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"Nicolae Simionescu"

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ORGANISING COMMITTEE

Dr. Maya Simionescu, SERA Coordinator
Dr. Anca V. Sima
Dr. Doina Popov

WELCOME

Dear Friends and Colleagues,

On behalf of ICBP team, it is my great pleasure to welcome all the participants to our Workshop held within the frame of our European Community – SSA – SERA project.

The topic we choose for this meeting reflects our major concern for maladies that affect today a large part of the world population – cardiovascular diseases and diabetes – and the strive to uncover the deviations from normal of subtle cellular mechanisms leading to these disorders.

The challenging task to put together an interesting and appealing program was possible due to you all, outstanding scientists from Europe and from Romania participating at this conference. On behalf of the Organizing Committee, a heartfelt thanks to all of you.

We have amass together basic and clinical data, hopping that this may promote translation of information "from bench to bedside" and exploration of new directions of research that may lead us faster "on route" to correct, ameliorate, prevent and treat these maladies.

After all, this is what biomedical science is all about.

We hope that you will enjoy this scientific event, and that the outcome will be increased knowledge, exchange of information, new collaboration and a general spirit of cohesion through Science and for Science.

Welcome to Romania / Bine ați venit în România
Welcome to ICBP / Bine ați venit în IBPC

Maya Simionescu
Director of ICBP "N. Simionescu"
Coordinator of the SERA project

PLATFORM PRESENTATIONS
HOW DO HDLs PROTECT AGAINST ATHEROSCLEROSIS?

CHRISTIAN EHNHOLM

Department of Biochemistry, National Public Health Institute, Helsinki, Finland

Cardiovascular disease (CVD) is the most significant cause of morbidity and mortality in the western world. In Finland CVD is the number one cause of death with almost twice as many people dying each year as a result of CVD than cancer. Many risk factor are associated with CVD and most can be managed.

Genetic, pathological, laboratory and interventional epidemiologic studies have clearly established the primary role of plasma lipoproteins in the development of CVD. A direct involvement of plasma cholesterol in the development and progression of atherosclerotic cardiovascular risk is one of the best-proven cases in modern medicine.

A strong positive relationship between the concentration of low density lipoprotein cholesterol (LDL-C) and the future risk of cardiovascular event has been observed in many large-scale population studies and the benefits of reducing LDL-levels has been proven beyond doubt in intervention studies. Yet, despite the impressive cardioprotective effects of aggressive LDL-C lowering, it is apparent that, the residual risk (~60%) in many people remains unacceptably high. Much of this residual risk relates to the presence of a low level of high density lipoprotein cholesterol (HDL-C).

It has been known for many years that the concentration of HDL-C correlates inversely with cardiovascular risk. The Framingham Heart Study showed that people whose HDL-C level was less than 35 mg/dl (0.91 mmol/l) at the beginning of the study had a future coronary risk more than eight times that in subjects whose HDL-C level was greater than 65 mg/dl (1.68 mmol/l).

This and other studies, strongly support the view that raising the level of HDL-C should be considered a therapeutic target of importance comparable to that of lowering LDL-C.

HDL is not homogeneous but of a spectrum of particles of distinct structure, function and composition. This heterogenicity which is the result of continuous remodeling of HDL by plasma factors, has important implications in terms of HDL metabolism and antiatherogenicity. These factors include lecithin: cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP), hepatic lipase (HL). As the remodeling of HDL by plasma factors is rapid relative to the 3–5 day plasma half life of their main apolipoproteins it dominates the metabolism of HDL. The origin, metabolism and transport functions of HDL will be discussed. Also the non-lipid functions of HDL and their role as anti-atherogenic factors will be dealt with.

The best known of the protective functions of HDL is their role in promoting efflux of cholesterol from macrophage foam cells in the artery wall thus inhibiting the progression of atherosclerosis. Studies concerning the role of PLTP in cholesterol efflux from macrophages will be reported.

ENDOTHELIAL CELL: YESTERDAY NEGLECTED, TODAY ON THE CENTRAL STAGE

MAYA SIMIONESCU

Institute of Cellular Biology and Pathology “Nicolae Simionescu”, Bucharest, Romania

Major human maladies (atherosclerosis, diabetes, obesity, neurodegenerative disorders) are associated with, or are due to vascular diseases that entail the direct participation of endothelial cells (EC). Initially considered a gratuitous cellophane-like sheet, the EC have earned the respect of biologists, pathologists and pharmacologists.

The **cell biologists** discovered that EC have a large array of paracrine, endocrine and autocrine functions: they monitor transcytosis of molecules from the blood to tissues, synthesize basal lamina and extracellular matrix components, guard vascular tone (by balanced synthesis of PGI2, NO, EDHF, endothelin), administer haemostasis (via vWf and PAI-1). We have reported that EC perform transcytosis of macromolecules (LDL, albumin, etc.) by a fluid phase, adsorbtive or receptor-mediated mechanism and caveolae (caveolin-rich EC microdomains) are the cargo-carrier for endocytosis and transcytosis. In addition

EC along the vascular tree are endowed with an innate heterogeneity that confers the ability to sense, monitor, command and modulate various functions, as well as to respond to various aggressive factors.

The **cell pathologists** revealed that EC dysfunction as a "syndrome" consists of perturbed transcytosis, disturbed synthetic capacity, corrupt cross-talk with neighbouring cells, unbalanced antithrombogenic capacity, impaired regulation of the vascular tone, disturbed proliferative capacity, finally leading to EC injury and death. Our data obtained on hyperlipemic rabbit and hamster, hyperlipemic and hyperglycemic hamster, as well as on human early atherogenesis, demonstrated that to these insults, the initial vessel wall response is the modulation of EC constitutive function: (1) increased LDL transcytosis that together with decreased efflux and interaction with matrix proteoglycans lead to subendothelial accumulation of modified and reassembled lipoproteins (mLp) and (2) increased synthetic capacity. The dual assault of hyperlipemia and mLp on endothelium generate a multipart inflammatory process that at the EC level is expressed by alteration of plasma membrane components, endoplasmic reticulum stress, increased adhesiveness for monocytes, anoikis, changes of the integrity of EC junctions, deficiency in lysosomal acid lipase, and ultimately to cholesterol ester accumulation and endothelial-derived foam cell formation.

The **pharmacologists** found that, directly or indirectly, EC dysfunction may be corrected by drugs such as Ebselen, ACE inhibitors, statins, etc. We have reported that in EC dysfunction induced by high glucose, enoxaparin has an antioxidant effect, aspirin and PPAR activators reduce MCP-1 expression and nebivolol improves EC performance. Since EC dysfunction is a gradual process, early markers and a combination of agents and drugs tailored for a specific EC dysfunctionality hold the future for therapeutic interventions. We have reasons to hypothesize that restoration of endothelial functions could be the target and the efficient mean to counteract the adverse effect of hyperlipemia / hyperglycemia. Together, the wealth of data on the role of EC in health and disease may add up to a complex discipline that includes **endotheliology, endotheliopathy and endotheliotherapy.**

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TARGETING THE CELL CYCLE MACHINERY TO TREAT CARDIOVASCULAR DISEASE

GAVIN BROOKS

Cardiovascular Research Group, School of Pharmacy, University of Reading, Whiteknights, Reading, Berkshire, UK

Cardiovascular disease represents a major clinical problem affecting a significant proportion of the world's population and it remains the major cause of death in the EU and the rest of the Western world. Furthermore, the burden on healthcare systems is increasingly high; the overall cost of cardiovascular disease to the EU economy is estimated to be in excess of 192 billion Euros per year. The majority of therapies currently available for the treatment of cardiovascular disease do not cure the problem, but merely treat the symptoms. Furthermore, many cardioactive drugs have serious side effects and have narrow therapeutic windows that can limit their usefulness in the clinic. Thus, the development of more selective and highly effective therapeutic strategies that could cure specific cardiovascular diseases would be of enormous benefit both to the patient and to those countries where health care systems are responsible for an increasing number of patients. There is increasing evidence to suggest that targeting the cell cycle machinery in cardiovascular cells (*e.g.* cardiac myocytes, vascular smooth muscle cells (VSMCs), endothelial cells) provides a novel approach for the treatment of certain cardiovascular diseases, including post-infarct heart failure, restenosis, in-stent stenosis and bypass graft failure.

It has been demonstrated that certain cell cycle molecules that are important for regulating terminal differentiation in cardiac myocytes (*e.g.* cyclins, cyclin-dependent kinases [CDKs], CDK inhibitors, E2F transcription factors) can be targeted to reinitiate cell division and repair in the myocardium post-infarction. Furthermore, cell cycle molecules that control excessive VSMC proliferation in disorders such as restenosis, in-stent stenosis and bypass graft failure have also been targeted effectively in recent laboratory and clinical studies. The results of these studies illustrate the exciting possibility of targeting components of the cell cycle machinery to improve cardiac function and prognosis for patients with heart failure and for patients with atherosclerosis.

ENDOPLASMIC RETICULUM: THE KEY FOR THE PATHOGENESIS OF DIFFERENT DIABETES PHENOTYPES

CONSTANTIN IONESCU-TÎRGOVIŞTE

Institute of Diabetes, Nutrition and Metabolic Diseases "N. Paulescu", Bucharest, Romania

Based on our clinical and epidemiological data, we have sustained for a long time the unitary character of the various phenotypes of the diabetic syndrome. In this study, we add several arguments sustaining that the unitary character of diabetes is related to a common primary defect in the function of the beta cell endoplasmic reticulum (ER), leading to an inadequate processing of the two main secretory molecules: pre-proinsulin and pre-proamylin. The post-translational changes of these molecules might explain the main proapoptotic and anti-regenerative pathogenic mechanisms leading to a progressive decrease in the β cell mass/function.

Apart the β cell, in the pathogenesis of diabetes there are also three other cells involved in diabetogenesis: the adipocyte (with storage energy function), the hepatocyte (the place where the metabolic pathways are intersecting) and the myocyte (the main energy consumer). All these cells have a special functional relation with the β cell, using their ER capacity to sense the circulating nutrients and reacting adequate to all changes in their concentrations. In our view, the increased proinsulin levels encountered in various diabetes phenotypes could be not only a marker of beta cell dysfunction, but also could indicate the main β cell defect, suggesting also its location in ER, not only from β cell, but also in the other three main cells involved in maintaining the homeostasis in the human body.

REGULATION OF HDL AND VLDL METABOLISM BY APO A-II, A MAJOR HDL APOPROTEIN, AS ASSESSED BY STUDIES IN TRANSGENIC MICE EXPRESSING HUMAN APO A-II

MICHELE CHABERT, SONIA DUGUE-PUJOL, XAVIER ROUSSET, JEAN CHAMBAZ, ATHINA KALOPISSIS

UMRS 872, Centre de Recherche des Cordeliers, Paris, France

The atheroprotective effects of HDL have been generally attributed to apo A-I, its major apoprotein, whereas the role of apo A-II, its second major apoprotein, was little studied. Therefore, we generated transgenic mice with various levels of expression of human apo A-II (hapo A-II), to study its role in vivo. Surprisingly, hapo AII transgenic mice presented several features of the Metabolic Syndrome (MS), such as postprandial hypertriglyceridemia, low plasma HDL levels, and glucose intolerance.

We showed that hapo A-II associates in plasma with triglyceride-rich lipoproteins (TRL) of intestinal and hepatic origin (chylomicrons and VLDL, respectively). The presence of hapo A-II on the surface of TRL transiently blocked their catabolism by inhibiting the activities of lipoprotein lipase (LPL) and hepatic lipase (HL). This resulted in postprandial hypertriglyceridemia that was proportional to the plasma concentration of hapo A-II.

Human apo A-II is more hydrophobic than apo A-I (human and murine) and is thus able to displace apo A-I from the surface of HDL, in vitro and in vivo. We showed that hapo A-II displaced apo A-I from the surface of HDL and greatly increased its catabolism in kidney, resulting in a very low plasma apo A-I concentration. Concomitantly, the size of HDL particles and their plasma concentration decreased and hapo A-II became the major HDL apoprotein.

We tested the properties of hapo A-II-rich HDL of transgenic mice in terms of two well established antiatherogenic properties of HDL: i) protection of TRL from oxidation; ii) stimulation of cholesterol efflux, the first step of reverse cholesterol transport from peripheral tissues to liver for recycling/ excretion into bile.

We showed that hapo A-II-rich HDL, which contain only trace amounts of apo A-I, carried normal amounts of paraoxonase and PAF-acetylhydrolase, two antioxidative enzymes of HDL. Moreover, hapo A-II-rich HDL protected TRL from copper-induced oxidation more efficiently than control mouse HDL rich in apo A-I.

Regarding cholesterol efflux, hapo A-II exhibited two opposite effects depending on the membrane protein involved. In Fu5AH cells, which highly express Scavenger Receptor BI (SR-BI), plasma from hapo A-II transgenic mice induced a lower SR-BI-mediated cholesterol efflux than control mouse plasma. Conversely, plasma from hapo A-II transgenic mice (containing pre-β HDL carrying hapo A-II) elicited a higher cholesterol efflux from J774 macrophages than plasma from wild-type mice, via stimulation of ATP Binding Cassette Transporter A1 (ABCA1).

Lipid-free hapo A-II was as effective as hapo A-I in stimulating ABCA1-mediated cholesterol efflux from macrophage foam cells.

Thus, apo A-II controls in vivo several key steps in lipoprotein metabolism: it retards TRL catabolism by inhibiting the activities of LPL and HL, it probably stabilizes HDL by retarding remodeling by HL (hydrolyzing HDL-phospholipid) and SR-BI (allowing selective uptake of HDL-cholesterol), and stimulates cholesterol efflux via ABCA1, the major protein involved in efflux from macrophage foam cells at the vascular wall. The role of hapo A-II in inflammation remains to be determined.

MODIFIED LIPOPROTEINS AS RISK FACTORS IN ATHEROSCLEROSIS AND DIABETES; *IN VIVO* AND *IN VITRO* MODELS

ANCA V. SIMA, CAMELIA STANCU

Institute of Cellular Biology and Pathology, "Nicolae Simionescu", Bucharest, Romania

Alteration of lipoproteins (Lp) is believed to play an important role in atheroma formation. This report focuses on the structural-functional modifications that occur in the vasculature of an animal model of fat-diet induced atherosclerosis. Golden Syrian hamsters were fed standard chow supplemented with 1% cholesterol +15% butter, hyperlipemic (HL), or by additional injection of streptozotocin, hyperlipemic/ /diabetic (HD). In HD hamsters plasma cholesterol, triglycerides, glucose and LDL increased, while HDL decreased after 2 weeks, the dyslipidemia and hyperglicemia being correlated with the extent and gravity of arterial lesions. The HL diet induces an increase in serum, LDL and beta-VLDL cholesterol, triglycerides, and a decrease in HDL-cholesterol and total peroxyl-radical trapping potential. Interestingly, in HL hamsters, after 4 weeks of fat diet, plasma glucose concentration increased significantly. Electron-microscopic examination of lesion-prone areas from HL hamsters' arteries showed that the alteration of plasma Lp fractions and peroxidation status leads to sequential modifications in the endothelial cells (EC), smooth muscle cells (SMC), monocyte-derived macrophages, and in the extracellular matrix. In order to visualize the pathway of LDL into the arterial wall, we isolated LDL from human subjects, labeled it with either DiI, or Au_{5nm} and injected it into HL hamsters. LDL- DiI injected in vivo for 24 h was taken up by the grossly normal intima, shoulders of the atherosclerotic plaques, insertion of sigmoid valves and by ostia of the aortic branches; the fibrous cap of the atheroma was barely labeled. Perfusion of LDL-Au_{5nm} in HL hamsters vasculature showed particles trancytosed through endothelial plasmalemmal vesicles or endocytosed by coated pits and vesicles of the endothelium. In the subendothelium, LDL-Au_{5nm} were present as aggregates, associated with proteoglycans, collagen and elastin fibers. After 1 year of HL diet, LDL-Au_{5nm} passed through openings of inter-endothelial junctions and accumulated in the subendothelium of coronary arteries and valves. In order to detect altered LDL accumulation, we used monoclonal antibodies against LDL oxidation products, 4-hydroxynonenal (HNE) and advanced glycation end-products (AGE), and localized them within the areas of intens LDL uptake on cryosections obtained from aortic arch and valves. We have also immunolocalized LDL oxidation products in the atherosclerotic plaques from diabetic, coronary artery diseased patients. HNE and AGE colocalized on cryosections from human atheroma, being present both intracellularly, in macrophage-derived foam cells from the shoulder areas and in the smooth muscle cells of the fibrous cap, and extracellularly, in lipid deposits near the internal elastic lamina. Cultured human EC incubated with HL and HD sera presented a 2-3 fold increase in cholesteryl esters (CE) content and in CD36 expression, while incubated with human in vitro gLDL accumulated CE, in parallel with the increase of CD36 and LOX-1 expression; gLDL significantly enhanced CD36 gene and protein expression in SMC compared with nLDL. An increase in the gene expression of SR-BI, MCP-1 and a decrease in LDLR gene expression was also detected. In addition, gLDL stimulated the proliferation rate of SMC. Together these results suggest that the hyperlipemic hamster is a reliable model to unravel cellular alterations leading to atheroma formation, and to test the effect of drugs in this process.

LIPID TRANSFER PROTEINS IN AN ANIMAL MODEL OF ATHEROSCLEROSIS: THE HYPERLIPIDEMIC HAMSTER

MARIUS R. ROBCIUC, ANCA V. SIMA

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania

Phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) are key enzymes in the lipid metabolism and play critical

role in cardiovascular diseases. Our goal was to evaluate the activities of PLTP and CETP in the serum of hyperlipidemic (HL) hamsters and the effect of alpha-tocopherol administration in this animal model. Male Golden Syrian hamsters received either standard chow (control) or chow supplemented with 20% butter and 0.1% or 0.5% cholesterol. Alpha-tocopherol was administrated by gavage for 4 weeks to HL and control hamsters. Sera from animals were analyzed for changes in: lipid parameters, apoE concentration, PLTP and CETP activity. Hamsters fed hyperlipidemic diets for 8 weeks developed significantly increased cholesterol, triglycerides and LDL-C levels, while the ratio of HDL/total cholesterol was significantly decreased. PLTP and CETP activities were significantly increased in all HL hamsters, but PLTP activity was less modulated by the high serum cholesterol levels. ApoE levels were also increased in the sera from HL hamsters, but were not correlated with the increased PLTP activity. Alpha-tocopherol administration had no significant effect on PLTP activity or apoE concentration, but significantly inhibited CETP activity, probably by reducing serum cholesterol.

OXIDATION OF PROTEIN-TYROSINE PHOSPHATASES IN CELLULAR SIGNAL TRANSDUCTION

FRANK-D. BÖHMER

Institute of Molecular Cell Biology, Center for Molecular Biomedicine, Friedrich-Schiller-University Jena, Jena, Germany

Protein-tyrosine phosphatases (PTPs) regulate cellular signal transduction by dephosphorylating phosphotyrosine containing proteins, thereby antagonizing the actions of protein-tyrosine kinases. The members of this family are encoded by more than 100 genes in the human genome. The 38 “classical PTPs” have exclusive specificity for phosphotyrosine, whereas the other family members are dual-specificity phosphatases (DUSPs). PTPs have a common cysteine-based mechanism of catalysis. The catalytic cysteine is susceptible to oxidation. Reversibly oxidized PTPs are inactive but can be reactivated by reduction. Evidence is accumulating that the reversible oxidation of PTPs may be an important aspect of signal transduction of many membrane receptors, for example growth factor receptors. In addition, PTP oxidation may also be involved in the cellular actions of adverse agents, such as UV irradiation, or oxidative stress.

We have previously reported inactivation of PTP1B upon UV-A irradiation of cells with subsequent ligand independent activation of receptor tyrosine kinases. Irradiation leads both to an increase of reactive oxygen species – thereby oxidizing protein tyrosine phosphatases – and a release of intracellular Ca^{2+} causing an activation of calpain. These independent signals are integrated and result in degradation of the oxidized phosphatase [Gulati *et al.*, EMBO Rep., 5, 812-7 (2004)]. The mechanism and physiological role of degradation of reversibly oxidized PTPs by calpain demand further clarification. *In-vitro,* reversible oxidation of PTP1B at its catalytic cysteine has been shown to lead to formation of a so-called sulfenylamide. This five-membered ring exerts strong constraints to the catalytic cleft which result in conformational changes in this particular region of the phosphatase, whereas the overall conformation remains mostly unchanged. We have identified the oxidation-specific cleavage site of PTP1B by calpain *in vitro.* It is localized in a loop at the side of the PTP opposite to the catalytic center. Oxidation of PTP1B leads to an enhanced association with calpain, thereby facilitating cleavage. Calpain-mediated cleavage irreversibly inactivates the enzyme. Mutations in the neighbourhood of the cleavage site redirect cleavage specificity. Ongoing work will reveal if alterations in calpain sensitivity of PTP1B can alter the cellular response to growth factors.

Different PTPs appear to differ in their sensitivity to oxidation, suggesting that oxidation may be more relevant for some members of the family than for others. We compared SHP-1 and SHP-2, two structurally related cytoplasmic protein-tyrosine phosphatases with different cellular functions and cell-specific expression patterns, for their intrinsic susceptibility to oxidation by H_2O_2. The extent of oxidation was monitored by detecting the modification of the PTP catalytic cysteine by three different methods, including a modified in-gel PTP assay, alkylation with a biotinylated iodoacetic acid derivative, and an antibody against oxidized PTPs. The dose-response curves for oxidation of the catalytic domains of SHP-1 and SHP-2 were similar. Both PTPs are only moderately sensitive to oxidation. Interestingly, the SH2 domains had a protective function with respect to oxidation. Furthermore, we observed strong differences in PTP oxidation when analyzing different cell lines. In EOL-1 cells, SHP oxidation by exogenous H_2O_2 in general, and

SHP-2 oxidation in particular were strongly diminished compared to HEK293 cells, at least partially related to a generally lower oxidant sensitivity of the EOL-1 cells. The data suggest that the differential cell functions of SHP-1 and SHP-2 are not related to differences in oxidation sensitivity. The modulating effects of SH2 domains for oxidation of these PTPs are in support of an enhanced oxidation-susceptibility of activated SHPs.

PHENOTYPIC CHANGE OF VASCULAR CELLS IN HIGH GLUCOSE CONDITIONS: FROM AFFECTED MOLECULES TO DYSFUNCTION AND POTENTIAL MODULATION

DOINA POPOV

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania

The diabetic vascular wall continuously exposed to high glucose concentration undergoes morphological, physiological, and biochemical changes that trigger vessel dysfunction. In this study we questioned on the effect of hyperglycemic insult on the phenotype of arterial endothelial cells (ECs) and smooth muscle cells (SMCs), and tried to identify the activated signaling molecules involved. Experiments were conducted on human aortic ECs and SMCs cultured in DMEM supplemented with 25 mM glucose (simulating diabetes) (control: 5.5 mM glucose, as physiological condition), and in vivo on streptozotocin-injected Golden Syrian hamsters (plasma glucose concentration: 440±20 mg/dL) (control: normal hamsters) followed by electron microscopy, immunofluorescence, and immunoblotting.

In ECs, 25 mM glucose induced (*vs*. control): (i) change from the quiescent to the secretory phenotype; this modification is similar to that encountered at the ECs in the aorta and the mesenteric resistance arteries of diabetic hamsters; (ii) augmented expression and reorganization of focal adhesion protein a-actinin, suggesting remodeling of actin filaments and crosslinking to the plasma membrane; (iii) ~2 fold increase in phosphorylation of STAT-3 and ERK1/2, contributing to cells intense proliferation and differentiation occurring in the hyperglycemic milieu.

Arterial SMCs exposed to high glucose concentration (both *in vitro* and *in vivo*) showed: (i) the modification of the contractile phenotype to either a biosynthetic one, displaying numerous copies of rER and a thickened extracellular matrix intensely immunostained for type IV collagen, and/or transdifferentiation into osteoblast-like cells. The latter nucleated calcification centers with the characteristic profile of hydroxyapatite around small membranous vesicles (~100 nm in diameter) extruded by viable vascular SMCs, as well as around larger vesicles (250 nm in diameter) arising from dead or dying SMCs; both these deposits contribute to the impeded arterial distension associated with diabetes, (ii) organization of vinculin plaques at the SMCs periphery, supporting cells spreading and focal adhesions formation (in controls, vinculin showed mainly an intracytoplasmic distribution), (iii) proliferation (as demonstrated by the presence of centriols) associated with increased protein expression of pSTAT-3 (~ 2.54 fold) and pERK1/2 (~ 2.12 fold above the levels in controls). Together, these data show that chronic exposure to high glucose is associated with abnormal signaling in arterial ECs and SMCs that may explain the impeded arterial wall reactivity.

Vascular wall dysfunction is a systemic disorder that affects microvasculature, coronary arteries, conduit arteries and small resistance arteries (Ø ≤ 300 μm). The mesenteric vascular bed of diabetic hamsters displayed a reduced NO-dependent vasodilation in response to acetylcholine and bradykinine, a process due to augmented hyperglycemia (~2.8 fold *vs*. normal), oxidative and carbonyl stress (~3.2× fold), and NO inactivation by increased deposition of AGE-collagen, and pentosidine. We document the potential reversal of defective endothelial-dependent relaxation by oral administration of L-arginine (eNOS substrate), and exposure to enoxaparin (*in vitro*).

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IDENTIFICATION OF NOVEL BIOMARKERS OF CARDIOVASCULAR DISEASES

JESUS EGIDO, JOSE LUIS MARTIN-VENTURA, LUIS MIGUEL BLANCO-COLIO

Fundación Jiménez Diaz, Autonoma University, Madrid, Spain

Assessment of vascular risk in asymptomatic patients is a major challenge for prevention of cardiovascular events. These events arise from the disruption of atherosclerotic plaques that contain

numerous inflammatory cells. Inflammatory and resident cells (endothelial and vascular smooth muscle cells) release different proteins that can generate a chronic inflammatory response in the injured artery. Measurement of circulating markers of inflammation may provide some insights into this process.

Interaction between members of the tumor necrosis factor (TNF) superfamily and their receptors elicits diverse biologic actions that participate in atherosclerosis development. Fas and its ligand are typical members of the TNF superfamily. Proteins secreted by cells implicated in atherosclerotic lesions, including soluble Fas (sFas) and soluble Fas ligand (sFasL), circulate in small, but detectable, amounts. We have observed that sFas concentrations are increased and sFasL are decreased in subjects at high cardiovascular risk, suggesting that these proteins may be novel markers of vascular injury. In addition, patients with familial combined hyperlipidemia or carotid atherosclerosis have decreased circulating sFasL levels, probably indicating endothelial dysfunction. To confirm this hypothesis, we have recently analyzed whether the forearm vasodilatory response to reactive hyperemia (an indicator of endothelial function), is associated with soluble sFasL plasma concentrations in subjects with coronary artery disease. There was a linear trend for the increase of sFasL and forearm reactive hyperemia which suggest that sFasL plasma concentrations could be a potential biomarker of endothelial function.

Another member of the TNF superfamily is the TNF-like weak inducer of apoptosis (TWEAK/ TNFS12) and its receptor Fn14. We have observed that Fn14 and TWEAK are expressed in macrophages and smooth muscle cells in carotid atherosclerotic plaques, and could be novel mediators of atherosclerosis. In addition, we have observed that soluble TWEAK (sTWEAK) is released in lower amount by carotid plaques than normal endarteries. Subsequent measurement of sTWEAK in plasma showed a reduced concentration in subjects with carotid stenosis compared with healthy subjects. Furthermore, in a test population of 106 asymptomatic subjects, we showed that sTWEAK concentrations negatively correlated with the carotid intima-media thickness, suggesting that sTWEAK could be a potential biomarker of subclinical atherosclerosis.

The identification of novel biomarkers along with traditional risk factors and imaging techniques, could help to target vulnerable patients and monitor the beneficial effects of pharmacological agents.

HIGH GLUCOSE CONDITIONS INDUCE AN INFLAMMATORY UP-REGULATION OF FRACTALKINE AND MCP-1 IN HUMAN SMOOTH MUSCLE CELLS BY A MECHANISM INVOLVING THE MAPK SIGNALING PATHWAY

ELENA DRAGOMIR, ILEANA MANDUTEANU, MANUELA CALIN, ANA MARIA GAN, DANIELA STAN, RORY RYAN KOENEN*, CHRISTIAN WEBER*, MAYA SIMIONESCU

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania
*Institute for Cardiovascular Molecular Research (IMCAR), Aachen, Germany

Objective

The major complication of diabetes mellitus is accelerated atherosclerosis that entails an inflammatory process, in which fractalkine and MCP-1 have key roles. We investigated the effect of diabetes-associated high glucose (HG) on these chemokines and the signaling mechanisms involved in human aortic smooth muscle cells (SMC).

Experimental design and methods

SMC exposed to HG were assayed for gene and protein expression of fractalkine and MCP-1 by RT-PCR and Western blot. The role of MAPK in the chemokines regulation was assessed employing inhibitors of ERK1/2 and p38 MAPK and the involvement of AP-1 and NF-kB by cell transfection with decoy oligodeoxynucleotides. The direct interaction between HG-activated SMC and monocytes was tested by adhesion assay.

Results

HG induced an increase in the mRNA and protein expression of fractalkine and MCP-1 and activated MAPK signaling pathway, a process associated with augmented oxidative stress. Transfection with decoy oligodeoxynucleotides revealed the involvement of transcription factors AP-1 and NF-kB in chemokine up-regulation. The MAPK inhibitors hinder IkBα and c-jun phosphorylation, indicating the role of MAPK in NF-kB and AP-1 activation. Up-regulation of

chemokines correlated well with the increased adhesive interaction between HG-activated SMC and monocytes. PPARα activators (fenofibrate and clofibrate) reduced mRNA and protein expression of chemokines in HG-activated SMC.

Conclusion

HG up-regulates the expression of fractalkine and MCP-1 in SMC leading to increased monocyte–SMC adhesive interactions by a mechanism involving activation of MAPK, AP-1 and NF-kB. This may account for the enhanced SMC accumulation, the increased adhesion between SMC and monocytes and consequently, to accelerated plaque formation in diabetes.

APOLIPOPROTEIN E: FROM DYSLIPIDEMIA, TO ATHEROSCLEROSIS, TO OBESITY

KYRIAKOS E. KYPREOS

University of Patras Medical School, Department of Medicine, Pharmacology Unit, Rio-Patras, Greece

Apolipoprotein E (apoE) is probably one of the most important proteins of the lipoprotein transport system and is responsible for maintaining normal plasma cholesterol and triglyceride levels in the circulation. Despite the beneficial effects of wild-type apoE, it has a reduced therapeutic value for the correction of dyslipidemias, because it acts as a double-edged sword: at physiological concentrations, it maintains lipid homeostasis and is atheroprotective; at high concentrations, apoE causes high triglyceride levels (hyper-triglyceridemia) and fails to clear cholesterol from the circulation.

We have used transient gene transfer methodologies in apoE-deficient mice to correct the apoE deficiency. These studies provided the following new insights on the role of apoE in cholesterol and triglyceride homeostasis in the circulation and the molecular etiology of type III hyperlipoproteinemia. a) Truncated apoE forms can clear efficiently cholesterol from the plasma of apoE-deficient mice; full-length apoE induces hypertriglyceridemia; b) Residues L261, W264, F265, L268 and V269 of apoE can account for the apoE-induced hypertriglyceridemia in vivo, and they also affect formation of HDL. Substitution of these residues by Ala provided a recombinant apoE form with improved biological functions which may have therapeutic applications in the future; c) The amino terminal domain 1-185 of apoE is sufficient to mediate efficient clearance of the apoE-containing lipoprotein remnants *in vivo* via the LDL receptor; d) ApoE-induced hypertriglyceridemia is due to increased hepatic VLDL triglyceride secretion and reduced activity of lipoprotein lipase *in vivo*; e) The LDL receptor deficiency or apoE mutations increase the sensitivity to apoE-induced hypertriglyceridemia.

Based on these findings we were able to generate a recombinant full-length apoE form (apoE4 [L261A/W264A/F265A/L268A/V269A] designated as apoE4mut1) with improved biological functions. Specifically, apoE4mut1 can efficiently correct the high cholesterol levels of apoE-deficient mice and promote the formation of HDL, even when expressed at high plasma concentrations. We believe that this recombinant apoE form is a potential new lead compound for the cure of dyslipidemia and possibly atherosclerosis in the future.

Recently we have also started investigating the role of apoE in the development of diet-induced obesity and related metabolic disorders. Obesity is a central feature of the metabolic syndrome and is associated with increased risk for type II diabetes. Our data establish that in addition to other previously identified mechanisms of obesity, apoE and the chylomicron pathway are also important contributors to the development of obesity and related metabolic dysfunctions in mice.

Overall, apoE appears to play pivotal role in a number of biochemical processes ranging from lipid and lipoprotein metabolism, to atherosclerosis, to obesity and more studies are needed in order to delineate all the mechanisms underlying these very important functions of apoE.

THE ROLE OF CELLULAR SENESCENCE FOR THE DEVELOPMENT OF CARDIOVASCULAR DISEASES

PIDDER JANSEN-DÜRR

Institute for Biomedical Ageing Research, Austrian Academy of Sciences, Innsbruck, Austria

Age is considered the single most important risk factor for the development of cardiovascular

diseases. However the molecular mechanisms, by which ageing processes provide the ground for the development of cardiovascular diseases is not known. Initially discovered by Leonard Haylfick in the 1960s, cellular senescence has now received a lot of attention as a potential mechanism of tumour suppression; moreover, increasing evidence suggests that cellular senescence contributes to both extrinsic and intrinsic ageing processes. We have studied replicative and stress-induced senescence of human endothelial cells as a model system for vascular ageing. Using biochemical and molecular biology techniques as well as systematic assays of gene expression changes via RNA profiling and proteomics technology, we identified a set of candidate genes that are differentially regulated in senescent human endothelial cells. The suitability of such candidate genes as targets for intervention or as markers for diagnosis are currently been investigated. This is relevant since it has been shown by several groups that normal ageing, and in particular stress induced premature ageing is associated with the accumulation of cells that exhibit the senescence phenotype in various human tissues including the vascular endothelium. Our current activities in this research area will be discussed.

MACROPHAGE-SPECIFIC GENE REGULATION OF apoE

ANCA GAFENCU, ELENA FUIOR, MARIUS ROBCIUC, ALEXANDRA ROBCIUC, DIMITRIS KARDASSIS*, MAYA SIMIONESCU

Institute of Cellular Biology and Pathology "N. Simionescu" Bucharest, Romania
*University of Crete, Heraklion, Greece

Objective

Based on the fact that apoE secreted by resident macrophages in the artery wall exerts an important protective role in atherogenesis, we investigated the mechanism of apoE gene repression by LPS in mouse macrophages RAW264.7.

Methods

The modulation of apoE expression in RAW264.7 under stress condition was tested by RT-PCR. To search for the LPS signaling mechanism involved in this gene expression modulation, transient transfection experiments were performed. ApoE proximal promoter alone or together with the multienhancer 2 (ME2) cloned in pGL3 basic vector containing the luciferase gene and different expression vectors for kinases and TF were used.

Results

LPS repressed the apoE expression, acting on the promoter, as well as on the ME2. Two upstream kinases are involved in this downregulation: Tpl-2 and MEKK1. Using IκB dominant negative expression vectors, we established that Tpl-2 and MEKK1 signaling pathways are convergent to NF-κB acting on apoE promoter; overexpression of IKKβ and p65 in RAW cells inhibit the activity of the apoE promoter. In addition, AP-1 complex activated by LPS via Tpl2 and MEKK1, downregulates apoE expression in macrophages, inhibiting the apoE promoter activity.

Conclusion

LPS acts *via* a dual mechanism in the repression of apoE gene expression in macrophages: via Tpl2 and MEKK1, LPS activates AP1 and NFκB transcription factors that inhibit the apoE expression acting directly on the promoter or indirectly, on the ME2.

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ROLE OF NADPH OXIDASE IN CARDIOVASCULAR DISEASE: FROM THE GENE TO THE PATIENT

GUILLERMO ZALBA

Fisiopatología Cardiovascular, Facultad de Medicina, Pamplona, Spain

Oxidative stress plays a key role in the pathophysiology of several major cardiovascular diseases, including atherosclerosis, hypertension, heart failure, stroke, and diabetes. The reactive oxygen species (ROS) affect multiple tissues, either directly or through nitric oxide depletion. ROS induce cardiovascular dysfunction by modulating cell contraction/dilation, migration,

growth/apoptosis, and extracellular matrix protein turnover, which contribute to vascular and cardiac remodelling. Of the several sources of ROS within the cardiovascular system, a family of multi-subunit NADPH oxidases appears to be a predominant contributor of superoxide anion. Although these enzymes respond to stimuli such as vasoactive and metabolic factors, growth factors and cytokines, recent data suggest a significant role of the genetic background in NADPH oxidase regulation. Common genetic polymorphisms within the promoter and exonic sequences of *CYBA*, the gene that encodes the $p22^{phox}$ subunit of the NADPH oxidase, have been characterized in the context of cardiovascular diseases.

ALTERATION ON CALCIUM HAEMOSTASIS AND PLATELET DISFUNCTION IN NON-INSULIN-DEPENDENT DIABETES MELLITUS (NIDDM)

PEDRO COSME REDONDO LIBERAL

Dept. of Physiology, Veterinary Faculty of University of Extremadura, Cáceres, Spain

Diabetes Mellitus is a syndrome characterized by a low or non-production of insulin by the β-pancreatic cell, so there are low insulin levels in the blood which reduce the ability of the cell to uptake glucose. Two type of Diabetes Mellitus have been described, the *Insulin-Dependent Diabetes Mellitus or "type I"* (*IDDM*), which required external insulin injections and *Non-Insulin-Dependent Diabetes Mellitus or "type II"* (*NIDDM*). The NIDDM is associated to metabolic disorder and can derivate into the IDDM. Several diabetes-associated disorders and thrombosis complications have been described to be linked to this syndrome, including retinopathy, neuropathy, nephropathy and arteriosclerosis. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) controls a variety of platelet functions, including aggregation. Agonists increase $[Ca^{2+}]_i$ by inducing Ca^{2+} release from intracellular stores or Ca^{2+} entry. Ca^{2+} removal from the cytosol and the maintenance of low resting $[Ca^{2+}]_i$ is mediated by Ca^{2+} sequestration into intracellular compartments by SERCA and Ca^{2+} extrusion across the plasma membrane. Several studies in different cell types, including platelets, have reported an altered Ca^{2+} homeostasis in NIDDM subjects.

The aim of the present communication is to describe some of the molecular bases involved in the abnormal Ca^{2+} homeostasis responsible for human platelet dysfunction in NIDDM patients.

IMPLICATIONS OF THE CEREBRAL BLOOD VESSELS IN AGING AND HYPERLIPEMIA, AS RISK FACTORS FOR NEURODEGENERATION

ELENA CONSTANTINESCU, FLORENTINA SAFCIUC, ANCA V. SIMA

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania

Degenerative diseases of the brain, like Alzheimer's disease (AD), Parkinson, senile dementia, etc., are devastating disorders, incurable at present, with a strong impact on the cognitive defficiencies and society integration capacities of the senescent individuals. Although the triggers of the neurodegeneration are still the topic of extensive debate, the possible implication of the brain blood vessels has been vigorously promoted in recent years, highlighting the cerebral (Cer) circulation as a novel, unexplored target for therapeutic intervention. Our studies were focused on the implications of the Cer vessels in two incriminating risk factors for neurodegeneration: the senescence and hyperlipemia.

Senescence

When not genetic in origin, the incidence of neuro-degenerative diseases increases dramatically after the sixth decade of life, when the brain cells become highly susceptible to damage by oxidative stress. We evaluated the contribution of the Cer microvessels to the oxidative stress during aging, on rat isolated capillaries, by: (i) assessment of precursors for advanced glycation end products (AGE) formation, (ii) activities of antioxidant enzymes, namely superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione disulfide reductase (GR), and (iii) the activities of metalloproteinases (MMPs), MMP-2 and MMP-9. The results showed that, by comparison with young rats, aged Cer microvessels contain: (i) ~106 % increase of protein carbonyls production; (ii) ~ 68% higher GPx activity, unmodified activities of SOD and GR; (iii) ~ 30%

diminishment in MMP-2 activity, and the specific occurrence of MMP-9 enzyme. The data suggest that the age-related changes of microvessels could increase the propensity for Cer diseases and might represent a prerequisite for the deterioration of mental status in the elderly.

Hyperlipemia

Recent data implying cholesterol (Chol) metabolism in the pathophysiology of AD prove the ability of Chol to modulate amyloid-beta (Aß) production, although the functional connection between Chol and Aß remains to be investigated. At present, no appropriate animal model for the study of neurodegeneration and hyperlipemia was validated. We used the hamster as an experimental model created in our institute, which simulates the human atherosclerosis. We evaluated the effects of the hyperlipemic diet on the structure of the Cer blood vessels and on the Aß deposition in the brain tissue. We found significant morphological changes on Cer capillaries, arterioles and meningeal vessels, such as: large perivascular spaces, branched thickened basal lamina (BL), endothelial developed Golgi apparatus and fibrillar inclusions and a large number of vesicles lining the plasmalemma in smooth muscle cells. Some meningeal vessels from hamsters with plasma Chol levels over 1000 mg/dl presented an intense autofluorescence in the adventitia, where the electron microscopy (EM) showed the presence of lipid-loaded perivascular cells. Specific thioflavin and Congo Red stainings of Aß gave a scarced reaction, compared to the strong signal obtained on control kidney sections from the same animals. However the EM images showed some fibrillar patterns in the capillary endothelium and in the thickened BL.

Conclusion

The studies could contribute to the medical warning for the risks implicated by a hyperlipemic diet, not only in cardiovascular diseases, but also in the alteration of the mental health, suggesting new potential therapeutic approaches for neurodegeneration.

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STORE-OPERATED CALCIUM ENTRY CHANNELS: HISTORY AND CURRENT KNOWLEDGE

JUAN A. ROSADO

Department of Physiology, University of Extremadura, Caceres, 10071, SPAIN.

Store-operated or “capacitative” calcium entry (SOCE) is a major mechanism for Ca^{2+} influx in non-excitable cells. SOCE, which was first proposed in 1986, is regulated by the filling state of the intracellular Ca^{2+} stores. As the Ca^{2+} concentration in the stores falls, a signal, generated in the stores, opens store-operated Ca^{2+} (SOC) channels in the plasma membrane. Several SOC currents have been identified. The best characterized is I_{CRAC}, but other I_{SOC} with different biophysical properties have been described in different cell types. In the past few years there has been considerable interest in the possibility of transient receptor potential (TRP) channels and Orai proteins functioning as SOCs. Orai1 has been presented as the pore of the channel mediating I_{CRAC}; however, the characteristics of SOCE in a number of cell types differ from that of the highly Ca^{2+}-selective I_{CRAC} and canonical TRPs (TRPC) or other TRPs might be responsible for conducting the non-selective cation current (I_{SOC}) activated by Ca^{2+} store depletion in such cells. TRPC proteins can form heteromeric cation channels with different biophysical and biochemical properties. Interestingly, TRPC1, which has long been suggested as a SOC channel candidate, associates with Orai1 and the Ca^{2+} sensor of the intracellular stores STIM1 in a ternary complex that contributes to enhance the variability of SOC currents available to regulate cellular functions.

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EMBO – PROMOTING THE MOLECULAR LIFE SCIENCES IN EUROPE

JAN TAPLICK

European Molecular Biology Organization, Heidelberg, Germany

The European Molecular Biology Organization (EMBO) promotes excellence in the molecular life sciences in Europe through targeted programs and activities. Established in 1964, the founders of EMBO showed an incredible vision when they

established the organization. Keeping faith with their vision, their principles and standards are a guide to our activities today.

The EMBO membership includes some of the leading researchers in Europe and represents a highly dynamic cross-section of the life sciences community. The organization elects new members annually on the basis of proven excellence in research. Members number more than 1300 today with a further 80 associate members worldwide.

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POSTERS

EFFECT OF HOMOCYSTEINE ON CALCIUM MOBILIZATION AND PLATELET FUNCTION IN TYPE 2 DIABETES MELLITUS

NICOLETA ALEXANDRU, ISAAC JARDÍN[1], DOINA POPOV, MAYA SIMIONESCU, JOAQUÍN GARCÍA- ESTAÑ[2], GINÉS M. SALIDO[1], JUAN A. ROSADO[1]

Institute of Cellular Biology and Pathology "N. Simionescu", Bucharest, Romania
[1]Department of Physiology, University of Extremadura, Cáceres, Spain
[2]Department of Physiology, Faculty of Medicine, University of Murcia, Spain

Background

Hyperhomocysteinemia is considered a risk factor in the development of thrombosis although its effect on platelet function and the mechanisms involved are still poorly understood. The purpose of this study was to investigate endogenous reactive oxygen species (ROS) generation, intracellular Ca^{2+} mobilization, and aggregation induced by homocysteine in platelets from type 2 diabetics and healthy subjects.

Materials and Methods

The studies were performed on platelets isolated from blood obtained from 28 type 2 diabetics and from 26 age- and gender matched healthy (control) donors. Endogenous ROS production and intracellular free calcium concentration were assayed by spectrophoto-fluorimetry, and platelet aggregation was monitored in a Chronolog aggregometer.

Results

Homocysteine induced a concentration-dependent increase in the endogenous production of ROS which lead to Ca^{2+} release from the dense tubular system and the acidic stores and subsequently, to platelet aggregation. For the same concentrations of homocysteine all the parameters tested: ROS generation, Ca^{2+} mobilization and platelet agreggation were more elevated in platelets from diabetic donors than in controls, which indicates that platelets from diabetic donors are more sensitive to homocysteine levels.

Conclusion

These findings, together with the hyperhomocysteinemia reported in diabetic patients, strongly suggest that homocysteine might be considered a risk factor in the development of cardiovascular complications associated to type 2 diabetes mellitus.

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OVEREXPRESSED $P21^{CIP1}$ AND $P27^{KIP1}$ CONTRIBUTE TO THE RENAL CELLS HYPERTROPHY AND PREMATURE SENESCENCE IN L-NAME-INDUCED HYPERTENSIVE HAMSTERS

MADALINA DUMITRESCU, GABRIELA COSTACHE, EMANUEL DRAGAN, DOINA POPOV

Institute of Cell Biology and Pathology "Nicolae Simionescu", Bucharest

Background

Cellular senescence is the molecular program that limits the finite proliferative potential of a cell. Senescent cells undergoing a permanent cell cycle arrest accumulate in aged tissues. The cyclin-dependent kinase inhibitors (CDKIs) p21Cip1 and

p27Kip1 coordinate the progression through the cell cycle, and p21Cip1 is up-regulated in senescent cells. This study aims to examine the role of p21Cip1 and p27Kip1 in the renal hypertrophy and premature cell senescence in an original model of hypertension induced by chronic NO inhibition using the endothelial NO synthase (eNOS) inhibitor NG-nitro-L-arginine methyl ester (L-NAME).

Methods

Forty Golden Syrian hamsters were divided into 4 groups: (i) hypertensive (HT, 13 mo old when sacrificed), which received L-NAME (40 mg/kg bw) daily, for 6 month; (ii) control group (CHT), made up by age-matched animals; (iii) aged (20 mo.old), and (iv) young (3 mo old) hamsters. Mean arterial pressure (MAP) was determined using the PowerLab acquisition system. Fragments from renal cortices from hamsters within the 4 groups were processed for: (i) histologic examination (after hematoxylin-eosin, and Trichromic Masson staining), (ii) frozen sections, used to check for the appearance of the senescence-associated β-galactosidase (SA-β-gal) activity, and (iii) Western blotting, to quantify the expression of p21Cip1 and p27Kip1, pAkt/Akt, eNOS and the dinitro-phenylhydrazine (DNPH)-derivatives of the carbonyl residues in lysates of kidney cortices.

Results

L-NAME-treated hamsters became hypertensive (HT), as their MAP raised ~ 2 fold above the values of MAP in the other groups. Our findings showed that, as compared to their related controls: (i) the HT and aged hamsters displayed an enlarged renal extracellular matrix accumulation, as well as a high percent of renal cells positive for the SA-β-gal activity; (ii) the oxidative stress was increased in aged and HT hamsters, as the quantitated carbonyls was elevated, while eNOS expression was decreased, amplifying the NO deficit; (iii) the protein expressions of p21Cip1 and p27Kip1 were enhanced in aging and HT hamsters, possibly related to the cell-cycle arrest; (iv) the Akt activity raised in aged and HT hamsters, suggestive for the involvement of the Akt signaling pathway in the senescence-induced cell-cycle arrest.

Conclusions

Renal oxidative stress in L-NAME-induced hypertensive hamsters might cause the overexpression of the CDKI p21Cip1 and p27Kip1, triggering the development of the renal hypertrophy together with the premature senescence phenotypic features.

This work was supported by the Romanian Academy Grant nr. 210/2006-2007

EFFECTS OF ADIPOCYTE-DERIVED PROINFLAMMATORY MOLECULES ON ENDOTHELIAL CELLS AND THEIR MODULATION BY ROSIGLITAZONE

ALINA CONSTANTIN, DELIA BUCULEI, MADALINA DUMITRESCU, GABRIELA COSTACHE

Institute of Cellular Biology and Pathology "N. Simionescu", Bucharest, Romania

Background

Adipose tissue enlargement is accompanied by alteration in adipokine production (*i.e.*, overexpression of TNF-α, IL-6, or underexpression of adiponectin). The proinflammatory status associated with these changes contributes to endothelial dysfunction in obese individuals. A novel role for thiazolidinediones, including rosiglitazone, in regulating the inflammatory response is recently emerging. We aimed to examine the intracellular signaling activated by the proinflammatory adipokines in vascular endothelial cells (ECs), and the effect of rosiglitazone on the expression of these fat-cell derived molecules.

Methods

Adipose depots from healthy subjects were used to obtain the stromal-vascular fraction (SVF) and adipocytes. SVF cells were cultured to confluence, and then preadipocytes were differentiated using adipogenic inducers. Postconfluent human ECs (EAhy926 cell line) were incubated with: (i) leptin or rosiglitazone, in the presence of TNF-α, or (ii) conditioned media from confluent or differentiated preadipocytes, preincubated with rosiglitazone. EC were lysed and protein expression and phosphorylation were analyzed by Western blotting, using antibodies against NF-kB or NADPH oxidase subunits,

MMPs, AMPK, and eNOS. Proteins with gelatinolytic activity (MMP-2, MMP-9) in supernatant media conditioned from ECs were identified by gelatin zymography. NOx metabolites were estimated by Griess reagent.

Results

The effects that leptin had on the EAhy926 were the following: (i) the overexpression of proteins known to be involved in inflammatory processes, such as NF-kB (p50 and p65 subunits), NADPH oxidase (the p47phox and p67phox subunits), and MMP2; and (ii) enhanced gelatinolitic activity of MMPs. Similar results were obtained when ECs were in the presence of media conditioned from mature adipocytes or differentiated preadipocytes, suggesting that proinflammatory factors secreted by these fat cells triggered the endothelial activation. Rosiglitazone interfered with: (i) NF-kB expression, yielding a diminished expression of the p50 subunit; (ii) TNF-α-evoked decrease of AMPK activity, stimulating the AMPK phosphorylation. Rosiglitazone diminished the gelatinolitic activity of MMPs, and increased NO synthesis.

Conclusions

The adipocyte-endothelium interaction is an important mechanism of inflammation linking the pathogenesis of obesity and cardiovascular disease. The antiinflammatory action of rosiglitazone shed a new light on its therapeutic use in vasculopathies.

This work was supported by the Romanian Ministry for Education and Research (CNCSIS) Grant nr. 448/2006-2007

IDENTIFICATION OF MOLECULES UNDERLYING HUMAN AORTIC SMC PHENOTYPIC CHANGE IN HYPERGLYCEMIA AND HYPERLIPIDEMIA

DOINA POPOV, ELENA CONSTANTINESCU

Institute of Cellular Biology and Pathology "N. Simionescu", Bucharest, Romania

Aims

We questioned on: (i) phenotypic changes of human aortic smooth muscle cells (SMCs) induced in vitro by high glucose concentration (as model for hyperglycemic insult) and by sera of obese type 2 diabetic patients (as model for obesity associated with diabetes), and (ii) identification of underlying signaling molecules.

Materials and Methods

The SMCs were cultured to confluence in: (i) DMEM with 10% fetal calf serum, supplemented with 30 mM glucose (controls: cells with 5 mM glucose), and (ii) DMEM with 10% serum of obese Type 2 diabetic patients (controls: cells in 10% serum of healthy subjects). The techniques used were: electron microscopy, immunofluorescence, and immunoblotting (to investigate expression and phosphorylation level of STAT-3, ERK1/2, IRS-1 and hormone-sensitive lipase (HSL).

Results

30 mM glucose induced in SMCs: (i) an enrichment in biosynthetic organelles, indicating a secretory phenotype (control: contractile phenotype), (ii) extended organization of vinculin plaques at the cells periphery, supporting cells spreading and focal adhesions formation (control: intracytoplasmic distribution), (iii) production of intensely immunostained type IV collagen (control: very faint labeling), (iv) proliferation (as demonstrated by the presence of centriols) associated with ~ 2.54 fold increased protein expression of pSTAT-3 (Tyr 705) and ~ 2 fold of pERK1/2 (Thr202/Tyr204) (vs. controls). Addition of 10-100 nM insulin reduced IRS-1(Tyr) phosphorylation in a time-dependent process, suggesting negative regulation of insulin receptor-mediated signaling. 30 mM glucose did not induced lipid loading of SMCs. The presence of serum of obese Type 2 diabetic patients conducted to: (i) accumulation of Oil red O stained lipid droplets, indicating a phenotypic switch towards differentiation into SMC-derived foam cells, and storage of triglycerides and cholesteryl esters (vs. controls), (ii) protein expression of HSL (not detected in controls), and (iii) expression and activation of the long isoform of leptin receptor, underlying metabolic dysregulations in lipid laden-cells.

Conclusions

The results support the biosynthetic metabolism of SMCs in high glucose conditions and the active lipid metabolism associated with cells differentiation into SMC-derived foam cells.

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ULTRASTRUCTURAL AND AUTOFLUORESCENT FINDINGS INDUCED BY HYPERLIPEMIA ON THE CEREBRAL VESSELS IN HAMSTER

ELENA CONSTANTINESCU, FLORENTINA SAFCIUC, ANCA SIMA

Institute of Cellular and Molecular Biology "Nicolae Simionescu", Bucharest, Romania

Background

Hyperlipemia is an established risk factor for the cardiovascular system diseases and recent studies have suggested a link between increased circulating cholesterol (Chol) levels and neurodegeneration. The aim of this study was to evaluate the effect of a hyperlipemic (HL) diet on the morphology of cerebral larger arteries and cortical microvessels in hamster, an animal model which simulates the human atherosclerosis.

Methods

Thirty male Golden Syrian hamsters were fed a HL diet (3% Chol and 15% butter), tested for plasma biochemical parameters (Chol, triglycerides) and sacrificed after 3 and 6 months respectively. After aldehyde fixation by perfusion, the carotid arteries were stained with Oil Red for the presence of lipid deposits, or processed for routine electron microscopy (EM). One cerebral emisphere was embedded in paraffin for the examination of lipid autofluorescence. Cortical samples with meningeal areas from the second emisphere were prepared for routine EM.

Results

After 6 months diet, the plasma Chol and triglycerides levels were significantly increased in HL hamsters, as compared to control animals. The Oil Red staining showed extended areas of fatty streaks on carotid arteries, containing a foamy lipid load as shown by thick or thin electron microscopy sections. In some HL hamsters not blood-washed by perfusion, the vessel lumen appeared filled with lipoprotein particles. The capillaries presented large perivascular spaces and branched thickened basal lamina (BL) containing a fibrillar material and some lipid deposits on the external side. The endothelial cells of larger arteries presented a cytoplasm rich in organelles, a developed Golgi apparatus and some fibrillar inclusions. The BL was thickened and sometimes contained vesicular formations. The smooth muscle cells had a large number of vesicles lining their plasmalemma. Some meningeal vessels from HL hamsters with plasma Chol levels over 1000mg/dl presented an intense lipid autofluorescence in the adventitia, where the EM preparations showed the presence of lipid-loaded perivascular cells.

Conclusion

The significant morphological changes in hamster cerebral blood vessels induced by hyperlipemia could represent a serious impairment for the normal functions of the brain. The study brings another medical warning about the risks of the HL diet, not only in cardiovascular diseases, but also in the neurodegenerative disorders and alteration of the mental health.

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GLYCATED LDL AND HIGH GLUCOSE CONCENTRATION INDUCE ENDOTHELIAL CELL DYSFUNCTION IN DIABETES

LAURA TOMA, GABRIELA BOTEZ, CAMELIA STANCU, ANCA SIMA

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania

Aim

To evaluate the endothelial cell dysfunction, known marker of diabetes, induced by high glucose and glycated lipoproteins.

Methods

Human endothelial cells (EAhy926) were incubated with human glycated LDL (gLDL) in DMEM 1‰ glucose supplemented with 2% fetal calf serum, or in DMEM with 4.5‰ glucose, for 24 h and 72 h. Glycated LDL was obtained by incubating LDL with 0.5 M glucose at 37°C, under

sterile conditions, in the presence of antioxidants (EDTA, BHT). Gene and protein expression of eNOS and SR-BI (Real Time PCR, Western Blot), intracellular free cholesterol (FC), the reactive oxygen and nitrogen species (ROS, RNS) and the total antioxidant potential (TRAP) of the medium were quantified.

Results

Showed that increased concentrations of glucose and gLDL in the medium determined a statistically significant decrease of eNOS mRNA and protein expression. SR-BI gene expression was also decreased in cells incubated in high glucose concentration, but not in the cells incubated with gLDL. FC was significantly increased in the cells incubated with gLDL, as compared with cells incubated with native LDL. Endothelial ROS and RNS concentrations were significantly increased after 72 h incubation with gLDL, as compared with control cells, a result which correlates with the decrease of TRAP in the culture medium.

Conclusion

Our data indicate that gLDL and high glucose concentrations might be considered risk factors in diabetes, because they determine the increase of the oxidative stress in the endothelial cells, accompanied by a decrease of the proteins implicated in nitric oxide synthesis.

The present study was supported by a grant from the Romanian Ministry for Education and Research (CNCSIS).

GLYCATED LDL INDUCE SCAVENGER RECEPTORS CLASS B EXPRESSION IN HUMAN SMOOTH MUSCLE CELLS

GABRIELA BOTEZ, CAMELIA STANCU, ANCA SIMA

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania

Aim

The purpose of this study was to investigate the contribution of LDL-AGE in development of atherosclerosis by evaluating its biological effects in human vascular smooth muscle cells (VSMC) in culture.

Methods

LDL from human plasma, separated by single-spin ultracentrifugation, was incubated in vitro with 0.5M glucose, with antioxidant protection, for 4 weeks at 37ºC (LDL -AGE). The degree of LDL glycation was assessed by an improved hydroxyl-methylfurfural assay. The formation of thiobarbituric acid-reactive substances was used to measure lipid peroxides. Protein modification was measured as pentosidine and furoyl furanyl imidazole formation. Agarose gel electrophoresis was used to assess LDL purity and alteration of the negative charge by glycation. LDL-AGE was incubated with VSMC for 24 hours in DMEM. Cell proliferation was measured by DNA fluorimetric assay. Gene expression of CD36 and SR-BI, LDL receptor (LDLR) and monocyte chemotactic protein-1 (MCP-1) was determined by Real-Time PCR. Protein expression of CD36 was assessed by Western blotting. As control, 24 hours starved cells incubated with native LDL (nLDL) were used.

Results

LDL-AGE significantly enhanced CD36 gene (69%, $p<0.05$) and protein (3.7 fold, $p<0.05$) expression in VSMC compared with nLDL. An increase in the gene expression of SR-BI (46%, $p<0.05$), MCP-1 (2.5 fold, $p<0.05$) and a decrease in LDLR gene expression (72%, $p<0.01$) was detected. LDL-AGE have stimulated the proliferation rate of VSMC (57%, $p<0.05$).

Conclusion

LDL-AGE induce cell proliferation and lipid loading of VSMC upon binding to scavenger receptors.

The study was supported by a grant from the Romanian Ministry for Education and Research (CNCSIS).

FELODIPINE AND ENALAPRIL MODULATE $p22^{PHOX}$ GENE EXPRESSION IN PERICYTES

MONICA RAICU, ADRIAN MANEA

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania

Background

Clinical and experimental data demonstrate the involvement of oxidative stress in the

development of vascular complications associated with diabetes, such as retinopathy, nephropathy, neuropathy and atherosclerosis. However, the precise molecular mechanisms responsible for the increased production of reactive oxygen species are not completely defined. Among others, NADPH oxidase is one of the most important sources of superoxide anion that induce dysfunction of vascular cells. Pericytes have an essential role in the capillary stability and function and the selective loss of this cell type represent a hallmark of diabetic retinopathy.

Aim

To investigate the regulation NADPH oxidase activity and $p22^{phox}$ gene expression by calcium channel blockers and angiotensin converting enzyme inhibitors in pericytes exposed to diabetic conditions (high glucose, angiotensin II).

Methods and results

NADPH oxidase activity was measured by DCF fluorescence assay and the $p22^{phox}$ mRNA expression was evaluated by real time PCR. The results showed that: (i) diabetic conditions augmented the NADPH oxidase activity and $p22^{phox}$ gene expression; (ii) nifedipine, felodipine, captopril and enalapril diminished significantly the up-regulated oxidase activity and $p22^{phox}$ mRNA level; (iii) the efficiency of the drugs was: felodipine > enalapril > nifedipine = captopril.

Conclusions

The present study provides evidence that calcium channel blockers (nifedipine, felodipine) and angiotensin converting enzyme inhibitors (captopril, enalapril) are important regulators of the NADPH oxidase in pericytes. Modulation of cellular redox state suggests the benefic effect of these drugs in the treatment of diabetic complications, such as retinopathy.

This work was supported by the Romanian Academy, Grant no. 56/2006.

RETINOIDS INDUCE apoE GENE UPREGULATION IN MACROPHAGES

IOANA M. FENYO, ELENA V. FUIOR, ANCA V. GAFENCU

Institute of Cellular Biology and Pathology "N. Simionescu", Bucharest, Romania

Background

Retinoids are key regulators of inflammation by mechanisms that are not completely understood. We postulated that retinoids influence apoE expression in macrophages and searched for the molecular mechanism involved**.**

Methods

Modulation of apoE expression in RAW 264.7 murine macrophages treated 24h with 1μM 9-cis retinoic acid (RA) and in mouse peritoneal macrophages (MPM) of C57BL/6 mice treated with vitamin A was tested by quantitative PCR. To identify the region involved in apoE gene regulation, transient transfection experiments were performed using plasmids encoding the proximal apoE promoter in the presence or absence of multienhancer 2 (ME2) or its deletion mutants, fused to the luciferase gene.

Results

RA increased endogenous apoE gene expression in RAW 264.7 cells and vitamin A increased apoE expression in MPM. Transient transfections showed that apoE promoter activity was not affected by RA, consistent with the lack of binding sites for RXR in this region. In the presence of ME2, the promoter activity was significantly increased by RA. Analysis of a series of ME2 deletion mutants indicated that a RXR binding site is between nucleotides 321-407.

Conclusion

Retinoids upregulate apoE expression in macrophages, suggesting their potential therapeutic use for the regulation of lipid metabolism and prevention of atherosclerosis.

This study was funded by CEEX Grant 130/2005 from the Romanian Ministry of Research and from a bilateral cooperation project co-financed by the Romanian and Greek Ministries of Research.

THE MISSENSE GLU298ASP VARIANT OF THE ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE IS POSITIVELY ASSOCIATED WITH TYPE 2 DIABETIC NEUROPATHY

SIMONA-ADRIANA MANEA, ANDREI CONSTANTINESCU, CONSTANTINA HELTIANU

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania

Aim

To investigate a possible association of the G894T polymorphism in eNOS gene with microvascular disorders in type 2 diabetes mellitus (T2DM), particularly in those patients with metabolic syndrome (MS).

Methods

The 450 subjects (150 controls and 300 patients with T2DM) were genotyped for the eNOS G894T polymorphism. The T2DM group was divided in T2DM without (n=112) and with (n=188) MS. The relationship of eNOS G894T variant with quantitative variables (age, duration of diabetes, BMI, total-cholesterol, triglycerides) was analyzed by Kruskal-Wallis and Bonferroni test. In the multivariate binary logistic regression a gender-adjusted model was used and the variables were log transformed.

Results

The frequencies of alleles and genotypes were not different in T2DM patients as compared with control. The distribution of 894T allele was higher in patients without MS (OR=0.66; 95%CI=0.46–0.95; P=0.02) than in those with MS. In the whole group of T2DM patients the eNOS G894T was significantly associated with men (F=5.34; P=0.005) and at the border of significance with diabetic neuropathy (F=2.17; P=0.12). The relationship with gender remained significant in patients with MS (F=4.6; P=0.011) while the correlation with diabetic neuropathy became significant (F=4.13; P=0.018). Multivariate regression analysis showed an association of eNOS G894T with diabetic neuropathy in MS subgroup which remained significant (P=0.05) in the presence of mentioned quantitative variables.

Conclusion

The data indicate that the eNOS G894T gene polymorphism represents an independent risk factor for diabetic neuropathy in patients with metabolic syndrome.

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ENDOTHELIAL-DERIVED FOAM CELLS POTENTIAL CONTRIBUTORS TO PLAQUE VULNERABILITY

LUMINITA IVAN, FELICIA ANTOHE

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania

Background

Subendothelial accumulation of extracellular lipids leads to the formation of foam cells primarily derived from monocytes/ macrophages, but also from endothelial cells (EC) in the late stages of atherosclerosis. The purpose of this study was to find out if hypelipidemic condition, that triggers the formation of endothelial-derived foam cells, modifies the expression of endothelial cell surface adhesion molecules and heat shock proteins. With time, the profound changes of EC, associated with the local inflammatory response, may lead to plaque rupture, thrombosis and the ensuing myocardial infarction or stroke (Ross, 1999; Libby, 2002). In this complex process, EC-derived foam cells could be the main candidate responsible for plaque vulnerability and rupture.

Materials and Methods

As a model system, human endothelial cell line EA hy926 derived from human umbilical vein endothelial cells was used (Edgel, 1983). Cells in culture were activated by incubation with 10%

serum from hyperlipidemic human subjects for 48 hours. Postconfluency, the normal or activated cells were tested for the expression of cell adhesion molecules (VCAM-1, E-selectin, VLA-4) and heat shock proteins (Hsp27, Hsp 90), using ELISA and Western blotting techniques.

Results

We report here that cells exposed in culture to hyperlipidemic condition become gradually loaded with modified lipids, express adhesion molecules (VCAM-1, E-selectin, VLA-4), show enhanced intracellular Ca^{2+} release, and demonstrate high level of heat shock proteins (Hsp27, Hsp90), which *in vivo* correlates positively with the severity of atherosclerosis (Pockley, 2002).

Conclusion

In the current study, we established a cell culture system that induces foam cells from endothelial cells after treatment with human serum from hyperlipidemic patients having high concentration of atherogenic components (cholesterol, triglyceride, oxidized LDL).The EC-foam cells model proved to be useful to identify the multiple changes induced in activated endothelial cells under hyperlipidemic stress.

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EXPRESSION OF HMGB1 NUCLEAR PROTEIN IN NORMAL AND HYPERLIPIDEMIC HAMSTER

RALUCA HARABA, VIOREL SUICA, ELENA UYY, FELICIA ANTOHE

Institute of Cellular Biology and Pathology "Nicolae Simionescu", Bucharest, Romania

Background

High mobility group box 1 (HMGB1) is the major component of the non-histone nuclear protein group and is known as a transcriptional regulator and an active factor in DNA repair, differentiation, and development. The purpose of the present study is to analyze the expression of HMGB1 in normal and experimental induced hyperlipidemic hamsters.

Methods

Thirty healthy male golden Syrian hamsters were divided into three experimental groups: 1) normal (N) fed with standard diet, 2) hyperlipidemic (H) fed with standard diet supplemented with 3% cholesterol and 15% butter and 3) treated hyperlipidemic (Ht) hamsters which after three months of high lipid diet received normal diet and fluvastatin sodium (0.075 mg fluvastatin administered by gavage per day) for another three months. At the end of the experiment, 1.5 ml of blood sampling were collected from the retro-orbital venous sinus for biochemical assays and the heart and lung fragments were harvested for morphological, Western blot or Rt-PCR **(**reverse transcription-PCR) analysis. The bands evidenced on Kodak autoradiographic film by ECL analysis and those obtained on agarose gel were quantified by densitometry using a computerized image analysis program (Scion Image). Results are expressed as means ± SD and differences among groups were analyzed by ANOVA test.

Results

As demonstrated by quantitative assays, serum levels of cholesterol, triglyceride and protein in the control group were lower than in the other two groups. Hyperlipidemic and treated hamsters, but no controls developed atherosclerotic plaques or lipids deposit evidenced on the cryosections of the mitral, aortic or ticuspide valves. The expression of HMGB1 in lung homogenate analyzed by Western blotting showed that the expression of HMGB1 protein in the H and Ht groups is higher then in normal group. The mRNA gene expression of HMGB1 in lung tissue showed differences between the groups.

Conclusion

The preliminary results of this study demonstrate that in the lungs of hyperlipidemic hamsters the gene and protein expression of HMGB1 is modified in comparison with the control groups.

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