significant (p=0.044). Concerning the allele frequencies of the E298 to D transition in the MI and control group were calculated at 36.3% and 28.7% respectively. In the univariate analyses of the data (Table 3), TT homozygous, compared with the group of homozygous and heterozygous individuals (GG+GT), were at higher risk for MI (OR=2.06, 95%CI=1.06-4.00, p=0.032). Even if TT homozygous were compared with E298 homozygous GG alone, they were still more likely to develop MI (OR=2.34, 95%CI: 1.17-4.68, p=0.016). We found no statistical significant evidence for a protective effect against the risk for MI among homozygous GG as compared to heterozygous GT (p=0.220), whereas when comparing them to GT+TT the result was of borderline significance (p=0.068).

Table 3
Logistic regression-derived, crude odds ratios (ORs) and 95% confidence intervals (CIs) for the risk of myocardial infarction (MI) in carries of the 298D allele among the study participants

eNOS E298D	Crude analysis		
	ORs	95% CI	P-value
TT vs GG+GT	2.06	1.06-4.00	0.032
TT vs GG	2.34	1.17-4.68	0.016
TT vs GT	1.82	0.91-3.64	0.090
T allele vs G allele	1.42	1.06-1.89	0.022
GG vs GT	0.78	0.52-1.16	0.220
GG vs TT+GT	0.70	0.48-1.03	0.068

DISCUSSION

We performed a case-control study of the common E298D polymorphism of the eNOS gene. Similarly to recent studies [4-6, 11-14], we found a significant association between homozygous carriers of the T allele and the occurrence of MI in the Greek population. The risk of developing MI was found to be about two-fold higher for 298D (homozygous carriers of the T allele) as compared with individuals carrying the wild type E298 allele, either heterozygous (GT) or homozygous (GG) (p=0.032).

Lack of an increased risk of MI in the eNOS GT heterozygous suggests that the risk of MI posed by the eNOS T allele is not dominantly expressed and that the increased risk is confined to eNOS TT homozygous. This finding confirms previous presumptions of a recessive gene effect. Patients who are homozygous for the E298D polymorphism are genetically predisposed to MI (Table 3). MI is a multifactorial disease. Further genetic and environmental risk factors, such as age, gender, BMI, smoking habits and family history as well as accompanying disorders like high blood pressure and hyperholesterolemia contribute significantly to the susceptibility of MI. The homozygous T allele carriers, as suggested by the univariate analysis of the present study, have an increased risk to develop MI. The association between eNOS E298D polymorphism and MI is strongly affected by cigarette smoking. Smokers, eNOS TT homozygous have greater risk of MI than eNOS GT heterozygous and GG homozygous smokers. These data, possibly suggest, that presence of established underlying endothelial dysfunction, as observed among cigarette smokers, may be necessary for this polymorphism to attenuate endothelial function and predispose patients to increased cardiovascular risk. This study suggests that an alteration in the activity of the vascular NO system and the decreased amount of endothelial NO, due to the E298D mutation, may promote atherosclerosis and thrombosis that lead to MI. However, the relation of the E298D mutation to the severity of coronary atherosclerosis has not been proven yet [4]. This study indicates that E298D polymorphism of the eNOS gene seems to be associated with MI occurrence in the Greek population. We found evidence that homozygous TT is positively related to the risk of MI and this association is independent of possible effects of other known MI risk factors. Smokers, hypertensive and those with a family history of CAD are more likely to develop MI.

REFERENCES

1. Moncada S., Higgs A.: The L-arginine-nitric oxide pathway. *N. Engl. J. Med. 329*: 2002-2012 (1993)
2. Marsden P.A., Heng H.H., Scherer S.W., Stewart R.J., Hall A.V., Shi X.M., Tsui L.C., Schappert K.T.: Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J. Biol. Chem. 268*:.17478-17488 (1993)
3. Hingorani A.D., Jia H., Stevens P.A., Hopper R., Dickerson J.E., Brown M.J.: Renin-angiotensin system gene polymorphisms influence blood pressure and the response to angiotensin converting enzyme inhibition. *J. Hypertens. 13(12 Pt 2)*: 1602-1609 (1995)
4. Hibi K., Ishigami T., Tamura K., Mizushima S., Nyui N., Fujita T., Ochiai H., Kosuge M., Watanabe Y., Yoshii Y., et al.: Endothelial nitric oxide synthase gene polymorphism and acute myocardial infarction. *Hypertension 32*: 521-526 (1998)
5. Hingorani A.D., Liang C.F., Fatibene J., Lyon A., Monteith S., Parsons A., Haydock S., Hopper R.V., Stephens N.G., O'Shaughnessy K.M., et al.: A common variant of the endothelial nitric oxide synthase (Glu298->Asp) is a major risk factor for coronary artery disease in the UK. *Circulation 100*: 1515-1520 (1999)
6. Gardemann A., Lohre J., Cayci S., Katz N., Tillmanns H., Haberbosch W.: The T allele of the missense Glu(298)Asp endothelial nitric oxide synthase gene polymorphism is associated with coronary heart disease in younger individuals with high atherosclerotic risk profile. *Atherosclerosis 160*: 167-175 (2002)
7. Morray B., Goldenberg I., Moss A.J., Zareba W., Ryan D., McNitt S., Eberly S.W., Glazko G., Mathew J.: Polymorphisms in the paraoxonase and endothelial nitric oxide synthase genes and the risk of early-onset myocardial infarction. *Am. J. Cardiol. 99*: 1100-1105 (2007)
8. Jo I., Moon J., Yoon S., Kim H.T., Kim E., Park H.Y., Shin C., Min J., Jin Y.M., Cha S.H., et al.: Interaction between -786TC polymorphism in the endothelial nitric oxide synthase gene and smoking for myocardial infarction in Korean population. *Clin. Chim. Acta 365(1-2)*: 86-92 (2006)
9. Schmoelzer I., Renner W., Paulweber B., Malaimare L., Iglseder B., Schmid P., Schallmoser K., Wascher T.C.: Lack of association of the Glu298Asp polymorphism of endothelial nitric oxide synthase with manifest coronary artery disease, carotid atherosclerosis and forearm vascular reactivity in two Austrian populations. *Eur. J. Clin. Invest. 33*: 191-198 (2003)
10. Poirier O., Mao C., Mallet C., Nicaud V., Herrmann S.M., Evans A., Ruidavets J.B., Arveiler D., Luc G., Tiret L., et al: Polymorphisms of the endothelial nitric oxide synthase gene -no consistent association with myocardial infarction in the ECTIM study. *Eur. J. Clin. Invest. 29*: 284-290 (1999)
11. Agema W.R., de Maat M.P., Zwinderman A.H., Kastelein J.J., Rabelink T.J., van Boven A.J., Feskens E.J., Boer J.M., van der Wall E.E., Jukema J.W.: An integrated evaluation of endothelial constitutive nitric oxide synthase polymorphisms and coronary artery disease in men. *Clin. Sci. (London) 107*: 255-261 (2004)
12. Antoniades C., Tousoulis D., Vasiliadou C., Pitsavos C., Chrysochoou C., Panagiotakos D., Tentolouris C., Marinou K., Koumallos N., Stefanadis C.: Genetic polymorphism on endothelial nitric oxide synthase affects endothelial activation and inflammatory response during the acute phase of myocardial infarction. *J. Am. Coll. Cardiol. 46*: 1101-1109 (2005)
13. Antoniades C., Tousoulis D., Vasiliadou C., Pitsavos C., Toutouza M., Tentolouris C., Marinou K., Stefanadis C.: Genetic polymorphisms G894T on the eNOS gene is associated with endothelial function and vWF levels in premature myocardial infarction survivors. *Int. J. Cardiol. 107*: 95-100 (2006)
14. Andrikopoulos G.K., Grammatopoulos K., Tzeis S.E., Zervou S.I., Richter D.J., Zairis M.N., Gialafos E.J., Sakellariou D.C., Foussas S.G., Manolis A.S., et al.: Association of the 894G>T polymorphism in the endothelial nitric oxide synthase gene with risk of acute myocardial infarction. *BMC Med. Genet. 9*: 43 (2008)
15. Alpert J.S., Thygesen K., Antman E., Bassand J.P.: Myocardial infarction redefined--a consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. *J. Am. Coll. Cardiol. 36*: 959-969 (2000)

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Characterization of Model Systems for the Study of Smooth Muscle Cell Phenotype

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Key words: Smooth muscle, phenotype, myocardin, serum response factor (SRF)

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*Presenter

SUMMARY

Introduction: Modulation of the expression of Smooth Muscle Cell (SMCs)-specific genes is a key feature of major human pathologies, including hypertension, atherosclerosis, kidney or hepatic fibrosis, airway obstructive diseases and cancer metastasis.

The aim of this work was to characterize two *in vitro* models for the study of the molecular control of SMC phenotype.

Methodology: Mesenchymal Stem Cells (MSCs) were isolated from Wharton's Jelly of neonate umbilical cords, characterized for MSC markers and used at passages 2-3. Human Umbilical Vein Endothelial Cells (HUVECs) were used at passages 2-3.

Results and Discussion: By Western blotting analysis, naive MSCs expressed extremely low levels of the specific SMC proteins Smooth Muscle-α-Actin (SM-α-A), SM-Calponin (SM-CNN) and SM-Myosin Heavy Chain (SM-MHC), compared to differentiated SMCs. In addition, minimal promoters of the above genes and of SM-22α, another SMC marker, driving a luciferase reporter, also showed similar low activity in MSCs. Transforming Growth Factor-β1 (TGF-β1) increased, while Platelet-Derived Growth Factor-BB (PDGF-BB) decreased, SM-α-A protein levels and promoter activity.

SMC phenotype and specific gene expression depend on two crucial transcription regulators: Serum Response Factor (SRF) and Myocardin. Over-expression of Myocardin via an adenoviral vector induced both the activity of the SMC gene minimal promoters as well as the expression of SM-α-Actin and SM-Calponin protein in the MSCs. However, Myocardin was unable to induce minimal promoters of SM-Calponin and of SM-22α bearing mutated SRF-binding elements, indicating that cooperation between SRF and Myocardin is essential in inducing the expression of genes that establish SMC identity in Wharton's Jelly MSCs.

Moreover, in preliminary studies using HUVECs, over-expression of Myocardin resulted in striking cell shape changes and *de novo* expression of SMC markers such as SM-α-Actin. These results are compatible with a phenotypical change referred to as Epithelial-to-Mesenchymal Transition (EMT), a process that plays a crucial role in organ growth, physiological and pathological tissue remodeling and cancer.

Both cell systems are therefore useful in understanding how Myocardin can induce undifferentiated cells (MSCs) or epithelial-type cells (HUVECs) to acquire a Smooth Muscle Cell-like phenotype, and ultimately how its expression may modulate the onset and course of human disease.

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Pharmacodynamic Interaction of Norfloxacin and Theophylline with Peripheral $GABA_A$-Receptors

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Key words: GABA, theophylline, norfloxacin, pharmacodynamic interaction, guinea pig ileum

S u m m a r y. The present study investigates the effects of the fluoroquinolone norfloxacin, the methylxanthine theophylline, as well as of the combined norfloxacin-theophylline application on the responses of the guinea pig ileum to the inhibitory neurotransmitter γ-aminobutyric acid (GABA). According to the results, GABA (from $1.6x10^{-6}$ up to 10^{-3} M) causes transient contraction in the guinea pig ileum preparations with a pEC_{50} value of 5.04, followed by relaxation. Norfloxacin, at concentrations equal or higher than 10^{-5} M, and theophylline, at concentrations equal or higher than $3x10^{-4}$ M, inhibited the contractile responses of the ileum to GABA, without modifying its relaxing effect, whereas lower norfloxacin and theophylline levels were required to cause a similar degree of inhibition when the above drugs were combined. In conclusion, the inhibitory effect of either norfloxacin or theophylline on the contractile response of the guinea pig ileum to GABA is enhanced when the two drugs are combined. This amplification could be attributed to the pharmacodynamic interaction of norfloxacin and theophylline at the level of $GABA_A$-receptors.

INTRODUCTION

Theophylline, a methylxanthine and a central nervous system stimulant is widely used in the management of asthma. There is evidence to suggest that attenuation of GABAergic neurotransmission by blockade of $GABA_A$-receptors and/or enhancement of glutamatergic neurotransmission by activation of glutamic acid receptors underlie the theophylline-induced seizures (1-3). The presence of GABA in the mammalian gastrointestinal tract, where it behaves as a neurotransmitter in the myenteric plexus neurons, has been demonstrated (4). It has been shown that GABA evokes transient contraction in the guinea pig ileum which are thought to be mediated through $GABA_A$-receptors located on cholinergic post-ganglionic neurons (5,6). On the other hand, the antagonistic effect of fluoroquinolones on the $GABA_A$-elicited contraction in the guinea pig ileum has been shown (7,8), and it has been suggested that these antimicrobial agents act as antagonists not only on central, but also on peripheral $GABA_A$-receptors. Taking into account that concurrent administration of fluoroquinolones and theophylline has been shown to raise the incidence of seizures because of their pharmacodynamic interaction with central GABA-receptors (9), this study aims to explore the theophylline's effect on the responses of the intestine to GABA and to investigate whether a pharmacodynamic interaction of theophylline and norfloxacin exists with peripheral $GABA_A$-receptors.

METHODS

Ileum preparations

In this study, guinea pigs (*Hartley albino*) of either sex weighing approximately 500 g were used. After euthanasia and laparotomy of the guinea pigs, according to the experimental principles of laboratory animals, the ileum was removed and placed in Krebs solution (millimolar composition: NaCl 118.00, $NaHCO_3$ 24.88, KH_2PO_4 1.18, KCl 4.70, $MgSO_4$ 1.16, $CaCl_2$ 2.52 and glucose 11.10). Isolated whole segments from the ileum, 2-3 cm long, were cut and suspended in 20 ml organ baths (Hugo Sachs Electronic, Germany), containing Krebs solution,

which was bubbled constantly with a mixture of 95% O_2 - 5% CO_2 gas, while being maintained at a temperature of 37°C. The preparations were connected to isotonic myograph transducers (Narco Co., USA) under a resting tension of 0.5 g and the responses were recorded on a physiograph recorder (desk model type DMP-4A, Narco Co., USA). The preparations were allowed to equilibrate for 45 minutes (with intervening washings) before any compound addition.

The preparation of the strips, as well as the whole experimental procedure (equilibration period, resting tensions, aeration of the tissues), were based on *in vitro* methods previously described.

Chemicals: The following compounds were used: GABA (Aldrich Chemical Co., USA), theophylline and norfloxacin (Sigma Chemical Co., USA). GABA was dissolved in distilled water, while theophylline and norfloxacin were dissolved in distilled water and 0.1 M NaOH. Serial dilutions were made using the same vehicle. When tested, these solvent vehicles did not alter the resting activity of the preparations and did not alter the drug responses. All solutions were gently added directly into the organ bath fluid by use of a micropipette.

Procedures: In order to obtain full concentration-response curves for GABA, after the 45 min equilibration period the ileal preparations were exposed to single concentrations of GABA (from 10^{-6} to 10^{-3} M). The tissue was washed out after the contractile (lasting approximately 5 seconds) and the consecutive relaxing responses to GABA were obtained; the contact time of the tissue with every single GABA-concentration was about 30 seconds. The occurrence of desensitization to GABA was prevented by allowing 30 minutes to elapse between adding single concentrations of the above compound (5).

In a second series of experiments the effect of norfloxacin on the GABA-induced responses of the ileum was studied. For this reason, the ileal preparations were exposed to various single concentrations of norfloxacin ($3x10^{-7}$, 10^{-6} and 10^{-5} M) 20 minutes prior to the addition of single concentrations of GABA (from 10^{-6} to 10^{-3} M).

In a third series of experiments the effect of theophylline on the GABA-induced responses of the ileum was studied; thus, the ileal preparations were exposed to various single concentrations of theophylline ($3x10^{-5}$, 10^{-4} and $3x10^{-4}$ M) 10 min prior to the addition of single concentrations of GABA (from 10^{-6} to 10^{-3} M).

In a fourth series of experiments the ileal preparations were exposed to single concentrations of GABA in the presence of theophylline (at the concentrations of $3x10^{-5}$ and 10^{-4} M) 10 minutes after pretreatment with norfloxacin (at the concentrations of $3x10^{-7}$ and 10^{-6} M). From each curve the negative logarithm of the concentration necessary to induce 50% of the maximal contraction (pEC_{50}) was determined.

RESULTS

Responsiveness to GABA

The addition of GABA in the organ bath fluid caused transient contraction in the guinea pig ileal preparations, followed by relaxation. The contractile effect was concentration-dependent and the minimum effective concentration was $1.6x10^{-6}$ M.

Responsiveness to GABA in the presence of various concentrations of norfloxacin

Norfloxacin, at the concentrations of $3x10^{-7}$ and 10^{-6} M did not modify, while, at the concentration of 10^{-5} M, it significantly reduced the contractile response of the ileum to GABA (*Figure 1*). The pEC_{50} value for GABA alone was 5.04, while the corresponding pEC_{50} values for GABA in the presence of norfloxacin at the concentrations of $3x10^{-7}$, 10^{-6} and 10^{-5} M, were 5.05, 4.97 and 4.72, respectively.

The relaxing response of the ileum to GABA was not modified at any of the norfloxacin concentrations tested (data not shown).

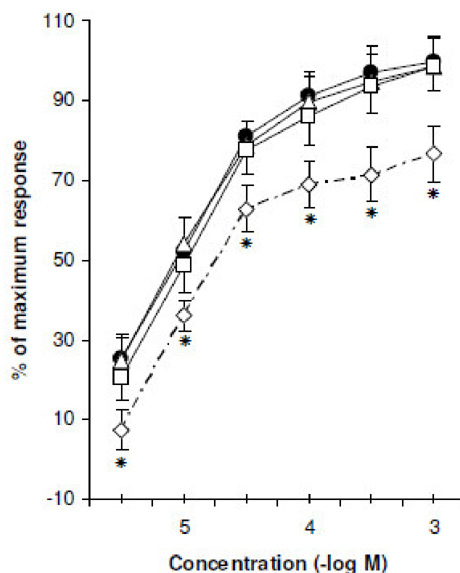

Figure 1. Concentration-response curves for the contractile response of the isolated guinea pig ileum to GABA (●, n=15-37;); to GABA in the presence of nor-

*floxacin at the concentration of $3x10^{-7}$ M (Δ, n=5-8, 10^{-6} M) (□, n=5-7) and 10^{-5} M (◊, n=5-6). The ordinate is expressed as a percentage of the mean maximum response induced by GABA alone (control). Each point represents the mean±SEM. * Indicates the significant values of GABA prevention caused by norfloxacin (p<0.05)*

Responsiveness to GABA in the presence of various concentrations of theophylline

Theophylline, at $3x10^{-5}$ and 10^{-4} M did not modify, while at $3x10^{-4}$ M, it antagonized the contractile response of the ileum to GABA (*Figure 2*). The pEC$_{50}$ values for GABA in the presence of theophylline at the concentrations of $3x10^{-5}$, 10^{-4} and $3x10^{-4}$ M, were 5.12, 5.15 and 4.82, respectively. As observed with norfloxacin, the relaxing response of the ileum to GABA was not modified at any of the above theophylline concentrations tested (data not shown).

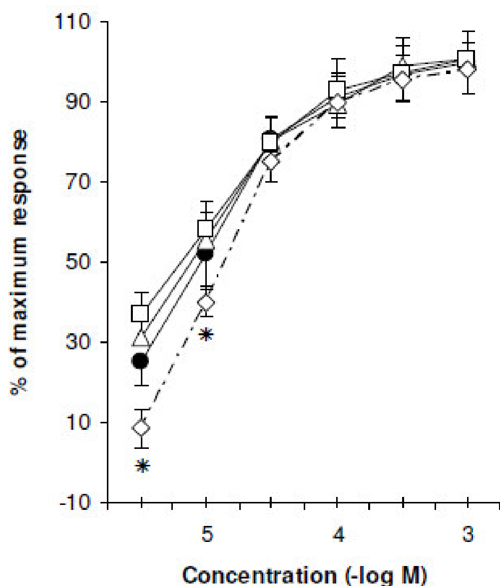

*Figure 1. Concentration-response curves for the contractile response of the isolated guinea pig ileum to GABA (●, n=15-37;); to GABA in the presence of theophylline at the concentration of $3x10^{-5}$ M (Δ, n=5-7, 10^{-4} M) (□, n=5-7) and $3x10^{-4}$ M (◊, n=5-8). The ordinate is expressed as a percentage of the mean maximum response induced by GABA alone (control). Each point represents the mean±SEM. * Indicates the significant values of GABA prevention caused by pheophylline (p<0.05)*

Responsiveness to GABA in the presence of both norfloxacin and theophylline

The combined application of theophylline and norfloxacin, at concentrations that did not modify the responses of the ileum to GABA when each drug was applied alone, produced a significant decrease in the contractile response of the ileal preparations to GABA (*Figure 3*). More specifically, the pEC$_{50}$ values for GABA in the presence of $3x10^{-5}$ M of theophylline plus $3x10^{-7}$ M and 10^{-6} M of norfloxacin, were, accordingly, 4.80 and 4.70, while in the presence of 10^{-4} M of theophylline plus $3x10^{-7}$ M and 10^{-6} M of norfloxacin, were 4.70 and 4.50, respectively.

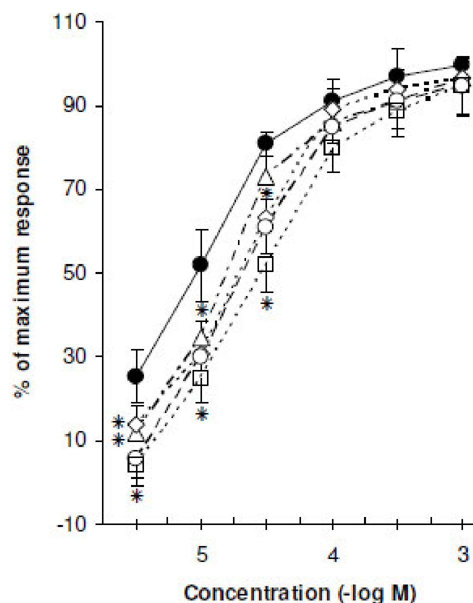

*Figure 1. Concentration-response curves for the contractile response of the isolated guinea pig ileum to GABA (●, n=15-37); to GABA in the presence of theophylline at the concentration $3x10^{-5}$ M after pre-treatment with norfloxacin at $3x10^{-7}$ M) (◊, n=5-12); to GABA in the presence of thephylline at the concentration of 10^{-4} M after pre-treatment with norfloxacin at $3x10^{-7}$ (O, n=5-8) M and 10^{-6} M (□, n=5-15). The ordinate is expressed as a percentage of the mean maximum response induced by GABA alone (control). Each point represents the mean±SEM. * Indicates the significant values of GABA prevention caused by pheophylline (p<0.05)*

DISCUSSION

In accordance with previous reports, results of the present study showed that GABA, at concentrations ranging from $1.6x10^{-6}$ M to 10^{-3} M, produces a transient contraction in the guinea pig ileum followed by relaxation. In addition, it has been shown that the fluoroquinolone norfloxacin

and the methylxanthine theophylline, above a certain concentration, inhibit the contractile effect of GABA on the ileum, without influencing its relaxing effect, while lower concentrations of each individual drug cause a similar degree of inhibition obtained after the combined use of the two drugs.

The contractile effect of GABA on the guinea pig ileum is thought to result mainly from activation of $GABA_A$-receptors, while activation of $GABA_B$-receptors is believed to yield a subsequent relaxing effect. Both receptor types are located on cholinergic enteric neurons and mediate the release of endogenous acetylcholine (5,6). As far as the effect of norfloxacin on the GABA-induced effects is concerned, results of this study are in agreement with previous studies showing that several fluoroquinolones, norfloxacin included, antagonize the $GABA_A$-elicited contraction in the guinea pig ileum, in a non-competitive manner, but do not influence the GABA-induced relaxation (7,8). Regarding the effect of theophylline on the GABA-receptors, evidence exists for its antagonistic effect on $GABA_A$-, but not on $GABA_B$-receptors. Indeed, binding studies have shown that theophylline inhibits the GABA-receptor binding of the $GABA_A$-agonist muscimol (10). Moreover, according to *in vitro* studies performed on *Xenopus laevis* oocytes, theophylline inhibits the $GABA_A$-induced currents in a competitive manner (2), while it does not modify the responses of the guinea pig isolated ileal preparations to $GABA_B$-agonists (11). Interestingly, the combination of ciprofloxacin (a fluoroquinolone) with theophylline is known to result to an additive reduction of muscimol binding to the GABA receptor (9), giving evidence for the interaction of the above drugs with the $GABA_A$-receptors. Considering the here observed antagonistic effect on the contractile responses of the guinea pig ileum to GABA following the combined application of norfloxacin and theophylline at concentrations that fail to produce any effect when the drugs are applied independently, the present study supports the hypothesis for a pharmacodynamic interaction between fluoroquinolones and theophylline.

In conclusion, this study gives evidence for a pharmacodynamic interaction of theophylline and norfloxacin with peripheral $GABA_A$-receptors.

REFERENCES

1. Nardone R, Buffone E, Covi M, Lochner PG, Tezzon F: Changes in motor cortical excitability in humans following orally administered theophylline. *Neurosci Lett.* 355:65-68 (2004)
2. Sugimoto T, Sugimoto M, Uchida I, Mashimo T, Okada S: Inhibitory effect of theophylline on recombinant GABA(A) receptor. *Neuroreport.* 12:489-493 (2001)
3. Amabeoku GJ: Gamma-aminobutyric acid and glutamic acid receptors may mediate theophylline-induced seizures in mice. *Gen Pharmacol.* 32:365-72 (1999)
4. Miki Y, Taniyama K, Tanaka C, Tobe T: GABA, glutamic acid decarboxylase, and GABA transaminase levels in the myenteric plexus in the intestine of humans and other mammals. *J Neurochem.* 40:861-865 (1983)
5. Giotti A, Luzzi S, Spagnesi S, Zilletti L: GABAA and GABAB receptor-mediated effects in guinea-pig ileum. *Br J Pharmacol.* 78:469-478 (1983)
6. Ong J & Kerr DI: Interactions between GABA and 5-hydroxytryptamine in the guinea-pig ileum. *Eur J Pharmacol.* 94:305-312 (1983)
7. Blandizzi C, De Bona M, Natale G, Agen C, Del Tacca M: Effects of quinolone carboxylic acid derivatives on GABAA receptor-mediated stimulation of gastric acid secretion and intestinal motility. *Eur J Pharmacol.* 201:35-39 (1991)
8. Koutsoviti-Papadopoulou M, Kounenis G, Elezoglou V: Inhibitory effect of flumequine, enoxacin and norfloxacin on the GABA-induced contractile effect on the guinea pig ileum. *Gen Pharmacol.* 26:1391-1395 (1995)
9. Imperatore C, Trimarchi GR, De Sarro A: Interaction between perfloxacin and aminophylline in genetically epilepsy-prone rats. *J Pharm Pharmacol.* 49, 1025-1029 (1997)
10. Segev S, Rehavi M, Rubinstein E: Quinolones, theophylline, and diclofenac interactions with the gamma-aminobutyric acid receptor. *Antimicrob Agents Chemother.* 32:1624-1626 (1988)

Ong J, Kerr DI, Berthelot P, Vaccher C, Flouquet N, Debaert M: Actions of thienyl analogs of baclofen in the guinea-pig isolated ileum. *Eur J Pharmacol.* 218: 343-345 (1992)

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Corticotropin Releasing Factor Affects the Expression of TLR4 Receptor in Adipocytes

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Key words: CRF, adipocytes, LPS, TLR4, interleukins

S u m m a r y. *Obesity is associated with chronic systemic low grade inflammation as well as inflame-mation of adipose tissue with ad hoc accumulation of macrophages. Like macrophages, the adipocytes have the ability to detect circulating lipopolysaccharides (LPS) via toll-like receptos-4 (TLR4) and produce inflammatory cytokines. Human adipocytes also ex-press the corticotrophin-releasing factor (CRF) family of neuropeptides and their receptors, a system affecting innate immunity at the level of macrophages. Aim of the present study was to examine the effects of CRF on the immune phenotype of adipocytes and specifically on their expression of the TLR4 receptor.*

INTRODUCTION

Obesity is associated with constant low level systemic inflammation as well as an ongoing inflammation within the visceral adipose tissue.

In obesity, adipocytes produce chemotactic factors which attract monocytes/macrophages from the blood stream. Upon entering the adipose tissue, monocytes are activated to macrophages which further activate adipocytes to produce more chemotactic factors and pro-inflammatory cytokines.

Lipo-poly-saccharide (LPS) stimulates macrophages, via the TLR4 receptor, to produce pro-inflammatory mediators (1). Interestingly, the expression levels of TLR4 control macrophage sensitivity to LPS. LPS transiently downregulates TLR4 promoting macrophage tolerance to further LPS stimulation while, the stress-related peptide corticotrophin-releasing factor (CRF) augments the efffect of LPS by inducing TLR4 gene expression in macrophages.

Adipocytes and macrophages share several common characteristics including the TLR4-NFkB-pro-inflammatory cytokine cascade. Furthermore, adipocytes and monocytes have a common precursor cells.

A complete CRF system exists within the visceral adipose tissue consisting of CRF, the Urocortins (UCNs), and their receptors CRF1 and CRF2 (2,3).

The aim of the present work was to examine the effect of CRF and CRF-related peptides in the production of adipogenic peptide Leptin and the pro-inflammatory cytokines (IL6, IL8) as well as the expression levels of TLR4 in adipocytes.

MATERIALS

We have used the mouse 3T3L1 pre-adipocyte cell line both as cultured pre-adipocytes and as fully differentiated adipocytes (4). Pre-adipocytes or fully differentiated 3T3L1 cells were incubated with CRF (10^{-8} M) and/or LPS (10 ng/ml) for 5h, 10h and 15h and levels of TLR4, Leptin, IL6 and IL8 were measured by FACS analysis, Real Time PCR or ELISA.

RESULTS

Our data show that 3T3L1 pre-adipocytes co-incubated for 5 or 10 h with CRF and/or LPS revealed a markedly decrease TLR4. In addition, fully differentiated 3T3L1 cells incubated with LPS plus CRF suppressed TLR4 by Real Time PCR.

CRF and UCN1 augmented LPS-mediated suppression of Leptin production from differentiated to adipocytes 3T3L1 cells.

CRF and the UCNs suppressed LPS-induced production of IL6 and IL8 from differentiated to adipocytes 3T3L1 cells.

DISCUSSION

LPS exerts a pro-inflammatory effect on differentiated 3T3L1 adipocytes suppressing the production of Leptin and inducing the production of the pro-inflammatory cytokines IL6 and IL8.

CRF1 and CRF2 receptor agonists exert a generalized suppressing effect on adipocytes suppressing their production of Leptin and of the pro-inflammatory cytokines IL6 and IL8.

CRF promotes TLR4 expression in undifferentiated adipocytes while it suppress its expression in differentiated adipocytes, suggesting that stress neuropeptides may have distinct effects on pre- and mature adipocytes.

In conclusion, CRF suppresses TLR4 expression in adipocytes, thus containing their pro-inflammatory activity.

REFERENCES

1. Hotamisligil G.S.: *Nature 444*: 860 (2006)
2. Seres J., et al.: *J. Clin. Endocrinol. Metab. 89*: 965 (2004)
3. Friedberg M., et al.: *J. Clin. Endocrinol. Metab. 88*: 385 (2003)
4. Gregoire F., et al.: *J. Physiol. Rev. 78*: 783 (1998)

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Region-dependent and Individual Differences in Glutamate Tissue Content Following Cannabinoids Administration

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Key words: Glutamate, cannabinoids, motor activity

S u m m a r y. Endocannabinoids modulate neurochemical processes in which a variety of neurotransmitters are involved. The excitatory neurotransmitter system, including glutamate and aspartate, partakes in cognitive function and neuroplasticity but is also involved in neurotoxicity processes. The aim of the present study was to in-vestigate the effects of cannabinoids on tissue levels of glutamate in two rat phenotypes previously differentiated as High Responders (HR) or Low Responders (LR), according to their response to a novel environment. Our results have shown that HR displayed increased motor activity as compared to LR rats. Cannabinoids modified motor activity in a dose-dependent manner, indicating a biphasic behavioral profile. Concerning glutamate tissue content, cannabinoids induced notable differences in glutamate content in a region-, phenotype- and drug dose- dependent manner. This study addresses the role of cannabinoids in modulating glutamate function and reveals a drug dose-, phenotype- and region- dependent effect on glutamate status. These results contribute to the emergent evidence indicating that cannabinoids are implicated in a variety of physiological functions such as cognition and neuroplasticity as well as pathological states such as neurotoxicity.

INTRODUCTION

Endocannabinoids modulate neurochemical processes in which a variety of neurotransmitters are involved. The neurochemical effects of exogenously administered cannabinoids and endocannabinoids are mediated via stimulation of cannabinoid receptors. Amongst the neurotransmitter systems with which the endocannabinoid system interacts (and plays a modulatory role), is the excitatory. The excitatory neurotransmitter system partakes in cognitive function and neuroplasticity but is also involved in neurotoxicity processes. Two key players involved in this modulation are the excitatory amino acids (EAAs), glutamate and aspartate. In the present study, we investigated the effects of Δ^9- tetrahydrocannabinol (THC) and WIN55,212-2 (WIN), two CB_1 receptor agonists with distinct pharmacological profiles, on tissue levels of glutamate in two rat phenotypes previously differentiated as High Responders (HR) or Low Responders (LR), according to their response to a novel environment.

METHODS

Male Sprague – Dawley rats were differentiated based on the vertical activity into HR and LR phenotypes. Either THC, WIN or vehicle were administered intraperitoneally (i.p.) to both phenotypes. Ambulatory and vertical locomotor activities were automatically registered. Another subset of rats received THC, WIN or vehicle and glutamate tissue levels were measured in discrete rat brain regions using High Performance Liquid Chromatography with electrochemical detection.

RESULTS

Our results have shown that HR displayed an increased motor activity as compared to LR rats. THC and WIN modified motor activity in a dose-

dependent manner, indicating a biphasic behavioral profile.

Concerning glutamate tissue content, THC modified glutamatergic status in a region-, phenotype- and dose-dependent manner. This neurochemical profile was similar to some extent following WIN administration.

DISCUSSION

This study addresses the role of cannabinoids in modulating glutamate function and reveals a drug dose-, phenotype- and region-dependent effect on glutamate tissue levels. These results demonstrate the ability of cannabinoids to produce distinct neurochemical events in EAA status which is greatly implicated in a variety of physiological functions such as cognition and neuroplasticitity as well as pathological states such as neurotoxicity.

REFERENCES

1. Brown T.M., Brotchie J.M., Fitzjohn S.M.: Cannabinoids decrease corticostriatal synaptic transmission via an effect on glutamate uptake. *J. Neurosci. 23*: 11073-11077 (2003)
2. Ferraro L., Tomasini M.C., Gessa G.L., Bebe B.W., Tanganelli S., Antonelli T.: The cannabinoid receptor agonist WIN 55,212-2 regulates glutamate transmission in rat cerebral cortex: an in vivo and in vitro study. *Cereb. Cortex 11*: 728-733 (2001)
3. Navarrete M., Araque A.: Endocannabinoids mediate neuron-astrocyte communication. *Neuron 57*: 883-893 (2008)
4. Polissidis A., Chouliara O., Galanopoulos A., Marselos M., Papadopoulou-Daifoti Z., Antoniou K.: (2009) Behavioural and dopaminergic alterations induced by a low dose of WIN 55,212-2 in a conditioned place preference procedure. *Life Sci. 85*: 248-254 (2009)

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Behavioural Effects of the Active Constituents of *Crocus Sativus* L., Crocins

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SUMMARY

Crocus Sativus L., (saffron) is a plant cultivated in various parts of the world. Saffron and its active constituents affect a number of neural processes (anxiety, depression memory etc). Crocins are among the active constituents of *Crocus Sativus* L. Ketamine is a non-competitive NMDA receptor antagonists with known psychotomimetic profile. mCPP is a 5-HT_{2c} receptor agonist which exacerbate self-grooming in rats and this is considered as an animal model of obsessive-compulsive disorder (OCD). The first aim of the present study was to investigate in the rat the effects of crocins on recognition memory deficits produced by ketamine. For this aim, the novel object recognition task was chosen. Subsequently, we evaluated whether or not crocins were able to reduce mCPP-induced excessive grooming. In the first study, post-training administration of crocins (15-30 mg/kg) reversed ketamine-induced performance deficits in the novel recognition task, suggesting that crocins modulate storage and/or retrieval of information. In a subsequent study, pre-training treatment with crocins (15-30 mg/kg) did not affect mCPP-induced excessive grooming. The present results a) support and extend the enhancing effects of crocins on memory and b) provide evidence that these active constituents of *Crocus Sativus* L., might not be involved in the mechanisms mediating the effects of mCPP on grooming.

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The Neurosteroid DHEA Protects the Retina against Chemical Ischemia

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Key words: Neurosteroids, neuroprotection, ischemia, retina, rat

INTRODUCTION

Ischemia is the underlying cause of many ocular diseases that lead to blindness. Many strategies have been employed to develop therapeutic agents for the successful treatment of ischemia induced retinopathies and the prvention of visual loss. Neurosteroids such as Dehydro-epiandrosterone (DHEA) have recently been shown to have anti-apoptotic properties (1), reminiscent of NGF actions. The aim of the present study was to investigate the putative neuroprotective properties of DHEA, and the involvement of NGF receptor signaling, in a model of retinal chemical ischemia.

METHODS

Eye cups of female Sprague-Dawley rats were incubated with PBS alone or in the presence of iodoacetic acid (5mM) and sodium cyanide (25mM) (Chemical Ischemia Mixture; C.I.M) or C.I.M. and DHEA (10^{-6}, 10^{-8}, 10^{-10} M) for 2x30 min, followed by incubation with PBS or DHEA for 2x30min, at 5%CO_2 / air, 37°C (2). In addition, eye cups were also co-incubated with the chemical ischemia mixture, DHEA (10^{-7} M), and inhibitor of the NGF (TrkA) receptor (10^{-6} M, Calbiochem 648450) or using a mixture of inhibitors - bicuculline/haloperidol/ketamine (all 10^{-6} M) - for GABA/Sigma-1/NMDA receptors, respectively. The eye cups were subsequently fixed and sectioned for PKC, ChAT, and bNOS immunoreactivity, retinal markers for rod bipolar, nitric oxide and cholinergic containing amacrine cells, respectively. TUNEL staining was also employed to examine apoptotic cell loss. In addition, eye cups were lysed and blotted for the detection of phospho- and total-isoforms of prosurvival protein kinases PI3K, Akt, MEK1/2 and ERK1/2.

RESULTS

Chemical ischemia resulted in significant decrease of ChAT, bNOS and PKC immunoreactivities. DHEA (10^{-6}, 10^{-8}, 10^{-10} M) protected the retina in a concentration-dependent manner. The TrkA inhibitor (10^{-6} M) blocked the DHEA (10^{7} M) dependent neuroprotection, whereas bicuculli-ne/haloperidol/ketamine had no effect. In addition, DHEA induced the phosphorylation/active-tion of the kinases examined, strongly indicating that its neuroprotective effects are mediated by these signaling cascades.

CONCLUSIONS

These results demonstrate for the first time that the endogenous neurosteroid DHEA protect the retina from ischemia. In addition, the neuroprotective effects appear to be mediated via the NGF receptor and its signaling cascades. Further studies are essential in order to elucidate the therapeutic relevance of these results.

REFERENCES

1. Charalambopoulos I., et al.: PNAS 2004. *J. Neurochem.* (2008)
2. Mastrodimou N., et al.: Naunyn-Schmied. Arch. *Pharmacol* (2005)

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Probing the structure of the extracellular portion of the third membrane-spanning segment of CRF_1 using the substituted-cysteine-accessibility method.

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Key words: Corticotropin-releasing factor type 1 receptor, structure, third membrane-spanning segment, binding-site crevice, methanethiosulfonate ethylammonium

SUMMARY

The type 1 receptor (CRF_1) for the corticotropin releasing factor (CRF) belongs to family B of G-protein coupled receptors (GPCRs) and, like the other GPCRs, consists of seven membrane-spanning segments (TMs), which have been proposed to bind small non-peptide ligands. Recently we have shown that, similar to family A, GPCRs, the TMs of CRF_1 form a water-accessible crevice, the binding-site crevice, which extends from the extracellular surface of the receptor into the plane of the membrane. The surface of this crevice must be formed by residues that contact ligands, as well as, by other residues that may play a structural role and affect binding indirectly. In this study we mapped the TM residues that form the surface of the binding-site crevice of CRF_1, starting from the extracellular portion of the third TM (TM3). We achieved this by applying the cysteine-substituted accessibility method (SCAM) and using as background the ΔCys mutant of CRF_1, which has near normal functional properties and it is relatively insensitive to the methanethiosulfonate (MTS) reagents. We mutated eight TM3 residues of CRF_1 to Cys and heterologously expressed the mutants in HEK 293 cells. Four of these mutants reacted with the hydrophilic, positively charged sulfhydryl-specific reagent, methane-thiosulfonate ethylammonium (MTSEA), added extracellularly. We therefore suggest that the side chains of the residues at the reactive loci (Thr192, Ala193, Tyr195, and Asn196) are on the water-accessible surface of the binding-site crevice of CRF_1. The pattern of accessibility is consistent with an alpha-helical conformation for this portion of TM3.

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Residues at Positions 211, 233 and 364 of Crf_1 Are Exposed in the Binding-Site Crevice of Receptor

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Key words: Corticotropin-releasing factor type 1 receptor, G-protein-coupled receptors, membrane-spanning segments, binding-site crevice, methanethiosulfonate ethylammonium

SUMMARY

The type 1 receptor (CRF_1) for the corticotropin-releasing factor (CRF) belongs to family B of G-protein-coupled receptors (GPCRs). The CRF_1, like all GPCRs, is a protein that spans the plasma membrane seven times thus forming seven membrane-spanning segments (TMs), which have been proposed to bind small non-peptide ligands, such as antalarmin. This leads to the hypothesis that similar to family A, rhodopsin-like, GPCRs, the membrane-spanning segments of CRF_1 as well as all family B GPCRs form a water-accessible crevice, the binding-site crevice, which extends from the extracellular surface of the receptor into the plane of the membrane. The surface of this crevice is formed not only by residues that can contact small ligands but also by residues that may play a structural role and affect binding indirectly. However, the lack of considerable structural information for the family B GPCRs precludes the support of this hypothesis. To test this hypothesis we started obtaining information about the structure of family B GPCRs, using as prototype the CRF_1 and testing its reaction with the positively charged sulfhydryl-specific methanethiosulfonate ethylammonium (MTSEA). We found that MTSEA inhibited the binding of the radiolabelled CRF analog, [^{125}I]-Tyr^0-sauvagine, to CRF_1, and that antalarmin protected against this irreversible inhibition. To identify the susceptible cysteine(s), we mutated, one at a time, four endogenous cysteines to serine. Mutation of Cys211, Cys233, and Cys364 to serine decreased the susceptibility of sauvagine binding to irreversible inhibition by the MTSEA. Thus, Cys211, Cys233 and Cys364 at the cytoplasmic ends of the third, fourth and seventh membrane-spanning segments are exposed in the binding-site crevice of CRF_1. These studies will ultimately provide us with the required information for the structure of CRF_1 and will be used to construct a CRF_1 molecular model. This model will be used as a prototype, for the understanding of the structure and function of all receptors belonging to the family B of GPCRs. This molecular model will also help us to determine the residues in the membrane-spanning segments of CRF_1 that interact with small non-peptide ligands, thus putting the basis for the rational design of new small CRF_1-selective ligands.

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Inhibition of Interleukin-1 Activity is related with Reduced Apoptosis and Improved Speckle Tracking Myocardial Deformation in Patients with Rheumatoid Arthritis

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Key words: Interleukin-1, apoptosis, myocardial deformation, rheumatoid arthritis

INTRODUCTION

Studies have shown that inhibition of interleukin-1 (IL-1) activity improves myocardial deformation through reduction of nitro-oxidative stress and reduces the size of experimental myocardial infarction through reduction of cell apoptosis. We investigated whether inhibition of Il-1 activity reduces apoptosis and thus, improves myocardial deformation in rheumatoid arthritis patients (RA).

METHODS

In an acute, double-blind trial, 43 patients with RA were randomized to receive a single injection of anakinra, a recombinant IL-1 receptor antagonist, (150 mg s.c.) or placebo and after 48-hours were crossed over to the alternate treatment. At baseline and 3-hours after the single injection, we assessed a) LV longitudinal, circumferential and radial strain and strain rate, using speckle tracking echocardiography and c) Fas and caspase-9 serum levels, as apoptotic markers. Patients were reassessed after 30 days of anakinra treatment.

RESULTS

At 3 hours and 30 days after treatment, there was an significant reduction in Fas (541±403 vs. 416±373 vs. 378±200 pg/ml), caspase-9 (2.63±2.79 vs. 2.01±1.82 vs. 1.66±1.63 ng/ml) and Longitudinal SR (-1.02±0.23 vs -1.125±0.20 vs -1.25±0.23 l/s) compared to baseline ($p<0.05$ for all comparisons). No changes were observed after placebo. Baseline Fas predicted the absolute and %change of Longitudinal SR after 3 hrs and 30 days post anakinra ($r = -0.578$, $r = -0.603$, $r=-0.523$, $r=-0.588$, $p<0.05$). Absolute and % changes of caspase-9, 3 hours post-anakinra were also related to the absolute and %change in Longitudinal SR ($r=-0.583$, $r=-0.555$, $r=-0.564$, $r=-0.538$, $p<0.05$) Similar association were observed after 30 days of anakinra treatment.

CONCLUSION

The reduction of apoptosis is a potential mechanism for the improvement of myocardial deformation after inhibition of IL-1 activity.

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The Olive Constituent Oleuropein Prevents the Doxorubicin-induced Heart Failure in Anesthetized Rats by Nitro-oxidative Stress Suppression and by Reversing Cardiac Remodeling.

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Key-words: Oleuropein, heart failure, nitro-oxidative stress, cardiac remodeling, doxorubicin

INTRODUCTION

Doxorubicin (DXR) is an anthracycline antibiotic generally used in the treatment of solid tumors, but its use is limited by a dose-dependent cardiomyopathy and heart failure. The pathogenesis of DXR-induced heart failure is complex and the role of iNOS, nitrosative and oxidative stress is not completely understood. Oleuropein (OLEU) is a natural phenolic antioxidant, which is present in elevated concentration in olives, olive oil and olive tree leaves conferring protection to the heart. The aim of the present study was to evaluate a possible protective role of OLEU in DXR-induced heart failure and to investigate a possible mechanism of action.

METHODS

Ninety rats were randomly divided into 6 groups and treated as follows: *Control* group with no treatment, *OLEU-1* and *OLEU-2* groups, treated with 70 and 140 mg/Kg^{-1} of OLEU respectively, given intraperitoneally (i.p.), for 14 consecutive days, *DXR* group treated with i.p. injection of 18mg/kg^{-1} of DXR, divided into 6 equal doses and given over a period of 2 weeks, *OLEU-1-DXR* and *OLEU-2-DXR* groups, rats treated with OLEU and DXR for 14 days as previously described. At the end of the injection protocols the rats were anesthetized and subjected transthoracic echocardiography examination (Vivid-i, GE Healthcare with a 12MHz probe). Then the rats were sacrificed and the hearts were rapidly excised for histological evaluation and for tissue assessment of malondialdehyde (MDA) and protein carbonyl concentration (PCs) as an index of oxidative stress, nitrotyrosine (NT) as indicator of *nitrosative* stress, for interleukin-6 (IL-6) and Big endothelin-1 (Big ET-1) which are important indicators of cardiac remodeling and apoptosis. Finally, cardiac tissue sample was used for qualitative and quantitative evaluation of inducible synthesis of nitric oxide (iNOS), as a marker of inflammation, both by immunohistochemistry method and Western-Blot.

RESULTS

Eighty two rats completed the study. The mortality in the *DXR* group was 18.7% by end of the injection protocol. Normal morphology of the cardiac tissue was seen in the *Control* group and in groups *OLEU-1* and *OLEU-2*. Myocardium exhibited morphological changes in *DXR* group only including edema, chronic inflammation and de-

generation of myocardial cells such as vacuolization. In the *OLEU-1-DXR* and *OLEU-2-DXR* groups mild hypertrophy without edema, inflammation and myocardial degeneration was observed. DXR induced a small decrease in wall thickness, a decrease in left ventricular (LV) mass, a decrease in fractional shortening (an index of systolic function), an increase in end-systolic LV diameter, and a trend towards adverse cardiac remodeling. Combined *OLEU-DXR* and *OLEU* alone groups did not cause any change and the animals did not differ from the normal *Control*. A statistically significant elevation in the levels of MDA, NT, PCs, IL-6 and Big ET-1 was observed in the DXR group whereas a significant reduction of the above mediators was observed in the control and in groups treated with OLEU. A significant expression of iNOS was detected in the cardiomyocytes in the DXR group compared to the control and to the groups treated with OLEU.

CONCLUSION

The present study shows that the olive constituent OLEU successfully treats the DXR-induced heart failure by reducing oxidative and nitrosative stress and reversing cardiac remodeling and apoptosis. DXR-induced heart failure produced a significant induction in iNOS and nitrotyrosine formation in the myocardium. Combined OLEU-DXR treatment may improve the therapeutic outcome and additional research should be conducted to prove if it could be used for clinical cardioprotection against DXR-induced toxicity,

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Comparison of Inflammatory Markers in Psoriatic Patients before and after Treatment with Etanercept

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Key words: Psoriasis, etanercept, inflammation, biological treatment

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S u m m a r y. Psoriasis is a chronic, systemic, inflammatory disease affecting mainly the skin and the joints. It is an immune cell-mediated disease in which T-lymphocyte activation and a subsequent inflammatory response are of great importance in its pathogenesis. We measured an array of inflammatory markers in psoriatic patients eligible for biological treatment. The comparison of this group at baseline and after twelve weeks of Etanercept treatment revealed a statistically important reduction in all markers as well as PASI score. This suggests that there might be an association between clinical improvement and reduced inflame-matory burden.

INTRODUCTION

Psoriasis is a chronic, systemic, inflammatory disease affecting mainly the skin and the joints. It is estimated to affect approximately 2% of the general population. It is often accompanied by an array of comorbidities, such as obesity, dyslipidemia, diabetes mellitus and coronary artery disease. Psoriasis is an immune cell-mediated disease in which T-lymphocyte activation is of major importance. It is widely accepted that the inflammatory response plays a central role in the pathogenesis of psoriasis (1,2). Several pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL) -2, IL-6, IL-8, IL-12, IL-23, as well as other substances contribute to an inflammation cascade of events that leads to the formation of the psoriatic lesion (3). Systemic inflammation could also be reflected on an array of markers e.g. white blood cell count (WBC), fibrinogen, ferritin, high sensitivity C-reactive protein (hs-CRP), erythrocyte sedimentation rate (ESR) etc. These are generally non-specific markers of acute inflammation that are used in daily practice in order to help in the diagnosis of the presence of an inflammatory process. They are also used as markers of treatment response. Researchers have explored in the past the possible link between some of them and psoriasis severity and response to treatment. High sensitivity CRP is the one that has attracted most of the attention (4-6) Several therapeutic agents that have been used for psoriasis aim at different targets of the underlying inflammation mechanisms. Methotrexate, cyclosporine and some of the newer biologic agents (TNF-α antagonists and IL-12/23 blockers) are among them.

METHODS

The study was approved by *Attikon* General University Hospital Ethics Committee. We recruited fifty patients with psoriasis vulgaris from the Dermatology outpatient clinic of our hospital. All patients were eligible for treatment with biologic agents and fulfilled the required recommendations according to the European guidelines (7). They had had a baseline PASI score of 10 or more and also they were intolerant or unrespon-

sive or had had a contraindication for standard systemic treatment. They were all started on Etanercept (a TNF-α antagonist) 50 mg subcutaneously injections twice a week for a period of twelve weeks. Patients had to be off treatment (topical or systemic) for at least a month prior to commencement of treatment. Other exclusion criteria were any sign of an acute or chronic inflammation (e.g. hepatitis, arthritis, and autoimmune diseases), previous exposure to biologic agents, psoriatic arthritis, increased alcohol consumption, immunosuppression, acute or chronic infections, liver or renal impairment, pregnancy and breast-feeding, history of cancer within the last 5 years and major trauma. We measured a set of inflammatory markers, i.e. WBC and netrophils, fibrinogen, ferritin, hs-CRP, ESR, haptoglobin, ceruloplasmin and α1-antitrypsin as well as PASI score at baseline and after twelve weeks of treatment. The non parametric Wilcoxon test (Wilcoxon signed-rank test) for paired observations was used to compare PASI score and inflammatory markers before and after treatment with Etanercept.

RESULTS

We gathered complete data for 41 patients. Statistically significant reduction from baseline to the end of the treatment was observed for all variables (PASI and inflammatory markers). Our results are summarized in Table 1.

DISCUSSION

We chose to measure a group of inflammatory markers that are common in daily practice and easy to measure in an ordinary laboratory. Although they are non-specific for a particular disease they certainly depict an inflammatory burden that in our case can be attributed to psoriasis, as long as other inflammations are excluded. We observed that all inflammatory markers are reduced after twelve weeks of treatment. This finding accompanies clinical improvement, which is reflected on the reduction of PASI score. Therefore, one could suggest that response to treatment in our psoriasis patients on Etanercept is associated with a reduced inflammatory burden, as the latter is outlined by the measured markers. Although it sounds logical that clinical assessment of psoriasis and laboratory findings assessing inflammation go hand in hand, solid conclusions are difficult to be drawn. Our study is limited by the relatively small number of patients. Further studies with larger number of participants and more therapeutic agents are needed in order these suggestions to be verified.

REFERENCES

1. Griffiths C.E.M., Barker J.N.W.N.: Pathogenesis and clinical features of psoriasis. *Lancet 370*: 263-271 (2007)
2. Gelfand J.M., Neimann A.L., Shin D.B.. et al.: Risk of myocardial infarction in patients with psoriasis. *JAMA 296*: 1735-1741 (2006)
3. Lowes M.A., Bowcock A.M., Krueger J.G.: Pathogenesis and therapy of psoriasis. *Nature 445*: 866-873 (2007)
4. Rashmi R., Rao K.S.J., Basavaraj K.H.: A comprehensive review of biomarkers in psoriasis. *Clin. Exper. Dermatol. 34*: 658-663 (2009)
5. Rocha-Pereira P., Santos-Silva A., Rebelo I. et al.: The inflammatory response in mild and in severe psoriasis. *Br. J. Dermatol. 150*: 917-928 (2004)
6. Strober B., Teller C., Yamauchi P., et al.: Effects of Etanercept on C-reactive protein levels in psoriasis and psoriatic arthritis. *Br. J. Dermatol. 159*: 322-330 (2008)
7. Pathirana D., Ormerod A.D., Saiag P., et al.: European S3-Guidelines on the systemic treatment of psoriasis vulgaris. *J. Eur. Acad. Dermatol. Venereol. 23(suppl 2)*: 34-38 (2009)

Table 1
PASI and inflammatory markers before and after treatment

	Baseline				after treatment				
	median	Q25	-Q75	IQR*	median	Q25	-Q75	-Q75	p-value**
PASI	17.00	15.00	-18.95	3.95	4.70	4.00	-7.95	3.95	<0.001
WBC	7.90	6.99	-9.39	2.41	6.40	5.88	-7.37	1.49	<0.001
Neutrophils	5.67	4.67	-6.08	1.41	4.23	3.78	-5.35	1.58	<0.001
Fibrinogen	370.00	296.00	-402.50	106.50	330.00	282.50	-376.00	93.50	<0.001
Ferritin	143.00	94.50	-188.50	94.00	125.00	88.50	-162.00	73.50	<0.001
hs-CRP	1.99	1.67	-2.24	0.57	1.02	0.66	-1.47	0.82	<0.001
ESR	16.00	9.50	-20.00	10.50	8.00	4.50	-10.00	5.50	<0.001
Haptoglobin	2.23	1.95	-2.55	0.60	2.04	1.65	-2.22	0.57	<0.001
Ceruloplasmin	405.00	300.00	-486.00	186.00	390.00	280.00	-465.00	185.00	<0.001
a1-antitrypsin	1.70	1.44	-1.87	0.43	1.49	1.25	-1.63	0.38	<0.001

*Inter Quartile Range; **Wilcoxon signed-rank test

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Association between Inflammatory Markers and Disease Severity Assessment in Psoriatic Patients Treated with Etanercept

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Key words: Psoriasis, inflammation, Etanercept, treatment

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S u m m a r y. Psoriasis is a common, inflammatory, chronic skin disease. Its precise aetiology is unknown. Immunological responses via T-cell activation as well as a cascade of inflammatory reactions play a central role in its pathogenesis. We measured an array of inflammatory markers in psoriatic patients at baseline and after twelve weeks of Etanercept treatment. Statistical analysis suggested that there might be an association between disease severity assessment, as this is clinically described by PASI, and inflammatory markers (fibrinogen at baseline and hs-CRP at the end of treatment).

INTRODUCTION

Psoriasis is a common, inflammatory, multifactorial, chronic, papulosquamous skin disease. Its precise aetiology is unknown. Immunological responses via T-cell activation as well as a cascade of inflammatory reactions play a central role in its pathogenesis (1). The inflammatory nature of psoriasis is indicated by cutaneous and systemic overexpression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL) -2, IL-6, IL-8, IL-12, IL-23 and IL-24 (2,3). Systemic inflammation could also be reflected on an array of markers e.g. white blood cell count (WBC), fibrinogen, ferritin, high sensitivity C-reactive protein (hs-CRP), erythrocyte sedimentation rate (ESR) etc. These are generally non-specific markers of acute inflammation that are used in daily practice in order to help in the diagnosis of the presence of any inflammatory process. Psoriasis Area and Severity Index (PASI) is a widely acceptable method to assess disease severity based on the extent, erythema, induration and scaling of the psoriatic lesions. Etanercept is a TNF-α blocker and belongs to the relatively new category of biological drugs. It is used against psoriasis, psoriatic arthritis and Crohn's disease.

METHODS

The study was approved by *Attikon* General University Hospital Ethics Committee. We recruited fifty patients with psoriasis vulgaris from the Dermatology outpatient clinic of our hospital. All patients were eligible for treatment with biologic agents and fulfilled the required recommendations according to the European guidelines (4). They had had a baseline PASI score of 10 or more and also they were intolerant or unresponsive or had had a contraindication for standard systemic treatment. They were all started on Etanercept 50 mg subcutaneously injections twice a week for a period of twelve weeks. Patients had to be off treatment (topical or systemic) for at least a month prior to commencement of treatment. Other exclusion criteria were any sign

of an acute or chronic inflammation (e.g. hepatitis, arthritis, and autoimmune diseases), previous exposure to biologic agents, psoriatic arthritis, increased alcohol consumption, immunosuppression, acute or chronic infections, liver or renal impairment, pregnancy and breast-feeding, history of cancer within the last 5 years and major trauma. We measured a set of inflammatory markers, i.e. WBC and neutrophils, fibrinogen, ferritin, hs-CRP, ESR, haptoglobin, ceruloplasmin and a1-antitrypsin as well as PASI score at baseline and after twelve weeks of treatment. Our objective was to reveal any possible association between disease severity, as the former is depicted by PASI, and the inflammatory burden, as the latter is outlined by the group of inflammatory markers. Spearman correlation coefficients were calculated between PASI and each of inflammatory markers at baseline and after treatment. Furthermore, for the purposes of the analysis a new variable, called PASI difference was constructed as the result of the equation: PASI difference=PASI (baseline)-PASI (end of the treatment). Linear regression analysis was applied in order to investigate whether PASI difference is associated with certain markers.

Table 1

Univariate linear regression models with dependent variable PASI difference and independent variables the inflammatory markers at baseline

	Unstandardized Coefficients		95% C. I. for B		
	B	Std. Error	Lower	Upper	p-value
hsCRP_b	-1,84	0,62	-3,09	-,58	0,005
ESR_b	0,27	0,08	0,10	0,44	0,003
a1-antitrypsin b	5,92	1,96	1,97	9,88	0,004

(b: baseline)

RESULTS

We gathered complete data for 41 patients. At baseline time PASI is significantly correlated with fibrinogen (Spearman r=0.359, p=0.021), while at the end of the treatment PASI is correlated with hs-CRP (Spearman r=0.417, p=0.007). Table 1 shows the regression coefficients and corresponding 95% confidence intervals from univariate regression models for PASI difference (only the statistically significant results are reported). All the possible univariate models were examined; each model including as an independent variable one of the set of the inflammatory markers. The hs-CRP, ESR and the a1 antitrypsin at baseline time are all significantly correlated with PASI reduction. Indeed, hs-CRP has a negative linear correlation with the dependent variable, while the other two have positive relationship. Specifically, for one unit increase in hs-CRP at baseline time, the PASI difference was reduced by 1.84 units. Similarly, for one unit increase in ESR at baseline time, the PASI difference was increased by 0.27 units, while for one unit increase in a1antitrypsin at baseline time, the PASI difference was increased by 5.92 units.

DISCUSSION

Association of fibrinogen with PASI at baseline and hs-CRP with PASI at end of treatment, as they are described by Spearman correlation coefficients, outlines a relationship between inflammatory burden and PASI. Increased inflammatory levels are reflected on increased PASI score. Therefore, one could suggest that inflammatory markers (fibrinogen and hs-CRP in our case) depict disease severity, as PASI does. So, we have a laboratory and objective measure of disease severity, along with PASI which is a clinical and somewhat subjective measure. Linear regression analysis showed that for every one unit increase in hs-CRP at baseline time, the PASI difference was reduced by 1.84 units. Therefore, the heavier the initial inflammatory burden is (as this is described by hs-CRP), the poorer response rate we have at the end of treatment. In other words, patients with a high inflammatory load at baseline have a less favorable response rate to treatment. Perhaps, that would justify a more aggressive approach in case we have patients with similar baseline PASI scores but differences in inflammatory markers. ESR and α1-antitrypsin results do not support these suggestions. In fact, they point to the opposite direction. Studies with larger number of participants are needed in order to verify these suggestions.

REFERENCES

1. Griffiths CEM, Barker JNWN. Pathogenesis and clinical features of psoriasis. Lancet 2007; 370: 263-71.
2. Lowes MA, Bowcock AM, Krueger JG. Pathogenesis and therapy of psoriasis. Nature 2007; 445: 866-873.
3. Nickoloff BJ. Cracking the cytokine code in psoriasis. Nat Med 2007; 13: 242-4.
4. Pathirana D, Ormerod AD, Saiag P et al. European S3-Guidelines on the systemic treatment of psoriasis vulgaris. J Eur Acad Dermatol Venereol 2009; 23 (suppl 2): 34-8

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Mechanisms of Interactions between Ethyl Alcohol and Pharmaceutical Substances

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Key words: Alcohol intolerance, Aldehyde dehydrogenase, Serotonin, Disulfiram-like reaction, Serotonin syndrome

S u m m a r y. The use of ethyl alcohol together with prescription drugs has been implicated in several pharmacodynamic and pharmacokinetic interactions with clinical consequences. Synergy is expected with sedative substances either as prescribed drugs, like benzodiazepines and tricyclic antidepressants, or as drugs of abuse, such as cannabis and opiates. On the contrary, pharmacokinetic interactions are unpredictable and difficult to recognize, as it is exemplified in the case of disulfiram. The so called "disulfiram reaction" has been attributed to the accumulation of acetaldehyde in the blood due to inhibition of the hepatic aldehyde dehydrogenase (ALDH). In addition to disulfIram, clinically similar toxic reactions have been reported for at least ten more pharmaceutical substances, which have been assumed to inhibit also ALDH. However, a more detailed investigation reveals that other mechanisms may be equally important. This article reports some examples of pharmaceutical substances with known "disulfiram-like reactions", which were scrutinized in order to clarify the underlying mechanism.

INTRODUCTION

Even moderate intake of ethanol may lead to remarkable interactions with other substances. To date there have been documented many such instances, when the organism is exposed to medicines and food constituents, or to substances of the occupational environment (1,2). The most common type of alcohol interactions are those attributed to a synergistic effect with drugs affecting the central nervous system, such as benzodiazepines, tricyclic antidepressants, opiates, antihistamines etc. Apart from these anticipated pharmacodynamic interactions, there are quite a few examples of toxic responses to alcohol which are often reported in the literature as *disulfiram-like reactions*, with the assumption that the underlying mechanism is an inhibition of acetaldehyde metabolism. Disulfiram, the prototype of these medicinal products, is known for the inhibition of the hepatic aldehyde dehydrogenase (ALDH) and mainly the low-K_m mitochondrial isozyme (ALDH2), which is most important for the metabolism of acetaldehyde (3,4,13). The accumulation of acetaldehyde produces very unpleasant symptoms with blurred vision, nausea, vomiting, anxiety and cardiovascular effects such as hypotension, palpitations, tachycardia and flushing of the face and neck (7-9).

In the present study, several medicinal products known to produce *disulfiram-like reaction* have been examined experimentally in the rat, in order to find out if they are able to alter the pharmacokinetic or the pharmacodynamic profile of ethyl alcohol. In addition to disulfiram, the substances tested were cefamandole, chloramphenicol, chlorpropamide, cotrimoxazole, furazolidone, griseofulvin, isoniazid, metronidazole, procarbazine, propranolol and quinacrine. These substances were given alone or in combination with ethanol. The parameters examined, included the enzymes involved in the metabolism of ethanol and acetaldehyde, the brain biogenic amines and the plasma concentrations of acetaldehyde.

METHODS

The methodology applied in this paper has been described in detail before (5,6).

Animal treatment

Male Wistar rats were used (Wistar/Af/Han/Mol/Io/RR), 4 months old and weighing 300 to 350 g, were used in this study. All animals were housed in groups of two to three in plastic cages (Macrolon) with a wood-chip bedding (Populus sp.) and had free access to tap water and pellet chow (Biozoe, Greece). All drugs were administered intraperitoneally in a single dose each day. The doses of the drugs used in the present study are pharmacological, which means that they are (per kg body weight) about 10 times higher than the therapeutic doses.

Hepatic enzyme activities

Alcohol dehydrogenase (ADH), cytochrome P-450-2E1 (CYP2E1), catalase (CAT) and aldehyde dehydrogenases ALDH1A1 and ALDH2.

Biogenic amines in the brain

Noradrenaline (NA), dopamine (DA), serotonin (5-HT), as well as their metabolites [3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA)] were determined in four brain subregions (hypothalamus, striatum, midbrain and frontal cortex), by high performance liquid chromatography (HPLC).

Determination of Blood Acetaldehyde

Quantitation of acetaldehyde was performed by hs-GC using acetonitrile as internal standard, according to the method described by Sarkola et al. (11).

RESULTS

When animals were given disulfiram, cefamandole, chloramphenicol and procarbazine, the mitochondrial low-K_m ALDH2 was significantly inhibited. In accordance, the combined intragastric administration of ethanol led to a significant increase of acetaldehyde levels in the blood (Table). Cotrimoxazole, griseofulvin, metronidazole, propranolol and quinacrine had no effect on the activity of ALDH2 (low and high-K_m) and ALDH1A1, with the exception of griseofulvin, which induced ALDH1A1 activity. Consequently, it seems that at least for the above mentioned five drugs (cotrimoxazole, griseofulvin, metronidazole, propranolol and quinacrine) the mechanism by which they provoke alcohol intolerance, is irrelevant to that of disulfiram

In addition, metronidazole inhibited also the activity of hepatic alcohol dehydrogenase (ADH), while metronidazole, quinacrine and cotrimoxazole inhibited the activity CYP2E1. Both enzymes are known to participate in the elimination of ethanol.

Apart from the effects on the metabolism of ethanol and acetaldehyde, the drugs tested in the present study, produced a number of significant changes on the levels of brain biogenic monoamines (Table), among which, those concerning the noradrenergic and the serotonergic system appear to be the most important for the explanation of the so called *disulfiram-like reaction*. Of these changes, the most uniform among the substances tested was the elevation of brain serotonin.

DISCUSSION

Disulfiram has been used for more than 50 years in the treatment of chronic alcoholism. The rationale behind its use relies on the alcoholics's supposed fear of the very unpleasant symptoms elicited upon drinking alcoholic beverages after having previously ingested the drug. The clinical syndrome arising by the interaction of disulfiram with ethanol is referred to as *disulfiram reaction* and has been attributed to the accumulation of acetaldehyde in the blood. Moreover, disulfiram inhibits dopamine-beta-hydroxylase (DBH), an enzyme responsible for the conversion of dopamine (DA) to noradrenaline (NA), leading to increased levels of DA and decreased levels of NA in the central (CNS) and peripheral nervous system. These changes of catecholamines and most importantly the reduction of NA have been proposed as another mechanism for the explanation of the *disulfiram reaction*, possibly via a sedative action on the CNS which follows the reduction of brain NA (5).

Table

Changes in acetaldehyde metabolism and brain neurotransmitters in alcohol intolerance

Substance	*ALDH2*	*Acetaldehyde*	*Noradrenaline*	*Serotonin*
Disulfiram	↓	↑	↓	-
Cefamandole	↓	↑	↓	↑
Chloramphenicol	↓	↑	-	↑
Chlorpropamide	↓	ND	-	-
Cotrimoxazole	-	-	↓	↑
Furazolidone	↓	ND	↑	↑
Griseofulvin	-	-	↓	↑
Isoniazid	↓	ND	↓	-
Metronidazole	-	ND	-	↑
Procarbazine	↓	↑	↑	↑
Propranolol	-	ND	-	↑
Quinacrine	-	-	↓	↑

↑ Increase, ↓ Decrease, — No change, ND Not determined

According to our results, disulfiram, cotrimoxazole, griseofulvin, quinacrine, cefamandole and isoniazid, decreased the central levels of NA.

This effect, in combination with a similar effect of ethanol, could be responsible for the produced alcohol intolerance, by a mechanism of true sedative synergism, as in the case of the concomitant use of other centrally acting drugs (14). Further support to this proposition, can be drawn from the fact that cotrimoxazole, griseofulvin and quinacrine, reduce brain NA, without affecting hepatic ALDH2, although they are known to precipitate a *disulfiram-like reaction*.

All tested drugs increased the levels of 5-HT in the CNS, with the exception of chlorpropamide and isoniazid. It should be pointed out, however, that the use of isoniazid for a long period of time, as it is prescribed for patients with tuberculosis, could lead to an increase of brain 5-HT levels due to its inhibitory action on MAO-A activity (12). Moreover, it is known that ethanol itself increases the levels of 5-HT in the CNS. Patients with a major elevation of brain 5-HT, show a toxic reaction to ethanol very similar to the *disulfiram reaction* (10). Therefore, co-administration of these drugs with ethyl alcohol could result in a substantial increase of central 5-HT levels, explaining the interaction provoked.

In the case of metronidazole, there was no change in the activity of mitochondrial ALDH2, but a significant inhibition of the cytosolic alcohol dehydrogenase (ADH). This could lead to elevated ethanol levels. Along these lines, it should be commented upon that metronidazole, quinacrine and cotrimoxazole, inhibited the activity of CYP2E1, a microsomal enzyme known to participate also in the elimination of ethanol. However, ethanol levels were not measured in the plasma of rats treated with metronidazole, quinacrine or cotrimoxazole.

In conclusion, the results of our study suggest that the *disulfiram-like reaction* produced by a number of drugs cannot be exclusively attributed to the inhibition of the hepatic ALDH2, since some of these agents do not exhibit any influence on this enzyme at all. It seems that, unlike disulfiram, the interaction of these pharmaceutical products with ethyl alcohol implies more complex mechanisms, concerning both ethanol metabolism and brain neurotransmitters. A rather common feature of the drugs tested in the present study is their ability to increase the levels of brain 5-HT. This central action, rather than ALDH2 inhibition, should be considered as the core mechanism of the *disulfiram-ethanol interaction*, even in the case of the conventional *disulfiram reaction* itself.

Unless elevated blood acetaldehyde and inhibition of ALDH2 have been documented, the term *alcohol intolerance* should be preferred to the term *disulfiram-like reaction*, when an incompatibility between ethanol and a pharmaceutical product is reported.

REFERENCES

1. Elovaara E., Marselos M., Vainio H.: N,N-Dimethylformamide-induced effects on hepatic and renal xenobiotic enzymes with emphasis on aldehyde metabolism in the rat. *Acta Pharmacol. Toxicol. 53*: 159-165 (1983)
2. Fraser A.G.: Pharmacokinetic interactions between alcohol and other drugs. *Clin. Pharmacokinet. 3*3: 79-90 (1997)
3. Hald J., Jacobsen E.: A drug sensitizing the organism to ethyl alcohol. *Lancet 2*: 1001-1004 (1948)
4. Hald J., Jacobsen E.: Formation of acetaldehyde in the organism after ingestion of Antabuse (tetraethylthiuram disulfide) and alcohol. *Acta Pharmacol. Toxicol. 4*: 305–310 (1948)
5. Karamanakos P.N., Pappas P., Stephanou P., Marselos M.: Differentiation of disulfiram effects on central catecholamines and hepatic ethanol metabolism. *Pharmacol Toxicol. 88*: 106-110 (2001)
6. Karamanakos P., Pappas P,, Boumba V,A,, Thomas C,, Malamas M,, Vougiouklakis T,, Marselos M.: Pharmaceutical Agents Known to Produce Disulfiram-Like Reaction: Effects on Hepatic Ethanol Metabolism and Brain Monoamines. *Intern. J. Toxicol. 26*: 423-432 (2007)
7. Kitson T.M.: The effect of disulfiram on the aldehyde dehydrogenases of sheep liver. *Biochem. J. 151*: 407-412 (1975)
8. Kitson T.M.: The disulfiram-ethanol reaction: a review. *J. Stud. Alcohol 38*: 96-113 (1977)
9. Larsen V.: The effects on experimental animals of antabuse (tetraethylthiuram disulfide) in combination with alcohol. *Acta Pharmacol. Toxicol. 4*: 321-332 (1948)
10. McBride W.J., Bell R.L., Rodd Z.A., Strother W.N., Murphy J.M.: 2005. Adolescent alcohol drinking and its long-range consequences. Studies with animal models. *Recent Dev. Alcohol. 17*: 123-142 (2005)
11. Sarkola T., Iles M.R., Kohlenberg-Mueller K., Eriksson C.J.: Ethanol, acetaldehyde, acetate, and lactate levels after alcohol intake in white men and women: Effect of 4-methylpyrazole. *Alcohol. Clin. Exp. Res. 26*: 239-245 (2002)
12. Self T.H., Chrisman C.R., Baciewicz A.M., Bronze M.S.: Isoniazid drug and food interactions. *Am. J. Med. Sci. 317*: 304-311 (1999)
13. Vasiliou V., Malamas M., Marselos M.: The mechanism of alcohol intolerance produced by various therapeutic agents. *Acta Pharmacol. Toxicol. 58*: 305-310 (1986)
14. Weinshenker D., Schroeder J.P.: 2007. There and back again: a tale of norepinephrine and drug addiction. *Neuropsychopharmacology 32*: 1433-1451 (2007)

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Role of Serotonin 5-HT_{2A} and 5-HT_{2C} Receptors on the Reward-facilitating Effect of Cocaine

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Key words: Intracranial self-stimulation, psychostimulants, 5-hydroxytryptamine, mesolimbocortical system, reinforcement

INTRODUCTION

The serotonin 5-HT_{2A} and 5-HT_{2C} receptors, which are found in abundance in the mesolimbocortical dopaminergic system, appear to modulate the behavioral effects of cocaine. The present series of studies set out to investigate the role of 5-HT_{2A} and 5-HT_{2C} receptors on the reward-facilitating effect of cocaine and localize the neural substrates within the mesolimbo-cortical dopaminergic system that are responsible for these effects.

METHODS

Male Sprague-Dawley rats were implanted with stimulating electrodes and bilateral cannulae for the experiments involving microinjections and were trained to respond for electrical stimulation. In the first study we examined the effectiveness of systemic administration of selective 5-HT_{2A} and 5-HT_{2C} receptor agonists (TCB-2 and WAY-161503) and antagonists (R-96544 and SB-242084) at blocking the reward-facilitating effect of cocaine. In the second study we examined the effects of intra-medial prefrontal cortex (mPFC), intra-nucleus accumbens (NAC) and intra-ventral tegmental area (VTA) injection of WAY-161503 on the reward-facilitating effect of cocaine.

RESULTS

Systemic WAY-161503 attenuated the reward-facilitating effect of cocaine. This effect was reversed by pre-treatment with the selective 5-HT_{2C} receptor antagonist SB-242084. Intracranial micro-injections of WAY-161503 into the mPFC and the NAC shell/core, but not the VTA, attenuated the reward-facilitating-effect of cocaine.

CONCLUSION

These data indicate that 5-HT_{2C} receptors within the mPFC and the NAC modulate the reinforcing effects of cocaine and provide evidence that 5-HT_{2C} receptor agonists could be a possible drug discovery target for the treatment of psychostimulant addiction.

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DHEA and NGF Protect the Retina from AMPA Excitotoxicity *in vivo*

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Key words: Excitotoxicity, neuroprotection, neurosteroids, NGF, retina, rat

INTRODUCTION

Ischemia has been proposed to play a prominent role in retinal cell death ensuing from several retinal diseases such as glaucoma and diabetic retinopathy. Excitotoxicity, resulting from ischemia, is a pathological process, where increase of glutamate and over activation of glutamate receptors, initiates a cascade of events that lead to visual deficits and blindness. Even today there are no therapeutic agents for the treatment of the neurodegenerative component of retinal diseases. Neurosteroids such as DHEA (Dehydroepiandrosterone) have recently been shown to have antiapoptotic actions via a mechanism involving NGF receptor, TrkA. The aim of the present study was to investigate whether DHEA and NGF could protect retinal cells from cell death in an *in vivo* model of AMPA excitotoxicity.

METHODS

Intravitreal administration of AMPA (42 nmol per eye) was earlier shown to result in retinal cell loss (ChAT, bNOS and calbindin immunoreactivity (1). The excitotoxicity model was employed in the present study as follows: Male and female Sprague-Dawley rats (250-300 g) were administered: AMPA (42 nmol per eye) or AMPA and DHEA (10^{-6},10^{-7} M) or NGF (60 pg/eye) or vehicle, intravitreally. In addition, a TrkA receptor inhibitor (Calbiochem 648450, 10^{-6} M) was co injected with AMPA (42 nmol per eye) and DHEA (10^{-6} M). Twenty four hours after treatment, eye cups were removed and prepared for immunohistochemistry. Antibodies for retinal markers nitric oxide (NO) and cholinergic containing amacrine cells (ChAT-, and bNOS-containing, respectively), and calbindin-containing horizontal and cone bipolar cells, were employed.

RESULTS

Intravitreal administration of AMPA (42 nmol per eye) led to retinal cell loss as previously reported (1) twenty four hours after administration. Co- administration of AMPA and DHEA protected the retina in a dose dependent manner. In addition, NGF (60pg/eye) mimicked the DHEA effects, thus protecting the retina from AMPA toxicity. The TrkA antagonist (10^{-6} M) employed reversed the neuroprotection afforded to the retina by DHEA (10^{-6}M).

CONCLUSIONS

The present results support that the endogenous neurosteroid DHEA protects the retina from AMPA excitotoxicity via a mechanism involving the TrkA receptor. This was sub-stantiated by the reversal of the DHEA mediated neuroprotection by the TrkA antagonist and by the NGF neuroprotective effects in the retina. Further studies are essential in order to characterize further the down stream signaling events and to evaluate the therapeutic relevance of these results.

1. Kiagiadaki & Thermos: IOVS 49: 3080-3089 (2008)

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Coagulation Factors Changes Induced by Stress and Saturated Lipid Diet

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Key words: stress, saturated fats, coagulation factors

S u m m a r y. Coagulation factors are associated with lipid profile. Chronic stress is reported to promote the risk for atherothrombotic disease with increase in the coagulation activity.The aim of the study was, to assess in an experimental model of swimming stress in association to diet rich in saturated fat (butter), the serum coagulation factors such as prothrombine time, activated partial thromboplastin time (a-ptt), fibrinogen, protein C, antithrombine III and plasminogen.Stress and saturated fat diet increased Fibrinogen and Antithrombine III and lowered protein C levels. It may be concluded that saturated lipid diet under stress is negatively correlated with coagulation process and predisposes to thromboembolic disease.

INTRODUCTION

It is already known that there are strong associations between the fibrinolysis parameters, tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI-1), and the serum lipids (total and very low density lipoprotein (VLDL), triglycerides etc). Fat dietary loads result in postprandial high lipid responses: VLDL, high density lipoprotein (HDL) and triglycerides reached maximum at 4 h after the meal (1). n-3 PUFAs supplemented diet leads to reduced serum triglycerides and very-low-density lipoproteins and increased high-density-lipoprotein cholesterol. These effects as well as a significant decrease in platelet aggregation can be considered beneficial in terms of cardiovascular risk (2,3). Low-fat, high-fiber diet reduces not only the atherogenic but also the thrombogenic tendency of an individual compared with a diet corresponding to the average Danish diet (4). Factor VII activity is increased by the fat content of the diet, 6 and 8 h after the fatty meals, whereas a decrease was observed after a fat-free meal (5). Chronic stress is reported to cause increased synthesis of PAI-1, thus promoting the risk for atherothrombotic disease by decreasing the likelihood of sponta-neous fibrinolysis and increasing the likelihood of fibrin deposition (6). It has been referred that patients at risk to develop cardiovascular diseases are more sensitive to venous thrombosis after long air travel. Stress, infection, or air pollution is involved in the development of a prothrombotic state in air travellers. After long haul air travel, this state is more pronounced in patients with risk factors, leading to coagulation induction and degranulation of platelets (7).

Total hip replacement surgery causes an increase in coagulation parameters activated by serum glucose increase by stress. This suggests a possible role of glucose in the activation of the coagulation system during hip surgery (8-10). Adverse catecholamine effects by sympathetic system overstimulation during critical illness have been observed in many organs with increase in the coagulation activity (hypercoagulability, thrombus formation) (11-13). Antithrombine III (AT III) is a small protein molecule (MW 58kDa) that inactivates several enzymes of the coagulation system (mainly thrombin). Although it is less responsive functionally to some physiological stimuli, it may be associated with a thrombotic tendency (14). Protein C is a major physiological anticoagulant that is activated by thrombin into activated protein C (APC). The activated protein C, provides physiologic antithrombotic activity and exhibits both anti-inflammatory and anti-apoptotic activities. It also plays a role in the development of thrombosis and ischemic stroke (15). The aim of the study was, to assess in an experimental model of swimming stress in association to diet rich in saturated fat (butter), the

serum coagulation factors such as prothrombine time, activated partial thromboplastin time (a-ptt), fibrinogen, protein C, antithrombine III and plasminogen.

METHODS

32 Wistar male rats, aged 5-6 week, weighing 200±50 mg were divided in four groups of 8 animals (A1, A2, B1, B2). Group A1 was fed cereals as usual, group A2 fed cereals and was exposed to daily swim stress in cold water 4°C for 10 min during 20 days.

B1 and B2 were fed a mixture of cereals and bovine butter 60/40, for 20 days and B2 was exposed to swim stress as A2.

Animals were kept 12 h in light and 12h in darkness and were cared in accordance with the principles of the *Guide for the Care and Use of Experimental animals* (21). Group A served as control and Group B as experimental group.

Statistical analysis was performed by t-test.

RESULTS

- Fibrinogen was increased in stress groups and further under rich fat diet.
- Antithrombine III rised under fat intake and stress combination.
- Stress and saturated fat diet lowered protein C levels which remained high in polyunsatutated fat diet of A2 in spite of stress.

DISCUSSION

The results are in agreement with other investigators.

High plasma fibrinogen levels is an important coronary risk factor, for those at high risk of heart attacks. Fibrinogen is considered at least as important as serum cholesterol, blood pressure or cigarette smoking. In men with high cholesterol or high systolic blood pressure levels, the incidence of heart attacks was respectively 6 times and 12 times greater in those with high plasma fibrinogen levels than in those with low fibrinogen levels (17). Moreover diet rich in saturated lipids is negatively correlated with coagulation factors as is has been relatively documented (18). Exposure to short-term mental stress affects hemoconcentration with associated increases in serum lipid concentrations, hemostatic factors, and blood viscosity. Stress and rich fat diet decreased protein C and in accordance to the literature, unsaturated acids (n-3 fatty acids), including alpha-linolenic acid, may have antithrombotic effects by enhancing protein C activity (19). Plasminogen was markedly decreased under stress and is in accordance with other reports referring similar results in dogs submitted in experimental septic stress with endotoxin (20).

It may be concluded that saturated lipid diet under stress is negatively correlated with coagulation process and predisposes to thromboembolic disease. Moreover dietary intake of unsaturated n-3 PUFAs can counterbalance the deleterious effects of the stressful sympathetic overstimulation and become the means to prevent cardiovascular and other related diseases, as it has been shown in previous studies mainly with olive oil (21-24).

REFERENCES

1. Salomaa V., Rasi V., Pekkanen J., Jauhiainen M., Vahtera E., Pietinen P., Korhonen H., Kuulasmaa K., Ehnholm C.: The effects of saturated fat and n-6 polyunsaturated fat on postprandial lipemia and hemostatic activity. *Atherosclerosis 103*: 1-11 (1993)
2. Fumeron F., Brigant L., Ollivier V., de Prost D., Driss F., Darcet P., Bard J.M., Parra H.J., Fruchart J.C., Apfelbaum M.: n-3 polyunsaturated fatty acids raise low-density lipoproteins, high- density lipoprotein 2, and plasminogen-activator inhibitor in healthy young men. *Am. J. Clin. Nutr. 54*: 118-122 (1991)
3. Tholstrup T., Marckmann P., Jespersen J., Sandstrom B.: Fat high in stearic acid favorably affects blood lipids and factor VII coagulant activity in comparison with fats high in palmitic acid or high in myristic and lauric acids. *Am. J. Clin. Nutr. 59*: 371-377 (1994)
4. Marckmann P., Sandstrom B., Jespersen J.: Low-fat, high-fiber diet favorably affects several independent risk markers of ischemic heart disease: observations on blood lipids, coagulation, and fibrinolysis from a trial of middle-aged Danes. *Am. J. Clin. Nutr. 59*: 935-939 (1994)

Table
Coagulation factors levels

	Prothrombine (sec)	Aptt(sec)	Fibrinogen mg/dl	Protein C%	Antithrombine III%	Plasminogen%
A_1	9,09±0,47	44,27±10,42	129±9	12,94±4,5***	115,45±7,3	9,26±1,8
A_2	8,98±0,51	45,72±11,4	148±11,7*	14,68±8,9	116,21±8,7	3,49±1,4
B_1	8,6±0,38	49,6±12,13	214±13,5*	7,82±1,57	124,9±2,52*	8,2±0,5
B_2	8,7±0,46	44,57±11,58	267±36*	8,76±3,18	130,9±1,03**	6,73±3

*p<0,05 **p<0,01 ***p<0,001

5. Hornstra G.: Influence of dietary fat type on arterial thrombosis tendency. *J. Nutr. Health Aging. 5*: 160-166 (2001)

6. Räikkönen K., Lassila R., Keltikangas-Järvinen L., Hautanen A.: Association of chronic stress with plasminogen activator inhibitor–1 in healthy middle-aged men. *Arterioscl. Thromb. Vasc. Biol. 16*: 363-367 (1996)

7. Schreijer A.J., Hoylaerts M.F., Meijers J.C., Lijnen H.R., Middeldorp S., Büller H.R., Reitsma P.H., Rosendaal F.R., Cannegieter S.C.: Explanations for coagulation activation after air travel. *Thromb. Haemost.*: (2010)

8. Hermanides J., Huijgen R., Henny C.P., Mohammad N.H., Hoekstra J.B., Levi M.M., DeVries J.H.: Hip surgery sequentially induces stress hyperglycaemia and activates coagulation. *Neth. J. Med. 67*: 226-229 (2009)

9. Kotsiou A., Anagnostou M., Mourouzis C., Rallis G., Chantzi C., Tesseromatis C.Impact of experimental trauma and niflumic acid administration on antimicrobials' concentration in serum and mandible of rats. *J. Musculoskelet. Neuronal. Interact. 6*: 242-246 (2006)

10. Saranteas T., Voukena V., Zotos N., Stranomiti J., Kondadaki A., Tachmintzi A., Pantos C., Tesseromatis C., Dimitriou V.: Lipid kinetics in obese patients undergoing laparoscopy. The impact of cortisol inhibition by etomidate. *Eur. J. Drug Metabol. Pharmacokinet. 29*: 187-192 (2004)

11. Dünser M.W., Hasibeder W.R.: Sympathetic overstimulation during critical illness: adverse effects of adrenergic stress. *J. Intens. Care Med. 24*: 293-316 (2009)

12. Ni M., Wang Y., Zhang M., Zhang P.F., Ding S.F., Liu C.X., Liu X.L., Zhao Y.X., Zhang Y.: Atherosclerotic plaque disruption induced by stress and lipopolysaccharide in apolipoprotein E knockout mice. *Am. J. Physiol. Heart Circ. Physiol. 296*: H1598-1606 (2009)

13. Tan S., Tillisch K. Mayer E.: Functional somatic syndromes: Emerging biomedical models and traditional Chinese Medicine Review Evidence-based Compl. and Alt. Medicine. eCAM 1: 35-40 (2004)

14. Zucker M.L., Gomperts E.D., Russel D., Joffe B., Feesey M., Kuschke R., Seftel H.: Antithrombin functional activity after saturated and unsaturated fatty meals and fasting in normal subjects and some disease states. *Thromb. Res. 15:* 37-48 (1979)

15. Björn Dahlbäck; Bruno O. Villoutreix: Regulation of blood coagulation by the protein C anticoagulant pathway novel insights in to structure-function relationships and molecular recognition *Arterioscl. Thromb. Vasc. Biol.* 25: 1311 (2005)

16. Mutanen M., Freese R.: Fats, lipids and blood coagulation. *Curr. Opin. Lipidol. 12*: 25-29 (2001)

17. Committee on Care and Use of Laboratory animals. Guides for the care and use of laboratory animals: Institute of Laboratory Animals and Resources. National Research Council, 1985 p.83, Washington, DC

18. Stone M.C., Thorp J.M.: Plasma fibrinogen - a major coronary risk factor. *J. R. Coll. Gen. Pract. 35(281)*: 565-569 (1985)

19. Muldoon M.F., Herbert T.B., Patterson S.M., Kameneva M., Raible R., Manuck S.B.: Effects of acute psychological stress on serum lipid levels, hemoconcentration, and blood viscosity. *Arch. Intern. Med. 155*: 615-620 (1995)

20. Aasen A.O, Gallimore M.J, Ohlsson K., Amundsen E.: Alterations of Plasmin Activity, Plasminogen Levels and Activity of antiplasmins during endotoxin shock in dogs. Haemostasis 7: 164-169 (1978)

21. Kotsiou A., Potamianou A., Vovou J., Daskala J., Tesseromatis C.: The influence of various lipid patterns on rats under stress and nicotine exposure. *Rev. Clin. Pharmacol. Pharmacokinet. 22(2 Suppl.)*: 401-406 (2008)

22. Kotsiou A., Potamianou A., Messari J., Tesseromatis C.: The influence of antioxidant agents on lipidaemic profile of stressed rats. *Rev. Clin. Pharmacol. Pharmacokinet. 21*: 31-37 (2007)

23. Kotsiou A., Tesseromatis C.: Olives and olive oil in health and disease prevention. Victor R. Preedy and Ronald Watson, 2010

24. Kotsiou A., Chatzigianni E., Pitsicali A., Tesseromatis C.: 2008. Cholesteryl Ester Transfer Protein (CETP) changes under the influence of saturated fat diet and anabolic treatment in rats. *Rev. Clin. Pharmacol. Pharmacokinet. 22*: 198-200 (2008)

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Cell Surface Expression of Nucleolin Is Maintained by $\alpha_v\beta_3$ Integrin and is required for Pleiotrophin-induced Cell Migration

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Key words: Heparin affin regulatory peptide, heparin-binding growth associated molecule, angiogenesis, tumour, casein kinase 2

SUMMARY

Pleiotrophin (PTN) is a secreted heparin-binding growth factor with roles in many different processes, such as cell growth and survival, neurite outgrowth, endothelial cell migration and angiogenesis, as well as tumour growth and metastasis. We have previously shown that PTN induces tumor and endothelial cell migration through its receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ) that forms a functional complex with $\alpha_v\beta_3$ integrin on the cell surface. The multifunctional protein nucleolin (NL) has been also mentioned to be a low affinity cell surface receptor for PTN. Nucleolin acts as a shuffle between cytoplasm and nucleolus, is increased on the surface of angiogenic endothelial cells and binds a variety of ligands that play critical role(s) in tumorigenesis and angiogenesis. In the present work, we studied whether NL plays a role in PTN-induced cell migration. Down-regulation of NL by siRNA or blockage of cell surface NL by its ligand 5(KPR)TASP in human endothelial cells completely abolished PTN-induced cell migration. NL was found to directly interact not only with PTN, but also with both RPTPβ/ζ and $\alpha_v\beta_3$ on the membrane of human endothelial and cancer cells. Interaction of both PTN and RPTPβ/ζ with NL was also observed inside the cell, while $\alpha_v\beta_3$ was only detected on the cell surface. Although NL was not detected on the surface of cells that do not express integrin $\alpha_v\beta_3$, both PTN and RPTPβ/ζ appeared in the nucleus of these cells, suggesting the existence of an alternative transport pathway. It seems that PTN is a casein kinase 2 (CK2) substrate, with CK2 being implicated in PTN and RPTPβ/ζ nuclear transport mechanism. Inhibition of CK2 activity did not influence the effects of PTN on cell migration, implying that nuclear translocation of PTN and RPTPβ/ζ does not affect cell migration and that cell surface NL participates in the transduction of a yet unknown signal that induces cell migration. Collectively, our data suggest that cell surface expression of NL is maintained by $\alpha_v\beta_3$ integrin and is required for PTN-induced cell migration.

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Application of the Genotoxicity Assay Comet as a Test for Evaluating Occupational Exposure to Chemotherapeutic Agents

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S u m m a r y. The expansion of anti-cancer chemotherapy has lead to a significant concern about the health and safety work conditions of the personnel handling these drugs, that have been characterized by the International Agency for Research on Cancer as hazardous to human. However, no reliable monitoring test of occupational exposure is currently clinically applied for the medical surveillance of these employees. Based on a descriptive self-evaluation survey, we have previously shown inadequate health and safety design and practices related to chemotherapeutic drug handling in Greek hospitals. In the present study, we use a quantitative and objective genotoxicity method, the comet assay, to evaluate occupational exposure to chemotherapeutics. DNA single strand breaks as revealed by comet in peripheral mononuclear blood cells were significantly higher in a group of employees involved in the preparation, handling or administration of cancer chemotherapeutic agents that in a control group. Comet data were correlated to demographic/epidemiological health data of the subjects as well as to the self-evaluation subjective occupational exposure data. Our results demonstrate clearly genotoxic effects related to occupational exposure to chemotherapeutics, indicating insufficient safety design and practices. They also suggest that SSBs could be an appropriate biomarker for evaluating genotoxicity in these employees. The comet assay in peripheral blood could provide an easy tool of the Health and Safety Department in hospitals for the exposure monitoring.

INTRODUCTION

The widely use of chemotherapeutic drugs with strong anti-cancer cytotoxic effects has lead to concerns about the possible health hazards caused to the hospital personnel involved in their use (1-3). As they are characterized as dangerous occupational and environmental factors, special alerts/guidelines have been published for their safe handling (4,5). In accordance, special department design and equipment is necessary as well as personal protective measures and safety practices, in order to minimize occupational exposure (2). Acute and/or chronic toxicity has been reported by hospital employees (7) and regular monitoring of occupational exposure as part of their medical surveillance is critical for their prevention and in order to provide a safe and healthy hospital environment for both patients and health workers (8,9). However, apart from routine blood and urine biochemical examinations, no other test reflecting genotoxicity and exposure levels is currently applied to these employees. In a self-evaluation risk assessment study conducted in the Greek hospitals, we have previously reported the safety conditions of health-care workers in positions involving handling of chemotherapeutic drugs (10,11). We have shown that compliance with regulations is largely inadequate and toxic side effects are often experienced and that training and surveillance of the staff should be a vital priority of the hospital management. Aim of the present study was to evaluate a common test for genotoxicity, the comet assay, as a measure of occupational exposure to chemotherapeutics.

METHODS

The study was conducted in a public hospital in Greece, following approval from the Scientific Committee and Administration. Blood samples were collected from a total of 32 subjects: 16 nurses involved in the preparation, handling or administration of cancer chemotherapeutic agents (experimental group) and 16 nurses from the same hospital who do not use chemotherapeutic agents in their work (control group). Samples were analyzed for DNA damage by using the single cell electrophoresis assay (comet assay). The incidence of DNA single strand breaks (SSB) in peripheral mononuclear blood cells was evaluated by using the parameter of %DNA in the comet tail. In parallel, self-evaluation data were collected by a specially designed questionnaire, covering a series of demographic characteristics, information about existing knowledge about the safe use of chemotherapeutic drugs, the protective working practices and safety procedures adopted by themselves and the hospital, and the adverse effects experienced. Data analysis was conducted using the statistical program SPSS15.0 for Windows.

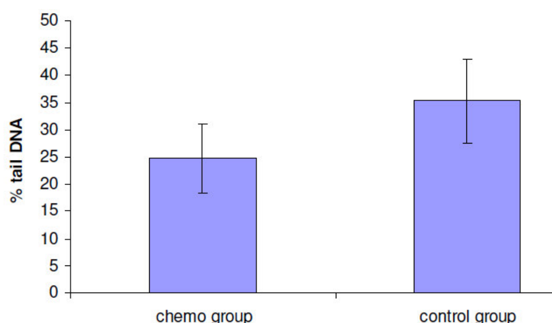

Figure1. DNA single strand breaks in peripheral mononuclear blood cells as analyzed by comet assay in blood samples from hospital personnel handling chemotherapeutics (treated) and other hospital employees (untreated)

RESULTS

The incidence of DNA single strand breaks (SSB) in peripheral mononuclear blood cells was analyzed in blood samples from both the experimental and the control groups. Comet results were initially checked for normality, homoscedasticity and homogeneity of variance. An unpaired Student's *t*-test between the median values of the two groups revealed a statistically significant increase of SSB in the group involved in the preparation, handling or administration of cancer chemotherapeutic agents as compared to the control group ($P<0.01$). Different epidemiological health data of the subjects (e.g. age, smoking and dietary habits) were correlated with the observed DNA damage as possible confounding variables. Comet results were also correlated to the self-evaluation subjective occupational exposure data.

DISCUSSION

In the present study, we evaluate a common test for genotoxicity, the comet assay, as a measure of occupational exposure to chemotherapeutics. Our data demonstrate clearly genotoxic effects related to occupational exposure to chemotherapeutics, indicating insufficient safety design and practices. These findings, using a quantitative and objective method, confirm our previous data based on a descriptive self-evaluation survey, showing inadequate health and safety design and practices related to chemotherapeutic drug handling in Greek hospitals, resulting in genotoxic harm of the employees. These data further stretch the need for training and medical surveillance by a specialized Health and Safety Department. They also suggest that DNA damage revealed by comet assay could be an appropriate and reliable biomarker for evaluating genotoxicity in employees handling chemotherapeutics. The comet assay in peripheral blood could provide an easy tool of the Health and Safety Department in hospitals for the exposure monitoring.

REFERENCES

1. Jochimsen P.R.: Handling of cytotoxic drugs by health-care workers: A review of the risks of exposure. *Drug Safety 7*: 374-380 (1992)
2. Veccio D., Sasco A.J., Cann C.I.: Occupational risk in health care and research. *Am. J. Med. 43*: 369-97 (2003)
3. Σουρτζή Π., Βελονάκης Ε.: Η χημειοθεραπεία ως επαγγελματικός κίνδυνος για τους νοσηλευτές. *Νοσηλευτική 43*: 435-441 (2004)
4. NIOSH Alert: Preventing Occupational Exposures to Antineoplastic and other Hazardous Drugs in Healthcare Settings DHHS (NIOSH) Publication No. 2004-165, 2004)
5. OSHA: Controlling Occupational Exposure to Hazardous Drugs. Technical Manual. Section VI. Health Care Facilities (1999)
6. Krstev S., Perunicic B., Vidakovic A.: Work practice and some adverse health effects in nurses handling antineoplastic drugs. Med Lav. Sep-Oct; 94(5): 432-9 (2003)
7. Fucic A., Jazbec A., Mijic A. Seso-Simic, Tomek R.: Cytogenetic consequences after occupational exposure to antineoplastic drugs. *Mutat. Res. 416*: 59-66 (1998)
8. Χατζάκη Α. Επαγγελματική Έκθεση σε Επικίνδυνα Φάρμακα. Πρόγραμμα Μεταπτυχιακών Σπουδών Υγιεινής και Ασφάλειας της Εργασίας. (2006)
9. Κωνσταντινίδης Θ.Κ. Τα πεδία πολυεπιστημονικών δράσεων της Υγιεινής και Ασφάλειας της Εργασίας. Σημειώσεις Μεταπτυχιακού Προγράμματος Σπουδών Υγιεινής και Ασφάλειας της Εργασίας. Δ.Π.Θ. (Τμήμα Ιατρικής) σε σύ-

μπραξη με το ΤΕΙ Αθήνας(Τμήμα Δημόσιας Υγιεινής και Τμήμα Νοσηλευτικής Α). Αλεξανδρούπολη. (2005)
10. Constantinidis T.C., Vagka E., Dallidou P., Basta P., Drakopoulos V., Kakolyris S., Chatzaki E.. Occupational Health and Safety of personnel handling chemotherapeutic agents in Greek hospitals. *Eur. J. Cancer Care*. (2008)
11. Constantinidis TC, Vagka, E., Dallidou, P., Basta, P., Drakopoulos, V., Chatzaki, E. Occupational exposure of health care workers to chemotherapeutic agents: A study in Greek Hospitals. *Rev. Clin. Pharmacol. Pharmacokinet. 22*: 126-128 (2008)

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Cyclin-dependent Kinase 5 Interacts with RPTPβ/ζ and Mediates Pleiotrophin-induced Endothelial Cell Migration

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Key words: Heparin affin regulatory peptide, heparin-binding growth-associated molecule, angiogenesis, glioma

SUMMARY

Cyclin-dependent kinase 5 (cdk5), a proline-directed serine/threonine kinase, belongs to the cyclin-dependent kinase (CDK) family. Unlike other members of this group, cdk5 does not have a known role in cell-cycle progression and requires the regulatory subunits p35 or p39 for activation. Cdk5 is primarily expressed in neuronal cells and plays an important role in processes like neuronal migration and neurite outgrowth, but its functions in non-neuronal cells are unclear. There are a growing number of molecules that exhibit the capacity to interact with cdk5. Pleiotrophin (PTN), also known as heparin affin regulatory peptide or heparin-binding growth-associated molecule is an 18-kDa secreted growth factor that has high affinity for heparin. PTN is expressed in various cancer cell lines, takes part in many different processes, such as cell growth and survival, cell migration and angiogenesis, exerting diverse functions in different cell lines. We have previously shown that PTN induces migration of endothelial cells through binding to its receptor protein tyrosine phosphatase β/ζ (RPTPشد/شد and $\alpha_v\beta_3$ integrin. In the present study, we investigated the role of cdk5 in PTN-induced human endothelial cell migration. Roscovitine, a synthetic inhibitor of cdks with selectivity towards cdk2 and cdk5, as well as down-regulation of cdk5 by siRNA completely attenuated PTN-induced migration of endothelial cells. PTN increased cdk5 kinase activity with the maximum increase observed within 5 min after stimulation of cells with PTN. Interestingly, by immunoprecipitations followed by Western blot or mass spectroscopy analyses, cdk5 was found to directly interact with RPTPβ/ζ. Similar results were obtained in human glioblastoma U87MG cells, which are known to express both RPTPβ/ζ and $\alpha_v\beta_3$ and migrate in response to PTN. These data suggest that cdk5 is a significant regulator of the PTN/RPTPβ/ζ/$\alpha_v\beta_3$ signaling pathway that leads to increased cell migration.

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Self-Prescribing: A common Phenomenon especially among Young Greek Doctors

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Summary. Self-prescribing among doctors is common, but no studies have documented this issue in Greece. We conducted a study about the self-prescribing behavior among Greek physicians using a questionnaire. According to our results, a quite large percentage of the participants self-treated themselves for less or more severe health problems, while the majority of them were young doctors. Although self-prescribing is acceptable in some situations, physicians should seek professional help for illness. Efforts to inculcate more rational help-seeking behavior should probably start in medical schools.

INTRODUCTION

Unlike to the rest of the population, when doctors becomes patients they have the capability to prescribe medicine for themselves. The medical knowledge in combination with the access to prescription medication, increase the possibility of self-treatment. Many physicians find it difficult to enter the patient role for various reasons such as time pressure, nature of sickness, worries about bothering colleagues, fear of showing weakness or lack of medical knowledge, concerns about confidentiality, and fear of restrictions of medical licensing (1-3). The Aim of the present study was to examine the extent of self-treatment, as it is presented in the bibliography, the multiple attempts of controlling the phenomenon, that are developed within regulations, recommendations or deontologies/ethics and finally to evaluate the consequences that arise from the practice of self-treatment. Furthermore we present the results obtained from the given answers of a reliable sample of Greek doctors (specialists and residents).

METHOD

A questionnaire based survey was conducted using a 3-pages self-reported anonymous questionnaire. The questionnaire was completed by a reliable sample of doctors from the Region of Thessaloniki. Initially the questionnaire was created based on others that were used or manufactured in similar studies. It included closed questions that examined demographic elements (age, sex, familial situation, etc), as well as questions that concerned the place and the years of exercise of medical profession. The physicians were asked by asked to answer in questions concerning the drugs and diseases for which they are self-prescribing, as well as in 13 proposals based on the 5grade scale Likert, with the following choices of answers: I absolutely agree, I agree, neither agree-neither disagree, I disagree, I disagree absolutely. The statements concerned the attitude and the behavior opposite in the treatment of their own health, but also their relatives and they had as aim to investigate the more general tendency of the doctors to self prescribing. The statistical analysis was conducted with the statistical package SPSS 16.0 for Windows using the tests x^2 analysis, t-test, analysis of variance and Pearson correlation (3-6).

RESULTS

The response rate was 76.71% (145 out of 189 distributed questionnaires). The majority of doctors (82 persons - 56,6%) had taken some kind of drug during the last month, and analgesics were the most commonly taken drugs (32.1%). From the 82 individuals, the 65.8% had self-treated

themselves and only 34.25% had contacted another doctor for the prescribing. The care and the administration instructions were given by themselves in percentage of 78%. Finally, 55.2% of the doctors replied that the medical knowledge they have, is adequate for the treatment of several health problems. According to our results there is a statistical significant positive correlation of attitudes and behavior with the age-related category ($p = 0.003$), that means that the older physicians disagree with the self-prescribing.

DISCUSSION

A quite large percentage of the participants self-treat themselves for less or more severe health problems. The relatively high level of self-prescribing in our study concurs with the findings in other countries (4-8). Previous studies have shown that physicians often self-prescribe medications, most practise self-treatment when they are ill, many have problems accepting their own illness, and many tend to avoid taking sick leave during an illness for which they would have sick-listed their patients (7,8). This may indicate that their health is getting worse or that they have started to take better care of their own health. In Greece there is inadequate literature regarding the phenomenon of self-treatment, so it is difficult to generalize the results and there is a need to perform more studies. The discussion of the problem might help doctors to evaluate with a different and maybe a more rational way the practice of self-treatment, establishing new and more functional attitudes and behaviors.

REFERENCES

1. Thompson W.T., Cupples M.E., Sibbett C.H., Skan D.I., Bradley T.: Challenge of culture, conscience, and contract to general practitioners' care of their own health: qualitative study. *BMJ 323*: 728-731 (2001)
2. Hendin H., Maltsberger J.T., Haas A.P.: A physician's suicide. *Am. J. Psychiatry 160*: 2094-2097 (2003)
3. Center C., Davis M., Detre T., Ford D.E., Hansbrough W., Hendin H., Laszlo J., Litts D.A., Mann J., Mansky P.A., Michels R., Miles S.H., Proujansky R., Reynolds C.F., 3rd, Silverman M.M.: Confronting depression and suicide in physicians: a consensus statement. *JAMA 289*: 3161–3166 (2003)
4. Christie J.D., Rosen I.M., Bellini L.M., Inglesby T.V., Lindsay J., Alper A., Asch D.A.: Prescription drug use and self-prescription among resident physicians. *JAMA 280*: 1253-1255 (1998)
5. Clarke J., O'Sullivan Y., Maguire N.: A study of self-care among Irish doctors. *Ir. Med. J. 91*: 175-176 (1998)
6. Richards J.G.: The health and health practices of doctors and their families. *N. Z. Med. J. 112*: 96-99 (1999)
7. Campbell S., Delva D.: Physician do not heal thyself. Survey of personal health practices among medical residents. *Can. Fam. Physician. 49*: 1121-1127 (2003)
8. Runeson B., Dahlin M., Åsberg M., Nygren A.: Stress and depressive symptoms in physicians and medical students in Sweden [abstract]. Eur. Psychiatry 2004;19: 49s (2004)

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Regulator of G Protein Signaling 4: A Novel Regulator of μ- and δ-Opioid Receptor Signaling

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Key words: Regulator of G protein signaling, G protein, internalization, Extracellular signal-regulated kinases, protein-protein interactions

S u m m a r y. In vitro studies have shown that the Regulator of G protein Signaling 4 (RGS4) interacts with the C-termini of μ- and δ-opioid receptors (μ-OR, δ-OR) (1). Herein we demonstrate that RGS4 associates with these receptors in living cells and forms selective complexes with Gi/o proteins in a receptor dependent manner. This interaction occurs within the predicted fourth intracellular loop of μ, δ-ORs as part of a signaling complex consisting of the opioid receptor, activated Gα and RGS4. Expression of RGS4 in HEK293 cells attenuated agonist-mediated ERK1,2 phosphorylation for both receptors and accelerated agonist-induced internalization of the δ-OR. Our findings demonstrate that RGS4 plays a key role in G protein coupling selectivity and signaling of the μ- and δ-ORs.

INTRODUCTION

Regulators of G protein signaling (RGS) proteins which comprise a large and diverse family of proteins can bind directly to Gα subunits to attenuate their signaling (2-4). Although the molecular basis of RGS-Gα interactions has been extensively studied, little is known on how RGS selectivity for G protein signaling events is determined in living cells. Growing evidence indicates that RGS proteins, by their ability to shorten the lifetime of activated Gα, confer selectivity for signaling pathways. However, it is still unclear to what degree G protein activation influences RGS binding, how stable RGS-G protein association is and how GPCRs might influence RGS-G protein association.

Recent observations employing pull down assays utilizing the C-terminal domains of the μ-OR and δ-OR (μ-CT, δ-CT) demonstrated the ability of purified RGS4 to interact directly with these receptor subdomains and modulate DAMGO-mediated adenylyl cyclase inhibition in HEK293 cells (1). This interaction was part of a signaling complex consisting of the receptor, Gα, Gβγ and RGS4 (1). In the present study we provide evidence for the selectivity of RGS-Gα pairs formed upon opioid receptor stimulation, indicate the structural determinants responsible for RGS4 and RGS4-Gα complex association and demonstrate that RGS4 by interacting with the μ- and δ-ORs confers selectivity to the activated receptors to couple with a specific subset of G proteins and subsequently to alter their signaling pathways.

METHODS

Constructs and Reagents: The rat myc-tagged μ-OR (in the pcDNA3 vector) was generously provided by Dr S.George, University of Toronto, Toronto, Canada. GtαGDP purified from bovine retina was kindly provided by H. Hamm, Vanderbilt University, Nashville, TN, USA. Hemagglutinin (HA)-tagged human RGS4 (HA-RGS4), anti-Goα and anti-Giα$_2$ antibodies were kindly provided by Dr G. Milligan University of Glasgow, Glasgow, Scotland. Hexahistidine (6xHis)-tagged RGS4 (His-RGS4) and 6xHis-RGS domain of RGS4 (His-4Box) in pQE60 vector were kindly provided by Dr T.M. Wilkie, University of Texas, TX, USA.

Preparation of GST fusion constructs and GST pull-down assays: GST fusion peptides genera-

tion and pull-down experiments were performed as described (5,6).

Cell cultures and transient transfections: HEK293 cells stably expressing either a EYMPME (EE)-tagged or a myc-tagged version of the μ-OR, or the flag-tagged δ-OR, were grown in Dulbecco's modified Eagle's medium as described previously (1,6,7). Transient transfections were performed according to (6)

Co-immunoprecipitation assays: HEK293 cells stably expressing the myc-tagged μ-OR or the flag-δ-OR were transiently transfected with empty vector or the HA-RGS4. 48 hours post transfection, the cells were stimulated or not with opioid agonists for the indicated times and rinsed in PBS buffer containing 1 mM PMSF and 1 mM sodium orthovanadate. Cells were lysed in lysis buffer A as described by (8,9).

Detection of MAPK phosphorylation: Measurement of MAPK phosphorylation was performed as described by (5).

Fluorescent-activated Cell Sorting (FACS) analysis: HEK293 cells expressing the flag-δ-OR were transfected with RGS4. Cells were treated for 15 min and 1 h with 1μM DSLET. Samples of 500.000 cells were acquired and incubated overnight with a polyclonal anti-flag serum (1:300) as described by (6).

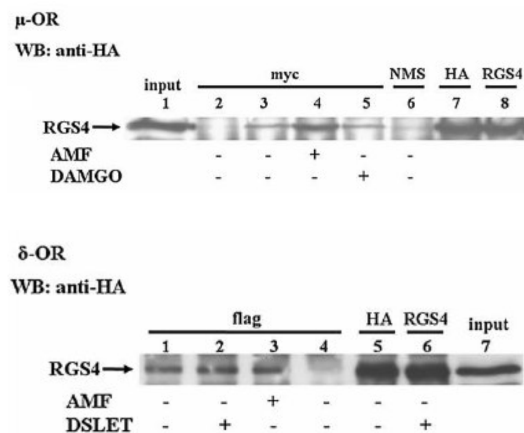

Figure 1. RGS4 interacts with the μ-OR and δ-OR in HEK293 cells

RESULTS AND DISCUSSION

In an attempt to examine whether RGS4 interacts with the δ-OR and μ-OR co-immunoprecipitaion studies were performed in HEK293 cellls expressing these receptors and RGS4. As shown in Fig. 1, RGS4 interacts directly with both μ- and δ-ORs. Mapping the sites of interaction demonstrated that the conserved region encompassing helix VIII at the δ-CT and μ-CT is the structural determinant responsible for RGS4 binding to these receptors. Binding of RGS4 to both opioid receptors occurs spontaneously and is not influenced by the activated state of these receptors. These results suggest that μ-OR and δ-OR physically interact with RGS4 in living cells and activation of these receptors is not a prerequisite for RGS4 association.

Taking into account that RGS4 and Gα subunits form *in vitro* a heterotrimeric complex with the C termini of μ-OR and δ-OR and that RGS4 interacts directly with the opioid receptors in HEK293 cells, we sought to determine, whether RGS4 confers selectivity to the activated μ- and δ-ORs for coupling with a particular subset of Gα proteins. To answer this question, the ability of specific Gα subunits of G proteins to interact with RGS4 upon agonist stimulation of the opioid receptors in HEK293 cells was tested. Our results have shown that in cells expressing the μ-OR, RGS4 preferentially interacted with $Gi\alpha_{1,3}$, $Gi\alpha_2$ and Goα, only upon receptor stimulation. We can thus conclude that RGS4 equipotently pairs with any of the endogenous G proteins tested in HEK293 cells upon μ-OR stimulation. In contrast, the δ-OR exhibits a differential selectivity profile for RGS4-Gα complex formation in δ-HEK293 cells. In the absence of agonist, RGS4 interacts with $Gi\alpha_{1,3}$ and Goα but not with $Gi\alpha_2$ (Fig. 2). Agonist activation of the δ-OR enhances coupling between RGS4 with $Gi\alpha_2$, whereas it attenuates RGS4-$Gi\alpha_{1,3}$ association. In contrast to agonist stimulation, AMF treatment forces RGS4 to couple with all Gα subunits tested. These results suggest that the agonist-activated δ-OR promotes selective RGS4-Gα interactions and suggest that RGS4 can dynamically regulate δ-OR selectivity for specific Gα subunits even in the absence of agonist. This is the first indication for an RGS-driven selection coupling with a specific G protein population depending on the presence or the activation state of a given receptor in living cells.

Opioid receptors stimulate ERK1/2 activity via PTX-sensitive and insensitive G protein signaling mechanisms (10-12) and growing evidence indicates that RGS proteins, and among them RGS4, are implicated in MAPK signaling through GPCRs (13). Indeed, RGS4 presence attenuates MAPK phosphorylation of both activated μ- and δ-ORs.

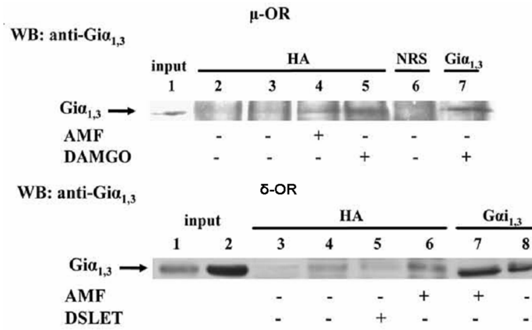

Figure 2. RGS4 interacts with specific Gαi/Gαo subunits in HEK293 cells

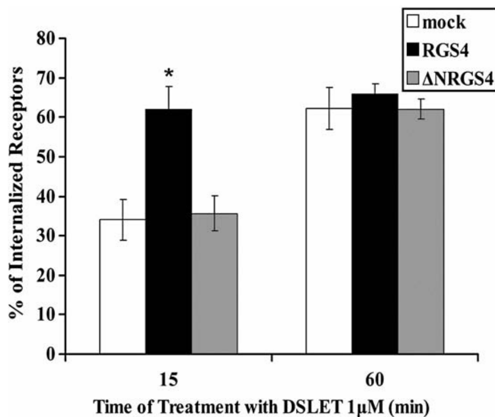

Figure 3. Effect of RGS4 expression on the internalization rate of δ-OR

Ample experimental evidence suggests that opioid analgesia and tolerance development involve complex cellular and molecular mechanisms as a result for the loss of membrane surface opioid receptors (14). In view of these considerations someone would think that RGS proteins may be implicated in opioid receptor internalization. Measurements of the δ-OR internalization fate indicated that the presence of RGS4 accelerated the early rate of the δ-OR endocytosis, whereas, on the other hand, an RGS mutant, lacking its N-terminus (ΔNRGS4), was unable to regulate the endocytotic pathway of the δ-OR; thus, demonstrating that the RGS4-N-terminus possesses a significant functional key role in opioid receptor mediated signaling (Fig. 3). We can therefore conclude that RGS4 can be an attractive target to selectively manipulate G protein pathways and regulate the potency, selectivity and duration of action of opioids in order to prevent the adverse effects of tolerance and dependence.

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REFERENCES

1. Georgoussi Z., Leontiadis L., Mazarakou G., Merkouris M., Hyde K., Hamm H.: *Cell. Signal. 18*: 771-782 (2006)
2. Hollinger S., Hepler J.R.: *Pharmacol. Rev. 54*: 527-559 (2002)
3. Chidiac P., Roy A.A.: *Receptors Channels 9*: 135-147 (2003)
4. Neitzel K.L., Hepler J.R.: *Semin. Cell Dev. Biol. 17*: 383 (2006)
5. Morou E., Georgoussi Z.: *J. Pharmacol. Exp. Ther. 315*: 1368-1379 (2005)
6. Leontiadis L.J., Papakonstantinou M.P., Georgoussi Z.: *Cell. Signal. 21*: 1218-1228 (2009)
7. Douris V., Swevers L., Labropoulou V., Andronopoulou E., Georgoussi Z., Iatrou K.: *Adv. Virus Res. 68*: 113-156 (2006)
8. Mazarakou G., Georgoussi Z.: *J. Neurochem. 93*: 918-931 (2005)
9. Swevers L., Morou E., Balatsos N., Iatrou K., Georgoussi Z.: *Cell. Mol. Life Sci. 62*: 919-930 (2005)
10. Georgoussi Z., Zioudrou C.: *Biochem Pharmacol. 45*: 2405-2410 (1993)
11. Megaritis G., Merkouris M., Georgoussi Z.: *Recept. Channels 7*: 199-212 (2000)
12. Georgoussi Z.: Molecular Aspects of G Protein-Coupled Receptors. In: (Ciruela F., Lujan R., eds). Pp. 169=206, Nova Science Publishers Inc, 2008
13. Yan Y., Chi P.P., Bourne H.R.: *J. Biol. Chem. 272*: 11924 (1997)
14. Kieffer B.L. Evans C.J.: *Cell 108*: 587 (2002)

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Regulation of Aromatase Expression by Hormones, Drugs Pesticides and Environmental Pollutants in Canine Mammary CMT-U27 Cells

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Key words: Aromatase, breast cancer, regulation, inducers, inhibitors

S u m m a r y. Aromatase, the enzyme that coverts testosterone to 17β-estradiol, has attracted attention lately because of its involvement in some forms of breast cancer in women. Studies are now undergoing on a variety of species to elucidate the effect of various agents on aromatase expression. Dog is an important species, widely used by the pharmaceutical industry for many study types, including those that will impact decision on drug development. In the present study, a wide variety of agents, including steroid hormones, drugs, pesticides, and environmental pollutants, were used to investigate their effect on aromatase expression in the canine mammary CMT-U27 cell line using quantitative real-time polymerase chain reaction (qPCR) on DNA synthesized from RNA extracted from the cells.

INTRODUCTION

Dogs are widely used by the pharmaceutical industry for assessing the metabolism, pharmacokinetics, safety, and efficacy of drugs and drug candidates in research and development. Compared to rat and human cytochrome P450s, however, relatively little is known about specific canine P450 enzymes. Aromatase is a member of the P450 family (CYP19, cytochrome P450arom) and is the enzyme responsible for the conversion of testosterone to 17β-estradiol. P450arom is present in many tissues, including the gonads, brain, placenta, bone and adipose tissue (1). Aromatase expression is affected by a wide range of agents, such as steroid hormones, drugs, pesticides and environmental pollutants i.e. pesticides commonly found in surface waters as contaminants (2). These agents are capable of altering aromatase, either directly as competitive inhibitors of the catalytic activity, or indirectly *via* regulation of its expression in cell-based assays. In the present study, we investigate the effect of a wide variety of agents (including hormones, drugs, pesticides and antioxidants) on aromatase expression in the canine mammary CMT-U27 cell line. Aromatase expression was assessed using quantitative real-time PCR. Corticosterone, rhapontin and Bisphenol A greatly reduced aromatase expression, whereas dexamethasone and propamocarb greatly upregulated aromatase. The results suggest that aromatase expression is regulated by a number of chemically and mechanistically diverse agents and further investigation is needed to further clarify themrole of these agents in aromatase activity.

MATERIAL AND METHODS

Canine mammary tumor CMT-U27 cells were a generous gift of Dr. E. Hellmen (Department of Anatomy, Physiology and Biochemistry, SLT, Uppsala, Sweden). All chemical agents were supplied by Sigma and Alexis Biochemicals. Cells were grown in RPMI 1640 medium (Biosera, UK) containing 10% Fetal Calf Serum (Sigma, UK), and 1% Antibiotic-antimycotic (Biosera, UK). The cells were allowed to grow to the full extent of the plate surface, after which the agents were added and the plates were incubated in 5% CO_2 for 24 hours at 37º C. Total RNA was extracted from the cells using Trizol LS. Synthesis of cDNA was performed with 1 ug total RNA, 100 pmol random primer, 100 nmol deoxynucleotide triphosphates, 10 units RNase inhibitor and using MMLV reverse transcriptase (200 units per reaction, Finnzymes, Espoo, Finland). Prior to cDNA synthesis, RNAs were treated for 5 min at room temperature with

DNase I-RNase free (1 U/reaction, Invitrogen, Carlsbad, CA) and the reaction was stopped by incubation at 65º C for 15 minutes. Quantitative real-time PCR was performed on a Roche LightCycler 2.0 system using KAPA SYBR Fast qPCR Kit. Primers were chosen with the assistance of the *Beacon Designer 7* software (PremierBiosoft, Palo Alto, CA) from sequences of *CYP19* (AJ854107) and 18S rRNA (NR003286). The thermal cycling comprised a denaturation step at 95º C for 10 min, 60 cycles at 95º C for 10 sec, 57º C for 10 sec and 72º C for 15 sec, and a melting step from 65º C to 95º C. Varying lengths of oligonucleotides produce disassociation peaks at different melting temperatures. Consequently, at the end of the PCR cycles, the PCR products were analyzed using the heat disassociation protocol to confirm that one single PCR product was detected by SYBR Green dye. Quantitative values were obtained from the threshold PCR cycle number (C_t) at which the increase in signal associated with an exponential growth for PCR product starts being detected. The level of expression of mRNA for CYP19 in each sample was calculated with the equation $1/2^{Ct}$ and the value obtained was divided by $1/2^{Ct}$ the value of 18S rRNA of the same sample.

RESULTS

A number of drugs and chemical agents were used to investigate their role in regulation of aromatase gene expression.

The agents were used on this study were the following.

(a) Hormones and vitamins: progesterone (10 μM), estradiol (10 μM), corticosterone (10 μM), 3,3',5–triiodo-L-thyronine (T3) (10 μM), colcalciferol (vitamin D3) (1 μM), retinoic acid (100 nM).

(b) Drugs, agonists and antagonists of intracellular functions: rofecoxib (DFU) (50 μM), dexamethasone (10 μM), forskolin (10 μM), [*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride (H-89) (10 μM), rapamycin (50 nM), metformin (100 μM), UDP4 (500 μM), UDP7 (500 μM), dichloroacetate (DCA) (10 μM), rosiglitazone (10 μM), roscovitine (10 μM), sodium butyrate (BuNa) (2 μM), 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) (100 μM

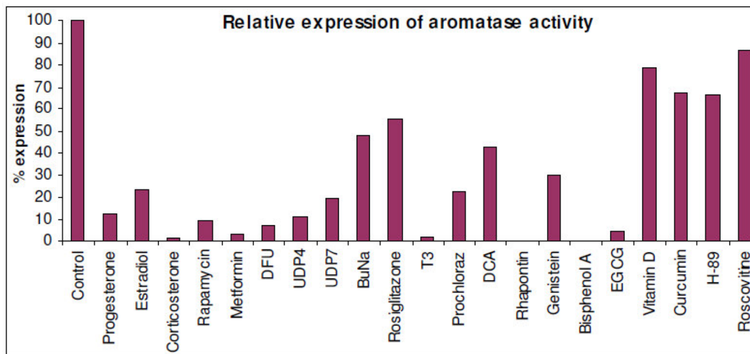

Figure 1. Inhibitors of aromatase expression

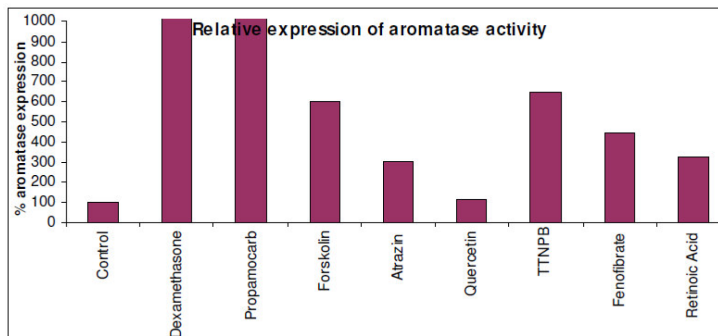

Figure 2. Inducers of aromatase expression

(c) Pesticides and environmental pollutants: bisphenol A (50 μM), prochloraz (50 μM), propamocarb (50 μM), atrazine (10 μM).
(d) Flavonoids and antioxidants: rhapontin (10 μM), genistein (10 μM), epigallochetin gallate (EGCG) (100 μM), curcumin (10 μM), quercetin (20 μM).

Progesterone, estradiol, corticosterone, rapamycin, metformin, DFU, UDP4, UDP7, T3, prochloraz, DCA, rhapontin, genistein, bisphenol A, and epigallochetin gallate (EGCG) inhibited the expression of aromatase ($p<0.05$). BuNa, rosiglitazone, vitamin D3, curcumin, H-89 and roscovitine also inhibited aromatase expression, but the results were not statistically significant ($p>0.05$).

Dexamethasone, propamocarb, forskolin, atrazine, quercetin, TTNPB, fenofibrate, and retinoic acid induced aromatase expression. ($p<0.05$)

As shown in Figure 1, hormones are moderate to potent aromatase inhibitors, except retinoic acid which induced aromatase expression. Dexamethasone, a glycocorticosteroid, significantly induced aromatase expression by 76815820% (Figure 2), whereas metformin, a biguanide, reduced aromatase expression by 97%. Pesticides act either as repressors (bisphenol A, prochloraz) or inducers (propamocarb, atrazin).

DISCUSSION

Regulation of the transcription of aromatase is complicated. Many factors habe been documended to induce the transcriptional activity, such as AMP, phorbol esters, dexamethasone, prostaglandin (PG) E2, transforming growth factor-β and γ-interferon (3), whereas cyclooxygenase inhibitors suppress CYP19 expression and inhibit the enzyme activity (4). Aromatase plays important roles during pregnancy as it is the key enzyme involved in estrogen production. Estrogens also play a crucial role in some disease states, particularly in breast cancer where, through binding to their target receptor, they promote proliferation of breast cancer cells (5).

In this study, we investigated the factors that influence the expression of aromatase. We used a number of agents that inhibit or activate signal transduction pathways in order to determine the factors that regulate the expression of aromatase in breast cancer cells. Our results have shown thatprogesterone, corticosterone and estradiol down-regulated (6,7) and dexamethasone strongly upregulated arpmatase (8). T3 and vitamine D3 down-regulated aromatase but *all*-trans-retinoic acid and TTNPB (RAR and RXR antagonists) up-regulated aromatase (9). Forskoli, an activator of adenyl cyclase, up-regulated aromatase (8) but H-89, an inhibitor of PKA and PKG, down-regulated aromatase (10). Rapamycin, a mTOR agonisr and rofecoxid (DFU), a COX inhibitor, reduced aromatase mRNA levels (11,12). PPARγ antagonist bisphenol A down regulated aromatase (13), rosiglitazone, a PPAPγ agonist, down-regulated (14) and fenofibrate (PPARα agonist) up-regulated expression of aroamatase. Other agents, such as genistein (a PPARγ ligand that also binds to estrogen receptors and inhibits EGF receptro (15), rhapontin,roscovitine, epigallocatechin (16), curcumin (17) and metformin (18), down-regulated aromatase, quercetin (19) slightly increased aromatatase expression. Agents that inhibit histone deacetylation, such as sodium butyrate, also dwon-regulate aromatase (20). Inducers of differentiationof neoplastic cells, such as UDP4 and UDP7, and dichloroacetic acid (21), a pyruvate dehydrogenase kinase inhibitor, down-redulated aromatase. Finally, pesticides alter aromatase expression; propamcarb and atrazine upregulated and prochloraz down-regulated down-regulated aromatase expression (22-24). These results suggest that aromatase expression was regulated by a number of chemically and mechanistically diverse agents, its regulation is in fact very complex and further investigationis needed to delineate the mechanisms via each one of these agents described in this study act.

REFERENCES

1. Katsiabas D.S., Mavridis S.K., Pappas I.S.: Expression of Cytochrome P450s and Transctiption Factors Pxr and Car in Canine Tissues. *Rev. Clin. Pharmacol. And Pharmacokinetics, 22:* 189-192 (2008)
2. Bretveld R.W., Thomas C.M.G., Scheepers P.T.J., Zielhuis G.A., Roeleveld N.: Pesticide exposure: the hormonal function of the female reproductive system disrupted? *Reprod. Biol. Endocrinol. 4:* 30-58 (2006)
3. Simpson E.R., Zhao Y., Agarwal V.R., Michael M.D., Bulun S.E., Hinshelwood M.M., Graham-Lorence S., Sun T., Fisher C.R., Qin K., Mendelson M.D.: Aromatase expression in health and disease: Recent Prog. Horm. Res. 52: 185-213 (1997)
4. Diaz-Cruz E.S., Shpiro C.L., Brueggemeier R.W.: Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. J. Clin. Endocrinol. Metab. 90: 2563-2570 (2005)
5. Hiscox S., Davies E.L., Barrett-Lee P., Aromatase inhibitors in breast cancer. Maturitas. 63: 275-279 (2009)
6. Hardy D.B., Janowski B.A., Chen C-C., and Mendelson C.R.: Progesterone Receptor Inhibits Aromatase and Inflammatory Response Pathways in Breast Cancer Cells via Ligand-Dependent and Ligand-Independent Mechanisms. *Mol. Endocrinol. 22*: 1812–1824 (2008)
7. Srinivasan R., Karundevi B.: Corticosterone Induces Steroidogenic Lesion in Cultured Adult Rat Leydig Cells by Reducing the Expression of StAR Protein and Steroidogenic Enzymes. *J. Cell. Biochem. 103*: 1472-1487 (2008)

8. Watanabe M., Ohno S., Nakajin S.: Forskolin and dexamethasone synergistically induce aromatase (CYP19) expression in the human osteoblastic cell line SV-HFO. *Eur. J. Endocrinol. 152:* 619-624 (2005)
9. Yanase T., Mu Y-M., Nishi Y., Goto K., Nomura M., Okabe T., Takayanagi R., Nawata H.: Regulation of aromatase by nuclear receptors. *J, Steroid Biochem. & Mol. Biol. 79*: 187–192 (2008)
10. Watanabe M., Mariko Noda M., Nakajin S.: Effect of epidermal growth factor and prostaglandin on the expression of aromatase (CYP19) in human adrenocortical carcinoma cell line NCI-H295R cells. *J. Endocrinol. 188:* 59–68 (2006)
11. Kopelovich L., Fay J.R., Sigman C.C., Crowell J.A.: The Mammalian Target of Rapamycin Pathway as a Potential Target for Cancer Chemoprevention. *Cancer Epidemiol. Biomarkers Prev. 16:* 1330-40 (2007)
12. Davies G., L.-A. Martin L.-A., N. Sacks N., Dowsett M.: Cyclooxygenase-2 (COX-2), aromatase and breast cancer: a possible role for COX-2 inhibitors in breast cancer chemoprevention. *Ann. Oncol. 13:* 669–678 (2002)
13. Kim J.I., Han E.H., Kim H.G., Oh K.N, Kim S.K., Lee K.Y., Jeong H.G.: Bisphenol A-induced aromatase activation is mediated by cyclooxygenase-2 up-regulation in rat testicular Leydig cells. *Toxicol. Lett. 193:* 200–208 (2010)
14. Talbert D.R., Allred C.D., Zaytseva Y.Y., Kilgore M.W.: Transactivation of ERa by Rosiglitazone induces proliferation in breast cancer cells. *Breast Cancer Res. Treat. 108:* 23–33 (2008)
15. Yeb L., Chanb M.Y., Leung L.K.: The soy isoflavone genistein induces estrogen synthesis in an extragonadal pathway. *Mol. Cell. Endocrinol. 302*: 73-80 (2009)
16. Qiao Y., Jinyan Cao J., Liangqun Xie L., and Xiaolin Shi X.: Cell Growth Inhibition and Gene Expression Regulation by (-)-Epigallocatechin- 3-Gallate in Human Cervical Cancer Cells. *Arch. Pharm. Res. 32:* 1309-1315 (2009)
17. Sun M., Estrov Z., Ji Y., Coombes K.R., Harris D.H., Kurzrock R.: Curcumin (diferuloylmethane) alters the expression profiles of microRNAs in human pancreatic cancer cells. *Mol. Cancer Ther. 7:* 464 (2008)
18. Rice S., Pellatt L., Ramanathan K., Whitehead S.A., Mason H.D.: Metformin inhibits aromatase via an ERK (extracellular signal-regulated kinase) - mediated pathway. *Endocrinol. 150:* 4794–4801 (2009)
19. Sanderson J.T., Hordijk J., Denison M.S., Springsteel M.F., Nantz M.H., van den Berg M.: Induction and Inhibition of Aromatase (CYP19) Activity by Natural and Synthetic Flavonoid Compounds in H295R Human Adrenocortical Carcinoma Cells. *Toxicol. Sci. 82:* 70–79 (2004)
20. Deb S., Zhou J., Amin S.A., Imir A.G., Yilmaz M.B., Lin Z., Bulun S.E.: A Novel Role of Sodium Butyrate in the Regulation of Cancer-associated Aromatase promoters I.3 and II by Disrupting a Transcriptional Complex in Breast Adipose Fibroblasts. *J. Biol. Chem. 281*: 2585–2597 (2006)
21. Wong J.Y.Y, Huggins G.S., Debidda M., Munshi N.C., De Vivo I.: Dichloroacetate Induces Apoptosis in Endometrial Cancer Cells. *Gynecol. Oncol. 109:* 394–402 (2008)
22. Sanderson J.T., Boerma J., Lansbergen G.W.A., van den Berg M.: Induction and Inhibition of Aromatase (CYP19) Activity by Various Classes of Pesticides in H295R Human Adrenocortical Carcinoma Cells. *Toxicol. Appl. Pharmacol. 182:* 44–54, (2002)
23. Andersen H.R., Vinggaard A.M., Rasmussen T.H., Gjermandsen I.M., Bonefeld-Jørgensen E.C.: Effects of Currently Used Pesticides in Assays for Estrogenicity, Androgenicity, and Aromatase Activity in Vitro. *Toxicol. Appl. Pharmacol. 179:* 1–12 (2002)
24. Fan W., Yanase T., Morinaga H., Gondo S., Okabe T., Nomura M., Komatsu T., Morohashi K.I., Hayes T.B., Takayanagi R., Nawata H.: Atrazine-Induced Aromatase Expression Is SF-1 Dependent: Implications for Endocrine Disruption in Wildlife and Reproductive Cancers in Humans. *Environ Health Perspect. 115:* 720–727 (2007)

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The Induction of Apoptosis by Labd-14-ene-8,13-diol (Sclareol) and the Suppression of Tumour Growth of Human Colon Cancer Cells is p53-Independent

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SUMMARY. The labdane diterpene sclareol has demonstrated significant cytotoxicity against human tumor cell lines and colon cancer xenografts. However, there is further need to study the mode of action of this compound as very little information is known for the anticancer activity of sclareol and other labdane diterpenes, in general. Sclareol-induced cell cycle arrest and apoptosis were assessed by flow cytometry and Western blot analyses. Finally, the anticancer ability of sclareol *in vivo* was assessed by using human colon cancer xenograft/mouse models. Sclareol arrested further *in vitro* the growth of human colon cancer and induced apoptosis by activating both caspases-8 and -9 in p53-deficient ($HCT116^{p53-/-}$) colon cancer cells. Intraperitoneal administration of liposome-encapsulated sclareol at the maximum tolerated dose induced a marked growth suppression of $HCT116^{p53-/-}$ tumors established as xenografts in NOD/SCID mice. Concluding sclareol kills human tumor cells by inducing arrest at the G_1-phase of the cell cycle and apoptosis via a novel, as yet unknown, mechanism that involves activation of caspases-8, -9 and -3, and it is p53-independent. These findings further suggest that liposomes-encapsulated sclareol may possess chemotherapeutic potential for the treatment of colorectal and other types of human cancer regardless of the p53-status.

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Behavioural and Neurochemical Changes of Etoricoxib: A Possible Implication of Serotonin Receptors 5-$HT_{1A/2A}$

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S u m m a r y. Etoricoxib, a selective inhibitor of cyclooxygenase-2 (COX-2) has been found to provoke behavioural changes in the rat. In the present study, we examined the possible involvement of serotonin receptors (5-HT_{1A} and 5-HT_{2A}) on etoricoxib-induced effects on behaviour. Male Wistar rats were used in all experiments. Selective agonists and antagonists of 5-HT_{1A} and 5-HT_{2A} receptors were injected separately, or in combination with etoricoxib. Spontaneous activity was recorded for one hour and was found to be increased when etoricoxib was given alone. The levels of brain serotonin were also determined by HPLC and were found significantly increased after etoricoxib administration. The increased behavioural activity produced by etoricoxib was inhibited by either a selective 5-HT_{1A} antagonist or a 5-HT_{2A} agonist. Our results indicate that behavioural changes after etoricoxib treatment are mediated by central seroto-ninergic mechanisms.

INTRODUCTION

Cyclooxygenase is the key enzyme that converts arachidonic acid (AA) to prostaglandins (PGs) and exists in two isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Although COX-2 is induced by inflammation and cell proliferation, it is constitutively expressed in the rat brain predominantly, in discrete population of neurons (1).

So far, several studies have demonstrated an implication of serotonin receptors (5-HT_{1A}, 5-HT_{2A}) in fever, analgesia and inflammation (2-4). Mackowiak et al., (2002) also reported a co-localization of 5-HT_{2A} receptors and expression of cyclooxygenase-2 in cortical regions of the brain (5). In a previous work we demonstrated that etoricoxib (a cyclooxygenase-2 inhibitor) induced neurochemical changes and alterations on behavioural activity even at doses similar with those used in humans (6). Therefore, it was of interest to examine further a possible implication of specific serotonin receptors (5-HT_{1A}, 5-HT_{2A}) in the actions of etoricoxib.

MATERIALS AND METHODS

Male Wistar rats (3-months old) were included in all experimental protocols. A 5-HT_{1A} selective agonist (8-OH-DPAT) and antagonist (WAY-100135) were injected separately, at doses of 0.3 mg/kg and 1 mg/kg respectively, or in combination with etoricoxib (1 and 10 mg/kg). In another experimental protocol, a 5-HT_{2A} selective agonist (DOI) and antagonist (ketanserin) were injected separately, at doses of 0.3 mg/kg and 3 mg/kg respectively, or in combination with etoricoxib (1 and 10 mg/kg).

Spontaneous behaviour was recorded for one hour, using a computerized activity monitoring system (ENV515, Activity Monitor, version 5, Med. Associates, USA). At the end of experiment several brain tissues were removed for the evaluation of serotonin (5-HT) and dopamine (DA) levels in discrete brain regions. A High Pressure Liquid Chromatography (HPLC) method was used to determine the levels of brain neurotransmitters (7, 8).

RESULTS

Even though 8-OH-DPAT had no impact on the increased mobility observed after etoricoxib administration, the 5-HT_{1A} agonist completely inhibited the increased levels of serotonin after administration of etoricoxib, in all brain regions studied. On the other hand, WAY-100135 abolished the action of etoricoxib only on the vertical mobile activity while abolished the action of etoricoxib on

serotoninergic function in specific rat brain regions.

With regard to 5-HT_{2A} receptors, it appears that DOI inhibited the effects of etoricoxib on rat mobility as well as on the serotoninergic function in the brain regions included in the study. On the contrary, ketanserin had no effect on etoricoxib-induced changes on the rat mobility while inhibited partially the etoricoxib action on serotoninergic function.

CONCLUSIONS

Etoricoxib induced changes on the behaviour of the rat as well as on neurochemical parameters of the brain, mainly referring to the serotoninergic transmission. Further investigation on the action of etoricoxib revealed an involvement of the serotoninergic receptors 5-HT_{1A} and 5-HT_{2A}, of which the 5-HT_{2A} seems to have a more important role. The involvement of 5-HT_{2A} in the expression of cyclooxygenase-2 in the brain (5) is also supported by our results. The effects of etoricoxib on the serotoninergic system in the rat brain indicate a possible interaction of this drug with central prostaglandins and other inflam-mation factors. This phenomenon needs to be further investigated.

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REFERENCES

1. Yamagata K., Andreasson K.I., Kaufmann W.E., Barnes C.A., Warley P.F.: Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron 11*: 371-386 (1993)
2. Blessing W.W.: 5-Hydroxytryptamine 1A receptor activation reduces cutaneous vasoconstriction and fever associated with the acute inflammatory response in rabbits. *Neuroscience 123*: 1-4 (2004)
3. Sufka K.J., Schomburg F.M., Giordano J.: Receptor mediation of 5-HT-induced inflammation and nociception in rats. *Pharmacol. Biochem. Behan. 41*: 53-56 (1992)
4. Sadrini M.L., Vitale G., Pini L.A.: Effect of rofecoxib on nociception and serotonin system in the rat brain. *Inflamm. Res. 51*: 154-159 (2002)
5. Mackowiak M., Chocyk A., Sanak M., Czyrak A., Fizal K., Wedzony K.: DOI, an agonist of 5-$HT_{2A/2C}$ serotonin receptor, alterss the expression of cyclooxygenace-2 in the rat parietal cortex. *J. Physiol. Pharmacol. 53*: 395-407 (2002)
6. Nikolaidou M., Pappas P., Antoniou K., Marselos M.: Effects of a selective cyclooxygenase inhibitor on behavioural and neurochemical parameters. *Rev. Clin. Pharmacol. Pharmacokinet. 22*: 263 (2008)
7. Sharp T., Zetterström T., Series H.G., Carlsson A.,A., Grahame-Smith D.G., Ungerstedt U.: HPLC-EC analysis of catechols and indoles in rat brain dialysates. *Life Sci. 41*: 869-872 (1987)
8. Dalla C., Antoniou K., Kokras N., Drossopoulou G., Papathanasiou G., Bekris S., Daskas S., Papadopoulou-Daifoti Z.: Sex differences in the effects of two stress paradigms on dopaminergic neurotransmission. *Physiol Behav. 93*: 595-605 (2008)

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Immunohistochemical Study of Steroid Receptors in Human Ovary

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Key words: Estrogen receptors, progesterone receptors, human ovary, granulosa cells, theca cells, ovarian stroma

S u m m a r y. The immunohistochemical technique of abidin-biotin was used for the detection of estrogen and progesterone receptors in normal and cystic human ovaries. Cystic ovaries present differences from normal ovaries in the expression of estrogen and progesterone receptors.

INTRODUCTION

In the past it was thought that estrogens do not play an important role in follicular maturation, as estrogen receptors (ER) had not been detected in theca (TC) and granulosa cells (GC) of the follicle. After the identification of a second ER (ERβ), its predominance in human ovary was established; but most studies failed to localize ERα in human ovaries. Data concerning the presence of ERα in human ovary are rather controversial and there are no studies concerning the influence of ovarian cysts in the expression of ERs in human ovarian cells and in the development of ovarian follicles.

Concerning progesterone (PRG), its role in the maintenance of pregnancy is well established, but only recently it was suspected that PRG may play a role in the development of ovarian follicles. Studies on the bovine and primate ovary showed that progesterone receptors (PR) were present not only in luteal cells but also in other cells of the ovary, supporting an autocrine/paracrine role of progesterone in the ovary. There are also few studies with controversial results concerning the presence of PR in human ovarian cells. But there are no studies concerning the influence of ovarian cysts in the expression of PR in human ovarian cells and in the development of ovarian follicles.

In this study we tried to distinguish differences in the expression of ERα and PR in normal and cystic human ovaries.

METHODS

27 ovaries obtained from 20 patients of reproductive age undergoing ovariectomy were tested. The cause of ovariectomy was the presence of uterine leiomyomata and ovarian cysts. The immunohistochemical technique of abidin-biotin was used. ERs and PRs were immunolocalized using a mouse monoclonal antibody directed against the A/B domain of the receptors (Novocastra, UK).

RESULTS

Localization of ER

ERα were immunolocalized in GC of preovulatory follicles in all samples tested but the expression of ERs varied greatly. Half of the samples of normal ovaries were immunopositive and half of them immunonegative for ERα in TC. All samples of cystic ovaries presented ERα in TC. Ovarian stroma cells were stained strongly positive in normal ovaries and moderately positive in cystic ovaries. ERα were also expressed in preantral follicles of all samples.

Localization of PR

PR were immunolocalized in GC and TC of all samples obtained from cystic ovaries but not in all samples obtained from normal ovaries. GC were stained moderately positive in most samples from normal or cystic ovaries. TC cells were stained moderately positive in most samples from normal ovaries but slightly positive in most samples from cystic ovaries. Ovarian stroma cells were stained positive in all samples and the

strength of staining was moderate in most samples either from normal or from cystic ovaries. Preantral follicles were stained moderately positive in normal ovaries but the strength of staining was diverse in cystic ovaries.

A characteristic photograph is depicted in Figure 1.

DISCUSSION

According to our results, ERα were present in GC of antral and preantral follicles and in ovarian stroma cells. TC expressed ERα in half of the cases, and this may reflect their transient presence during the follicular cycle. Ovarian stroma cells, which were stained strongly positive, may play a supportive role in follicular growth and maturation. According to our results, estrogens seem to play a local role in human ovary, not only through ERβ but also through ERα.

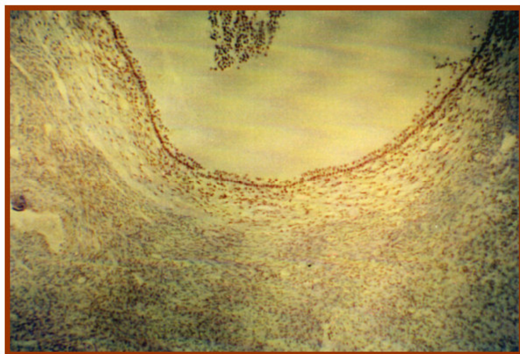

Figure 1. Progesterone receptors in human ovary (Magnification x107): A moderate staining in GC, in TI and in ovarian stroma cells is observed

Cystic ovaries presented stronger staining for the presence of ERα in TC and weaker staining in ovarian stroma cells than normal ovaries. The presence of ER in human ovarian cells reflects a role of estrogens in follicular maturation. This role may be differentiated in presence of ovarian cysts.

Progesterone plays a central coordinating role in regulating reproductive events associated with the establishment and maintenance of pregnancy, including ovulation, uterine and mammary gland development and tumorigenesis, as well as neurobehavioral expression associated with sexual responsiveness. But only recently it was suspected that it may play a role in ovarian folliculogenesis.

In our study there was no difference in the immunolocalization of PR in ovarian stromal cells of normal and cystic ovaries. PR may be expressed transiently in GC and TC of the developing antral follicles of normal ovaries but show permanent presence in cystic ovaries. The staining for the presence of PRs was lighter in TC and showed greater diversity in preantral follicles of cystic ovaries. These differences in the expression of PR in the follicular cells of cystic ovaries probably imply that the presence of simple ovarian cysts may influence the development of ovarian follicles.

In conclusion, cystic ovaries present differences from normal ovaries in staining for the presence of ERα and PR. The presence of ER and PR in human ovarian cells reflects a possible role of estrogens and progesterone in follicle development. This role may be differentiated in presence of ovarian cysts.

REFERENCES

1. Byers M., Kuiper G.G.J.M., Gustafsson J.A., Park-Sarge O.K.: Estrogen receptor-β mRNA expression in rat ovary: down regulation by gonadotropins. *Mol. Endocrinol. 11*: 172-182 (1997)
2. Chaffkin L.M., Luciano A.A., Peluso J.J.: Progesterone as an autocrine/paracrine regulator of human granulosa cell proliferation. *J. Clin. Endocrinol. Metab. 75*: 1404-1408 (1992)
3. Chandrasekher Y.A., Melner M.H., Nagalla S.R., et al.: Progesterone receptor but not estradiol receptor, messenger ribonucleic acid is expressed in luteinizing granulosa cells and the corpus luteum in rhesus monkeys. *Endocrinology 135*: 307-314 (1994)
4. Couse J.F., Lindzey J., Grandien K., Gustafsson J.A., Korach K.S.: Tissue distribution and quantitative analysis of estrogen receptor α (ERα) and estrogen receptor β (ERβ) messenger ribonucleic acid in the wild-type and ERβ Knockout mouse. *Endocrinology 138*: 4613-4621 (1997)
5. Drummond A.E., Findlay J.K.: The role of estrogen in folliculogenesis. *Mol. Cell. Endocrinol. 151*: 57-64 (1999)
6. Economou L., Papamitsou T., Papaioannidou P., Manthos A., Alvanou A., Miliaras D.: Estrogen receptors alpha are present in human ovarian cells. *Fertil. Steril. 78*: S163-S164 (2002)
7. Hild-Petito S., Stouffer R.L., Brenner R.M.: Immunocytochemical logalization of estradiol and progesterone receptors in the monkey ovary throughout the menstrual cycle. *Endocrinology 123*: 2896-2905 (1989)
8. Hurst Bradley S., Leslie Kimberly K.: Uses of plasmid technologies 1. The role of oestrogen in follicular development. *Mol. Human Peprod. 3*: 643-645 (1997)
9. Isola J., Korte J.-M., Tuohimaa P.: Immunocytochemical localization of progesterone receptor in the chick ovary. *Endocrinology 121*: 1034-1040 (1987)
10. Iwai T., Nanbu Y., Iwai M., Fujii S., Mori T.: Immunohistochemical localization of oestrogen receptors and progesterone receptors in the human ovary throughout the menstrual cycle. *Virchow Arch. Pathol. Anat. 417*: 369-375 (1990)
11. Khan M.S., Dodson A.R., Heatley M.K.: Ki-67, oestrogen receptor, and progesterone receptor protein human rate ovarii and in endometriosis. *J. Clin. Pathol. 52*: 517-520 (1999)

12. Kuiper G.G.J.M., Enmark E., Pelto-Hnikko M., Nilsson S., Gustafson J.-A.: Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA 93*: 5925-5930 (1996)

13. Kuipper G.G.J.M., Carlsson B., Grandien K., EnMark E., Haggblad J., Nilsson S., Gustafsson J.-A.: Comparison of the ligand binding specificity and transcipt tissue distribution of estrogen receptors alpha and beta. *Endocrinolog 138*: 863-870 (1997)

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Safety/Tolerability of $\alpha_2\delta$ Ligand Pregabalin in the Treatment of Fibromyalgia: An Evidence Based Evaluation

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Key words: Pregabalin, fibromyalgia, safety, tolerability, meta-analysis

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S u m m a r y. $\alpha_2\delta$ ligands gabapentin and pregabalin are approved for the treatment of pain from diabetic neuropathy and post-herpetic neuralgia in adults and as adjunctive therapy of partial seizures in children and pregabalin has been also approved for treatment of anxiety disorders. Fibromyalgia (FBM) is a common chronic pain disorder affecting up to 2% of the general population. Aim of our study was to estimate the safety and the tolerability of pregabalin in the treatment of fibromyalgia and for that purpose a systematic review of relevant randomized double-blind placebo-controlled trials was performed.

INTRODUCTION

Fibromyalgia (FBM) is a chronic pain condition. It is usually accompanied by other symptoms like fatigue, sleep disturbance, anxiety and depresssion. Recently, pregabalin (Lyrica, Pfizer) has been approved for the treatment of FBM. A recent meta-analysis clearly indicated its efficacy in the treatment of FBM (1).

Pregabalin is an $\alpha_2\delta$ ligand, related structurally to neurotransmitter c-aminobutyric acid GABA. Pregabalin appears to be a promising drug for many chronic diseases like neuropathic pain in spinal cord injury, hot flashes in women with natural or tamoxifen-induced menopause and essential tremor (2-4).

Since FBM is a chronic relapsing disorder, safety/tolerability issues are of high importance. Adverse effects could affect adhesion to therapy or lead to withdrawals and dropouts, thus reducing efficacy.

In order to efficiently integrate valid information about safety/tolerability profile of pregabalin in FBM treatment, the literature was systematically reviewed and a meta-analysis of all randomized controlled trials was conducted.

METHODS

Search strategy

Main search was conducted in the electronic databases MEDLINE, EMBASE, PubMed, CINAHL and Cochrane Central Register of Controlled Trials (CENTRAL), using the terms *fibromyalgia* and *pregabalin*. The main search as well as screening of titles was completed independently by two reviewers.

Eligibility of relevant studies

Eligible studies for the meta-analysis were randomized double-blind controlled trials, which compared the efficacy of GP or PB to placebo in the treatment of FBM.

Data extraction

Information from each study was extracted independently by two reviewers using a standardized data extraction form. Study general characteristics, methodology and outcomes for both intervention and control groups were recorded, where available, and double-checked.

Statistical analysis

Safety / tolerability profile was assessed on the basis of the adverse events and the percentage

of dropouts due to them. Comparison of the incidence of adverse events and dropouts due to then between treatment and control group were expressed as odds ratio (OR) with 95% confidence interval (CI). To further evaluate safety / tolerability profile, Number Needed to Harm (NNH were calculated. Meta-analysis was conducted using REVIEW MANAGER 4.2 software (REVMAN).

RESULTS

From the 48 publications initially identified, 3 RCTs were included in the meta-analysis (5-7).

Safety/tolerability of pregabalin 300, 450 and 600mg/day

Dropouts due to side effects had a higher incidence in pregabalin groups compared to control group. For 300 mg/day, OR (95% CI) = 1.65 (1.12 to 2.42), P = 0.01. For 450 mg/day, OR (95% CI) = 2.28 (1.58 to 3.29), $P < 10^{-4}$. For 600 mg/day, OR (95% CI) = 3.57 (2.4 to 5.31), $P < 10^{-5}$. NNH for 300, 450 and 600 mg/day were 10, 11 and 6, respectively. Dizziness, somnolence, dry mouth and weight gain were found significantly higher in all treatment groups compared to control group. Dizziness and somnolence were the most frequent side effects of pregabalin treatment. Dizziness risk was for 600 mg/day, OR (95% CI) = 9.1 (5.95 to 13.92), $P < 10^{-5}$, NNH=3, for 450 mg/day, OR (95% CI) = 7.92 (5.54 to 11.32), $P < 10^{-5}$, NNH=3 and for 300mg/day, OR (95% CI) = 4.60 (3.20 to 6.61), $P < 10^{-5}$, NNH=5. Somnolence risk was for 600mg/day, OR (95% CI) = 7.0 (4.08 to 12.02), $P < 10^{-5}$, NNH=5, for 450mg/day, OR (95%CI) = 6.43 (4.03 to 10.26), $P < 10^{-5}$, NNH=6 and for 300mg/day, OR (95%CI) = 5.17 (3.22 to 8.29), $P < 10^{-5}$, NNH=7.

DISCUSSION

Overall, pregabalin was well tolerated with no serious side effects. Most side effects were of mild to moderate severity. The most common side effects and most frequent reasons for dropouts were dizziness and somnolence. Adverse events' incidence was dose related. This study also indicates that titration period is of great importance. Longer titration periods are correlated with better tolerability. Recommendations for future studies should include assessment of the best schema employed and of ideal titration period for pregabalin in order to reduce side effects and maximize efficacy and tolerability.

REFERENCES

1. Tzellos T., Toulis K., Goulis D., Papazisis G., Zampeli V., Vakfari A., Kouvelas D.: Gabapentin and pregabalin in the treatment of fibromyalgia: a systematic review and a meta-analysis. *J. Clin. Pharm. Ther.* 2010. In press.
2. Tzellos T.G., Papazisis G., Toulis K.A., Sardeli C., Kouvelas D.: A2δ ligands gabapentin and pregabalin: future implications in daily clinical practice. *Hippokratia 2010*. In press.
3. Tzellos T.G., Papazisis G., Amaniti E., Kouvelas D.: Efficacy of pregabalin and gabapentin for neuropathic pain in spinal-cord injury: an evidence-based evaluation of the literature. *Eur. J. Clin. Pharmacol. 64*: 851-858 (2008)
4. Toulis K.A., Tzellos T., Kouvelas D., Goulis D.G.: Gabapentin for the treatment of hot flashes in women with natural or tamoxifen-induced menopause: a systematic review and meta-analysis. *Clin. Ther. 31*: 221-235 (2009)
5. Crofford L.J., Rowbotham M.C., Mease P.J., et al.: Pregabalin for the treatment of fibromyalgia syndrome: results of a randomized, double-blind, placebo-controlled trial. *Arthritis Rheum. 52*: 1264-1273 (2005)
6. Mease P.J., Russell I.J., Arnold L.M., Florian H., Young J.P.,Jr, Martin S.A., Sharma U. A randomized, double-blind, placebo-controlled, phase III trial of pregabalin in the treatment of patients with fibromyalgia. *J. Rheumatol. 35*: 502-514 (2008)
7. Arnold L.M., Russell I.J., Diri E.W., et al.: A 14-week, randomized, double-blinded, placebo-controlled monotherapy trial of pregabalin in patients with fibromyalgia. *J. Pain 9*: 792-805 (2008)

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Estrogen and Progesterone Receptors are Present in Rat Fetal Cartilage Tissue

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Key words: Estrogen receptors, progesterone receptors, cartilage tissue, chondrocyte, rat, fetus

S u m m a r y. The immunohistochemical technique of abidin-biotin was used for the detection of estrogen and progesterone receptors in muscle cells and in the chondrocytes of the maturing epiphyseal growth plate in the femur and tibia of Wistar rat fetuses at the late stage of fetal life. Negative nuclear immunohisto-chemical staining was observed in rat fetal muscle tissues. Estrogen receptors alpha and progesterone receptors beta were immunolocalized in resting, proliferating and hypertrophic zones of cartilage tissue.

INTRODUCTION

The last years it was observed that estrogen and progesterone receptors are present in tissues other than the reproductive ones (1-4). In rat, estrogen and progesterone receptors were localized in various tissues (5,6). ERα has been predominantly found in anterior pituitary, uterus, vagina, testis, liver and kidney, while ERβ is predominant in thyroid, ovary, prostate, skin, bladder, lungs, gastro-intestinal tract, cartilage and bone (4-6). But there is no study investi-gating the presence of estrogen and progesterone receptors in rat fetal tissues.

The aim of this study was to investigate the existence of estrogen and progesterone recaptors in muscle and cartilage tissue of the rat, at the late stage of fetal life.

METHODS

The immunohistochemical technique of abidin-biotin was used. To increase ER and PR detection sensitivity, the ovarian sections were subjected to microwave irradiation. ERs and PRs were immunolocalized using spesific mouse monoclonal antibodies directed against the A/B domain of the receptors (Novocastra, UK).

RESULTS

Negative nuclear immunohistochemical staining for the presence of estrogen and progesterone receptors was observed in rat fetal muscle tissues. The staining was positive to both estrogen and progesterone receptors in the chondrocytes of the maturing epiphyseal growth plate in the femur and tibia of Wistar rat fetuses at the late stage of embryogenesis. The staining was positive in resting, proliferating and hypertrophic cells. A variety in the strength of staining was observed in resting, proliferating and hypertrophic zones of cartilage tissue.

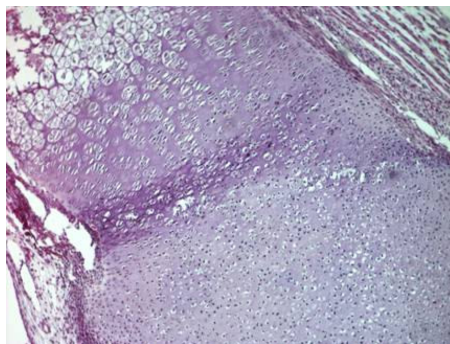

Figure 1. Simple hematoxylin – eosin staining in the chondrocytes of the maturing epiphyseal growth plate in tibia of Wistar rat fetus. Cells of resting, proliferating and hypertrophic zones of cartilage tissue can be observed

Characteristic photographs of the immunohistochemical preparations are presented in figures 1-3.

DISCUSSION

Estrogens and progesterone play a central role in regulating reproductive events associated with the establishment and maintenance of pregnancy (7); they are implicated in ovulation, uterine and mammary gland development and tumorigenesis, and in neurobehavioral expression associated with sexual responsiveness (7). Recently it was also suspected that they may play a role in ovarian folliculogenesis (8-14).

Estrogens and progesterone have important biological effects (15-18) and their receptors have been localized in various tissues, including cartilage and bone (1-6,19-21), but there are no reports on their presence in rat fetal tissues. To our knowledge, our study is the first study that localized estrogen and progesterone receptors in cartilage tissue of rat fetus at the late stage of endometrial development.

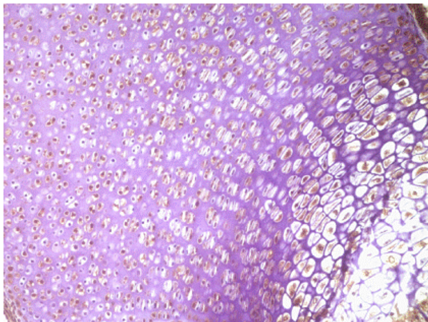

Figure 2. Staining for nuclear estrogen receptors in the chondrocytes of the maturing epiphyseal growth plate in tibia of Wistar rat fetus. Positive staining in the cells of resting, proliferating and hypertrophic zones of cartilage tissue can be observed

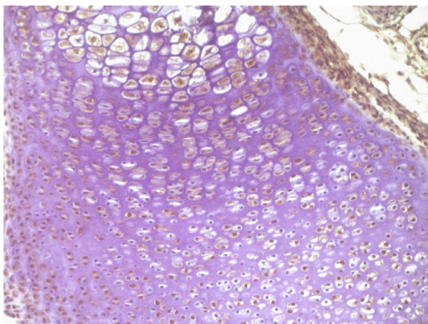

Figure 3. Staining for nuclear progesterone receptors in the chondrocytes of the maturing epiphyseal growth plate in tibia of Wistar rat fetus. Positive staining in the cells of resting, proliferating and hypertrophic zones of cartilage tissue can be observed

REFERENCES

1. Couse J.F., Lindzey J., Grandien K., Gustafsson J.A., Korach K.S.: Tissue distribution and quantitative analysis of estrogen receptor a (ERa) and estrogen receptor b (ERb) messenger ribonucleic acid in the wild-type and ERb knockout mouse. *Endocrinology 138*: 4613-4621 (1997)
2. Kuipper G.G.J.M., Carlsson B., Grandien K., EnMark E., Haggblad J., Nilsson S., Gustafsson J.-A.: Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology 138*: 863-870 (1997)
3. Passanen S., Ylikomi T., Hsyvala Heimo, Pentti Tuohimaa: Distribution of progesterone receptor in chicken: novel target organs for progesterone and estrogen action. *Mol. Cell. Endocrinol. 135*: 79-91 (1997)
4. Taylor A.H., Al Azzawi F.: Immunolocalisation of estrogen receptor beta in human. *J. Mol. Endocrinol. 24*: 145-155 (2000)
5. Saunders P.T.K., Maguire S.M., Gaughan J., Millar M.R.: Expression of oestrogen receptor beta (ERb) in multiple rat tissues visualized by immunohistochemistry. *J. Endocrinol. 154*: 13-16 (1997)
6. Pelletier G.: Localization of androgen and estrogen receptors in rat and primate tissues. *Histol. Histopathol. 15*: 1261-1270 (2000)
7. Warner M., Nilsson S., Gustafsson J.A.: The estrogen receptor family: Curr. Opin. Obstet. Gynecol. 11: 249-254 (1999)
8. Drummond A.E., Findlay J.K.: The role of estrogen in folliculogenesis. *Mol. Cell. Endocrinol. 151*: 57-64 (1999)
9. Khan M.S., Dodson A.R., Heatley M.K.: Ki-67, oestrogen receptor, and progesterone receptor protein human rate ovarii and in endometriosis. *J. Clin. Pathol. 52*: 517-520 (1999)
10. Kuiper G.G.J.M., Enmark E., Pelto-Hnikko M., Nilsson S., Gustafson J.-A.: Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA 93*: 5925-5930 (1996)
11. Posenfeld C.S., Yuan X., Manikkam M., Calder M.D., Garverick H.A.: DB: Cloning, sequencing and localization of bovine estrogen beta within the ovarian follicle. *Biol. Reprod. 60*: 691-697 (1999)
12. Sar Madhabananda, Welsch Frank: Differential expression of estrogen receptor-b and estrogen receptor-a in the rat ovary. *Endocrinology 140*: 963-971 (1999)
13. Tomanek M., Pisselet C., Monget P., Madigou T., Thieulant M.L., Monniaux D.: Estrogen receptor protein and mRNA expression in the ovary of sheep. *Mol. Reprod. Dev. 48*: 53-62 (1997)
14. Wu T.C.J., Wang L., Wan Y.J.Y.: Detection of estrogen receptor messenger ribonucleic acid in human oocytes and cumulus-oocyte complexes using reverse transcriptase-polymerase chain reaction. *Fertil. Steril. 59*: 54-59 (1993)
15. Karakiulakis G., Thomas P.J., Papaioannidou P.G., et al.: The effects of endogenous and exogenous sex-hormones and of maturation on cGMP phosphodiesterase activity in the brain. *Meth. Find. Exp. Clin Pharmacol. 6*: 437-443 (1984)
16. Papaioannidou P., Kyriakidis I., Pantelidou V., et al.: Sexual dimorphism of second to fourth digit ratio (2D:4D) in Greek population. Proceedings of the 8th Congress of EACPT. Medimond Srl 2007: 229-233

17. Kyriakidis I., Papaioannidou P.: Epidemiologic Study of the Sexually Dimorphic Second to Fourth Digit Ratio (2D:4D) and Other Finger Ratios in Greek Population. *Coll. Antropol. 32*: 1093-1098 (2008)
18. Papaioannidou P., Kyriakidis I.: 2D:4D ratio is higher in older people in Greece. May this be attributed to environmental influence? *Pharmacoepidemiol. Drug Safety 17*: S261-S262 (2008)
19. Papamitsou T., Dermentzopoulou-Theodoridou M., Papaioannidou P., Barlayannis D., Manthos A.: Expression of AR in the adult rat testis after an every day treatment with testosterone enanthate. Proceedings of the 13th World Congress on In Vitro Fertilization, Assisted Reproduction & Genetics. Medimond Srl pp. 27-30, 2005
20. Economou L., Papamitsou T., Papaioannidou P., Manthos A., Alvanou A., Miliaras D.: Estrogen receptors alpha are present in human ovarian cells. *Fertil. Steril. 78*: S163-S164 (2002)
21. Oshima Y., Matsuda K., Yoshida A., Watanabe N., Kawata M., Kubo T.: Localization of Estrogen Receptors alpha and beta in the Articular Surface of the Rat Femur. *Acta Histochem. Cytochem. 40*: 27-34 (2007)

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Regulation of Pathologic Homeostasis of Hyaluronic Acid in Airway Smooth Muscle Cells from Patients with Asthma by Corticosteroids and β_2 agonists: Therapeutic Relevance

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Key words: Hyaluronic acid, asthma, corticosteroids, beta2 agonists, airway smooth muscle cells

INTRODUCTION

Asthma is characterized by reduced lung tissue flexibility which is mainly controlled by extracellular matrix molecules including glycosaminoglycans such as hyaluronic acid (HA). We have recently shown that airway smooth muscle cells (ASMC) from patients with asthma exhibit reduced synthesis and secretion of HA, as compared to ASMC from healthy donors. In this study, we investigated the effect of corticosteroids and β_2-agonists on HA homeostasis by ASMC.

METHODS

Primary cultures of ASMC were established from dissected airway muscle bundles obtained from isolated bronchi of control subjects (organ donors), or from endobronchial biopsies of patients with asthma. Cells were incubated with corticosteroids (budesonide), long acting beta2 agonists (LABA) (formoterol) or their combination. HA secretion was measured by ELISA while gene and protein expression for HA synthases (HAS), hyaluronidases (HYAL) and the HA receptors CD44 and RHAMM was assessed by real time PCR and western blotting, respectively.

RESULTS

In diseased and healthy ASMC budesonide and formoterol significantly induced HA secretion and their combination showed a synergistic effect. The latter effect was associated with a significant increase in HAS-1 and a significant decrease in HYAl-1 gene and protein expression. Formoterol but not budesonide induced gene expression of the HA receptor CD44. These effects were more prominent for ASMC from patients with asthma, as compared to healthy donors.

CONCLUSION

Our results indicate a novel mechanism underlying the beneficial effect of combined corticosteroids and LABA by regulating the abnormal HA turnover associated with inflammatory lung diseases.

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Association Study of the Single Nucleotide Polymorphism (SNP), rs6983267, at Region 3 of Chromosome 8q24, with Prostate Cancer in the Greek Population

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Key words: genetic variants, prostate cancer, chr8q24 and SNPs, prevalence of rs6983267.

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S u m m a r y. Recently, common variants on human chromosome 8q24 were found to be associated with prostate cancer risk. While conducting an association study at the Greek population to investigate the frequency and the susceptibility of one SNP, rs6983267, located at the region 3 of chromosome 8q24, to the prostate cancer, we found highly significant correlation (Odds ratio=2.83 and p-value=0.002). Comparing the findings to other studies, conducted in northern Europeans, the population attributable risk (PAR%) and the frequency of the at risk allele (G vs T) were higher at the Greek population with an independent risk for the carriers to develop the disease.

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer in men and represents the second most common cause of cancer-related death in men in the European Union (1). The only firmly established risk factors for prostate cancer are age, family history of prostate cancer and ethnicity (2). Men older than 65 y, with a first degree relative with the disease and of African descent are at greater risk than those with no family history and of European descent. African Americans are 1.5-2 times more likely to develop prostate cancer, and 2.4-3 times more likely to die from this disease, than European Americans (3). Genetic factors probably con-tribute to such differences, along with a combination of environmental factors.

In men with European ancestry the locus, marked by rs6983267 at the region 3 of chromosome 8q24, has showed the higher odds ratio and PAR (population attributable risk) with an overall population frequency in northern Europeans of 50% for the at risk allele (6, 11). We examined the frequency of this SNP (single nucleotide polymorphism) in the Greek population in a prostate cancer case-control study to conclude whether it is significant or not.

MATERIALS AND METHODS

Study subject

A total of 109 patients with prostate cancer participated in the study. They were identified and recruited from the General Hospital of Athens *Laiko* (n=68) and from the General *Hospital* of Athens George *Gennimatas* (n=41) in Greece, with an age distribution between 48-87 years. The inclusion criterion for case subjects was biopsy-confirmed adenocarcinoma of the prostate cancer. Whole blood samples, tumor-node-metastasis (TNM) stage, Gleason score as determine by biopsy, and levels of prostate-specific antigen (PSA) at diagnosis were available for all the patients. Case subjects were classified as having advanced (A) disease if they met any of the following criteria: a grade 3 or 4 tumor, spread to nearby lymph nodes and metastasis, a Gleason score ≥8, or a PSA level ≥50 ng/ml, otherwise, subjects were classified as having localized disease (L).

Control subjects (total number 99) were randomly selected from The General Hospital of Attica *KAT*. They were all healthy individuals, without any kind of cancer-history, and had PSA levels <4 ng/ml.

Genotyping of SNP rs6983267

DNA extraction from total blood samples was performed with the Wizard® Genomic DNA Purification Kit (Promega, USA). The analysis of rs6983267, was done using a real time PCR platform Lightcycler® 480, Roche

Diagnostics. Extension primers and oligonu-cleotide markers (probes) were specifically designed by TIB MOLBIOL (Berlin, Germany).

Statistical Analysis

The power of the statistical analysis was estimated with the method of Cohen (14). Differences in allele frequencies between case subjects and control subjects were tested with the use of a chi-square test with 1 degree of freedom and allelic odds ratios and 95% confidence intervals were estimated. For genotypes, a series of tests assuming a co-dominant, dominant, or recessive genetic model were performed with the use of unconditional logistic regression with adjustment for age. Association of the SNP with aggressiveness of prostate cancer (advanced or localized), gleason score, TNM stages and family history (yes or no) were tested only among case subjects with the use of a chi-square test of a 2×K table, in which K is the number of possible categories within each variable. A test for trend was used to assess the proportion of genotypes associated with prostate cancer with the two age group >65y and ≤65y. Association of SNP with the mean age at diagnosis were tested only among case subjects with the use of a two-sample t-test. Because serum PSA levels were not normally distributed, a nonparametric analysis (Wilcoxon rank-sum test) was used to assess the association between SNP and preoperative serum PSA levels in case subjects. All reported p values are based on a two-sided test.

RESULTS

The evaluation of rs6983267 at region 3 of chromosome 8q24 revealed significantly different frequencies (p value <0.05) in genotypes and in alleles between prostate cancer cases and control subjects (Table 1).

Table 1
Odds Ratio for Genotype and Alleles (T: wild type allele, G: mutant allele)

Genotype	*Odds Ratio*	*95% C.L.*	*p*
TTvsGT+GG	2.83	1.38-6.00	0.002
Allele T vs G	2.06	1.33-3.02	0.001

The use of unconditional logistic regression with adjustment for age indicated that the best fitting inheritance model is the dominant model, which showed the highest likelihood and the best significance (Table 2).

Table 2
Best fitting inheritance model

Inheritance model	*Odds Ratio for SNP*	*95% C.L.*	*P*	*Log-likelihood*
Dominant	2.82	1.43-5.56	0.003	-122.361
Recessive	2.52	1.19-5.34	0.016	-124.112
Co-dominant	1.28	0.71-2.28	0.410	-126.794

Table 3
Adjusted Odds Ratios and Population attributable Risks for rs6983267

	Frequency of Associated Factors		*Odds Ratio (95% C.L.)*	*p*	*PAR%*
	Cases	*Controls*			
Age			1.02 (0.985-1.049)	0.308	
Family History	0.19	0.09			9.89
rs6983267 GT+GG	0.81	0.61	2.82 (1.431-5.557)	0.003	37.42

Adjusted Odds Ratios and Population attributable Risks for rs6983267 was calculated using multivariate logistic regression model (Table 3). In order to combine several risks such as men carrying the rs6983267 and have positive family history, we estimated the joint population attributable risk, joint PAR=43.61%, with the use of the following equation: jointPAR%=1 – [Πi(1 – PARi)]. For the last calculation we used the information of PAR% for family history from the association study of the Sweden population (11).

Finally, we investigated the association of the presence of rs6983267 with the aggressiveness of the disease and none of the clinical characteristics in case subjects were significantly associated with the rs6983267 (Table 4).

Table 4
Association of the SNP with Clinical Characteristics in case subjects

Variable	*Total No of Sujects*	*Reference TT Cases/frequency*	*Associated GT+GG Cases/frequency*	*p value*
Aggressiveness of disease – no (%)				
Localized	59	12 (20.34)	47 (79.66)	0.755
Aggressive	27	4 (14.82)	23 (85.18)	
Gleason score – no (%)				
≤4	1	0 (0)	1 (100)	0.357
6	34	8 (23.53)	26 (76.47)	
7	35	5 (14.29)	30 (85.71)	
8	5	1 (20.00)	4 (80.00)	
9	3	1 (33.33)	2 (66.67)	
10	1	1 (100)	0 (0)	
Biopsy – no (%)				
T1	1	1 (100)	0 (0)	0.281
T2	50	10 (20.00)	40 (80.00)	
T3	17	2 (11.76)	15 (88.24)	
T4	5	1 (20.00)	4 (80.00)	
Tx	13	2 (15.38)	11 (84.62)	
Family History – no (%)				
NO	59	10 (16.95)	49 (83.05)	0.538
YES	14	4 (28.57)	10 (71.43)	
Median PSA level		9.00	8.05	0.833
Mean Age at diagnosis		68.50	67.21	0.307
Age – no (%)				
>65y	56	13 (23.21)	43 (76.79)	0.226
≤65y	30	3 (10.00)	27(90.00)	

DISCUSSION

In genomewide studies, multiple chromosomal loci at 8q24 have been associated with prostate cancer (4-11). The highly significant findings in our study confirm the association of rs6983267 at region 3 of 8q24 with prostate cancer in the Greek population and indicate the independent risk for carriers to develop the disease. This risk, has probably, a cumulative effect with positive family history and with other chromosomal regions reported in the literature, which supports the validity of genetic association studies in complex diseases such as cancer. In our study the PAR% for the Greek population is 37.42% and the frequency for the G allele is 61.85%, which indicates the greater significance of this SNP to our population.

The mechanism by which rs6983267 affects the risk of prostate cancer is not known yet. However, no correlation between SNPs located in the c-MYC gene and SNPs located generally in the chromosomal region 8q24 and especially in the unknown gene has been reported (5). Nevertheless, it is possible that the risk variants identified in the chromosomal region 8q24 modify c-MYC regulation by predisposing to genomic instability or altering long-range regulation of expression.

REFERENCES

1. Fitzpatrick J.M.: Optimizing the management of prostate diseases: prostate cancer. *BJU International 101(sup2)*: 1 (2008)

2. Crawford, E.D. Epidemiology of prostate cancer. *Urology 62*: 3-12 (2003)

3. NCI SEER Program, Ries LAG et al editors: Cancer of the Prostate (Invasive) SEER Incidence and U.S. Death Rates, Age-Adjusted and Age-Specific Rates, by Race 2002-2006 Annual SEER Cancer Statistics Review (CSR), 1975-2006

4. Xu J., Dimitrov L., Chang B.-L., Adams T.S., Turner A.R., Meyers D.A., et al.: A combined genomewide linkage scan of 1,233 families for prostate cancer-susceptibility genes conducted by the international consortium for prostate cancer genetics. *Am. J. Hum. Genet. 77*: 219-229 (2005)

5. Amundadottir L.T., Sulem P., Gudmundsson J., et al.: A common variant associated with prostate cancer in European and African populations. *Nat. Genet. 38*: 652-658 (2006)

6. Yeager M., Orr N., Hayes R.B., et al.: Genome-wide association study of prostate cancer identifies a second risk locus at 8q24. *Nat. Genet. 39*: 645-649 (2007)

7. Haiman C.A., Patterson N., Freedman M.L., et al.: Multiple regions within 8q24 independently affect risk for prostate cancer. *Nat. Genet. 39*: 638-644 (2007)

8. Zheng S.L., Sun J., Cheng Y., et al.: Association between two unlinked loci at 8q24 and prostate cancer risk among European Americans. *J. Natl. Cancer Inst. 99*: 1525-1533 (2007)

9. Robbins C., Torres J.B., Hooker S., et al.: Confirmation study of prostate cancer risk variants at 8q24 in African Americans identifies a novel risk locus. *Genome Res. 17*: 1717-1722 (2007)

10. Al Olama A.A., Kote-Jarai Z., Giles G.G., et al.: Multiple loci on 8q24 associated with prostate cancer susceptibility. *Nat. Genet. 41*:1058-1060 (2009)

11. Zheng S.L., Sun J., Wiklund Fr., et al.: Cumulative association of five genetic variants with prostate cancer. *N. Engl. J. Med.. 358*: 910-919 (2008)

12. Gudmundsson J., Sulem P., Steinthorsdottir V., et al.: Two variants on chromosome 17 confer prostate cancer risk, and the one in TCF2 protects against type 2 diabetes. *Nat. Genet. 39*: 977-983 (2007)

13. FitzGerald L.M., Patterson B., Thomson R., Polanowski A., Quinn S., Brohede J., Thornton T., Challis D., Mackey D.A., Dwyer T., Foote S., Hannan G.N., Stankovich J., McKay J.D., Dickinson J.L.: Identification of a prostate cancer susceptibility gene on chromosome 5p13q12 associated with risk of both familial and sporadic disease. *Eur. J. Hum. Genet. 7*: 368-377 (2009)

14. Cohen J.: Statistical Power Analysis for the Behavioural Sciences. 2nd ed., Hillsdale, NJ: Lawrence Erlbaum, 1988

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Development of a new series of Aryl Ureido Derivatives as Anticancer Agents against Breast Cancer Cells

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Key words: Breast cancer, aryl ureido derivatives, cell growth inhibitors, cancer therapy

S u m m a r y. Breast cancer is the second cause of cancer mortality worldwide in humans and half of all tumours in dogs and there is an unmet need for novel anticancer agents. I have developed a new series of aryl ureido derivatives as anticancer agents. These compounds have a strong antiproliferative effect against canine breast cancer CMT-U27 cells at concentrations (1-10 μM) comparable to drugs that have already been used in chemotherapy such as gefitinib, soferanib and lapatinib. These results suggest that aryl ureido derivatives were potent inhibitors of proliferation of breast cancer cells.

INTRODUCTION

Mammary gland tumours accounts about half of all tumours in dogs and are much more common in countries where ovariohysterectomy is not routinely performed (1). The duration of exposure to steroid hormones early in life determines the overall mammary cancer risk. The risk of developing mammary gland tumours increases from 0.5% to 8%, and to 26%, depending on whether the ovariohysterectomy is performed before the first, second, or after many estrus periods, respectively (2). Dogs with malignant mammary gland tumours are known to have a significantly shorter survival time than dogs with benign tumors with mean survival time ranging from 4 to 17 months and it can vary significantly depending on histological type and differentiation, stage of disease, and type of treatment (3).

Currently, surgery is the only commonly practiced treatment for dogs with mammary tumours, and there were no established adjuvant chemotherapy or hormonal therapies as practiced in human medicine (4). Doxorubicin was used and found to be effective in dogs with pulmonary metastasis, and a preliminary report showed improved survival in dogs with high-risk mammary gland tumours receiving adjuvant doxorubicin compared with (5) dogs treated with surgery alone (6).

Since the surgical excision of malignant mammary tumours in not curative, effective therapies are needed. In this study, I developed a new series of aryl ureido derivatives as anticancer agents against human and canine breast cancer cells. The aryl ureido derivatives share common structural features with Sorafenib (Nevaxar, Bayer) (7) that blocks RAF/MEK/ERK pathway and ABT-869 (linifanib) that is a novel multi-targeted inhibitor of the vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) receptor tyrosine kinase family members (8). There were synthesized a number of compounds that were assessed for its antiproliferative activity and among those there were compounds that they have IC_{50} at pharmaceutical concentrations 1-10 μM.

MATERIAL AND METHODS

Materials

Gefitinib, lapatinib, sorafenib, praclitaxel and bexarotene were purchased from LC Laboratories (Boston, MA, USA), amines, isocyanates, MTT and other chemicals were purchased from Alfa Aesar (Belgium). Culture medium, FCS, antibiotic/antimycotic were purchased from Biosera

(UK), plasticware was purchased from Kisker, Germany.

Synthesis of aryl urea derivatives

Amines (0.5 mmol) were dissolved in dichloromethane (10 ml) and then 0.5 mmol of the appropriate isocyanate was added dropwise. Next day, the formed precipitate was filtered. TLC was performed to assess the purity of the compounds. NMR and IR spectra are now in progress.

Cell culture

The canine mammary tumor cell lines CMT-U27 was used in this study (9). Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 1% penicillin-streptomycin and fungizone, in an atmosphere of 5% CO2/95% humidified air at 37 °C.

Cell growth assay

7.5×10^3 cells were seeded into each well of 96-well plates (Kisker, Germany). Next day, cells were incubated with various concentrations of newly synthesized compounds. After 48 hrs, using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously with minor modification to the original protocol (10), the cells were incubated with MTT (0.5 mg/ml of final concentration) for 3 hours. After 3 hours the cell culture medium with MTT was removed, cells were lyzed with DMSO (100 μl/well) and the absorbance of the dissolved formazan was measured at 492 nm using a microplate reader (Organon Teknika Reader 230S, Germany). Growth inhibition was expressed as the ratio of the mean absorbance of treated cells to that of control cells. Each experiment was performed in triplicate.

RESULTS

The aryl ureido derivatives that were synthezised, shared common features with sorafenib and linitanib. The other substituent of nitrogen of ureas was included: substituted or not pyridines, pyrimidines, pyrrazoles, pyridazines, pyrazines, purines, thiazoles and isoxazoles. Some of them were tested for their antiproliferative effect on CMT-U27 cells (canine mammary carcinoma) using MTT assays. The results of this study have shown that some of them exerted their activity at very low concentrations (1-10 μM) (Figure 1). When the substituent of urea was pyrimidine or pyridine then it had very strong antiproliferative activity compared to other substituents (data not shown). In the aryl substituent usually the most effective were the compounds that have Cl, CF_3 or F in 3 and 4 position of the substituent. A comparison of the newly synthesized compounds with other drugs used in chemotherapy of cancer have shown that gefitinib (Iressa) (an EGFR tyrosine kinase inhibitor) (14) had IC_{50} to cells 10 μM, sorafenib (Nexavar) (a RAF/MEK/ERK pathway inhibitor) (15) 12.5 μM, lapatinib (Tykerb) [an epidermal growth factor receptor (EGFR) and HER2/neu (ErbB-2) tyrosine kinase inhibitor] (16) at >20 μM, praclitaxel (Taxol) (an antimicrotubule agent) <1 μM and bexatorene (Targretin)(a selective activator of retinoid X receprors) (17) <1 μM. These results have shown that the newly synthesized aryl ureido derivatives were very effective as antiproliferative agents against breast cancer cells and possibly against other cancer cell types.

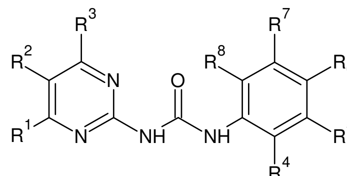

Figure 1. Structure and activity of aryl ureido derivatives of pyrimidine

DISCUSSION

Despite the remarkable progress achieved in the treatment of cancer over the last several years, many problems such as multidrug resistance (MDR), cellular heterogeneity, heterogeneous molecular abnormalities, karyotypic instability, and lack of selective action of antineoplastic agents still remain. Breast cancer is the second cause of cancer mortality worldwide in humans and lifetime risk of developing invasive breast cancer in USA is 12.6% (11). The *HER2* oncogene, or its protein receptor, HER 2, is amplified or overexpressed in approximately 25–30% of breast tumors (12) and is associated with decreased disease-free period and low overall survival rate (13). Current chemotherapy for breast cancer includes for HER2-overexpressing cancers trastuzumab or in combination with taxanes. Lapatinib is limited to HER2+ breast cancer in combination with capecitabine.

In this study, we have developed a new series of aryl ureido derivatives that share common features with sorefanib (7) and linifanib (8). These agents exerted significant cell growth inhibitory effect in CMT-U27 cells (Table 1). The IC50 values of cell growth inhibition were at pharmacological concentrations 1-10 μM. Additional studies are needed to delineate the mechanism of action of these molecules.

REFERENCES

1. Moe L.: Population-based incidence of mammary tumours in some dog breeds. *J. Reprod. Fertil. 57*: 439-443 (2001)
2. Schneider R., Dorn C.R., Taylor D.O.: Factors influencing canine mammary cancer development and postsurgical survival. *J. Natl. Cancer Inst. 43*: 1249-1261 (1969)
3. Misdorp W, Else R., Hellmen E.: Histological classification of mammary tumors of dog and cat. In: *WHO International Histological Classification of Tumors of Domestic Animals 2nd series Washington DC Armed Forces Institute of Pathology, Americal Registry of Pathology* (2001)
4. MacEwen E.G., Harvey H.J., Patnaik A.K., Mooney S., Hayes A., Kurzman I., Hardy W.D.Jr.: Evaluation of effects of levamisole and surgery on canine mammary cancer. *J. Biol. Response Mod. 4*: 418-426 (1985)
5. Hahn K.A., Knapp D.W., Richardson R.C., Matlock C.L.: Clinical response of nasal adenocarcinoma to cisplatin chemotherapy in 11 dogs. *J. Am. Vet. Med. Assoc. 200*: 355-357 (1992)
6. Simon D., Schoenrock D., Baumgartner W., Nolte I.: Postoperative adjuvant treatment of invasive malignant mammary gland tumors in dogs with doxorubicin and docetaxel. *J. Vet. Intern. Med. 20*: 1184-1190 (2006)
7. Liu L., Cao Y., Chen C., Zhang X., McNabola A., Wilkie D., Wilhelm S., Lynch M., Carter C.: Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res. 66*: 11851-11858 (2006)
8. Albert D.H., Tapang P., Magoc T.J., Pease L.J., Reuter D.R., et al., Preclinical activity of ABT-869, a multitargeted receptor tyrosine kinase inhibitor. *Mol. Cancer Ther. 5*: 995-1006 (2006)
9. Hellmen E.: Characterization of four in vitro established canine mammary carcinoma and one atypical benign mixed tumor cell lines. *In Vitro Cell. Dev. Biol. 28A*: 309-319 (1992)
10. Mosmann T.: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth 65*: 55-63 (1983)
11. Jemal A., Siegel R., Ward E. *et al.*: Cancer statistics. *CA Cancer J. Clin. 58*: 71-96 (2008)
12. Slamon D.J.: Proto-oncogenes and human cancers. *N. Engl. J. Med. 317*: 955-957 (1987)
13. Robinson A.G., Turbin D., Thomson T. *et al.*: Molecular predictive factors in patients receiving trastuzumab-based chemotherapy for metastatic disease. *Clin. Breast Cancer 7*: 254-261 (2006)
14. Tanno S., Ohasaki Y., Nakanishi K., Toyoshima E., Kikuchi K.: Small cell lung cancer cells express EGFR and tyrosine phosphorylation of EGFR is inhibited by gefitinib (*Iressa*, ZD1839). *Oncol. Rep.* ***12*:** 1053-1057 (2004)
15. Wilhelm S.M., Adnane L., Newell P., Villanueva A., Llovet J.M., Lynch M.: Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol.* Cancer Ther. *7*: 3129–3140 (2008)
16. Burris H.A.: Dual kinase inhibition in the treatment of breast cancer: initial experience with the EGFR/ErbB-2 inhibitor lapatinib". Oncologist 9 *(Suppl 3)*: 10–15 (2004)
17. Qu L., Tang X.: Bexarotene: a promising anticancer agent. *Cancer Chemother. Pharmacol. 65*: 201-205 (2010)

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Long Term Effects of Oxcarbazepine in Brain Hypoxia-ischemia: A Study in Neonatal Rats

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Key words: oxcarbazepine, rats, brain hypoxia-ischemia, neuroprotection

S u m m a r y: Brain hypoxia–ischemia induces neuronal damage in several brain regions, especially hippocampus. The toxic action of the excitatory amino acids may contribute to this damage, a process called excitotoxicity. Previous studies showed that drugs inhibiting presynaptic glutamate release, like oxcarbazepine and lamotrigine, had short-term neuroprotective effects in a rat model of brain hypoxia-ischemia. Aim of our study was to evaluate if oxcarbazepine has a long-term impact on the hippocampal histological outcome and also on anxiety-like behaviour of the rat suffering from hypoxic-ischemic encephalopathy.

INTRODUCTION

Brain hypoxia-ischemia induces damage in several regions including the cortex, the hippocampus and the striatum. Oxidative stress and excitotoxicity may contribute to this neuronal damage (1). Previous studies demonstrated that drugs acting in different stages of this process could exert neuroprotective effects, such as the antioxidant deferoxamine (2) and drugs modulating presynaptic voltage-sensitive sodium channels like lamotrigine (3) and oxcarbazepine (OXC) (4). In a previous study we evaluated the effect of lamotrigine on anxiety-like behaviour and memory in asphyxiated newborn rats (5). The purpose of the present study was to investigate if OXC, which has a similar mechanism of action with lamotrigine, has a long term impact on the hippocampal histological outcome and also on anxiety-like behaviour of the rat suffering from hypoxic-ischemic encephalopathy.

METHODS

In 7-day old rats a hypoxic-ischemic injury to the left cerebral hemisphere by left common carotid artery ligation was induced, followed by a one-hour exposure to hypoxia (8% oxygen). Immediately after hypoxia animals received intraperitoneally saline (n = 8, control group) and OXC at 80 mg/kg (n = 8, OXC group). Three months after HI rats were exposed to an elevated plus-maze and then they were sacrificed. For the histological evaluation brain sections were taken at the coronal plate according to Paxinos and Watson coordinates. Coronal sections (8 mm) were obtained from each brain at the level of the anterior hippocampus and stained with haematoxylin and eosin. The severity of damage was assessed in CA1, CA3, CA4 regions of the hippocampus and the dentate gyrus (DG) by using light and electron microscopy. The parameter used in the evaluation of the results was the total percentage of damaged neurons. For the evaluation of anxiety like behaviour the elevated plus-maze test was performed, a behavioural test reflecting spatio-temporal indexes of anxiety (6). It consisted of an elevated Plexiglas maze with two open white-coloured arms and two black-coloured enclosed arms of the same dimensions. *Ratio of entries* denotes the number of entries into open arms divided by the total number of entries. *Ratio of time* denotes the time spent in open arms divided by the time spent in both arms.

RESULTS

Calculation of the percentage of damaged neurons in the regions of the neonatal hippocampus of the left hemisphere revealed that the CA1 region suffered the greatest damage followed by DG and CA3, while the CA4 region displayed the least damage. Histological evaluation revealed that OXC administration led to a distinct reduction in the percentage of injured neurons in the CA1 region ($p = 0.038$) but this reduction was not observed in the rest of the hippocampus.

In the elevated plus maze test, rats of the OXC group entered the open arms more often than the asphyxiated rats ($p < 0.03$). The ratio of entries reflected the decrease of anxiety-like avoidance behaviour and impaired spatial learning performance. The ratio of time confirmed these results.

DISCUSSION

Our histological results suggest that OXC administration induces a long term neuroprotective effect in the CA1 region of the hippocampus and this is in accordance with previous data that i.p. administration of OXC immediately after asphyxia reduces neuronal damage observed in the CA1 region of the hippocampus a short period (7 days) after the insult (4). This neuroprotective effect of OXC is similar with the effect observed after acute lamotrigine administration in the same rat model (2).

The elevated plus-maze test is mainly used for investigating anxiolytic-/anxiogenic-like behaviours, since open areas are naturally avoided by rodents. Both entries into and time spent on the open arms of the elevated plus-maze test were increased in OXC-treated rats pointing to an anxiolytic-like effect of OXC. This result confirms the finding that drugs inhibiting presynaptic glutamate release acting on voltage-sensitive sodium channels, like OXC and lamotrigine (5), could decrease anxiety induced by hypoxic-ischemic brain damage.

REFERENCES

1. Vannucci S., Hagberg H.: Hypoxia-ischemia in the immature brain. *J. Exp. Biol. 207*: 3149-3154 (2004)
2. Papazisis G., Pourzitaki C., Sardeli C., Lallas A., Amaniti E., Kouvelas D.: Deferoxamine decreases the excitatory amino acid levels and improves the histological outcome in the hippocampus of neonatal rats after hypoxia-ischemia. *Pharmacol. Res. 57*: 73-78 (2008)
3. Papazisis G., Kallaras K., Kaiki-Astara A., Pourzitaki C., Tzachanis D., Dagklis T., Kouvelas D.: Neuroprotection by lamotrigine in a rat model of neonatal hypoxic-ischaemic encephalopathy. *Int. J. Neuropsychopharmacol. 11*: 321-329 (2008)
4. Papazisis G., Kapoukranidou D., Kalpidis I., Albani M., Kouvelas D.: Histological outcome after oxcarbazepine treatment in the asphyxiated neonatal rat brain. *Eur. Neuropsychopharmacol. 19*: 263 (2009)
5. Papazisis G., Dagklis T., Pourzitaki C., Melios G., Kapoukranidou D., Kouvelas D.: Lamotrigine improves anxiety-like behaviour and memory in a rat model of hypoxic-ischaemic encephalopathy. *Eur. Neuropsychopharmacol. 19*: 48-49 (2009)
6. Kouvelas D., Pourzitaki C., Papazisis G., Dagklis T., Dimou K., Kraus M.M.: Nandrolone abuse decreases anxiety and impairs memory in rats via central androgenic receptors. *Int. J. Neuropsychopharmacol. 11*: 925-934 (2008)

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Cadmium Effects on the Expression of Inflammatory and Angiogenic Mediators of Skeletal Muscles after Sustained Ischaemia/Reperfusion Injury

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Key words: Rat peripheral skeletal muscles, Cd, ischaemia-reperfusion injury, interleukins, angiogenic mediators

S u m m a r y. Cadmium (Cd) is a very toxic metal affecting almost every organ of the body. Due to its increasing presence in the environment, food and tobacco smoking has been attracted much attention. As Cd antagonizes Ca in cell metabolism it is considered as a xenobiotic implicated in the pathophysiology of cardiovascular disease (hypertension, atherosclerosis, and peripheral arterial disease (PAD). The present study is the first one that was conducted in a rat model of unilateral limb ischaemia where inflammatory or angiogenic mediators were measured post hypoxic stimuli, in peripheral skeletal muscle extracts. It was hypothesized that hypoxic stimuli such as ischaemia reperfusion injury with/without either acute or chronic cadmiosis alters the profile of proinflammatory cytokines (interleukins IL-6, IL-1b) derived from peripheral skeletal muscles. It was also hypothesized that inflammatory molecules such as m-RNA IL-6 or angiogenic mediators such as m-RNA VEGF or m-RNA Ang-2 will be influenced by acute or chronic Cd exposure. The results indicate that Cd in acute or chronic exposure of rat skeletal muscles increases the expression of the inflammatory mediators m-RNA IL-6, IL-6, IL-1b proteins, following hypoxic stimuli. In addition a decrease of the angiogenic mediators m-RNA VEGF and m-RNA Ang-2 was observed in the same muscle extracts, where their expression is considered part of the adaptation response to hypoxia. Therefore it may be of clinical interest to understand the molecular mechanisms by which smoking causes vascular disease or to link specific constituents of tobacco smoke such as Cd to individual manifestations of cardiovascular toxicity.

INTRODUCTION

The extended use of Cadmium (Cd) in industry after 2nd world war was classified this divalent heavy metal as an internal and external environmental toxic element. Eventually Cd increases its presence in the soil, water, air, food and living organisms (1-3). Cd antagonizes calcium (Ca) in cell metabolism and calmodulin does not differentiate Ca from Cd (4), thus Cd might contribute to the changes of ionic content of the sarcoplasm that are observed in ischaemia of peripheral muscles. In addition, there is a growing body of evidence that Cd *per se* produces an ischaemia type injury via endothelial cell damaging (5) which results to hypoxia type injury in affecting tissues (6). Consequently, Cd can be considered as an inflammatory signal for the vessel wall via its ability to increase free radical production and could be particularly toxic for heart and blood vessels (7). In view of the above association, various pathological processes such as atherosclerosis (8), hypertension (9), and cardiac conductivity disturbances have been linked in experimental and clinical studies with Cd exposure. Smokers present 4-5 times higher blood concentrations of Cd than no smokers (10) and this may be linked to the high prevalence of Peripheral Arterial Disease (PAD) in smokers that suffer from chronic ischaemia of peripheral muscles (7). Cd indeed is referred to be a significant mediator of smoking-induced PAD because adjustment for Cd decreased the association of smoking with PAD (11).

Hypoxia, due to ischaemia or Cd intoxication, causes reactive oxygen species generation (7,12-

15) which triggers the inflammatory reaction with the consequent production of inflammatory mediators such as cytokines (7) or angiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang2) (16).

The present study is the first one that was conducted in a rat model of unilateral limb ischaemia where inflammatory or angiogenic mediators were measured post hypoxic stimuli, in peripheral skeletal muscle extracts. It was hypothesized that hypoxic stimuli such as ischaemia reperfusion injury with /without either acute or chronic cadmiosis alters the profile of proinflammatory cytokines (interleukin IL-6 and IL-1b) derived from peripheral skeletal muscles. Furthermore, it was hypothesized that inflammatory molecules such as m-RNA IL-6 or angiogenic mediators such as m-RNA VEGF or m-RNA Ang2 will be influenced by acute or chronic Cd exposure.

MATERIAL AND METHODS

Seventy Wistar type male rats 200-250 g BW were used. The animals were housed in constant environmental conditions (12/12 hour light/dark cycle, 23-24 ^{o}C room temperature and 45% humidity) with food and water access ad libitum and according to the Principles of the Guide to the care and Use of Experimental Animals (1993). Initially a group of 15 animals served as preliminary experiment-received 2 mg/kgr b.w. $CdCl_2$ and then sacrificed at 1, 3, 6, 12 and 24 h post toxicosis. Tissue harvesting included the soleus and gastrocnemious muscles burned and the solid remnant diluted in $HN0_3$.The concentration of Cd per mg of muscle tissue was estimated by atomic absorption analysis and the highest concentration of the metal was noticed at 12 h post toxicosis. Three groups of animals were treated as follows. (i) The acute cadmiosis group (15 rats) received 2 mg/kg b.w. $CdCl_2$ subcutaneously and twelve hours post toxicosis, they sustained ischaemia reperfusion of their left limb. (ii) The chronic cadmiosis group (30 rats) received 0.5 mg/kg b.w. $CdCl_2$ subcutaneously every other day for 16 weeks. (iii) The control group (10 rats) received the same volume of 0.9% NaCl subcutaneously every other day also for 16 weeks. All 3 groups sustained ischaemia reperfusion injury on their left leg. The right limb served as an internal control for each rat. In the end of each experimental period the rats were anesthetized via intraperitoneal ketamine and xylazine administration and then sustained ischemia reperfusion injury of their left leg. The gastrocnemious and soleus muscles were removed en block, immediately frozen in liquid nitrogen and stored at -70 ^{o}C, for further analysis.

Whole Protein Extraction: 100 mg of frozen tissue (skeletal muscle) were homogenized in 500 μl of Protein Extraction Buffer (PEB) (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA,1% Triton X-100,10% glycerol , protease inhibitor cocktail (Complete Mini, Roche, USA) using a tissue tearor.

ELISA for rat IL6,IL1b,IL10, and rat tumor necrosis factor (TNF)-a: The IL6,IL1b,IL10, and TNF-a protein levels in the skeletal muscle crude extracts were evaluated by performing the appropriate developmental ELISAs (DuoSet) (RnD Systems).

Total RNA extraction and cDNA synthesis: The RNA extracts (DNase I treated) from 30 mg of frozen tissue (skeletal muscle) were prepared using the RNeasy Mini Kit (QIAGEN), according to the modified protocol for fibrous tissue provided by the manufacturer.

Comparative quantitative-PCR: Rat IL6 cDNA (mRNA) levels of treated animals were evaluated in comparison to controls. Normalisation was achieved by L32 cDNA (mRNA)levels. Amplimers for rat IL6(M26744) and rat L32 (BC061562) mRNA were designed using the Oligo6.71 software(Molecular Biology Insights Inc):

L32-forward: TGAAGCCCAAGATCGTCAA,
L32-reverse:TTCATTCTCTTCGCTGCGTAG,
IL6-forward: TCTTCTGGAGTTTCCGTTTCTA,
IL6-reverse: CTAGGTTTGCCGAGTAG
VEGF-forward: CACGACAGAAGGGGAGCAGAA,
VEGF-reverse: CTGCAGGAAGCTCATCTCTC,
Ang2-forward: GACCAGTGGGCATCGCTACG,
Ang2-reverse: CTGGTTGGCTGATGCTACTG

Statistics: SPSS version 14(SPSS Inc) software and R v2.10 were used for the statistical analysis. Due to many zero values non normal variables, was not able to be transformed, so Non Parametric analysis was performed. Differences between two (2) groups were analyzed with the Mann-Whitney U test differences between three (3) groups were analyzed using the Kruskal-Wallis one –way analysis of variance by ranks. A value of $P<0.05$ was considered statistically significant. Box-Plots were used to graphically represent the difference between the groups.

RESULTS AND DISCUSSION

According to the findings of this study, acute and chronic cadmiosis alone or in combination with limb ischaemia-reperfusion injury are medi-

ated by an increase of inflammatory process in peripheral skeletal muscle tissue. This inflammatory reaction is reflected in the cytokine profile of IL-6 and IL-1 derived from rat skeletal muscle protein extracts. In addition, cadmiosis either acute or chronic, decreases angiogenic mediators in peripheral skeletal muscles following ischaemia-reperfusion injury. Although in chronic cadmiosis of the same set of muscles, there are some indicative results showing that angiogenic activity, as it is reflected in mRNA VEGF and Ang-2 production, presents a trend to relapse in chronic versus acute Cd exposure.

Skeletal muscle ischaemia and reperfusion injury remains an issue of concern because of the morbidity and mortality that is associated with PAD. Unlike healthy tissue, the microenvironment of ischaemic tissue is characterized by increased oxidative stress and the release of many cytokines (17,18). In acute and chronic exposure, Cd produces hypoxia in various tissues via endothelial cell damaging (5,6), which are integrated via oxidative stress in cell level (19).

Oxidative stress appears to be a common mechanism involved in the inflammatory reaction attributed either to Cd toxicity (12,15,19,20,) or to ischaemia-reperfusion injury (13). Multiple cytokines and growth factors are present at sites of inflammation, and each of these can potentially influence the nature of the inflammatory response.

In both inflammatory stimuli, a plasma increase of the activity of the inflammatory cytokines such as TNF-a, IL-1 and IL-6 is observed (7,21). Myocardial ischaemia leads to an increase of IL-6 synthesis in the cardiac myocytes as an integral part of the reaction to injury resulting from ischaemia and reperfusion (17,21). Similarly, in skeletal myocytes, in the present study, we found statistically higher levels ($p=0.06$) in mRNA IL-6 from muscles sustaining ischaemia/reperfusion versus non ischaemic muscles. These results are in accordance with previous reports that Cd exerts cytotoxic effects by inducing oxidative stress that leads to IL-6 induction in various tissues (19). Cadmiosis either in acute ($p=0.01$) or in chronic exposure ($p=0.02$) led to a further induction of IL-6 protein expression among rat skeletal muscles sustaining ischaemia – reperfusion. This statistically significant increase of IL-6 detection in acute cadmiosis presents a trend to relapse in chronic cadmiosis ($p=0.08$). Certain cytokines, such IL-6 and IL-1, also play a crucial role in the pathogenesis of the inflammation (22). However, in contrast to cytoprotective and antiapoptotic role attributed to IL-6 expression in cardiac myocytes sustained ischaemia (7), in skeletal muscles of ischaemic limbs, it is referred that IL-1 is predominantly expressed by myocytes with central nuclei, indicating that these cells are regenerating myocytes.

Nevertheless, hypoxia/reoxygenation specifically induce biosynthesis and release of IL-1.The presented data point out that both acute and chronic cadmiosis led to an increase of IL-1b among muscles that did not sustained ischaemia-reperfusion injury. This increase was strongly significant in acute cadmiosis non ischaemic muscles versus control ($p=0.03$) and indicative of significance in the comparison of IL-1b between chronic cadmiosis and control ($p=0.08$). Furthermore, acute cadmiosis versus control led to a statistical significant increase of IL-1b in muscles sustaining ischaemia-reperfusion injury ($p=0.04$).

Taking into consideration that IL-6 was also strongly induced in the same set of ischaemic muscles ($p=0.01$), it can be assumed that both inflammatory signals are expressed in parallel.

This assumption could be extended to the results among chronic cadmiosis muscles versus acute. A tendency to relapse IL-1b, even though non statistical significant in protein measurements, is observed in chronic versus acute cadmiosis in non ischaemic muscles, an analogous trend to decline as it is observed in IL-6 production in chronic versus acute cadmiosis, but in ischaemic muscles ($p=0.08$).

According to these findings, it can be postulated that Cd toxicity and ischaemia-reperfusion injury both as hypoxic stimuli, act in concert increasing inflammatory mediators as reflected by IL-1 and IL-6 expression. The inflammatory reaction seems to be more intense in muscles undergoing acute rather than chronic cadmiosis.

Even so through the expression of cytokines and growth factors in tissue level, inflammatory signals are integrated that influence angiogenic activity, which are expressed in cytokines that are considered strong angiogenic factors (23). In particular, the inflammatory cytokine IL-1b is characterized as an essential initiating trigger of VEGF-dependent angiogenesis(23)According to our finding, IL-1b induction that is observed in acute cadmiosis muscles versus control either sustaining ischaemia-reperfusion injury or not did not lead to triggering of VEGF production. In particular IL-1b that is referred to inhibit a number of angiogenic processes in myocardial level is over expressed in peripheral skeletal muscles in acute cadmiosis, whereas VEGF production was down regulated. In the present study, a significant decrease of VEGF production was demonstrated in acute cadmiosis versus control ($p=0.02$) among muscles sustaining ischaemia-reperfusion injury,

while a decrease indicative of significance (p=0.1) was found among non ischaemic muscles.

Chronic hypoxia in experimental models of rat hind limb ischaemia is referred to lead to VEGF upregulation. Likewise in the present study, chronic hypoxia produced by chronic cadmiosis led to an increase of mRNA VEGF production among muscles not sustaining ischaemia-reperfusion injury. Specifically, mRNA VEGF production was found significantly increased (p=0.02) in chronic cadmiosis compared with that of acute cadmiosis and was elevated in a level indicative of significance (p=01) in chronic cadmiosis compared with that of control. It is not known if the observed VEGF induction in chronic cadmiosis represents a skeletal myocytes protection against ischaemic injury as it has been proved for cardiac myocytes. On the other hand, acute hypoxia produced by ischaemia-reperfusion injury, which acts as a strong inflammatory stimulus, led to a VEGF up regulation (p=0.06) among either acute cadmiosis or control muscles. This finding is justified from previous studies in which VEGF is considered as a potent factor of increasing permeability of endothelial cells and therefore contribute to the inflammatory response. Moreover inflammatory tissue is prone to induce new vessel formation in different diseases such as rheumatoid arthritis, skin wounds and PAD

The transient increase of total cytokines in the acute ischaemia tissue is considered beneficial to cell-based therapeutic angiogenesis. In compliance local hypoxia triggers initiation of VEGF mediated augmentation of angiogenesis in pancreatic islet grafts (24).

A physiological and transient upregulation of Ang2 can stimulate revascularization, as it was seen in a mouse hind limb ischaemia model (25). Ang2 is upregulated only at sites of vascular remodelling to allow the vessel to revert to a more plastic state(25). In accordance with this report the present study shows that in skeletal muscles sustaining ischaemia-reperfusion injury, acute cadmiosis decreases dramatically (p=0.006) mRNA Ang-2 levels versus control. In addition in the same set of muscles, a significant decrease of mRNA VEGF production (p=0.02) in acute cadmiosis versus control was observed. Taking under consideration that VEGF up regulation and Ang-2 expression are both prerequisites for active angiogenesis (16) it can be concluded that the strong inflammatory signal of acute cadmiosis decreases angiogenic mediators such as mRNA VEGF and mRNA Ang-2 in peripheral skeletal muscle despite an ischaemic stimulus being present. Although it was found that angiogenic activity, as mirrored in Ang-2 levels, presents a trend indicative of relapse (p=0.1) in chronic cadmiosis ischaemic muscles versus acute cadmiosis ischaemic muscles process in human muscle (26).

In conclusion, this study shows that Cd, a well known smoke constituent, increases the expression of the inflammatory mediators mRNA IL-6,IL-1 AND IL-1b proteins of rat skeletal muscles following hypoxic stimuli after acute and chronic Cd exposure. Moreover in the same muscle extracts, a decrease of the angiogenic mediators mRNA VEGF and mRNA Ang-2 is also observed where their expression is considered part of the adaptation response to hypoxia.

REFERENCES

1. Carageorgiou H., Liapi C., Messari I., Tyligada C., Papadopoulos G., Sitaras N.: Environmental Pharmacology. University of Athens, Athens, pp. 195-200, 2004

2. Tzotzes V., Tzilalis V., Giannakakis S., Saranteas T., Papas A., Mourouzis I., Mourouzis C., Zarros A., Pantos C., Cokkinos D., Carageorgiou H.: Effects of acute and chronic cadmium administration on the vascular reactivity of rat aorta. *Biometals 20*: 83-91 (2007)

3. Satarug S., Garrett S.H., Sens M.A., Sens D.A.: Cadmium, environmental exposure, and health outcomes. *Environ. Health Perspect. 118*: 182-90 (2010)

4. Sutoo D., Akiyama K.: Effect of cadmium or magnesium on calcium-dependent central function that reduces blood pressure. *Arch. Toxicol. 74*: 1-4 (2000)

5. Kuester R.K., Waalkes M.P., Goering P.L., Fisher B.L., McCuskey R.S., Sipes I.G.: Differential hepatotoxicity induced by cadmium in Fischer 344 and Sprague-Dawley rats. *Toxicol. Sci. 65*:151-159 (2002)

6. Nolan C.V., Shaikh Z.A.: The vascular endothelium as a target tissue in acute cadmium toxicity. *Rev. Life Sci. 20*: 1403-1409 (1986)

7. Bhatnagar A.: Environmental cardiology: studying mechanistic links between pollution and heart disease. *Rev. Circ Res. 29;99*: 692-705 (2006)
7. Bhatnagar A.: Environmental cardiology: studying mechanistic links between pollution and heart disease. *Rev. Circ Res. 29;99*: 692-705 (2006)
8. Revis N.W., Zinsmeister A.R., Bull R.: Atherosclerosis and hypertension induction by lead and cadmium ions: an effect prevented by calcium ion. *Proc. Natl. Acad. Sci. USA 78*: 6494-6498 (1981)

9. Houston M.C.: The role of mercury and cadmium heavy metals in vascular disease, hypertension, coronary heart disease, and myocardial infarction. *Altern. Ther. Health Med. 13*: S128-133 (2007)

10. Järup L., Akesson A.: Current status of cadmium as an environmental health problem. *Rev..Toxicol. Appl. Pharmacol. 238*: 201-208 (2009)

11. Navas-Acien A.: Lead, cadmium, smoking, and increased risk of peripheral arterial disease. *Circulation 109*: 3196-3201 (2004)

12. Ognjanović B.I., Pavlović S.Z., Maletić S.D., Zikić R.V., Stajn A.S., Radojicić R.M., Saicić Z.S., Petrović V.M.: Protective influence of vitamin E on antioxidant defense system in the blood of rats treated with cadmium. *Physiol. Res. 52*: 563-570 (2003)

13. Li C.: Reactive species mechanisms of cellular hypoxia – reoxygenation injury. *Am. J. Physiol. Cell. Physiol. 282*: C227-C241 (2002)
14. Atahan E., Ergün Y., Kurutaş E.B., Alıcı T.: Protective Effect of zinc aspartate on long-term ischemia-reperfusion injury in rat skeletal muscle. *Biol. Trace Elem. Res.*: (2009)
15. Shaikh Z.A., Vu T.T., Zaman K.: Oxidative stress as a mechanism of chronic cadmium-induced hepatotoxicity and renal toxicity and protection by antioxidants. *Toxicol. Appl. Pharmacol. 154*: 256-263 (1999)
16. Richardson R.S., Wagner H., Mudaliar S.R., Henry R., Noyszewski E.A., Wagner P.D.: Human VEGF gene expression in skeletal muscle: effect of acute normoxic and hypoxic exercise. *Am. J. Physiol. 277*: H2247-2252 (1999)
17. Takihara-Yammauchi K.: Hypoxic stress induces cardiac myocyte-derived interleukin-6. *Circulation 91*: 1520-1524 (1995)
18. Zachary Ian.: Vascular endothelial growth factor and anti-angiogenic peptides as therapeutic and investigational molecules. *Rev. Drugs 6*: 224-231 (2003)
19. Balharry D., Sexton K., BéruBé K.A.: An in vitro approach to assess the toxicity of inhaled tobacco smoke components: nicotine, cadmium, formaldehyde and urethane. *Toxicology 244*: 66-76 (2008)
20. Dong W., Simeonova P.P., Gallucci R., Matheson J., Flood L., Wang S., Hubbs A., Luster M.I.: Toxic metals stimulate inflammatory cytokines in hepatocytes through oxidative stress mechanisms. *Toxicol. Appl. Pharmacol. 151*: 359-366 (1998)
21. Ikonomidis I., Athanassopoulos G., Lekakis J., Venetsanou K., Marinou M., Stamatelopoulos K., Cokkinos D.V., Nihoyannopoulos P.: Myocardial ischemia induces interleukin-6 and tissue factor production in patients with coronary artery disease: a dobutamine stress echocardiography study. *Circulation 112*: 3272-3279 (2005)
22. Qin S.L., Li T.S., Kubo M., Ohshima M., Furutani A., Hamano K.: Transient increase of cytokines in the acute ischemic tissue is beneficial to cell-based therapeutic angiogenesis. *Circ. J. 72*: 2075-2080 (2008)
23. Scholz D., Cai W.J., Schaper W.: Arteriogenesis, a new concept of vascular adaptation in occlusive disease. Review. *Angiogenesis 4*: 247-257 (2001)
24. Namba T., Koike H., Murakami K., Aoki M., Makino H., Hashiya N., Ogihara T., Kaneda Y., Kohno M., Morishita R.: Angiogenesis induced by endothelial nitric oxide synthase gene through vascular endothelial growth factor expression in a rat hindlimb ischemia model. *Circulation 108*: 2250-2277 (2003)
25. Tressel S.L., Kim H., Ni C.W., Chang K., Velasquez-Castano J.C., Taylor W.R., Yoon Y.S., Jo H.: Angiopoietin-2 stimulates blood flow recovery after femoral artery occlusion by inducing inflammation and arteriogenesis. Arterioscler. *Thromb. Vasc. Biol. 28*: 1989-1995 (2008)
26. Gustafsson T., Rundqvist H., Norrbom J., Rullman E., Jansson E., Sundberg C.J.: The influence of physical training on the angiopoietin and VEGF-A systems in human skeletal muscle. *J. Appl. Physiol. 103*: 1012-1020 (2007)

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Histopatholgic Modifications of Rabbit Conjunctiva after the Administration of Latanoprost, Travoprost, Vimatoprost and Combinations with Timolol: Preliminary Report

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AIM AND INTRODUCTION

It is known that in case of glaucoma, chronic local administration of drugs lowering the intraocular pressure often results in modifications so in the stroma and the epithelium of conjunctiva as well. Histological studies have revealed an increase of multinuclear cells and change of subepithelial collagen. The aim of the present study was the investigation of the possible changes on rabbit conjunctiva by chronic administration of Latanoprost, Vimatoprost, and Travoprost alone and in combination with the non selective b-blocher Timolol. Latanoprost, vimatoprost, and travoprost are prostanoid selective prostaglandin F2 (FP) receptor agonists, which are believed to reduce the intraocular pressure by increasing the outflow of aqueous humour. Studies in animals and man indicate that the main mechanism of action is increased uveoscleral outflow.

MATERIAL AND METHODS

Thirty five New Zealand white rabbits divided in seven groups (5 rabbits in each) were used and treated as follows.

Group 1: One eye drop of latanoprost (xalatan) was administered every morning in both eyes of the rabbits for 12 months.

Group 2: One eye drop of vimatoprost (lumigan) was administered every morning in both eyes of the rabbits for 12 months.

Group 3: One eye drop of travoprost (travatan) was administered every morning in both eyes of the rabbits for 12 months.

Group 4: One eye drop of latanoprost plus timolol were administered every morning in both eyes of the rabbits for 12 months.

Group 5: One eye drop of vimatoprost plus timolol were administered every morning in both eyes of the rabbits for 12 months.

Group 6: One eye drop of travoprost plus timolol were administered every morning in both eyes of the rabbits for 12 months.

Group 7: That was the control group without any treatment in the rabbit's eyes leaving in the same conditions for the same period of time (12 months), according to the Principles of the Guide to the care and Use of Experimental Animals (1993).

In the end of the drugs administration conjunctiva from both eyes was removed under anaesthesia and the animals were sacrificed. Each conjunctiva was cut in two pieces. The first was put in 2.5% formaldehyde solution and 3% glutaric aldeide diluted in cacodilic sodium buffer pH 7.2 and 350 mosmol pressure followed by the processes of dehydration , sliding and enclosing in parafine.The other half of the conjunctiva samples was treated according to the current methods of electronic microscopy (Phosphovolframic haematoxyline method (P.T.A.H.) Photonic and electronic microscopy). The photonic microscopy study expresses the relation of the nucleus chromatin proteins with the nucleus DNA of the studied cells. Probable nucleus changes are also studied. The electronic microscopy informs us about the changes in the relation between euchromatine and condensed eterochromatine. In addition the samples that were in the buffer were put in a solution of 5% oxalic acid for 5 min. The

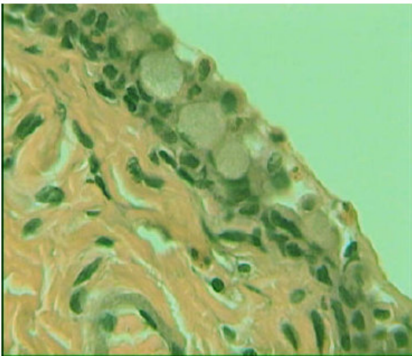
biopsies were washed 5 times in distilled water (4min each) and then were passing from a solution of 5% nitric silver in caustic ammonium 10 min. This method shows the basic aminoacids of chromatine in the shape of grains meaning that nitric silver had reacted with them and finally, when these basic aminoacids are many are expressed as conglomerates.

RESULTS

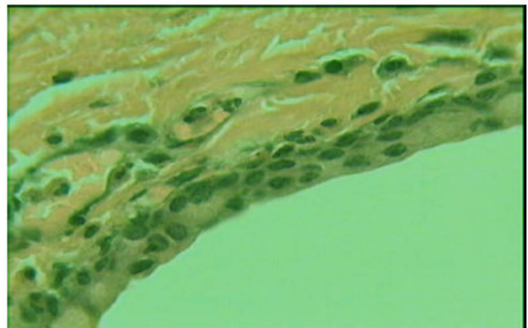
Phosphovolframic haematoxyline methodology expresses the structural and functional state of the chromatin in the nucleus in relation with its DNA. The plectonomic formation that is formed by the DNA with the nucleus chromatin express the functionality of the studied cells .In the results of the photonic microscopy is indicated the intense reaction of the stain with the basic nucleus aminoacids of the conjunctiva epithelial cells (Pictures 1, 2, 3) and the changes are referred mainly in the relation between DNA with istones and the acid proteins of the nucleus. Important role in relative data plays the structural and functional expression of conjunctival epithelial cells in normal and pathological situations.

The first pictures of the 3 groups represent the different functionality of the surface epithelium in response to the treatment with the above drugs.

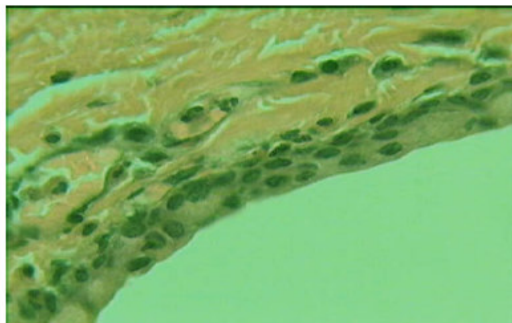
1. Ammar D.A., Kahook M.Y.: The effects of combination glaucoma medications on ocular surface epithelial cells. *Adv. Ther. 26*: 970-975 (2009)
2. Wierzbowska J., Łagocka A., Siemiatkowska A., Robaszkiewicz J., Stankiewicz A., Sierdziński: The impact of common classes of topical antiglaucoma medications on central corneal thickness - own observations. *Klin. Oczna 111*: 323-326 (2009)



Picture 1. Photoniography represents conjunctiva epithelial cells of the control group.The black arrow indicates the reaction of the stain with the nucleus chromatin in intense dark colour. P.T.A.H. Method X2.800



Picture 2. Photoniography represents conjunctiva epithelial cells of the Latanoprostl group. The black arrow indicates the reaction of the stain with the nucleus chromatin in intense dark colour. P.T.A.H. Method X2.800



Picture 3. Photoniography represents conjunctiva epithelial cells of the latanoprost plus timolol group.The black arrow indicates the reaction of the stain with the nucleus chromatin in intense dark colour. P.T.A.H. MethodX2.800

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Glutamine pretreatment alters cytokine levels in Corticotropin-Releasing Hormone- deficient (*Crh-/-*) mice during LPS-induced systemic inflammation

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Key words: CRH, LPS, Glutamine, Cytokines

INTRODUCTION

Glutamine (GLN) has been shown to protect against inflammatory injury and illness in experimental and clinical settings. The mechanism of this protection is unknown; however, laboratory and clinical trial data have indicated a relationship between GLN-mediated protection and enhanced heat shock protein 70 (HSP70). Heat Shock protein (HSP) expression is vital to cellular and tissue protection after stress or injury. Especially, the 70-kDa heat shock protein (HSP70) family is a group of proteins that are critical for protein assembly, folding, and transport. Glucocorticoids modulate the synthesis and expression of HSP70.

Aim of the present work was to study the GLN-mediated effect on HSP70 in glucocorticoid deficient animals. Hypothalamic Corticotropin Releasing Hormone (CRH), a major mediator of the stress response, is involved in the inflammatory response by exerting indirect anti-inflammatory effects via stimulation of glucocorticoid release, as well as, potent direct proinflammatory effects in a plethora of tissues. Previous studies have shown that mice deficient for *Crh* (*Crh-/-* mice) have also insufficient basal and stress-induced glucocorticoid release. In addition, *Crh-/-* mice show 2-3x higher plasma cytokine levels following LPS-induced systemic inflammation and increased mortality.

METHODS

For our studies we used adult (8-12 weeks old) male wild type (*Crh+/+*) and *Crh-/-* mice. GLN (750 mg/kg body weight) was administered intraperitoneally 45 min before the injection of LPS (80 μg/mouse). The control groups received only GLN at the same dose. Blood and lung tissue were collected 16 hr following the injection of LPS and were immediately frozen and stored at -80 °C. Plasma and lung TNFα and IL-6 concentrations were measured by ELISA.

RESULTS AND DISCUSSION

Our results showed that GLN administration decreased plasma IL-6 levels in LPS-treated *Crh -/-* mice, while it had no effect on plasma IL-6 levels in LPS-treated *Crh+/+* mice. In addition, GLN pretreatment significantly decreased lung IL-6 expression at 16 hr in both genotypes. Similarly, GLN administration significantly attenuated (50%) the plasma concentration of TNFα in *Crh -/-* mice compared to the group that received only LPS. However, *Crh+/+* mice showed no significant difference in the lung TNFα levels with or without GLN pretreatment. In order to test if the effect of GLN in both genotypes is mediated by HSP70, we measured the lung levels of HSP70 in LPS-treated *Crh+/+* and *Crh -/-* mice with or without pre-administration of GLN. To our surprise GLN did not alter HSP70 protein expression in LPS-treated *Crh-/-* mice. However, GLN significantly induced (4x) HSP70 protein levels in LPS-treated *Crh+/+* mice. In summary our data provide evidence that GLN can protect *Crh-/-* mice from LPS-induced systemic inflammation by lowering plasma and tissue cytokine levels. The mechanism through which GLN attenuates the cytokine response in LPS-treated *Crh -/-* mice is under investigation.

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Cocaine-induced effects are modulated by antagonism of cannabinoid CB1 receptors.

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Key words: Cocaine, cannabinoids, CB1 receptor, motor activity

S u m m a r y. A great amount of evidence has shown the stimulating and rewarding properties of cocaine. In particular, cocaine stimulates motor activity and induces place preference and self administration in experimental animals. The endocannabinoid system (ECS) is implicated in reward, addiction and the effects of psychostimulants. However the modulatory role of the ECS on cocaine-induced effects is far from clear. Thus, the aim of the present study was to investigate the effects of CB1 antagonism on cocaine-induced behavioural effects. Sprague-Dawley rats were observed for locomotor activity and place preference procedure after administration of vehicle, cocaine, a cannabinoid antagonist, or coadministration. Our results showed that cocaine induced a clear increase in motor activity and place preference. When coadministered with SR, these effects were modified. This study provides further evidence for the role of the ECS in cocaine- induced behavioural effects, and furthermore, emphasizes the involvement of the ECS in psychostimulant addiction.

INTRODUCTION

The ECS modulates many physiological processes including reward and cognition and consequently, it is involved in pathological states such as drug dependence and addiction. Interestingly, the ECS has been implicated in psychostimulant addiction and has even been considered a potential target for treatment, however, at present, this phenomenon is not completely understood. Cocaine is well known to modulate motor activity and induce place preference. In the present study, we chose to investigate the effects of SR, a CB_1 receptor antagonist, on these cocaine-induced behavioural effects.

METHODS

Male *Sprague-Dawley* rats were divided into two groups; the first group was intraperitoneally (i.p.) administered one of four treatments and locomotor activity was measured. The second group of rats underwent a place preference procedure. The four treatment groups were as follows: vehicle, cocaine, SR or SR + cocaine.

RESULTS

The results show that cocaine-administered rats exhibited increased motor activity and this effect was modulated by coadministration with SR. Likewise, cocaine produced strong place preference that was also modified by coadministration with SR.

DISCUSSION

This study addresses the effects of the CB1 antagonist, SR, on the universally known stimulatory effects of cocaine on locomotor activity and rewarding effects on place preference procedure. Our results show that SR was indeed able to modulate these behavioural effects, thus providing further evidence for a role of the ECS in the effects of cocaine. These findings contribute to the growing literature on the significance of the ECS in reward and addiction and its modulatory role on psychostimulants.

REFERENCES

1. Corbille A.G., Valjent E., Marsicano G., Ledent C., Lutz B., Herve D., Girault J.A.: Role of cannabinoid type 1 re-

ceptors in locomotor activity and striatal signaling in response to psychostimulants. *J. Neurosci. 27*: 6937-6947 (2007)
2. Gardner E.L.: Endocannabinoid signaling system and brain reward: emphasis on dopamine. P*harmacol. Biochem. Behav. 81*: 263-284 (2005)
3. Parolaro D., Rubino T.: The role of the endogenous cannabinoid system in drug addiction. *Drug News Perspect. 21*: 149-157 (2008)
4. Polissidis A., Chouliara O., Galanopoulos A., Papadopoulou-Daifoti Z., Antoniou K.: Behavioural and dopaminergic alterations induced by a low dose of WIN55,212-2 in a conditioned place preference procedure. *Life Sci 85*: 248-254 (2009)
5. Wiskerke J., Pattij T., Schoffelmeer A.N., De Vries T.J.: The role of CB1 receptors in psychostimulant addiction. *Addict. Biol. 13*: 225-238 (2008)

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Non-replication of Association between *MAPT-SNCA* Synergistical Interaction and Susceptibility to Parkinson's Disease in a Southern European population

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Key words: Parkinson's disease, MAPT, SNCA, a-synuclein, Case-control study

S u m m a r y. The combination of MAPT H1H1 genotype and SNCA (rs356219) GG genotype interaction has recently been identified as a possible factor to approximately double the risk for development of PD. The objective of our study was to test the association of the interaction of these two genetic variants with Parkinson's disease in a southern European case-control study. We analysed MAPT haplotypes and performed SNP genotyping with Taqman assays for the SNCA rs356219 marker in cohorts of 352 patients and 417 controls of Greek and Italian origin, respectively. Cases (n=352) were more often homozygotes for the MAPT H1 haplotype than controls (n=417). However, the association of the SNCA rs356219 G allele or GG homozygotes with Parkinson's disease was not confirmed. Furthermore the interaction of the SNCA GG genotype with MAPT H1H1 genotype was not proved to be increased among cases with Parkinson's disease compared to the controls. The data suggest that increase of PD risk by this specific combination of genotypes is not reproducible to all PD populations.

INTRODUCTION

One of the main neuropathological features of the disease consists of intracellular proteinaceous inclusions called Lewy bodies (1). Aggregation and fibrillization of the α-synuclein protein, which is the main component of Lewy bodies, represent key events in the pathogenesis of PD (2) and the disease is classified as an α-synucleinopathy.

Subsequently, the genetic association between the overall *SNCA* locus and PD has been examined in order to confine the genetic polymorphisms within the *SNCA* locus that may be associated with increased levels of a-synuclein protein and affect susceptibility to Parkinson's disease (PD). To that point, data from many studies add to the accumulating evidence that SNP rs356219 (A/G) in the 3' region of the *SNCA* gene confers increased risk to PD (3).

In addition, a disease mechanism based on the protein tau has been proposed in PD (4,5). Tau proteins are a group of phosphorylated neuronal microtubule-associated proteins that bind to microtubules and promote microtubule assembly and stabilization. They are expressed in neurons and they are particularly abundant in axons.[6] Due to the proposed interactions of α-synuclein and tau protein and their abnormal intracellular aggregation in neurodegenerative diseases (4,5), the genetic association of microtubule-associated protein tau (*MAPT*) gene and PD has been examined and the studies confirmed the significant effect of the H1 haplotype of *MAPT* gene on PD risk.

Furthermore, the combination of the *SNCA* rs356219 GG and *MAPT* H1H1 risk genotypes has been recently shown to approximately double the risk for development of PD [3] supporting the

hypothesis that tau and a-synuclein proteins might be implicated in a common PD pathogennetic pathway.

Since the meta analysis of previous studies in PD patients gave supporting results, (3) concerning the previously referred interaction between *SNCA* rs356219 and *MAPT* H1 haplotype we tested whether this interaction is associated with PD in a southern European group of patients. We conducted a study in a cohort of PD patients and controls from two sites, Greece (Athens) and Italy (Rome), and sought to add more data to the previous studies which were based on samples from Northern and Central Europe.

METHODS

We recruited 352 unrelated sporadic PD patients (mean age: 67.3±10.7 years, 37% female, mean age of diagnosis: 54.8 years). The patients were of Greek and Italian ancestry. The 122 PD Greek samples were selected from the Department of Neurology, *G. Gennimatas* General Hospital, Athens, Greece. A total of 230 Italian patients were recruited from a center in Rome (Centro Europeo di Ricerca sul Cervello (CERC) - Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS). The patients had idiopathic PD and did not suffer from other neurological diseases. The process of sample collection did not include any intervention that is not part of any common clinical practice. Idiopathic Parkinson's disease (PD) was diagnosed according to the criteria of the UK Parkinson's Disease Society Brain Bank. The use of the UKPDS standard diagnostic criteria has been shown to increase diagnostic accuracy reaching levels of up to 90% (7). The PD symptoms were quantified by applying Part III of the Unified Parkinson's Disease Rating Scale (UPDRS) (8) score. The control group consisted of 417 unrelated individuals (mean age: 65.8±17.2, 33.3% female) who were as well of Greek and Italian ancestry. The Greek control subjects donated blood during their treatment in Athens Trauma Hospital *KAT*, and in the *Onassis* Cardiac Surgery Center, Athens, Greece. The Italian control subjects were again recruited from Rome.

Genetic analysis of H1 and H2 haplotype

Blood samples were drawn for DNA extraction, using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) and following the manufacturer's protocol, from patients and controls.

The H1/H2 haplotype differentiation was based on the insertion/deletion polymorphism and has been performed as reported by Baker et al. (9) with minor modifications (Table 1).

Marker rs356219 was genotyped using Taqman SNP genotyping assay. The ABI Prism® 7000 SDS instrument in conjunction with the ABI TaqMan Universal Master Mix was used to perform the assay obtained from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the ABI Prism® 7000 SDS 1.0 Software (Perkin-Elmer, Applied Biosystems Division).

RESULTS

The *MAPT and SNCA* genotype distribution in PD patients and controls is summarized in Table 1. The two markers were in Hardy-Weinberg equilibrium. Overall the frequency of the H1H1 genotype was 63.35 % in PD cases and 56.6% in controls. H1H1 homozygotes had a greater risk of PD than H1/H2 and H2H2 carriers (Table 1). The G allele for the SNP rs356219 was not associated with an increased risk of the disease (prevalence: 40% in patients, 38.3% in controls) and moreover patients homozygous for the G allele did not have an increased disease risk (Table 1). The combination of MAPT H1H1 genotype and SNCA GG genotype did not also have any effect on disease susceptibility (Table 1), in contrary with Goris et al. study where these specific two genotypes were observed to interact, with the combination of both doubling the risk for PD.

Table 1
Genotype Analysis for MAPT and SNCA and their Interaction

Genotype		PD Cases%	Controls%
MAPT			
H1/H1		63.35%	56.6
H1/H2		30.97	38.85
		5.86%	4.55
Total		352	417
SNCA (rs356219)			
G/G		15.34	15.59
A/G		49.43	45.32
A/A		35.22	39.09
Total		352	417
MAPT-SNCA	*Interaction*		
MAPT H1/H1	*SNCA* G/G		
+	+	9.09	9.11
+	-	54.27	47.48
-	+	6.25	6.48
-	-	30.4	36.93
Total		352	417

DISCUSSION

Our study using a Southern European population provided some negative evidence for the possible *SNCA* rs356219 GG and *MAPT* H1H1 synergistic interaction which was found to increase PD risk in Goris et al. study. We report that in our population the SNCA rs356219 GG genotype and the G allele for the same SNP did not confer risk to PD. However our results confirm that the distribution of the H1 haplotype of *MAPT* is an important risk factor of PD, something observed by nearly all relevant studies (summarized in Refenes et al) (10).

Generally, data obtained from statistical analysis cannot alone be efficient to interpret complicated and multifactor biological phenomena. Another explanation for the differing results concerning the same examined associations is ethnic background influence. Our study was the first conducted in a Southern European population concerning the previously referred synergistic interaction and the SNCA rs356219 GG genotype role in PD. Even if the incidence of a disease is geographically rather uniform, the importance of different genetic factors could vary between different populations. The possibility of ethnic background influence among white Caucasians to explain the contradictory results have been discussed previously (11).

REFERENCES

1. Forno L.S.: Neuropathology of Parkinson's disease. *J. Neuropathol. Exp. Neurol. 55*: 259-272 (1996)

1.

2. Volles M.J., Lansbury P.T.,Jr.: Zeroing in on the pathogenic form of alpha-synuclein and its mechanism of neurotoxicity in Parkinson's disease. *Biochemistry 42*: 7871-7878 (2003)

3. Goris A., Williams-Gray C.H., Clark G.R., et al: Tau and alpha-synuclein in susceptibility to, and dementia in, Parkinson's disease. *Ann. Neurol. 62*: 145-153 (2007)

4. Jensen P.H., Hager H., Nielsen M.S., et al.: alpha-synuclein binds to Tau and stimulates the protein kinase A-catalyzed tau phosphorylation of serine residues 262 and 356. *J. Biol. Chem.* 1999, 274:25481-25489.

5. Giasson BI, Forman MS, Higuchi M, et al: Initiation and synergistic fibrillization of tau and alpha-synuclein. *Science 300*: 636-640 (2003)

6. Higuchi M., Lee V.M., Trojanowski J.Q.: Tau and axonopathy in neurodegenerative disorders. *Neuromolecular Med.* 2: 131-150 (2002)

7. Hughes A.J., Daniel S.E., Lees A.J.: Improved accuracy of clinical diagnosis of Lewy body Parkinson´s disease. *Neurology 57*:1497-1499 (2001)

8. Goetz C.G., Fahn S., Martinez-Martin P., et al: Movement Disorder Society-sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS): Process, format, and clinimetric testing plan. *Mov. Disord. 22*: 41-47 (2007)

9. Baker M., Litvan I., Houlden H., et al: Association of an extended haplotype in the tau gene with progressive supranuclear palsy. *Hum. Mol. Genet. 8*: 711-715 (1999)

10. Refenes N., Bolbrinker J., Tagaris G., Orlacchio A., Drakoulis N., Kreutz R.: Role of the H1 haplotype of microtubule-associated protein tau (MAPT) gene in Greek patients with Parkinson's disease. *BMC Neurol. 9*: 26 (2009)

11. Winkler S., Konig I.R., Lohmann-Hedrich K., et al: Role of ethnicity on the association of MAPT H1 haplotypes and subhaplotypes in Parkinson's disease. *Eur. J. Hum. Genet. 15:* 1163-1168 (2007)

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Acenocoumarol Pharmacogenetics: Correlation between CYP2C9 polymorphisms and dose requirements

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Key words: Vitamin K antagonists, thromboembolism, CYP2C9, polymorphism

*S u m m a r y. CYP2C9 is the major enzyme involved in the metabolism of oral anticoagulants warfarin, phenprocouman and acenocoumarol. Allelic variants, CYP2C9*2 and CYP2C9*3 have been reported to have reduced enzymatic activity in the metabolism of warfarin6. Carriers of CYP2C9*2 and CYP2C9*3 variants are poor metabolizers and therefore sensitive to coumarins. Significantly lower warfarin dose requirements have been reported for patients carrying either the CYP2C9*2 or the CYP2C9*3 polymorphism (6). Likewise, CYP2C9*3 has been related to a lower clearance and to an increased half-life of elimination of S-acenocoumarol. The influence of the CYP2C9 polymorphism on sensitivity to acenocoumarol is less known (40. Aim of this project was to investigate the relation of the CYP2C9*2 and CYP2C9*3 polymorphisms in Greek population, to the variability of acenocoumarol dose requirements.*

INTRODUCTION

The coumarins or Vitamin K antagonists (VKAs) have been the mainstay of oral anticoagulation for over 50 years. Acenocoumarol is a first line agent for the prevention and therapy of venous thromboembolism (1). However, optimal oral anticoagulation is usually hampered by significant interindividual variability in dose requirements for a given target level of anticoagulation. Acenocoumarol dosage regime determination exhibits a great variability and is affected by several environmental and congenital factors. The VKAs produce their anticoagulant effect by interfering with the cyclic interconversion of vitamin K and its 2,3 epoxide (vitamin K epoxide), thereby modulating the γ-carboxylation of glutamate residues on the N-terminal regions of vitamin K-dependent proteins. Acenocoumarol is available as a racemic mixture of R- and S-enantiomers. S-acenocoumarol is the more potent enantiomer, but as it undergoes an extensive first-pass metabolism, it has shorter elimination half-life ($t_{1/2}$=1.8 h) and less contribution to the anticoagulant effect than the R-enantiomer ($t_{1/2}$=6.6 h). Cytochrome CYP2C9 is the main enzyme implicated in aceno-coumarol metabolism. The CYP2C9 has been demonstrated to be polymorphic. Variant alleles, CYP2C9*2 and CYP2C9*3 have been related to reduced enzymatic activity, but their clinical relevance in acenocoumarol metabolism has not been established (1-3).

MATERIALS AND METHODS

Patients

Blood samples from 84 acenocoumarol-treated Greek patients were collected from the anticoagulant clinic of General Hospital KAT. Of the patients 38 were female and 46 were male with an age average of 58 years old. All patients were genetically unrelated. All, patients were monitorred by the international normalized ratio (INR). All the patients signed an informed consent form and fulfilled specific inclusion criteria. The patients studied, have been stable for at least 60 days and had an INR target within the therapeutic range 2.0-3.0.

Genotyping

DNA samples were extracted from human whole blood collected into tubes containing EDTA. The kit used for DNA extraction was Micromix 660, (Talent srl, Italy). Real time PCR was performed on the LightCycler® 480 Instrument (Roche, Germany). For the CYP2C9*2 and CYP2C9*3 polymorphisms analyzed, a 374 bp fragment and a 180 bp fragment of the human CYP2C9 genome were amplified with specific primers concluded in the LightSNip kit which was

designed by TIB-MolBiol, Germany. Every reagent vial contains all primers and probes to run 96 Lightcycler reactions. The PCR reaction mixture contained 7.0 µl PCR grade H20, 4.0 µl Reagent Mix 4.0 µl Lightcycler FastStart DNA Master Hyprobe and 5.0 µl DNA template. The LightCycler® 480 Instrument was programmed according to the manufacturer's parameters. Human CYP2C9*2 is detected with a SimpleProbe probe and CYP2C9*3 with probes labeled with LightCycler Red. The genotypes are identified by running a melting curve with specific melting points for the wildtype (wt) and for the mutant for CYP2C9*2 and CYP2C9*3 respectively.

RESULTS

The comparison of weekly maintenance dose in relation to body weight showed that patients carrying at least one CYP2C9*3 allele required lower mean weekly dose than patients with wild-type genotype. Furthermore, patients carrying CYP2C9*2 allele, had abnormally higher weekly dose requirements than wild-type patients (Table 1).

DISCUSSION

Optimal monitoring of acenocoumarol treatment is hampered by broad interindividual variability in maintenance dose requirements. This fact, as well as the narrow therapeutic index puts patients at risk of overanticoagulation during the period of establishing the individualized dose of anticoagulation.

This study showed that allelic frequencies of CYP2C9*2 and CYP2C9*3 were found to be 28% and 67% respectively in our group of patients. A positive correlation was found between acenocoumarol dose and non-genetic factors such as age, weight, body surface and concurrent medication. No association was found between acenocoumarol dose and gender. It was also found that patients carrying the following genotypes: heterozygous for CYP2C9*3 (het-3), homozygous for CYP2C9*3 (mut-3) and double heterozygous (d-het) required lower weekly dose of acenocoumarol than the wild-type patients. The later was statistically significant (p=0.001) for the following subgroups: i) female patients bearing het-3, mut-3 and d-het geno-types, ii) male patients with het-3 genotype, iii) patients >65 years with het-3, mut-3 and d-het genotypes, iv) patients <65 years carrying d-het genotype.

On the other hand, patients heterozygous for CYP2C9*2 (het-2) required higher weekly dose of acenocoumarol than wild-type patients. However, this result was not statistically significant. No association was found between bleeding episodes and CYP2C9 polymorphisms. Finally, it was found that the CYP2C9*3 polymorphism is more significant than CYP2C9*2 with respect to the dose of acenocoumarol required to provide the expected anticoagulation. Bearing the CYP2C9*3 allele was associated with the need for a lower acenocoumarol dose as shown in Table 1. The sensitivity to acenocoumarol in CYP2C9*3 carriers may be attributed to a diminished metabolization of S-acenocoumarol. S-acenocoumarol, despite the fact that is the more potent enantiomer, is clinically inactive because of its extremely short half-life compared to R-acenocoumarol. Consequently, polymorphisms diminishing the rate of metabolism of S-acenocoumarol may produce an accumulation of this potent enantiomer (4,5).

In addition to the CYP2C9*3 allele, older age was also an independent factor related to a lower acenocoumarol requirement. In conclusion, genotyping for the CYP2C9*3 polymorphism may be useful for predicting sensitivity to aceno-coumarol treatment. This may be important in selected group of patients, such as the elderly, or patients at high risk of bleeding.

REFERENCES

1. Ansell J. et al.: The pharmacology and management of the vitamin K antagonists, The seventh ACCP conference on antithrombotic and thrombolytic therapy. *Chest 126*: 204S-33S (2004)

Table 1

Association of CYP2C9 genotype and acenocoumarol dose in mg/w, mg/w/kg, mg/w/m²

	mg/w		*mg/w/kg*		*mg/w/m²*		
Total	*Mean*	*sdev*	*mean*	*Sdev*	*mean*	*sdev*	*N*
wt	17.90	6.73	0.22	0.08	9.02	2.99	20
het2	20.75	7.61	0.30	0.13	11.59	4.54	8
het3	16.93	11.42	0.22	0.16	8.96	6.15	30
mut3	16.85	8.60	0.20	0.10	8.66	4.25	10
dhet	16.22	7.41	0.21	0.09	8.43	3.73	16
dhet+het3+mut3	16.71	9.79	0.22	0.13	8.76	5.19	56

2. Markatos C., et al.: VKORC1 and CYP2C9 allelic variants influence acenocourol dose requirements in Greek patients. *Pharmacogenomics 9*: 1631-1638 (2008)
3. Tassies D., et al. Pharmacogenetics of acenocoumarol: cytochrome P450 CYP2C9 polymorphisms influence dose requirements ans stability of anticoagulation. *Haematologica 87*: 1185-1191 (2002)
4. Thijssen H.H.W., et al.: The possession of the CYP2C9*3 allele is associated with low dose requirements of acenocoumarol. *Pharmacogenetics 10*: 757-760 (2000)
5. Thijssen H.H.W., et al.: Altered pharmacokinetics of R- and S-acenocoumarol in a subject of heterozygous for CYP2C9*3. *Clin. Pharmacol. Ther. 70*: 292-298 (2001)
6. Aithal G.P., et al.: Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. *Lancet 353*: 717-719 (1999)

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Modulation of P-glycoprotein Mediated Drug Transport in Caco-2 Cells by *Aloe Vera* Phytotherapeutic Products: A Methodology to Evaluate Drug-Herb Interactions in Clinical Practice

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Key words: Drug-herb interactions, *Aloe vera*, Caco-2, P-gp, Rhodamine-123

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Summary. Nowadays, drug-herb interactions are considered as a potential factor affecting pharmacotherapy outcomes. In this work we evaluated the modulation of P-gp function by Aloe vera juice in Caco-2 cells. The data obtained thus far indicated that Aloe vera exhibited the capability to inhibit P-gp thus raising the possibility of the emergence of adverse drug reactions (ADRs).

INTRODUCTION

The increasing number of patients consuming herbal medicinal products (HMPs) has raised serious concerns about the emergence of potential drug-herb interactions in clinical practice. Although WHO has characterized HMPs as "active" medicinal products (1), this statement opposes with the public opinion about their safety, due to the general belief that no toxic effects can be produced from products of natural source. Indeed, clinical evidence now exists to support the notion that the concomitant use of HMPs and drugs from patients caused clinically-significant drug-herb interactions (2). Therefore, the capability of assessing drug-herb interactions has been an important issue in order to achieve improved drug delivery outcomes in clinical practice (3).

Drug transporters play a key role in absorption, distribution, metabolism and excretion (ADME) of several drugs. In particular, P-glycoprotein (P-gp) an ABC-transporter encoded by ABCB1 gene in humans localized in small intestine is responsible for the active efflux of several xenobiotics including a wide number of drugs (4). Especially, P-gp function is modulated via inhibition or induction, a fact influencing the bioavailability of drugs that are P-gp substrates. This seems to be causally related to several cases of clinically relevant drug-drug, drug-herb and/or drug-food interactions (5,6). To this respect and in trying to develop methodologies for the prediction and evaluation of such potential drug interactions, Caco-2 cells represent a well documented *in vitro* model for drug transport permeability assays with good correlation between the *in vitro* measurements and the obtained *in vivo* data (7,8). This approach gives satisfactory experimental results that pushed regulatory organizations to focus and also to propose such methodology as a prerequisite upon the processes of new drug development as well as drug interaction studies (9).

In this work, the capacity of commercially available *Aloe vera* juice (AV) to modulate P-gp is presented. *Aloe vera* juice is a herbal supplement widely known and consumed for treatment of heartburn, irritable bowel syndrome and generally for relieving gastrointestinal (GI) dysphoria problems (10). The effect of AV on P-gp function was assessed by using Rhodamine-123 as substrate of P-gp.

METHODS

Aloe vera sample preparation

A daily dose of 50 ml of commercial product of *Aloe vera* juice (AV) was centrifuged, decanted and evaporated to dryness through a rotary

evaporator. The dried extract (1580 mg) was then diluted with aqueous solution (10ml, 1% v/v DMSO) by sonication at room temperature and further dilutions of the AV stock solution were made with HBSS/HEPES buffer 10mM (pH=7.4) to obtain final working solutions. Transepithelial experiments in the presence of AV were conducted in concentrations of AV extract of 0.1, 0.05 and 0.01 mg/ml.

Cell culture and measurement of cellular integrity

Cells were seeded in 24-well 6.5 mm Transwells Polyester membrane inserts with 0.4 μm pore size (Corning Costar, Cambridge, MA, USA) at a density of $2x10^5$ cells/cm^2 and cultured for 20 days before the medium was replaced with HBSS/HEPES buffer 10mM (pH=7.4) for permeability assays. Transepithelial Electric Resistance (TEER) of Caco-2 monolayers was monitored using a Millicel-ERS® device (Millipore Brandford, USA) and wells with values over 400Ω/cm^2 were accepted for the assay. All measures were done in duplicates.

Measurement of transepithelial transport of Rhodamine-123

Rhodamine-123 (0.5 μM) was placed in the apical or basolateral side of the Transwell plate. *Vera*pamil (100 μM) was used as a positive control inhibitor to evaluate the function of P-gp in Caco-2 cells. For evaluation of the possible inhibition from AV, extracts were placed in both the receiver and the donor sides. Permeability of Rho-123 was estimated from its concentration in the apical side that corresponds to transport from the basolateral to apical chamber. At appropriate time points samples of the supernatant were withdrawn from the apical or basolateral side and were directly analyzed by a previous published developed HPLC method with slight modifications (11). Apparent permeability coefficient was calculated as Paap= (dQ/dt) x (1/A*Co) (cm/s) where (dQ/dt) (pmole/s) is the efflux rate, Co is the initial concentration of Rho-123 in the donor side and A is the surface area of the Transwell. All experiments were run in duplicates.

RESULTS

The results obtained indicate that AV extract in concentrations of 0.05 mg/ml and 0.1 mg/ml reduced TEER values of the Caco-2 membrane in a range up to 40% of the initial value upon exposure of cells for 120 min and/or 180min. However, after the removal of AV extract from cell cultures, the TEER values were seen to return to their initial values within a very short time (30 min).

Transport rate of Rho-123 from the basolateral to apical side of wells along with its cumulative amount it has been estimated to be near 81-84% higher than that seen upon evaluating transport rate from the apical to basolateral direction. This result indicates the polarized structure within the membrane organization of Caco-2 cell monolayer cultures (different number of *P-gp* molecules at the apical versus to basolateral side of the membrane), as expected. In the presence of verapamil (100 μM), a known drug that is inhibitor for P-gp, the transport rate and the cumulative amount of Rho-123 from basolateral to apical side was decreased about 90% as compared to the control values. These data have confirmed the expression of P-gp gene in Caco-2 cells used in our studies and, more importantly, that the produced protein is functional.

The application of AV extracts on Caco-2 cells lead to reduction of both the permeability and the cumulative transport of Rho-123 from basolateral to apical side with a dose-dependent manner. The magnitude of such inhibition was estimated to be about 70% at AV extract concentration of 0.1 mg/ml, about 47% at concentration 0.05 mg/ml and finally about 9% at concentration of 0.01 mg/ml. Furthermore, the permeability values indicating the total amount of Rho-123 passed through the Caco-2 membranes and being measured as percentage of the control-untreated Caco-2 cultures was seen to be about 65% at AV extract concentration of 0.1 mg/ml, about 45% at concentration of 0.05 mg/ml and finally about 20% at concentration of 0.01 mg/ml (Figure 1).

DISCUSSION

The analysis of the obtained data indicated that besides the decrease of the TEER values as reported above, paracellular transport of Rho-123 did not seem to occur. Since Rho-123 is been transported from the intracellular environment exclusively through the efflux function of P-gp, AV extract seems to contain compounds that are capable of inhibiting the function of P-gp. These results raise the possibility that drug-AV interactions could be occurred at the level of P-gp function in the intestine, although more data are needed to evaluate the clinical relevance of such interaction.

The clinical evaluation of drug-herb interactions seems to be a much more complex problem than that faced in drug-drug interactions. Apart of the active ingredients that are used to standardize and characterize HMPs, phytotherapeutic mix-

tures usually contain other secondary compounds that may not possess pharmacological actions, but they still exhibit the potential to modulate the pharmacokinetic parameters of drugs (2,12). As far as the evaluation of AV pharmacological properties is concerned, two previous experimental works suggested somehow controversial results. Indeed, it has been suggested that although AV use can loosen the tight junctions of Caco-2 cells, thus enhancing the paracellular transport of protein molecules like insulin (13), however the application of ^{3}H-digoxin as probe-substrate of P-gp did not reveal any inhibitory effect on P-gp function (14). To this respect, further work is needed in order to more thoroughly delineate the effects of AV on P-gp function and evaluate the clinical relevance of potential drug interactions.

REFERENCES

1. WHO, Research Guidelines for Evaluating the Safety and Efficacy of Herbal medicinal products., Organization, W. H., Ed., Regional Office for the Western Pacific, Manila, 1993

2. Vizirianakis S.I., Termentzi A., Niopas I., Kokkalou E.: Clinical and pharmacogenomic assessment of herb-drug interactions to improve drug delivery and pharmacovigilance. In: *Plants in Traditional and Modern Medicine: Chemistry and Activity* (Kokkalou E., ed.) Transworld Research Network ISBN 978-81-7895-432-5, 2010

3. Kennedy D.A., Seely D.: Clinically based evidence of drug-herb interactions: a systematic review. *Expert. Opin. Drug Saf. 9*: 79 (2010)

4. Li Y., Yuan H., Yang K., Xu W., Tang W., Li X.: The Structure and Functions of P-Glycoprotein. *Curr. Med. Chem.:* (2010)

5. Yu D.K.: The contribution of P-glycoprotein to pharmacokinetic drug-drug interactions. *J. Clin. Pharmacol. 39*: 1203 (1999)

6. Zhang L., Reynolds K.S., Zhao P., Huang S.M.: Drug interactions evaluation: an integrated part of risk assessment of therapeutics. *Toxicol. Appl. Pharmacol. 243*: 134 (2010)

7. Artursson P.: Cell cultures as models for drug absorption across the intestinal mucosa. *Crit. Rev. Ther. Drug Carrier Syst. 8*: 305 (1991)

8. Artursson P., Karlsson J.: Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem. Biophys. Res. Commun. 175*: 880 (1991)

9. FDA: Guidance for industry- Drug interactions Studies, study design, data analysis and implications for dosing and labeling, 2006

10. Reynolds T., Dweck A.C.: *Aloe vera* leaf gel: a review update. *J. Ethnopharmacol. 68*: 3 (1999)

11. Iqbal T., Kinjo M., Dowling T.C.: Determination of Rhodamine 123 in cell lysate by HPLC with visible wavelength detection. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 814*: 259 (2005)

12. Mueller S.C., Majcher-Peszynska J., Uehleke B., Klammt S., Mundkowski R.G., Miekisch W., Sievers H., Bauer S., Frank B., Kundt G., Drewelow B.: The extent of induction of CYP3A by St. John's wort varies among products and is linked to hyperforin dose. *Eur. J. Clin. Pharmacol. 62*: 29 (2006)

13.Chen W., Lu Z., Viljoen A., Hamman J.: Intestinal drug transport enhancement by *Aloe vera*. *Planta Med. 75*: 587 (2009)

14. Djuv A., Nilsen O.G.: Caco-2 cell methodology and inhibition of the P-glycoprotein transport of digoxin by *Aloe vera* juice: *Phytother. Res. 22*: 1623 (2008)

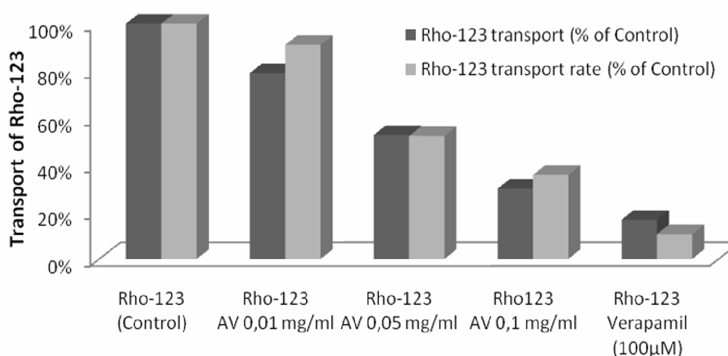

Figure 1: Extent of Transport of Rho-123 from basolateral to apical side of Caco-2 cell monolayer culture

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Incidence and Distribution of the Polymorphisms in the NRAMP1 (SLC11a1) Gene and susceptibility to Tuberculosis in Greek Population

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Key words: NRAMP1/SLC11A1, Tuberculosis, INT4, D543N, 3΄UTR

Summary. There is substantial evidence that host genetic factors affect the susceptibility to tuberculosis. The natural resistance macrophage protein 1 (NRAMP1, now known as SLC11a1, solute carrier family 11 member) seems to play a role in the pathophysiology of infections with mycobacteria. The aim of this study was to determine whether NRAMP1 polymorphisms affect the incidence of tuberculosis in Greek population. A case control study design was used to compare the frequency of 3΄UTR, D543N, INT4 of NRAMP1 among 142 tuberculosis patients and 144 ethnically matched healthy controls. All patients were diagnosed by X-ray, smear and culture tests from the laboratory of the general hospital of thoracic diseases "Sotiria". Out of the three NRAMP1 polymorphisms only the polymorphism in intron 4 (INT4) showed a significant association with tuberculosis, with CC homozygotes having higher risk of developing tuberculosis as compared with GG (odds ratio [OR]=7.31, 95% confidence interval [CI]=1.33-40.17, p value=0.022). Regarding the other two polymorphisms (D543N, 3΄UTR) there was no evidence for a linkage with tuberculosis as opposed to the INT4 polymorphism.

INTRODUCTION

In a global scale it is estimated that eight million new cases of tuberculosis occur per year from which 2.9 million eventually die. These high incidence rates worldwide can be explained by multiple factors such as the migration of individuals from regions with high tuberculosis incidence to those with low tuberculosis incidence, the increasing number of HIV positive individuals and the emergence of multidrug resistant tuberculosis due to unwise use of antibiotics (1). Amongst all infectious diseases, tuberculosis seems to be the main cause of human mortality. In Greece with a population of 11.100.000 people, the incidence of tuberculosis is estimated at 16 per 100.000 persons and the mortality rate at 2 per 100.000 (13).

These evolving conditions have lead researchers to explore the genetic factors that may play a role in the host defense system towards infection with mycobacteria. In 1981 a gene was found in mice that controls immunity to tuberculosis and was named NRAMP1 (5). The human homologue of NRAMP1 located on the long arm of chromosome 2 (2q35) and expressed only in reticuloendothelial cells seems to have an important function which is to pump out iron (Fe) from the macrophage cells thus affecting the growth of the mycobacteria (2-4). Several polymorphisms in the natural resistance macrophage protein 1 gene (NRAMP1, now known as SLC11A1) have been studied at different parts of the world in order to associate them with susceptibility to tuberculosis. The results of these studies have been controversial amongst different ethnic groups. Studies conducted in West Africans and Koreans showed a positive association of the gene with pulmonary tuberculosis while studies conducted in Denmark and Mexicans showed that the gene may play a role in the reproduction of the bacilli but is not associated with the risk of the disease among these populations (4-7). The purpose of this study was to explore the association between polymorphisms of NRAMP1 and susceptibility to tuberculosis among the Greek population.

MATERIALS AND METHODS

Patients and controls

Blood samples from 142 Greek patients with tuberculosis were collected from the General Hospital of Thoracic Diseases "Sotiria". Among them, 44 were female and 98 were male with an age average of 54 years old. 144 Greeks were used as controls of which 86 were female and 58 were male with an age average of 70 years old. All patients were genetically unrelated. The diagnosis was based on smear and culture tests and positive X-rays. All individuals with immunodeficiency or any systemic disease were ruled out of the study.

Genotyping

DNA samples were extracted from human whole blood collected into tubes containing EDTA. For DNA isolation, the "Genomic DNA PureLink™ kit extraction kit" was used. (Invitrogen, USA). The NRAMP1 polymorphisms investigated were: the INT4, a single nucleotide change in intron 4 (469+14G/C), the D543N, a non-conservative single-base substitution at codon 543 that changes aspartic acid to asparagine and the 3´UTR, a TGTG deletion in the 3´untralsated region. Real time PCR was performed on the LightCycler®480 Instrument, (Roche, Germany). For each of the three polymorphisms analyzed, a LightSNip kit was designed by TIB-MolBiol, Germany. The PCR reaction mixture contained 14.4-10.4 μl PCR grade H20, 1.0 μl Reagent Mix 2,0 μl Lightcycler FastStart DNA Master Hyprobe 1.6 μl $MgCl_2$ and 1.0-5.0 μl DNA template. The LightCycler®480 Instrument was programmed according to the manufacturer's parameters.

Statistical analysis

Initially, variants at three polymorphisms (3´ UTR, INT4, D543N) in the NRAMP1 gene were compared between tuberculosis cases and controls with chi-square test. To assess the differences in demographic characteristics we used chi-square and t test, as appropriate. Subsequently, all three NRAMP1 polymorphisms were, each independently, associated with tuberculosis through logistic regression analysis after adjustment for age and gender. The SAS statistical package (Version 9.1, SAS Institute Inc, Cary, NC) was used in all analyses.

RESULTS

A total of 142 patients diagnosed with tuberculosis and 144 healthy individuals participated in the study. Table 1 shows demographic characteristics and the frequency of several polymorphisms in the NRAMP1 gene by group of participants. Cases were more likely to be men ($p<0.0001$) and were significantly younger in age ($p<0.0001$) as compared to controls. The NRAMP1 allelic frequencies for the D543N and 3´ UTR variants were not found to differ significantly between cases and controls. However, we identified a borderline evidence for an association between tuberculosis and the polymorphism in the INT4 locus ($p=0.058$). The 3´ UTR del/del allele frequency was very low (1.4% of the total sample). Due to the low proportion of the less common alleles at the loci D543N and 3´ UTR the results should be interpreted with caution.

To further examine the relation of tuberculosis with the allelic frequencies in D543N, 3´ UTR and INT4 of NRAMP1, three separate logistic regression analyses were performed after adjustment for age and gender. There was no evidence for association between tuberculosis and the D543N allele ($p=0.977$). Concerning the 3´ UTR variants, no evidence of significant associations between tuberculosis were identified. A significant association was found between tuberculosis and polymorphism in the INT4 of the NRAMP1 gene. Specifically, when compared with their corresponding common alleles (GG), CC homozygotes for the INT4 variants were at increased risk for tuberculosis (OR=7.31, 95% CI: 1.33-40.17, $p=0.022$) whereas GC heterozygotes were not found to differ significantly.

Table 1
Demographic characteristics and polymorphisms in NRAMP1 among 142 tuberculosis cases and 144 controls

Variables	TB patients (n = 142)	Controls (n = 144)	P-value
Gender			<0.0001
Male	98 (69.0)	58 (40.3)	
Female	44 (31.0)	86 (59.7)	
Age, yrs	54.32 ± 16.44	69.63 ± 10.77	<0.0001
D543N			0.743
G/G	138 (97.2)	139 (96.5)	
G/A	4 (2.8)	5 (3.5)	
3´ UTR			1.00
TGTG/TGTG	137 (96.5)	139 (96.5)	
TGTG/del	4 (2.8)	4 (2.8)	
Del/del	1 (0.7)	1 (0.7)	
INT4			0.058
G/G	84 (59.2)	93 (64.6)	
G/C	48 (33.8)	49 (34.0)	
C/C	10 (7.0)	2 (1.4)	

Data are presented as N (%) or mean ± SD

DISCUSSION

Tuberculosis remains one of the leading causes of death due to infectious diseases as it is estimated that one-third of the world's population is infected with the Mycobacterium tuberculosis. Despite the large number of infected people, only 10% of them will eventually develop clinical disease (8). The factors contributing to an infectious disease such as tuberculosis are environmental and social, but there are other factors also, which may be as important and these are host genetics (9).

Many researchers have explored the relationship between the NRAMP1 polymorphisms and susceptibility to tuberculosis but the results vary among different studies. These discrepant results might be attributed to diverse genetic backgrounds among various populations (10).

Studies performed on populations of European descent showed no statistically significant association between NRAMP1 and susceptibility to tuberculosis (11). The present study, which involved Greek patients with well defined cases of tuberculosis, demonstrated that although the two polymorphisms (D543N and 3´UTR) show no association with susceptibility to tuberculosis, the third one (INT4) seems to be independently and significantly associated to the disease. The first two results are in compliance with previous studies but the last one is the first time that appears at a European population. The study design distinguishes susceptibility to infection with Mycobacterium tuberculosis and susceptibility to disease progression, since all the control subjects had been infected by the bacteria, but had not developed the disease. Their infection was confirmed by using PPD (purified protein derivative) skin tests.

The INT4 variants CC homozygotes are at increased risk for tuberculosis when compared to their corresponding common alleles GG. Nevertheless, it is not clear whether the INT4 variants affect the function of the NRAMP1 gene or whether another functional polymorphism exists in the gene. Further analysis of the mechanism of action of NRAMP1 and its genetic variants may become a strong ally in the therapeutic approach of tuberculosis along with vaccine and antibiotics in order to control an infectious disease that spreads widely and accounts of the death of more people than any other infectious disease (12).

REFERENCES

1. Ma X., et al.: 5´dinucleotide repeat polymorphism of NRAMP1 and susceptibility to tuberculosis among Caucasian patients in Houston. *Texas Int. J. Tuberc. Lung Dis. 6:* 818-823 (2002)

2. Blackwell J.M, et al.: Genomic organization and sequence of the human NRAMP gene: identification and mapping of a promot*er region polymorphism. Mol. Med. 1:* 194-205 (1995)

3. Cellier M., et al.: Human natural resistance-associated macrophage protein: cDNA cloning, chromosomal mapping, genomic o*rganization, and tissue*-specific expression. *J. Exp. Med. 180*: 1741-1752 (1994)

4. Søborg C., et al.: Natural resistance-associated macrophage protein 1 polymorphisms are associated with microscopy-positive tuberculosis. *J. Infect. Dis. 186*: 517-521 (2002)

5. Bellamy R., et al.: Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans. *N. Engl. J. Med. 338*: 640-644 (1998)

6. Ryu S., et al.: 3'UTR polymorphisms in the NRAMP1 gene are associated with susceptibility to tuberculosis in Koreans. *Int. J. Tuberc. Lung Dis. 4*: 577-580 (2000)

7. Niño-Moreno P., et al.: P2X7 and NRAMP1/SLC11 A1 gene polymorphisms in Mexican mestizo patients with pulmonary tuberculosis. *Clin. Exp. Immunol. 148*: 469-477 (2007)

8. Murray C.J.L., et al.: Tuberculosis in developing countries: burden, intervention and cost. *Bull IUATLD 65*: 6-24 (1990)

9. Bellamy R.: Susceptibility to mycobacterial infections: the importance of host genetics. *Genes and Immunity 4*: 4-11 (2003)

10. Liu W., et al.: VDR and NRAMP1 gene polymorphisms in susceptibility to pulmonary tuberculosis among the Chinese Han population: a case-control study. *Int. J. Tuberc. Lung Dis. 8*: 428-34 (2004)

11. Li H.T., et al.: SLC11A1 (formerly NRAMP1) gene polymorphism and tuberculosis susceptibility: a meta-analysis. *Int. J. Tuberc. Lung Dis. 10*: 3-12 (2006)

12. Murray C.J., et al.: Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet 349*: 1269-76 (1997)

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REVIEW OF CLINICAL PHARMACOLOGY AND
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Role of Pleiotrophin in Human Prostate Cancer Cell Growth *in vivo*

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Key words: Heparin affin regulatory peptide, heparin-binding growth associated molecule, angiogenesis, tumour

SUMMARY. Pleiotrophin (PTN) is a heparin-binding growth factor with diverse biological activities, the best known being those related to the nervous system, tumor growth and angiogenesis. We have previously used an antisense strategy for inhibition of PTN expression in the human prostate cancer cell line LNCaP, in order to study the role of PTN in cancer cell growth and angiogenic potential in vitro. By decreasing the expression of endogenous PTN, we found that PTN was essential for LNCaP cell migration, anchorage-dependent and independent growth, as well as their potential to induce angiogenesis. The aim of the present work was to study the role of endogenous PTN in human prostate cancer cell growth and angiogenic potential in vivo. For this purpose, we stably transfected human prostate cancer PC3 cells with an antisense plasmid vector, in order to express reduced amounts of PTN (AS-PC3). We observed a decreased migration capability of AS-PC3 cells compared with the mock-transfected (PC-PC3) or the corresponding non transfected (PC3) cells, in line with our previous results with LNCaP cells. We then implanted PC-PC3 and AS-PC3 cells subcutaneously in immunocompromised mice. Xenografts of AS-PC3 cells grew significantly slower compared with PC-PC3 xenografts and the size of the tumors was smaller, in correlation with the amounts of endogenous PTN. Moreover, AS-PC3 tumors had significantly more extended necrotic areas compared with PC-PC3 tumors. Finally, lethality as well as the number of metastases was significantly higher in PC-PC3 compared with AS-PC3 xenografts. Collectively, these data suggest that PTN has a role in human prostate cancer cell growth in vivo and may be a good target for developing new therapies.

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Nitric Oxide Levels in the Dental Pulp of Streptozocin Induced Diabetic Rats

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Key words: Diabetes, dental pulp, pioglitazone, nitric oxide, streptozocin

S u m m a r y. This study's purpose is to determine nitric oxide (NO) concentration in the dental pulp of streptozocin induced diabetic rats and to identify whether pioglitazone administration affects NO levels in the dental pulp of normoglycaemic and diabetic rats. Wistar rats were divided into four groups: N, non diabetic controls; NP, non diabetic rats receiving pioglitazone (3 mg/kg); DM, strepto-zocintreated diabetic rats (50 mg/kg); and DM+P, diabetic rats receiving pioglitazone (3 mg/kg) for 8 weeks. Nitric oxide concentration was higher in the dental pulp of diabetic rats (527 ± 23 μM) than in control rats (177 ± 2 μM) ($p < 0.001$). Pioglitazone treated diabetic rats did not exhibit further increase in NO levels (542 ± 13 μM) ($p = 0.315$ versus DM group). Treatment with pioglitazone also did not affect NO levels in non diabetic rats (162 ± 6 μM) ($p = 0.35$ versus N group). In conclusion, diabetes induced vascular changes in the dental pulp may be mediated by an increase in NO levels. Pioglitazone administration does not affect NO concentration either in normoglycaemic or in diabetic rats.

INTRODUCTION

Nitric oxide (NO) plays a significant role in the pathogenesis of dental pulp inflammation (1). It is likely that NO has an anti-inflammatory role in the healthy pulp and during the early stages of inflammation in the hyperaemic pulp. This protective effect may be mediated by inhibiting leukocyte and platelet adhesion/aggre-gation on the endothelial surface and by increasing tissue perfusion (2,3). On the other hand, it has been suggested that NO has detrimental effects as well, because of the toxic effects of its exaggerated production on the pulpal tissues (4). Furthermore, the excessive vasodilation and increased vascular permeability elicited by local high NO concentrations can raise the intrapulpal hydrostatic pressure, because the pulp is a low compliance system located in a closed and rigid dental chamber (5). Consequently, the increased intrapulpal pressure may compress the pulpal venules and thus significantly impair the pulpal perfusion leading to pulpal necrosis.

Vascular changes of the dental pulp have been reported in diabetes mellitus (6). They consist of micro-angiopathy with a thickened basement membrane and are more pronounced in the central pulp. Marked calcification of pulp blood vessels has also been described (7). Increased NO bioavailability in the early stages of diabetic angiopathy has been reported (8). Therefore, NO may mediate vascular changes of the dental pulp.

Pioglitazone is an insulin-sensitizer used for the treatment of type 2 diabetes mellitus. Its administration has been associated with anti-inflammatory effects although serum NO levels are not affected (9). Its eventual action on the dental pulp has not been investigated.

This study's purpose is to determine NO concentration in the dental pulp of streptozocin induced diabetic rats and to identify whether pioglitazone administration affects NO levels in the dental pulp of normoglycaemic and diabetic rats.

METHODS

Twelve-week-old male Wistar rats were fed standard lab rodent chow and had free access to drinking water. The animals were housed in a temperature-controlled (22±1°C) and humidity-

controlled (60±10%) room with a 12-hour dark/light cycle. Rats were group housed three per cage except when placed in individual metabolic cages. Animals were treated according to the Directive by the Council of the European Communities (86/609/CEE) and the Directive by the European Parliament (2003/65/CE) on the protection of animals used for experimental and other scientific purposes.

Diabetes was induced after an overnight fast with a single intraperitoneal injection of streptozocin (50 mg/kg freshly dissolved in 0.9% sterile sodium chloride). In order to confirm the diabetic state, blood glucose levels were measured three days after streptozocin injection with a portable glucose-measuring device (Accutrend® GCT, Roche Diagnostics GmbH, Mannheim, Germany) using blood obtained by tail prick of conscious rats. Rats with blood glucose >300 mg/dl were used as diabetic rats.

Rats rendered diabetic by streptozocin were randomized into two groups of five rats each: (i) no treatment (DM); (ii) pioglitazone 3 mg/kg per day (10) incorporated in chow (DM+P). Daily food intake was checked at regular intervals to affirm by weight the dose of administered drug. Ten age-matched male rats that had been injected with saline alone served as non-diabetic controls and were randomized into two groups of five rats each: (i) no treatment (N); (ii) pioglitazone 3 mg/kg per day incorporated in chow (NP). Rats were maintained on these treatments for 8 weeks. At the end of the study, rats were anaesthetized with an intraperitoneal injection of thiopental (40 mg/kg) and totally bled by cardiac puncture.

The dental pulp of the incisors was homogenized in 4 x w/v of phosphate buffered saline using the Ultra-Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany). The homogenate was centrifuged at 3,000 x g for 10 min at 4 ℃ in a refrigerated centrifuge (11). Nitric oxide (NO) concentration was determined in the supernatant with the Quantichrom Nitric oxide assay kit (Bioassay systems, Hayward, CA, USA) and the Plate Reader microplate reader (DASsrl, Rome, Italy).

RESULTS

Our diabetic rats developed the typical characteristics of diabetes (polyuria, polydipsia and weight loss).

Nitric oxide concentration was higher in the dental pulp of diabetic rats (527 ± 23 μM) than in control rats (177 ± 2 μM). The difference was statistically significant ($p < 0.001$). Pioglitazone treated diabetic rats did not exhibit further increase in NO levels (542±13 μM) ($p = 0.315$ versus DM group). Treatment with pioglitazone also did not affect NO levels in non diabetic rats (162 ± 6 μM) ($p = 0.35$ versus N group) (Figure 1).

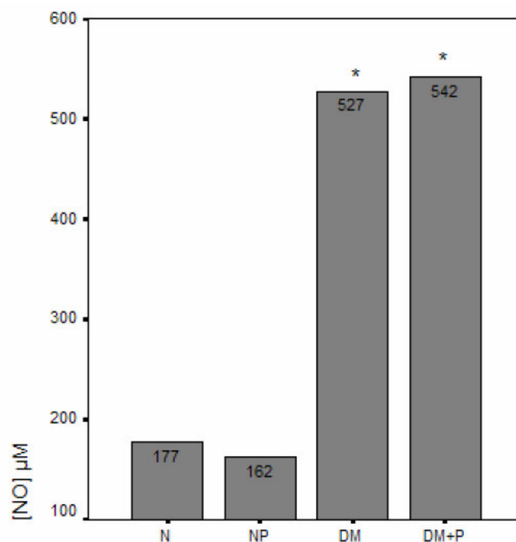

Figure 1. Nitric oxide concentration (μM) in the dental pulp of non-diabetic rats (N), pioglitazone-treated non-diabetic rats (NP), non-treated diabetic rats (D) and pioglitazone-treated diabetic rats (DM+P)
**, p < 0.001 versus N group*

DISCUSSION

We are the first to examine the effect of diabetes on NO levels in the dental pulp. We find that NO concentration significantly increases in the pulp of diabetic rats. Our study is also the first to investigate the effect of pioglitazone treatment on NO levels. We found that pioglitazone does not affect NO concentration either in normoglycaemic or in diabetic rats.

We directly measured NO in dental pulp homogenates rather than using non-specific nitric oxide synthase (NOS) inhibitors. Data were normalized for the dry weight of the homogenized pulp. However, NO is a gas and can escape easily if the tissue sample undergoes steps of purification. Therefore, our measure may not reflect the true tissue concentration as it took place after homogenisation and a 10 min-centrifugation.

In conclusion, diabetes induced vascular changes in the dental pulp may be mediated by an increase in NO levels. Pioglitazone administration does not affect NO concentration either in normoglycaemic or in diabetic rats.

REFERENCES

1. Di Nardo Di Maio F., Lohinai Z., D'Arcangelo C., Esposito De Fazio P., Speranza L., De Lutiis A., Patruno A., Grilli A., Felaco M.: Nitric oxide synthase in healthy and inflamed human dental pulp. *J. Dent. Res. 83*: 312-316 (2004)

2. Kubes P., Suzuki M., Granger D.N.: Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci USA 88*: 4651-4655 (1991)

3. Nathan C.: Nitric oxide as a secretory product of mammalian cells. *FASEB J. 6*: 3051-3064 (1992)

4. Lohinai Z., Szabó C.: Role of nitric oxide in physiology and pathophysiology of periodontal tissues. *Med. Sci. Monit. 4*: 1089-1095 (1998)

5. Lohinai Z., Balla I., Marczis J., Vass Z., Kovách A.G.: Evidence for the role of nitric oxide in the circulation of the dental pulp. *J. Dent. Res. 74*: 1501-1506 (1995)

6. Russell B.G.: The dental pulp in diabetes mellitus. *Acta Pathol. Microbiol. Scand. 70*: 319-320 (1967)

7. Bissada N.F., Sharawy A.M.: Histologic study of gingival and pulpal vascular changes in human diabetics. Egypt. Dent. J. 16: 283-296 (1970)

8. Sharma S.P.: Nitric oxide and the kidney. *Indian. J. Nephrol. 14*: 77-84 (2004)

9. Ishibashi M., Egashira K., Hiasa K., Inoue S., Ni W., Zhao Q., Usui M., Kitamoto S., Ichiki T., Takeshita A.: Anti-inflammatory and antiarteriosclerotic effects of pioglitazone. *Hypertension 40*: 687-693 (2002)

10. Yoshimoto T., Naruse M., Nishikawa M., Naruse K., Tanabe A., Seki T., Imaki T., Demura R., Aikawa E., Demura H.: Antihypertensive and vasculo- and renoprotective effects of pioglitazone in genetically obese diabetic rats. *Am. J. Physiol. 272*: E989-996 (1997)

11. Davila-Esqueda M.E., Vertiz-Hernandez A.A., Martinez-Morales F.: Comparative analysis of the renoprotective effects of pentoxifylline and vitamin E on streptozotocin-induced diabetes mellitus. *Ren. Fail. 27*: 115-122 (2005)

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Differential Gene Expression of Versican Isoforms in a Hypoxic-Ischemic Rat Brain Model of Perinatal Asphyxia

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Key words: Proteoglycan, Versican, perinatal asphyxia, Wistar rat

INTRODUCTION

Hypoxia-ischemia is a stressful condition for the organism and particularly for the nervous system. Immature brain is frequently exposed to combined hypoxic and ischemic insults during the perinatal period (1). Central nervous system response to hypoxia-ischemia is highly determined by molecules of the extracellular matrix (ECM). Extracellular matrix molecules act as a physical framework and exert profound effects on cell shape and behavior including cell adhesion, spreading, migration, proliferation and differentiation (2). The major components of ECM are proteoglycans, glycosaminoglycans and collagens. Lecticans is a subfamily of chondroitin sulfate proteoglycans (CSPGs) that include aggrecan, neurocan, brevican and versican (3). Versican is one of the major extracellular proteoglycans in the developing and adult brain (4). In the present study we tried to evaluate Versican isoforms 0, 1, 2 and 3 gene expression in the recovering hypoxic-ischemic rat brain.

MATERIALS AND METHODS

Animals used for the experiment

Seven days old male Wistar rats weighting 12-16 g were used for this experiment.

Induction of hypoxia-ischemia

Perinatal asphyxia in rats was studied according to the model by Vannucci et al. (5). Briefly, rats' brain became ischemic by permanent ligation of the left common carotid artery. Hypoxia was induced by exposing rats in 8% oxygen – 92% nitrogen gas mixture for 60 minutes.

Experimental setting

After 2, 24, 48, 72 hours and 7 days of the end of the hypoxia period the animals were sacrificed by decapitation. Animal's samples that had not been subjected to hypoxia-ischemia were used as controls. Five animals were used per group. Total RNA was extracted from both cerebral hemispheres. Total RNA was subjected to reverse transcription by using RT-PCR. cDNA samples were amplified by PCR using beta-actin gene expression as internal standard. In total 30 rats from three generations were used.

RESULTS

Versican 0 gene expression is differentially regulated in the hypoxia-ischemia model. Versican 0 gene expression at the different time points of the experiment is presented in Table 1 and Figure 1. Gene expression is presented as % of control. Two hours after the induction of hypoxia-ischemia, Versican 0 is significantly up regulated. Interestingly, despite of this initial up regulation, 12 hours after the hypoxia Versican 0 is significantly decreased. After that time point and up to the 7 days time point, the levels of Versican 0, although radually raised, remain steadily significantly down regulated compared to control.

Versican 1 gene expression is differentially regulated in the hypoxia-ischemia model. Versican 1 gene expression at the different time points of the experiment is presented in Table 1 and Figure 2. Gene expression is presented as % of control. Two hours after the induction of hypoxia-ischemia and up to the 48 hours time point, Versican 1 levels are highly, steadily and significantly down regulated compared to control. At 72 hours

time point Versican 1 levels return to control levels. However, after 1 week, Versican 1 levels remain slightly, but significantly, decreased compared to control.

Versican 2 gene expression is diffentially regulated following Versican 0 gene expression pattern. Versican 2 gene expression at the different time points of the experiment is presented in Table 1 and Figure 3. Gene expression is presented as % of control. Versican 2 gene expression is following the gene expression pattern of Versican 0. Two hours after the induction of hypoxia-ischemia Versican 2 is significantly up regulated. Like Versican 0, at the 12 hours time point and up to the 7 days time point, the levels of Versican 2 are steadily significantly decreased compared to control.

Versican 3 is not expressed in control animals whereas it is differentially regulated in hypoxia-ischemia model. Versican 3 gene expression at the different time points of the experiment is presented in Table 1 and Figure 4. Gene expression is presented as % of the 2 hours value. Versican 3 isoform is not expressed in control animals (no hypoxia-ischemia). Interestingly, two hours after the induction of hypoxia-ischemia V3 gene expression is highly induced whereas after this time point and up to 1 week it shows a decreasing pattern.

DISCUSSION

In the present study we investigated the gene expression of Versican isoforms in the rat brain after hypoxia-ischemia. Two hours after the induction of hypoxia-ischemia the expression of both genes for Versican 0 and 2 isoforms was up regulated. However, from this time point onwards their expression was significantly down regulated. V0 elevated expression immediately after hypoxia-ischemia may correlate with the need of GAGs, in order to promote inflammation response. The up regulation of Versican 2 in the initial phase following CNS injury has been associated with the failure of nerves to regenerate (6,7).

Versican 1 gene expression was significantly down regulated immediately after the induction of hypoxia-ischemia and for the whole duration of the experiment. The V1 decrease that we observed is in accordance with reports which support that V1 expression levels are down-regulated after CNS lesions, where the neurite extension is inhibited (8). It is worth mentioning that we did not detect V3 expression under normal conditions (control animals), but there was expression in hypoxic-ischemic samples. Interestingly, the expression of V3 isoform is highly induced immediately after the induction of hypoxia-ischemia followed by a decreasing pattern for the rest of the period studied. Wight et al, showed that the V3 expression promotes the formation of elastic fiber assembly in arterial muscle cell thus adversing the results of inflammation in vascular injuries (9) and correlates well with our data.

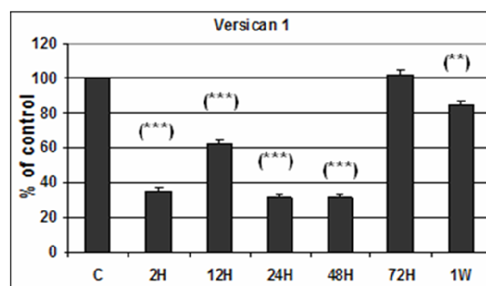

*Figure 1: Expression of Versican 0 in the left cerebral hemisphere at the different time points**

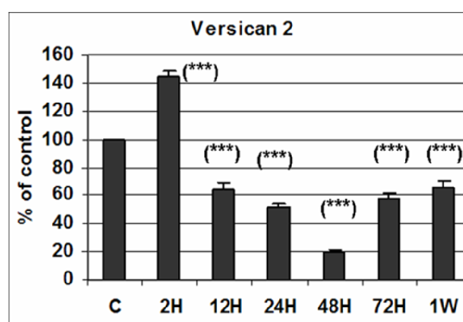

*Figure 2: Expression of Versican 1 in the left cerebral hemisphere at the different time points**

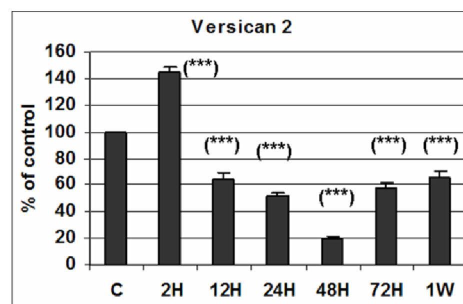

*Figure 3: Expression of Versican 2 in the left cerebral hemisphere at the different time points**

Table 1
Expression of Versican 0, 1, 2 and 3 in both cerebral hemispheres at the different time points of the experiment

Time	Versican 0*	Versican 1*	Versican 2*	Versican 3**	Versican 0 p-value	Versican 1 p-value	Versican 2 p-value	Versican 3 p-value
Control	100	100	100	No gene expression	-	-	-	-
2 hours	152±2	34.7±2.5	145.1±3.2	100	<0.001	<0.001	<0.001	-
12 hours	23.4±2.3	62±2	64.7±4.2	78.9±5.4	<0.001	<.001	<0.001	<0.001
24 hours	78±3	31.7±1.5	52.3±2	55.3±2.5	<0.001	<0.001	<0.001	<0.001
48 hours	86±2	31.6±1.5	20±2	39.9±2	<0.001	<0.001	<0.001	<0.001
72 hours	103.3±2.1	102.1±2.5	58±3.2	57.7±2.1	<0.001	0.28	<0.001	<0.001
1 week	64±2.1	85.2±1.6	65.7±4.2	47.4±2.5	<0.001	0.004	<0.001	<0.001

Values are presented as mean ± standard deviation, p-value was considered significant when p ≤ 0,05, * expression is presented as % of control, ** expression is presented as % of 2 hours value

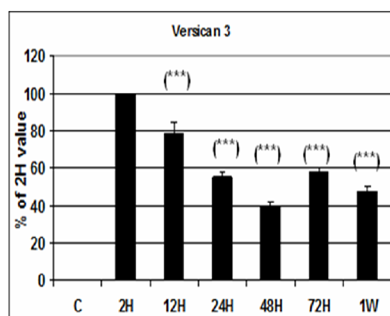

*Figure 4: Expression of Versican 3 in both cerebral hemispheres at the different time points of the experiment**

*Versican isoforms are differentially expressed at different time points after the induction of hypoxia. Values represent % of control (animals with no hypoxia) and are expressed as mean ± standard deviation. C: control, H: hours, W: week, (***)= p-value<0,001

Overall, our data suggest a distinct gene expression pattern of Versican isoforms in the recovering hypoxic-ischemic rat brain. Future studies should focus on examining protein levels and correlating gene and protein patterns with histological findings.

REFERENCES

1. Volpe J.J.: Perinatal brain injury: from pathogenesis to neuroprotection. *Ment. Retard. Dev. Disabil. Res. Rev. 7*: 56-64 (2001)

2. Kritis A., Kapoukranidou D., Michailidou B., Hatzisotiriou A., Albani M.: Sciatic nervcrush evokes a biphasic TGF-β and Decorin modulation in the rat spinal cord. *Hippokratia 14*: 37-41 (2010)

3. Gottschall P.E., Sandy J.D., Zimmerman D.R.: Substrates for metalloendopeptidases in the central nervous system. Matrix Metalloproteinases in the Central Nevous System. London Imperial College Press. Pp. 87-118, 2005

4. Kritis A., Tzimagiorgis G., Pesintzaki C., Karakiulakis G., Dimitriadou A., Papakonstantinou E.: Analysis of proteoglycans gene expression in non small cell lung carcinoma. *Rev. Clin. Pharmacol. Pharmacokinet. 16*: 83-84 (2008)

5. Vannucci R.C.: Experimental biology of cerebral hypoxia-ischemia: relation to perinatal brain damage. *Pediatr. Res. 27*: 317-326 (1990)

6. Fu S.L., Hu J.G., Li Y., Yin L., Jin J.Q., Xu X.M., et al.: Induction of rat neural stem cells into oligodendrocyte precursor cells. *Sheng Li Xue Bao 57*: 132-138 (2005)

7. Pizzorusso T., Medini P., Berardi N., Chierzi S., Fawcett J.W., Maffei L.: Reactivation of ocular dominance plasticity in the adult visual cortex. *Science 298*: 1248-1251 (2002)

8. Wu Y., Sheng W., Chen L., Dong H., Lee V., Lu F., et al.: Versican V1 isoform induces neuronal differentiation and promotes neurite outgrowth. *Mol. Biol. Cell. 15*: 2093-2104 (2004)

9. Wight T.N., Evanko S., Kinsella M., Chang M., Han C.Y., Sakr S., et al.: The pro-inflammatory nature of the vascular extracellular matrix. *Int. Congr. Ser. 1262*: 404-406 (2004)

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Effect of all-trans-Retinoic Acid and Conjugate with Spermine on Human Endothelial and Prostate Cancer Cell Growth *in Vitro* and Angiogenesis *in Vivo*

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SUMMARY.Retinoids constitute a large family of organic compounds structurally related to the naturally occurring vitamin A. All-trans-retinoic acid (atRA) is known to modulate a wide range of cellular biological processes through binding to and activation of specific receptors, the retinoic acid receptors (RAR) α, β and γ. Retinoids are being used for the treatment of various diseases, ranging from acne vulgaris to acute promyelotic leukemia. Their use however is limited due to serious adverse effects and there is a great need for analogues with minimized adverse effects. Towards this direction, we have previously synthesized a series of atRA conjugates with polyamines like spermine (SPM) and have tested them against RNase P activity. Among the molecules tested, the most potent inhibitor was the N^1,N^{12}-bis(*all-trans*-retinoyl)spermine (RA_2SPM). In the present work we studied the effect of RA_2SPM on angiogenesis in vivo and on human endothelial and prostate cancer cell growth in vitro. Both atRA and RA_2SPM dose-dependently inhibited angiogenesis in the chicken embryo chorioallantoic membrane model, with RA_2SPM being more effective and less toxic for the tissue. Similarly, both atRA and RA_2SPM decreased the number of human endothelial and prostate cancer LNCaP and PC3 cells in a concentration-dependent manner. In all cases, RA_2SPM was more effective and potent compared with atRA. Interestingly, the effect of RA_2SPM was mediated by RARα and was higher in PC3 cells that do not express RARβ and are considered more aggressive, while in cells that express RARβ it was less effective. Both atRA and RA_2SPM decreased expression of the growth factor pleiotrophin (PTN), which is known to be significant for prostate cancer cell growth. Further studies are in progress in order to elucidate whether the effect of atRA and RA_2SPM is related to their effect on the expression of PTN, as well as their mechanism of action on tumor and endothelial cells.

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Prediction of Bone Healing in Rats after Treatment with Parecoxib

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Key words: Bone healing, fracture healing, cyclogenase inhibitors, parecoxib

S u m m a r y. Parecoxib inhibits fracture healing in rats when administered postoperatively. The purpose of this study was to estimate the possibility of bone healing in rats after fracture and treatment with parecoxib for the first postoperative week. Closed mid-diaphyseal fractures of the left femoral shaft were generated, and parecoxib was administered intraperitoneally for one week, immediately after fracture. Bone healing was evaluated radiographically 2 and 6 weeks after fracture. Bone healing can be predicted by the equation f(x)=y=17.5x-25, where y is the percentage of bone healing, and x is time in weeks after fracture.

INTRODUCTION

Selective and non-selective cyclo-oxygenase (COX) inhibitors have demonstrated inhibitory effects on bone healing (1-7). In previous studies we have demonstrated that parecoxib, a new selective COX-2 inhibitor, delays early fracture healing in rats, when it is administered for one week after bone fracture (8-10) but we concluded that this effect was rather transient, as the long-term effects seemed to be non significant (11-14). We have also stressed the importance of the reversibility of this delay in fracture healing, as, from a clinical point of view, what seems to be more important with the use of COX inhibitors post-operatively, is not their transient inhibition on bone healing but the reversibility of this inhibition and their long-term effects on bone healing and rehabilitation (14).

The purpose of this study is to estimate the reversibility of the inhibitory effect of parecoxib on bone healing and to develop a model for the prediction of bone healing in *Albino Wistar* rats after a short treatment with parecoxib.

METHODS

A total of 40 adult male *Albino Wistar* rats, 12-week-old, weighing 250-350 g were used in this study. The animals were divided in two groups, before operation: group A (20 animals) and group B (20 animals). In all rats a closed transverse mid-diaphyseal femoral fracture was generated by external blunt trauma to the femur after its stabilization by insertion of an intramedullary pin, as described in previous works (8-15).

Parecoxib sodium (1.06 mg/kg) was administered intra-peritoneally in both study groups, immediately after the surgical procedure and fracture, and once daily for one week. Fracture healing was evaluated radiographically 2 weeks after fracture in group A and 6 weeks after fracture in group B.

T test, chi-square test and cross-tabs were used, in order to explore the relationship between the radiographic confirmation of bone healing and time.

RESULTS

Bone healing and callus formation was 10% and 80% at 2 and 6 weeks after fracture respectively. This difference was found to be significant (t test, $p<0.001$).

There was no violation of the assumption of the chi-square test concerning minimum expected cell frequency, as minimum expected count was

9 (>5), and Pearson chi-square test value had to be corrected by Yates' Correction for Continuity, because a 2 by 2 table was used. The conducted analysis confirmed the significance of the difference ($p<0.001$) that was also found by t test for possibilities (by means of z criterion) in bone healing between the second and the sixth week after fracture and administration of parecoxib.

Bone healing can be predicted as a function of time by the equation: $f(x)=y=17.5x-25$, where y is the percentage of bone healing, and x is time in weeks after fracture.

DISCUSSION

Parecoxib and other cyclooxygenase inhibitors prevent the healing of fractures by inhibiting prostaglandin synthesis and angiogenesis. As COX inhibitors are used as analgesics for bone fractures and for the relief of post-traumatic and postoperative pain in orthopaedics, it is of crucial meaning to investigate whether this inhibitory effect on bone reconstruction is reversible, and if it is safe to use these drugs in Orthopedics.

According to our previous studies, parecoxib, given in a high dose and for a short duration after bone trauma, seems to have a transient inhibitory early effect on bone healing in rats. Our present results confirm the reversibility of the inhibitory effect of parecoxib on bone healing, and offer a model for the prediction of long term bone healing in rats after treatment with parecoxib.

According to our model for the prediction of bone healing in rats treated with parecoxib for one week after fracture, no bone healing can be expected until the middle of the second week after fracture, while bone healing will be completed in all subjects by the beginning of the eighth week after operation and fracture.

REFERENCES

1. Endo K., Sairo K., Komatsubara S., et al.: Cyclooxygenase-2 inhibitor inhibits the fracture healing. *J. Physiol. Anthropol. 21*: 235-238 (2002)
2. Long J., Lewis S., Kuklo T., et al.: The effect of cyclooxygenase-2 inhibitors on spinal fusion. *J. Bone Joint. Surg. Am. 84*: 1763-1768 (2002)
3. Zhang X., Schwarz E.M., Young D.A., et al.: Cyclooxygenase-2 regulates mesenchymal cell differentiation into the osteoblast lineage and is critically involved in bone repair. *Clin. Invest. 109*: 1405-1415 (2002)
4. Gerstenfeld L.c., Thiede M., Seibert K., et al.: Differential inhibition of fracture healing by non-selective and cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs. *J. Orthop. Res 21*: 670-675 (2003)
5. Brown K.M, Saunders M.M., Kirsch T., et al.: Effect of COX-2-specific inhibition on fracture-healing in the rat femur. *J. Bone Joint. Surg. Am. 86*:.116-123 (2004)
6. Leonelli S.m., Goldberg B.a., Safanda J., et al.: Effects of a cyclooxygenase-2 inhibitor (rofecoxib) on bone healing. *Am J Orthop 35*: 79-84 (2006)
7. Gerstenfeld L.c., Al-Ghawas M., Alkhiary Y.m., et al.: Selective and nonselective cyclooxygenase-2 inhibitors and experimental fracture-healing. Reversibility of effects after short-term treatment. *J. Bone Joint. Surg. Am. 89*: 114-125 (2007)
8. Akritopoulos P., Papaioannidou P., Hatzokos I., et al.: Short term effects of parecoxib on fracture healing in rats. *Epitheor. Klin. Farmakol. Farmakokinet. 27*: 12-14 (2009)
9. Akritopoulos P., Papaioannidou P., Hatzokos I., et al.: Parecoxib inhibits early bone healing in rats. *Aristotle University Medical Journal 36 Suppl*: 29 (2009)
10. Akritopoulos P., Papaioannidou P., Hatzokos I., et al.: Early effects of parecoxib on bone healing in rats. *Bas. Clinic. Pharmacol. Toxicol. 105*: 89-90 (2009)
11. Akritopoulos P., Papaioannidou P., Kotoula M., et al.: Influence of Parecoxib on fracture healing in rats. *Aristotle University Medical Journal 34 Suppl*: 113 (2007)
12. Akritopoulos P, Papaioannidou P, Kotoula M et al.: Parecoxib does not inhibit the long term fracture healing in rats. *Helliniki Iatriki 74 Suppl*: 35 (2008)
13. Akritopoulos P., Papaioannidou P., Kotoula M., et al.: Parecoxib has no long-term effects on fracture healing in rats. 26th Triennial World Congress SICOT/SIROT, Hong Kong, 24-28 August 2008
14. Akritopoulos P., Papaioannidou P., Hatzokos I., et al.: Parecoxib has non-significant long-term effects on bone healing in rats when administered for a short period after fracture. *Arch. Orthop. Trauma Surg. 129*: 1427-1432, 2009 (DOI 10.1007/s00402-008-0707-6 (2008)
15. Akritopoulos P., Papaioannidou P., Hatzokos I., et al.: Influence of Parecoxib on bone healing in rats. Abstracts of the 24th Medical Congress of Northern Greece p 98, 2009

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Safety and Tolerability of $\alpha_2\delta$ Ligand Gabapentin in Treating hot Flashes: An Evidence-based Systematic Review and Meta-Analysis

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Key words: Gabapentin, safety, hot flashes, menopause, meta-analysis

Summary. About 70% of all postmenopausal women experience hot flashes. Hormonal therapy, although effective, is associated with serious side effects and its use raises important concerns. A recent meta-analysis has indicated that gabapentin is efficacious in treating hot flashes. However, the safety and tolerability profile of gabapentin for treating hot flushes has not been thoroughly investigated. In order to efficiently integrate valid information about the safety and tolerability profile of gabapentin, the literature was systematically reviewed and a meta-analysis of all randomized controlled trials was conducted. Overall, gabapentin is well tolerated with no serious side effects. Dizziness/unsteadiness and fatigue/somnolence are the two most common clusterings of side effects reported. Dropout risk due to side effects is 2-fold higher compared to placebo. A long titration period appears to be of great importance for minimizing the risk of side effects.

INTRODUCTION

Hot flashes is a term used to describe a clustering of symptoms associated with natural menopause. About 70% of all postmenopausal women experience hot flashes. Hormonal therapy, although effective, is associated with significant side effects, mostly caused by the estrogenic component and its use raises important concerns. A recent meta-analysis has indicated that gabapentin is efficacious in the treatment of hot flashes (1).

Gabapentin is a γ-aminobutyric acid analogue. So far, it has shown efficacy for many chronic diseases, like neuropathic pain in spinal cord injury, fibromyalgia, migraine prophylaxis and essential tremor (2-4).

However, the safety and tolerability profile of gabapentin prescribed to women experiencing hot flashes has not been thoroughly investigated. In order to efficiently integrate valid information about the safety and tolerability profile of gabapentin in these patients, the literature was systematically reviewed and a meta-analysis of all randomized controlled trials was conducted.

METHODS

Search strategy

The main search was conducted in the electronic databases MEDLINE, EMBASE and Cochrane Central Register of Controlled Trials. Key terms used were *gabapentin* and *hot flashes*. The main search, as well as screening of titles was performed independently by two reviewers.

Studies eligibility criteria

Randomized, controlled trials (RTCs) comparing gabapentin with placebo in women with natural or tamoxifen-induced menopause, experiencing hot flashes were eligible for inclusion.

Data extraction

Data from each study regarding general characteristics, methodology and outcomes, for both the intervention and control groups, were ex-

tracted independently by 2 reviewers, using a standardized extraction form.

Statistical analysis

Risk of dropouts and symptom clustering of dizziness/unsteadiness and fatigue/somnolence were evaluated from studies as risk ratios. Risk ratios were calculated for all studies using a fixed- or random-effects model. Meta-analysis was conducted using Review Manager Version 4.2 for Windows.

RESULTS

From the 40 publications initially identified, 4 RCTs were included in the meta-analysis (5-8),

Safety and tolerability of gabapentin compared to control

Both the risk of dizziness/unsteadiness and fatigue/somnolence were significantly higher in women treated with gabapentin than in controls. For dizziness/unsteadiness the risk ratio was RR = 6.94 (95% CI, 3.19-15.13), $p < 0.001$ and for fatigue/somnolence the risk ration was RR = 4.78 (95% CI, 2.23-10.25), $p < 0.001$. Regarding dropouts due to side effects the risk ratio was RR =2.09 (95% CI, 1.13-3.85), $p = 0.02$. Due to the small number of studies included no subgroup analysis or meta-regression modeling could be performed.

DISCUSSION

Overall, gabapentin is well tolerated with no serious side effects reported in any study included in this systematic review and meta-analysis. It is clearly indicated that the dropout risk due to side effects is 2-fold higher with gabapentin compared to placebo. Dizziness/unsteadiness and fatigue/somnolence are the two most common clusterings of side effects reported. A long titration period appears to be of great importance for minimizing the risk of side effects. Recommendations for future studies should include dose-response analysis, further exploration of the association between titration period and risk of side effects and direct comparison of gabapentin with pregabalin in the treatment of hot flashes.

REFERENCES

1. Toulis K.A., Tzellos T., Kouvelas D., Goulis D.G.: Gabapentin for the treatment of hot flashes in women with natural or tamoxifen-induced menopause: a systematic review and meta-analysis. *Clin. Ther. 31*: 221-235 (2009)
2. Tzellos T.G., Papazisis G., Amaniti E., Kouvelas D.: Efficacy of pregabalin and gabapentin for neuropathic pain in spinal-cord injury: an evidence-based evaluation of the literature. *Eur. J. Clin. Pharmacol. 64*: 851-858 (2008)
3. Tzellos T.G., Papazisis G., Toulis K.A., Sardeli C., Kouvelas D.: $A_2\delta$ ligands gabapentin and pregabalin: future implications in daily clinical practice. *Hippokratia* 2010. In press
4. Tzellos T., Toulis K., Goulis D., Papazisis G., Zampeli V., Vakfari A., Kouvelas D.: Gabapentin and pregabalin in the treatment of fibromyalgia: a systematic review and a meta-analysis. *J. Clin. Pharm. Ther.* 2010. In press.
5. Guttuso T.,Jr, Kurian R., McDermott M.P., Kieburtz K.: Gabapentin's effects on hot flashes in postmenopausal women: A randomized controlled trial. *Obstet. Gynecol. 101*: 337-345 (2003)
6. Butt D.A., Lock M., Lewis J.E., et al.: Gabapentin for the treatment of menopausal hot flashes: A randomized controlled trial. *Menopause 15*: 310-318 (2008)
7. Pandya K.J., Morrow G.R., Roscoe J.A., et al.: Gabapentin for hot flashes in 420 women with breast cancer: A randomized double-blind placebo-controlled trial. *Lancet 366*: 818-824 (2005)
8. Reddy S.Y., Warner H., Guttuso T., et al.: Gabapentin, estrogen, and placebo for treating hot flushes: A randomized controlled trial. *Obstet. Gynecol. 108*: 41-48 (2006)

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Greek Women's Diet during Pregnancy: Preliminary Results of a Research Study

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Key words: Nutrition, pregnancy, Greek diet

S u m m a r y. Background: The importance and significance of nutrition during pregnancy is well- recognized. Malnutrition can lead to birth defects as well as to maternal health problems. This paper focuses on recording the nutritional preferences of healthy Greek pregnant women, thus providing a guide for proper dietary consultation. Methods: This is a prospective study using a random sample of pregnant women (n=51, aged 26-38 years old) over a 9 month period. The American Food Frequency Questionnaire was used to record food consumption, after being appropriately modified and adjusted so as to apply to Greek diet. Results: Daily energy intake was estimated to be approximately 1.775 kcal. Carbohydrate, protein and fat intake was 42%, 18% and 40% of daily energy intake, respectively. Though fat intake levels were shown to be higher than recommended, these levels were not considered abnormal, as these ratios are common to the Mediterranean diet, due to the high consumption of olive oil. It was shown that milk and dairy products were lower in Greeks' daily preference (57%, 47% respectively) than recommended. Finally, meat and fruits intake on weekly basis were higher than recommended; i.e. 85% of the women consumed meat products more than twice a week and 87% of the women consumed more than 15 fruits per week. Conclusion: On the basis of these results, health-care professionals can early identify the presence of possible nutritional deficiencies that may potentially interfere with the good prognosis of pregnancy and well-being of the mother and fetus. In this way, they may give suitable advice concerning both quality and quantity of daily food intake.

INTRODUCTION

The importance of balanced nutrition during the different stages of life cannot be questioned. Nutrition represents one of the most important biological needs of human species. There are however special periods during human life where nutrition plays an even more important role, such as pregnancy (1). Pregnancy is considered one of the most important periods in a woman's life. During pregnancy a variety of anatomical and physiological changes occur in order to regulate woman's metabolism, adjust the female body and support the new life growing within the uterus as well as to prepare for the upcoming stage of lactation (1). Woman's nutritional status affects the different stages of fetus development, which concern the structure and function of the various organs and systems (2). An unbalanced nutrition with deficiencies in macronutrients (carbohydrates, proteins, fat) and micronutrients (vitamins, minerals) during pregnancy can lead to serious complications (3). Recent studies have shown that adopting nutritional habits based on Mediterranean diet in pregnancy, can have a positive effect on preventing atopy and wheezing in young children (4). Over the last years, the importance of healthy nutritional status during the pre-conception period has been well recognized concerning both the woman's ability to conceive as well as achieving proper fetal development during the first weeks of pregnancy (5).

This is a descriptive study assessing the food consumption of healthy Greek pregnant women, who live in the rural area of Athens. The aim of the study was to record the macronutrient and micronutrient intakes of these women and next to compare them with the Recommendations made for pregnant women from the Institute of Medicine.

MATERIALS AND METHODS

A random sample of 51 pregnant women, 26-38 years of age was selected from the clientele of a private antenatal clinic located in Athens. During their first visit to the clinic, a serial number was given to all women. Women with even serial number were considered as candidates for the

study. The first 60 of them were contacted by a member of the research team during their regular antenatal appointments at a private maternity hospital. The study requirements were clearly explained to the participants and they were given an information sheet concerning the goals of the study. They were asked to sign a written consent form. Ethical approval was obtained by Harokopio University Ethics Committee. The great majority of them (53/60) agreed to participate in the study. However, women with any metabolic illness, i.e. diabetes, or other chronic diseases, were excluded from the study. For this reason, two women initially recruited, were finally excluded from the study, when they occasionally developed gestational diabetes.

Data collection was performed over a 9 month period (April to December 2009). Women were given a Food Frequency Questionnaire to fill in during the eighth month of their pregnancy. The research tool used was Willet's Food Frequency Questionnaire (6), adjusted to Greek standards, by nutrition scientists of Harokopio Univeristy. The adjusted form of the questionnaire was validated by a pilot study done to a population of 20 female students of Harokopio University. This semi-quantitative questionnaire included 68 items and a number of questions concerning the frequency by which the items listed were consumed. It also contained *closed-type* questions about consumption of fat and adoption of certain dietary styles, i.e. vegetarian. Also, questions concerning fruit consumption and the use of dietary supplements were added, while questions about American type food were removed, i.e. consumption of phoenix oil. This semi-quantitative questionnaire was first used in 1985 by Willet et al. (6) to assess usual food consumption in a population of 173 female nurses aged 34-59 years old. Later, other researchers (7,8) also validated its use, while to date it has also been used in the evaluation of nutritional habits in pregnant populations (9). Participants were in the end asked to fill in a questionnaire with demographical and socio-economical data.

Data was analyzed using the Diet Analysis Plus 4.0 program. For the purpose of statistical analyses and graphical presentation, SPSS version 17.0 was used.

RESULTS

Participants' mean age was 32.5±3.1 years old and their Body Mass Index (BMI) was 22.2±4.1 kg/m^2. Most of the participants (41/51= 80.4%) were pregnant for the first time and most of them (35/51=68.6%) had acquired a university degree.

More than half of the participants consumed on a daily basis fat-free or low-fat milk, juices and white bread, while 34 out of 51 chose to consume at least one orange per day. Almost all of the participants consumed olive oil in their diet daily.

On a weekly basis, participants preferred to consume at least twice a week cheese (96.3%), vegetables i.e. tomatoes (92.6%) and fruits such as apples and pears (86.2%). Nevertheless, women frequently consumed products like coffee and sugar (62.3 and 65% respectively).

At least once a week, the great majority of the sample chose to consume green vegetables (96.3%). The animal protein of their choice was chicken (85.2%) and red meat (81.5%), while fish was high in their preference as well (63%). The most common carbohydrate products participants chose were pasta (85.2%) and rice (81.5%). Finally, it is interesting that two out of three pregnant women consumed at least once a week chocolate products.

Women avoid consuming during pregnancy. Alcohol was the first to be mentioned, with almost none of them having drunk any *hard liquor* (93%) and 60% of them avoid drinking beer throughout pregnancy. Concerning food, the great majority of women avoid consuming margarine and liver (77.8% and 67% respectively).

From the data already mentioned, it seems that Greek pregnant women consume less amounts of foods such as milk, vegetables and carbohydrates than what is officially recommended (10). However, fat consumption is slightly higher than recommended. This fact is basically due to the daily use of olive oil, a common trend in Mediterranean type diet. Meat and fruit consumption is also higher than recommended. It is worth noting that 85% of the participants consumed meat products more than twice a week. At the same time 87% of the participants consumed more than 15 portions of fruits per week. Analysis of questionnaires revealed that the mean daily Energy Intake in our sample was approximately 1775 kcal/day. The proportion of carbohydrates, proteins and fat were 42%, 18% and 40%, respectively. Table 2 shows the comparison of our data with the recommendations for pregnant women issued by the Institute of Medicine (2002/2005) (11).

Relatively to the micronutrient context of the sample's diet, it seems like there is an adequate intake of the majority of vitamins and minerals. At this point it is worth mentioning that concerning micronutrients, insufficient intake is considered to be the intake which is less than 75% of RDA (Recommended Dietary Allowances). Keeping

that in mind, 90% of participants had indeed sufficient intake of vitamins A and C, 80% had sufficient intake of vitamins D, B1, B3 and B6. However, ¼ of women were found to have lower intakes for vitamins E and folic acid than recommended.

The analysis of the data showed sufficient intake for most of the minerals. In particular, intakes according to recommendations were measured in 91.5% of the sample for Calcium, 60% for Zinc and 63.5% for Magnesium. It was impressive to find an insufficient intake of iron recorded in almost all pregnant women. The great majority of them (n=42/51, 82%) were receiving iron supplements, while (n=22/51, 43.1%) of the sample was taking supplementary folic acid, a fact that can justify and explain the absence of potential pathology attributed to iron and folic acid deficiencies. Figure 5 demonstrates the percentage of RDA according to mean intake of micronutrients by women of the sample.

DISCUSSION

These are the preliminary results of an ongoing study recording the daily consumption and intake of a sample of 51 women. On review of literature very few data was found concerning the dietary intake of Greek pregnant women. Energy intake recorded in this study seems to be lower than the energy intake reported for pregnant populations in Greece (12) and Cyprus (13). It is also lower than energy intake measured in populations in Holland (14) and Spain (15), while it is similar to that reported in Northern Ireland (16). Protein intake was according to recommendations and higher than what was previously reported for Greek pregnant women (12), while carbohydrate and fat intake was lower (12).

It is interesting to find that fat intake was higher than recommended. At the same time, an increased intake of mono-unsaturated fatty acids was observed (20% of Energy Intake), a fact which can be attributed to the frequent use of olive oil in the habitual diet of Greek women. Similar results are also recorded for Cypriot pregnant women (13). Recently, a positive correlation was found between the increased intake of mono-unsaturated fatty acids and increased birth weight (17).

Findings showing that pregnant women had low Vitamin E intake are in accordance with studies of pregnant populations in Cyprus, Spain and Northern Ireland (13,15,16). At this point it is worth mentioning that Black (18) commended that established recommendations for vitamin E intake are probably higher than necessary and that is why deficiency is unlikely to occur. Also, it is thought that estimates of vitamin E intake are underreported, due in part to underreporting of the amounts of dietary fat consumed and the lack of specificity of the vitamin E sources in diet (19). In any case, current dietary patterns appear to provide sufficient vitamin E to prevent deficiency symptoms such as peripheral neuropathy (19). In our study few pregnant women were found to have low vitamin E intake in comparison with other studies (13,15,16). A comment needs to be addressed, concerning the fact that foods containing a small amount of vitamin E but sufficient amounts of other antioxidants are more beneficial than foods that contain only a large amount of vitamin E (20). It has been found that populations consuming foods rich in saturated fatty acids demonstrate increased needs for vitamin E, a fact that can explain partially why pathology due to deficiency of vitamin E was not seen in our sample, since their diet was low in saturated fat (21). Just recently, cardiac problems in newborns have been correlated with very high intake of vitamin E (>14.9 mg/day) by their mothers during pre-conception and pregnancy (22). These contradictory facts prove the necessity for more research in order to define the ideal amount of vitamin E intake during pregnancy.

It has been found that maternal nutrition affects significantly vitamin concentration in the fetal blood, such as vitamin A levels that are necessary for proper visual acuity and for better functioning of the immune system (23) and vitamin D levels for osteogenesis (25). Additionally, vitamin B12 plays an important role, which is usually low in vegetarian pregnant women and thus in their infants' blood circulation. These low concentrations could lead to anemic, hypotonic and with slow rate of growth infants mainly at 3-6 months of age (25).

Literature shows that the extremely common occurrence of anemia, edema and cramps in pregnant populations are due to lack of important micronutrients in women's daily diet such as iron, calcium and magnesium (26). Unbalanced diet in general, is considered to be an aggravating factor for pathological complications during pregnancy such as hypertension and gestational diabetes (3,27). Recently, inadequate zinc intake during pregnancy was correlated with post partum depression (28). In this study inadequate iron intake was recorded. However that intake was similar with that recorded for Korean pregnant (21), was less than that for Cypriot (13) and higher than the intake recorded for pregnant in Northern Ireland (16). In all the above studies however, women were receiving iron supple-

ments. In the present study, most of the participants (82%) were receiving iron supplements as well. Adequate iron intake is very important during pregnancy, in order to avoid the occurrence of newborn's neurological problems that may have an impact in adult life, such as disorders in myelination of brain white matter, changes in metabolism of monoamines and disorders in the function of hippocampus (29). Concerning folic acid intake, our results are similar with those found in a study in U.S.A. (26), while folic acid intake seems to be lower for Cypriot pregnant women (13). In any case, a great proportion of our sample (42.3%) received folic acid supplements throughout pregnancy.

The importance of healthy nutrition during pregnancy has been demonstrated with several studies concerning both the well being of the fetus and pregnant woman. Specifically, it has been proven that fetuses that are driven to adjust to limited availability of nutrients are forced to permanently alterate their physiology and metabolism. So, although they might appear healthy at birth, these alterations are the source of the most degenerative diseases for their later on life (22). A recent study showed that these infants are in increased risk for cardiac disease, hypertension and diabetes during adulthood (27). Moreover, these neonates although born close to their due date, they have smaller size and weight than expected (2). Healthy nutrition has been proven to affect positively fetus' brain development, especially during the last three months of gestation (30). Foods rich in ω3 and ω6 fatty acids, like fish and vegetable oil, are necessary to improve brain functioning, visual acuity and learning ability (31). These important fatty acids cross placenta barrier (31) and supplementation during pregnancy increases their concentrations in umbilical blood (32). Finally, a recent study showed that adequate intake of ω3 and ω6 fatty acids during pregnancy, significantly reduces the risk of preterm labour and therefore the perinatal morbidity and mortality (33,34).

LIMITATIONS OF STUDY

The present study was conducted using a Food Frequency Questionnaire (FFQ), which is considered to be the method of choice in order to record dietary habits in large samples for long periods of time (35). FFQ are considered to be easier to complete and easier to be analyzed by researchers than dietary diaries, they have better response rates (36) and are more valid to lead to results about nutrition habits over long periods of time (35). The goal of the present study was to assess dietary preferences throughout pregnancy and since participants had limited time to spend, that is why this tool was selected. However, this tool has a number of restraints the most important of which is the lack of detailed recording and accuracy that food diaries usually achieve (37,38). Nevertheless, there is no perfect tool to use. Food diaries might lead to underreporting, due to subjectivism of the person who is completing it, and FFQ to over-reporting (38). In a recent study in United Kingdom, both methods were used and researchers concluded that they lead to similar outcomes concerning dietary habits of population (35). In the present study, in an attempt to limit the probable error lying within the method of choice, FFQ were given to participants after detailed briefing by the health care scientists explaining to them which was the correct way to fill them in.

CONCLUSION

Adequate intake of nutrients during pregnancy constitutes a positive prognostic factor of its outcome. It is of great importance that health professionals, who deal with pregnant women, to diagnose early any nutritional defects that can easily be addressed and therefore benefit pregnancy progress. This study assesses the current nutritional status of Greek pregnant women. It has been found that current dietary habits lead to adequate intake of some very important nutrients. However, some deficiencies were identified concerning intakes of essential micro-nutrients, such as iron, folic acid and in a less extent vitamin E. These inadequate intakes can easily be overcome by correct dietary advice and the use of proper dietary supplementation.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

REFERENCES

1. Zampelas A., Basilakou T., Bitoratos N., Yiannakoulia M., Dontas A., Risbas G., et al.: Η διατροφή στα στάδια της ζωής. (in Greek). Pasxalidis eds, Athens, 2003

2. Rhind S.M.: Effectos of maternal nutrition on fetal and neonatal reproductive development and function. *Ann. Reprod. Sci. 82-83*: 169-181 (2004)

3. ADA Reports. Position of the American Dietetic Association: Nutrition and lifestyle for a healthy pregnancy outcome. *J. Am. Diet. Assoc. 102*: 1479-1490 (2002)

4. Chatzi L., Torrent M., Romieu I., Garcia-Esteban R., Ferrer C., Vioque J., Kogevinas M., Sunyer J.: Mediterranean diet in pregnancy is protective for wheeze and atopy in childhood. *Thorax 63*: 507-513 (2008)

5. Blades M.: Nutrition before and during pregnancy. *Nutr. Food Sci 2*: 99-101 (1998)

6. Willett W.C., Sampson L., Stampfer M.J., Rosner B., Bain C., Witschi J., Hennekens C.H., Speizer F.E.: Reproducibility and validity of a semiquantitative food frequency questionnaire. *Am. J. Epidemiol. 122*: 51-65 (1985)
7. Olafsdottir A.S., Thorsdottir I., Gunnarsdottir I., Thorgeirsdottir H., Steingrimsdottir L.: Comparison of women's diet assessed by FFQs and 24-hour recalls with and without underreporters: associations with biomarkers. *Ann. Nutr. Metab. 50*: 450-460 (2006)
8. Manios G., Basilakou T., Yiannakoulia M., Faratzian P., Xanthopoulos D.: Nutritional evaluation. Harokopio University eds. Athens, 2004 (in Greek).
9. Fawzi W.W., Rifas-Shiman S.L., Rich-Edwards J.W., Willett W.C., Gillman M.W.: Calibration of a semi-quantitative food frequency questionnaire in early pregnancy. *Ann. Epidemiol. 14*: 754-762 (2004)
10. Goldy C.G., Hanss-Nuss H., Milani T.J., Freeland-Graves J.: Food choices of low income women during pregnancy and postpartum. *J. Am. Diet. Assoc. 105*: 899-907 (2005)
11. Institute of Medicine. Dietary Reference Intakes for energy, carbohydrate, fiber, fatty acids, cholesterol, protein and amino acids. Washington DC, National Academy of Sciences. 2002/2005
12. Schulpis K., Spiropoulos A., Gavrili S., Karikas G., Grigori C., Vlachos G., Papassotiriou I.: Maternal-neonatal folate and vitamin B12 serum concentrations in Greeks and in Albanian immigrants. *J. Hum. Nutr. Dietet. 17*: 443-448 (2004)
13. Hassapidou M., Papadopoulou N.: Assessment of the dietary intakes of healthy adult pregnant women in Cyprus. *Nutr. Food Sci. 30*: 111-115 (2000)
14. Van Raaij J.M.A.: Energy requirements of pregnancy for health Dutch women. *Eur. J. Obs. Gyn. Repr. Biol. 61*: 7-13 (1995)
15. Ortega R.M., Lopez-Sobaler A.M., Andres P., Maritnez R., Quintas M.E., Requejo A.M.: Maternal vitamin E status during the third trimester of pregnancy in Spanish women: influence on breast milk vit E concentration. *Nutr. Res. 19*: 25-36 (1999)
16. Wright M.: A case control study of maternal nutrition and neural tube defects in Nothern Ireland. *Midwifery 11*: 146-152 (1995)
17. Petridou E., Stoikidou M., Diamantopoulou M., Mera E., Dessypris N., Trichopoulos D.: Diet during pregnancy in relation to birthweight in healthy singletons. *Child Care Health Dev. 24*: 229-242 (1998)
18. Black A.E., Wiles S.J., Paul A.A.: The nutrient intakes of pregnant and lactating mothers of good socio-economic status in Cambridge, UK: some implications for recommended daily allowances of minor nutrients. *Br. J. Nutr. 56*: 59-72 (1986)
19. Institute of Medicine, Food and Nutrition Board: Vitamin E. Dietary reference intakes for vitamin C, vitamin E, selenium, and carotenoids. Pp. 186-283, National Academy Press, Washington D.C., 2000
20. Herrera E., Barbas C.: Vitamin E: action, metabolism and perspectives. *J. Physiol. Biochem. 57*: 43-56 (2001)
21. Gao X., Wilde P.E., Maras J.E., Bermudez O.I., Tucker K.L.: The Maximal Amount of α-Tocopherol Intake from Foods Alone in U.S. Adults (1994-1996 CSFII): An Analysis by Linear Programming. *Ann. N.Y. Acad. Sci. 1031*: 385-386 (2004)
22. Smedts H.P., de Vries J.H., Rakhshandehroo M., Wildhagen M.F., Verkleij-Hagoort A.C., Steegers E.A., Steegers-Theunissen R.P.: High maternal vitamin E intake by diet or supplements is associated with congenital heart defects in the offspring. *BJOG 116*: 416-423 (2009)
23. Olson J.A.: Benefits and liabilities of vitamin A and carotenoids. *J. Nutr. 126(Suppl. 4)*: 1208–1212 (1996)
24. Nicolaidou P., Hatzistamatiou Z., Papadopoulou A., Kaleyias J., Floropoulou E., Lagona E., Tsagris V., Costalos C., Antsaklis A.: Low vitamin D status in mother-newborn pairs in Greece. *Calcif. Tissue Int. 78*: 337-342 (2006)
25. Higginbottom M.C., Sweetman L., Nyhan W.L.: Syndrome of methylmalonic aciduria, homocystinuria, megaloblastic anemia and neurological abnormalities in a vitamin B12 deficient breast-fed infant of a strict vegetarian. *N. Engl. J. Med. 299*: 317-323 (1978)
26. Berg M.J., van Dyke D.C., Chenard C., Niebyl J., Hirankarn S., Bendich A., et al.: Folate, zinc and vitamin B-12 intake during pregnancy and postpartum. J. Am. Diet. Assoc. 101: 242-244 (2001)
27. Barker D.J.: Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition 13*: 807-813 (1997)
28. Hess S.Y., King J.C.: Effects of maternal zinc supplementation on pregnancy and lactation outcomes. *BMC Pregnancy Childbirth 7(Suppl. 1)*: 33-38 (2009)
29. Beard J.L.: Why iron deficiency is important in infant development. *J. Nutr. 138*: 2534-2536 (2008)
30. Clandinin M.T.: Brain development and assessing the supply of polyunsaturated fatty acid. *Lipids 34*: 131-137 (1999)
31. Uauy R., Mena P., Rojas C.: Essential fatty acids in early life: structural and functional role. *Proc. Nutr. Soc. 59*: 3-15 (2000)
32. Krauss-Etschmann S., Shadid R., Campoy C., Hoster E., Demmelmair H., Jiménez M., et al.: Nutrition and Health Lifestyle (NUHEAL) Study Group. Effects of fish-oil and folate supplementation of pregnant women on maternal and fetal plasma concentrations of docosahexaenoic acid and eicosapentaenoic acid: a European randomized multicenter trial. *Am. J. Clin. Nutr. 85*: 1392-1400 (2007)
33. Makrides M., Duley L. Olsen S.F.: Marine oil, and other prostaglandin precursor, supplementation for pregnancy uncomplicated by pre-eclampsia or intrauterine growth restriction. Cochrane Database Syst. Rev. 3: CD003402 (2006)
34. Horvath A., Koletzko B., Szajewska H.: Effect of supplementation of women in high-risk pregnancies with long-chain polyunsaturated fatty acids on pregnancy outcomes and growth measures at birth: a meta-analysis of randomized controlled trials. *Br. J. Nutr. 98*: 253-259 (2007)
35. Crozier S.R., Inskip H.M., Keith M.G., Robinson S.M.: Dietary patterns in pregnant women: a comparison of food-frequency questionnaires and 4d prospective diaries. *Br. J. Nutr. 99*: 869-875 (2008)
36. Henderson L., Gregory J., Swan G.: The National Diet and Nutrition Survey: Adults aged 19-64 years. H.M. Stationery Office, London, 2002
37. Kipnis V., Subar A.F., Midthune D., Freedman L.S., Ballard-Barbash R., Troiano R.P., et al.: Structure of dietary measurements error: results of the OPEN Biomarker Study. *Am. J. Epidemiol. 158*: 14-21 (2003)
38. Robinson S., Godfrey K., Osmond C., Cox V., Barker D.: Evaluation of a food frequency questionnaire used to assess nutrient intakes in pregnant women. *Eur. J. Clin. Nutr. 50*: 302-308