SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Ethanol accelerates vascular calcification in human aortic smooth muscle cells Quantitative Trait Locus mapping reveals alcohol consumption genes

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The Examination takes place at the Department of Biochemistry and Molecular Biology, University of Debrecen, the 11 th of February 2016, 12 PM.	
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1 INTRODUCTION

1.1 Vascular Calcification

Epidemiological studies suggested in early nineties that moderate consumption of alcohol in France (20-30 g alcohol intake per day) can reduce risk of coronary heart disease by at least 40%. Since then studies from several countries confirmed this finding demonstrating a 20-40% lower incidence for cardiovascular disease among drinkers of alcoholic beverages as compared to non-drinkers. The epidemiological consensus currently is that frequent moderate consumption of alcohol is associated with the lowest risk for coronary artery disease and mortality. Recently it has been observed that although moderate alcohol use has an apparent protective association with coronary heart disease, heavy alcohol consumption, in particular hard liquor, is associated with greater calcification in coronary arteries. Accordingly, binge or heavy episodic drinking in short periods of time, defined as 5 or more drinks, was shown to be associated with increased cardiovascular disease and subsequent mortality. Moreover, calciphylaxis, known to almost exclusively develop in patients with Stage 5 chronic kidney disease, was also reported to occur in heavy drinkers with physiological renal function. While we are gradually deciphering how alcohol might exert beneficial effects on atherosclerosis leading to prevention of cardiovascular and cerebrovascular disease, the molecular basis of the dichotomous effects of alcohol on vascular calcification have not been explored.

Vascular calcification is a common complication of conventional risk factors for cardiovascular morbidity, and the extent is predictive of subsequent mortality beyond the established conventional disease states. Calcification develops in two main distinct sites within arteries. While in atherosclerosis the intima is affected focally at predisposed areas of vessels, in medial sclerosis the media layers of the large- and medium-sized arterial wall are mineralized in a diffuse fashion. Intimal calcification in atherosclerosis is frequently accompanied by medial calcification and vice versa. They are strongly associated with aging, arterial remodeling including intimal-medial thickening and changes of the geometry and function of valves of the heart. Such calcifications have devastating clinical consequences related to an increased risk of cardiovascular morbidities and complications such as atherosclerotic plaque burden, myocardial infarction, coronary and cerebrovascular artery disease, postangioplasty dissection, and increased ischemic episodes in peripheral vascular disease. Calcification has been demonstrated to be a powerful independent marker of coronary heart disease events in patients with diabetes. Both the Framingham risk index and coronary calcification score - as measured by electron beam computed tomography - have been shown to have prognostic value for cardiovascular events. Studies also indicated that coronary calcification may be predictive of, or strongly associated with, sudden cardiac death. The principal pathologic effects of vascular calcification are the abnormal perfusion of organs and stiffening of the vessels leading to increased left ventricular afterload.

In calciphylaxis, the small cutaneous arterioles are affected. The main feature of calciphylaxis is the mineralization of vessels almost exclusively occurring in patients diagnosed with advanced chronic kidney disease (CKD). After medial calcification by smooth muscle cells, the endothelium acquires a procoagulant phenotype resulting in occlusion of small arterioles. The clinical picture is typically characterized by very painful skin necrosis that is life-threatening due to sepsis and concomitant cardio- and cerebrovascular diseases. The disease state was first described and studied by the famous Hungarian scientist, Hans Selye.

Hyperphosphatemia has been demonstrated to act as a nontraditional risk factor for cardiovascular disease and found to increase mineralization. Serum levels of phosphorus are significantly related to the presence of coronary artery calcification. Hyperphosphatemia is commonly observed in chronic kidney patients, and there is a strong association of serum phosphate level with mortality risk in chronic hemodialysis patients. Tight associations between arterial calcification and stiffness, pulse pressure, or mortality have also been found to contribute to the high rates of cardiac and peripheral ischemic disease and left ventricular hypertrophy in this population. Moreover, the phosphate level was also found to be independently related to aortic calcification in healthy individuals with physiological renal function.

1.2 Proteins and enzymes involved in vascular calcification

It is well established that inorganic phosphate is an important regulator of vascular calcification. Although the precise mechanisms of vascular calcification are not completely understood, abnormalities in mineral metabolism are considered important risk factors. In this regard, we should mention that acute ethanol ingestion has been shown to increase serum phosphate. It is thought to be an actively regulated multistep process in which interchangeable cellular phenotypes under certain pathological conditions are the driving force. One of the main contributors is the trans-differentiation of smooth muscle cells into osteoblast-like cells. After osteoblastic differentiation these cells lack characteristics of smooth muscle cells, and

develop osteoblast features. Jono et al. revealed that human smooth muscle cells cultured in media containing physiological serum phosphate levels do not mineralize, but these cells can be induced to mineralize by elevating phosphate level in culture medium to that typically observed in hyperphosphatemic individuals. Granular deposits associated with the extracellular matrix developed in a time- and dose-dependent fashion. Exposure of human smooth muscle cells to elevated phosphate was shown to provoke substantial phenotypic transition towards osteoblasts. At physiological phosphate levels, cells express smooth muscle lineage markers, including SM 22 α and SM α -actin. After exposure to elevated phosphate, a dramatic loss of the markers for smooth muscle cell lineage occurs and simultaneously a gain of osteogenic markers such as alkaline phosphatase, osteocalcin and core-binding factor alpha-1 (*Cbfa-1*) develops.

1.2.1 Phosphate co-transporter, Pit-1 and Core-binding factor-a-1, Cbfa-1

Cellular uptake of P_i occurs through a sodium-dependent phosphate co-transporter, Pit-1, which is essential for vascular smooth muscle cell calcification and phenotypic modulation in response to elevated phosphate. Three types of phosphate co-transporters have been identified based on structure and regulation. While types I and II transporters are restricted to the kidney and intestine, type III transporters are present in many tissues including kidney, heart, lung, and bone. Pit-1 and Pit-2 represent type III transporters. Of the known transporters, Pit-1 was found to be expressed in human smooth muscle cells as well as human aorta. Vascular calcification during which smooth muscle cells gain an osteoblastic phenotype is accompanied by increased expression of Cbfa-1 in cells exposed to high phosphate or platelet-derived growth factor. Cbfa-1 is a transcription factor that acts as an essential regulator of osteoblast differentiation and fulfills a dominant function for other gene products. Cbfa-1 is also crucial for chondrocyte differentiation. In Cbfa-1 null mice the intramembranous and endochondral ossification are completely blocked, owing to the maturational arrest of osteoblasts demonstrating that this transcription factor is essential for osteoblast differentiation, bone matrix gene expression and, consequently, bone mineralization.

1.2.2 Alkaline phosphatase (ALP)

Alkaline phosphatase is one of the phenotypic markers of osteoblasts. The expression of alkaline phosphatase occurs in type IV atherosclerotic lesions, areas of medial calcification, and valves of the heart. Alkaline phosphatase is expressed on the cell surface contributes to vascular calcification through cleaving organic phosphate compounds and releasing inorganic phosphate. The enzyme activity is indispensable in early osteogenesis. Inflammatory cytokines and vitamin D upregulate alkaline phosphatase activity and mineralization. Several *in vitro* studies suggested that vascular calcifying cells express high levels of alkaline phosphatase, and the capacity for mineralization of these cells is dependent on their alkaline phosphatase activity. Therefore, it is most likely that the induction of alkaline phosphatase in vascular cells accelerates the development of vascular calcification.

1.2.3 Osteocalcin (OC)

Osteocalcin, a skeletal member of the family of extracellular mineral binding Gla proteins, is the major noncollagenous protein in bone matrix. It is synthesized by the osteoblast and it is secreted into the bone matrix at the time of bone mineralization. The calcium binding properties of osteocalcin and its pattern of expression in bone suggests an important function during bone mineralization. Osteocalcin is a gamma-carboxyglutamic acid containing protein present in calcified atherosclerotic lesions and mineralized heart valves at high concentration. Upregulation of osteocalcin was shown to occur in vascular cells in response to elevated phosphate.

1.3 QTL mapping of alcohol self-administration

Nerves containing glutamate located close to bone cells exhibit functional glutamate receptor. It was revealed that glutamate alters bone resorption *in vitro* via a mechanically sensitive glutamate/aspartate transporter protein suggesting a function for glutamate in mechanical load and bone remodeling. Metabotropic glutamate receptor type 7 (*Grm7*) gene was also shown to serve as a promising candidate that might be implicated in altered alcohol preference. In the central nervous system, the excitatory amino acid glutamate serves as a potent neurotransmitter exerting its effects via various membrane glutamate receptors.

We studied the genetic background of alcoholism. Alcoholism has been shown to be a complex disorder determined by genetic background of several genes. Our interest was to expand genome scanning from 5 chromosomes (1, 2, 3, 9 and 15) to cover all autosomal chromosomes and find ethanol consumption locus/loci (QTLs) and Quantitative Trait Genes (QTGs) involved in alcoholism. Neurochemical processes underlying alcoholism and drug addiction are not well explored. One strategy to identify neurochemical mechanisms of addiction is to map the relevant genes. Strong evidence in quantitative genetics suggests that most traits and alcoholism are significantly affected by genetic factors. Genetic variation in alcohol drinking was revealed by McClearn and Rodgers half a century ago via comparing well established inbred mouse strains. In spite of intensive effort the biological basis of oral alcohol self-administration is not well revealed. New genetic tools, including high throughput

SNP genotyping and gene expression microarrays, provide hope that genes responsible for alcoholism can be identified. This is substantial because effective therapy can be obtained if the molecular relation between the genetic basis and the biochemical pathways involved are understood.

Alcohol preference in rodents serves as an important model for hedonic aspects of alcoholism. Since the early 1990s a substantial number of Quantitative Trait Loci (QTLs) have been mapped in genetic studies for alcohol preference, consumption, and acceptance.

Alcohol drinking behavioral phenotypes of C57BL/6ByJ, BALB/cJ, CXBI/ByJ, progenitors and RQI strains have been described previously. There was a novel trait gene mapping strategy Recombinant QTL Introgression, which was applied to map QTLs on five chromosomes for alcohol preference and consumption in 80 RQI strains of the b₅i₇ series . In that study five mouse chromosomes (1, 2, 3, 9, and 15) with polymorphic microsatellite markers for Quantitative Trait Loci (QTLs) for alcohol consumption were scanned. 44 B6.C and 36 B6.I inbred congenic Recombinant QTL Introgression (RQI) mouse strains of the b₅i₇ series carrying genes of BALB/cJ or CXBI origin on C57BL/6ByJ genetic background were used. In the B6.C set of strains, multiple regression analysis resulted a model with three microsatellite markers, which explained 32% of the genetic variance.

2 AIMS

Calcification of soft tissues develops under pathological conditions and has detrimental consequences, particularly when it occurs within vessel walls such as arteries, arterioles and heart valves. Calcification plays a crucial role in the pathogenesis of atherosclerosis and medial sclerosis, both leading to cardiovascular morbidity and mortality. It is also essence of a rare disease, calciphylaxis with very high mortality.

Epidemiologic studies suggest a complex association between alcohol consumption and cardiovascular disease.

Identification of processes involved in the complex interaction between alcohol consumption and vascular calcification should enable prediction of susceptibility to the disease and give potential targets to establish new preventive measures and effective therapies.

Alcohol preference in rodents serves as an important model for hedonic aspects of alcoholism, and alcoholism was shown to be a complex disorder determined by genetic background factors.

The objectives of our studies

- 1. To determine whether ethanol alters the mineralization of vascular smooth muscle cells and accumulation of calcium in extracellular matrix.
- 2. To determine whether ethanol affects the transition of vascular smooth muscle cells into osteoblast-like phenotype.
- 3. To determine the mechanism by which the osteoblastic transition of vascular smooth muscle cell occurs.
- 4. To expand genome scanning from 5 chromosomes (1, 2, 3, 9 and 15) to cover all autosomal chromosomes, and increased sample sizes in alcohol drinking preference tests of b_5i_7 RQI mouse strains. To analyze the combined data with composite interval mapping (CIM) and by multiple interval mapping (MIM).
- 5. To identify loci and genes that play a role in alcohol preference and drinking behavior.

3 MATERIALS AND METHODS

3.1 Cell culture and reagents

Human aortic smooth muscle cells were purchased from Cell Applications Inc. (San Diego, CA, USA) and fetal bovine serum (FBS) from Gibco (Paisley, UK). Unless otherwise mentioned, all other reagents were obtained from Sigma-Aldrich (Steinheim, Germany). Cell cultures were maintained in growth medium DMEM (GM) containing 15% FBS, 60 U/mL penicillin, 60 μ g/mL streptomycin, 120 μ g/mL neomycin, and 1 mM of sodium pyruvate. Cells were grown to confluence and used from passages 3 to 7. Primary human umbilical vein endothelial cells (HUVECs) were removed from human umbilical veins by exposure to dispase and cultured in medium 199 containing 15% FBS, antibiotics, L-glutamine, sodium pyruvate and endothelial cell growth factor. HepG2 cells were maintained in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin.

3.2 Histopathologic examination

For histopathologic examination, tissues and vessels were fixed in 10% formalin and embedded in paraffin. Five-micrometer sections were deparaffined with xylol for 8 minutes and rehydrated in a descending series of isopropyl-alcohol. Hematoxylin/eosin staining was performed (hematoxylin for 6 minutes, followed by a wash in distilled water for 8 minutes, staining with eosin for 2 minutes, dehydrating, and mounting on a coverslip). After rehydration, von Kossa staining was also performed - tissues in 5% silver-nitrate solution in front of a 60-watt lamp were incubated for one hour then rinsed in distilled water, exposed to 5% sodium thiosulfate for 5 minutes, staining fresh frozen sections were used (at 8 to 10 micrometer) after drying the section to the slides. Formalin fixation, a brief wash with tap water (1-10 mins) and rinse with 60% isopropanol were followed by staining with freshly prepared Oil Red-O working solution (15 mins). Tissues were rinsed with 60% isopropanol and then stained for nuclei with hemalaun solution. Stained slides were scanned with a Mirax Midi scanner (3D Histech, Budapest, Hungary) for digital documentation.

3.3 Induction of calcification

At confluence, cells were maintained in calcification medium which was prepared by adding 1 to 4 mmol/L of inorganic phosphate (P_i) to the growth medium. Both growth medium and calcification medium were changed every 2 days. The enhancement of P_i -provoked calcification by ethanol was most pronounced at a P_i concentration of 3 mmol/L. Therefore, we used 3 mmol/L P_i for inducing calcification in our experiments.

3.4 Quantification of calcium deposition

Cells grown on 48 well-plates were washed twice with PBS and decalcified with 0.6 N HCl for 30 minutes. Calcium content of the supernatants was determined by the QuantiChrome Calcium Assay Kit (Gentaur, Paris, France) as described by the protocol. After decalcification, cells were washed twice with PBS and solubilized with NaOH (0.1 mol/L) and SDS (0.1%), and the protein content of samples was measured with a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Calcium content of the cells was normalized to protein content and expressed as μ g/mg protein. For time-course experiment cells were treated for 5, 7, 10, 12 days before solubilizing. Mineral deposition in the extracellular matrix was also assessed by Alizarin Red staining. After staining, cells were washed twice with distilled water and once with 70% ethanol. To solubilize the stained extracellular matrix granules we incubated the cells with 100 mmol/L cetylpyridinium chloride for 1 hour, followed by measuring the absorbance of the dissolved dye at 570 nm.

3.5 Inorganic phosphate measurement

 P_i content of cell lysates was determined by the QuantiChrome Phosphate Assay Kit (Gentaur, Paris, France). After the ethanol treatment, cells were washed twice with PBS, solubilized with 1 % Triton X-100 and the cell lysates were assayed for P_i . Phosphate content of the cells was normalized to protein content and expressed as mmol/mg cell protein.

3.6 Alkaline phosphatase (ALP) activity assay

Cells grown on 6-well plates were washed with HBSS twice, cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and were assayed for ALP activity. Briefly, 130 μ l of Alkaline Phosphatase Yellow Liquid Substrate (Sigma-Aldrich, Steinheim, Germany) was combined with 50 μ g of protein samples. Kinetics of p-nitrophenol formation was followed for 30 minutes at 405 nm during incubation at 37°C. Maximum slope of the kinetic curves was used for calculation.

3.7 Western Blot and Osteocalcin assay

To detect osteocalcin expression, cells grown on 6-well plates were treated for 7 days. Extracellular matrix was dissolved in 200 μ L of EDTA (0.5 mol/L, pH 6.9) for osteocalcin and then cell lysate was obtained for glyceraldehyde-3-phosphate dehydrogenase assay. Equal loading of 30 μ l EDTA solubilized sample was electrophoresed on a 16.5 % Tris-Tricine Peptide gel (Bio-Rad, Hercules, CA, USA) and blotted onto nitrocellulose membranes (Hybond-ECL, Amersham Biosciences, Buckinghamshire, UK). After blocking, the membranes were incubated with polyclonal anti-osteocalcin antibody at 1:200 dilution (Santa

Cruz, Santa Cruz, CA, USA), followed by a peroxidase labeled anti-rabbit IgG antibody (Amersham Biosciences, Buckinghamshire, UK). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 30 µL of cell lysate was electrophoresed on a 12.5% SDS-PAGE then blotted onto a nitrocellulose membrane. The membrane was incubated with mouse monoclonal anti-GAPDH (Novus Biologicals, Inc., Cambridge, UK) followed by a peroxidase labeled anti-mouse IgG antibody (Amersham Biosciences, Buckinghamshire, UK). For alcohol dehydrogenase 1 expression, cell lysate was electrophoresed on a 12.5% SDS-PAGE and blotted onto a nitrocellulose membrane. After blocking, the membrane was incubated with rabbit monoclonal anti-alcohol dehydrogenase 1 antibody at 1:1000 dilution (Abcam, Cambridge, UK), followed by a peroxidase labeled anti-rabbit IgG antibody. After quantification, the membrane was reprobed for glyceraldehyde-3-phosphate dehydrogenase. Antigen-antibody complex was visualized with a horseradish peroxidase chemiluminescence (Amersham system according to the manufacturer's instructions Biosciences. Buckinghamshire, UK). Quantification of proteins was performed using computer-assisted video densitometry (Alpha DigiDoc RT, Alpha Innotech, San Leandro, CA, USA). Osteocalcin content of the same EDTA solubilized extracellular matrix samples were quantified by an enzyme-linked immunoabsorbent assay (Bender MedSystems, Vienna, Austria).

3.8 Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated, reverse transcribed and *Cbfa-1* mRNA was determined as described previously [28,29]. Briefly, for *Cbfa-1* mRNA levels the 25 μ l reaction mixture contained 5 μ l of reverse transcribed sample, 0.3 nmol/L of forward (5'-ATGGCGGGTAACGATGAAAAT-3') and reverse primers (5'-ACGGCGGGGAAGACTGTGC-3') and 12.5 μ l of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). PCRs were carried out using the iCycler iQ Real Time PCR System (Bio-Rad, Hercules, CA, USA). Results were normalized by Cyclophilin mRNA levels.

3.9 Cell viability assays

After treatment of cells with 20 to 80 mmol/L of ethanol in the presence or absence of calcification medium for 7 days. Following this, the monolayers were washed twice with HBSS and the test solutions were replaced with 550 μ l of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (0.5 mg/mL) solution in HBSS after which the cells were incubated for an additional 6 hours. After the MTT solution was removed, 550 μ l of

dimethyl-sulfoxide (DMSO) was added to the wells, and the optical density at 570 nm was measured.

3.10 Statistics

Data are shown as mean \pm SD. Statistical analysis was performed by one-way ANOVA test followed by a Newmann-Keuls test for multiple comparisons and two-way ANOVA test as necessary.

3.11 Recombinant QTL Introgression strains

Recombinant QTL Introgression was employed for the genetic analysis of complex quantitative straits. During this method we used short-term phenotypic selection, congenicity, recombination and inbreeding. For phenotype we selected a mesotelencephalic dopamine system related trait. QTLs from BALB/cJ and CXBI donor strains that are determining continuous variation of mesencephalic tyrosine hydroxylase (TH/MES) enzyme activity were introgressed onto B6 background strain from BALB/cJ and CXBI donor strains. CXBI is a recombinant inbred strain carrying B6 and BALB/cBy genes. We developed two types of F2s (B6XC and B6XI) and in each type replicate lines (α and β) were produced by equal division of each F2 litter. For the phenotype we tested at least 45 F2 males in each of the four lines and selected 15 for the first backcross to B6 females. We examined \geq 45 backcross1 (b₁i₀) male offspring and 15 males were chosen then intercrossed with nonlittermate females resulting in b₁i₁ generation.

3.12 Behavioral tests

Adult (11±2-week-old) male mice of C57BL/6By, BALB/cJ, CXBI, and RQI strains were utilized. We employed a "two-bottle choice" paradigm with enhancing ethanol concentration. To adapt the animals to the taste of ethanol, ethanol solutions were offered in increasing concentrations. The solutions were provided in custom-made drinking. Data were entered automatically employing A&D COLLECT software and QUATTRO spreadsheets.

3.13 DNA extraction, polymerase chain reaction, capillary gel electrophoresis

DNA was isolated from tail tips of mice derived from each RQI strain using the method of Miller et al.. We selected PCR primers as markers polymorphic for B6 and C strains with information from the Mouse Genome Database (Mouse Genom Informatics, The Jackson Laboratory). For QTL mapping 396 microsatellite markers were employed. To analyze PCR products using our ABI Prism 310 Genetic Analyzer, dye (FAM, TET or HEX)-labeled microsatellite markers were custom-synthesized, fluorescently labeled and, purified by Integrated DNA Technologies, Inc., Coralville, IA. Employing a 310 Genetic Analyzer one base size difference could be detected.

3.14 QTL Mapping

For mapping we used M-estimators, robust alternatives to sample mean and median for estimating the center of locations. The original phenotypic data were processed in the Explorer function of SPSS ver.13 to receive Tukey's biweight M-Estimators.

QTL position and effect size was judged by CIM using Windows QTL Cartographer version 2.5. We employed the standard model Zmapqtl 6 in the CIM procedure with a 10 cM window size and a 2 cM walking speed, forward and backward method of regression with probability of into or out of 0.1. Threshold values of significance for QTLs was determined by permutation analysis. Strain sets were also analysed by the MIM option for QTL Cartographer version 2.5.

4.15 Data analysis

Descriptive statistics, eta-squared, Post Hoc Test LSD 0.05 (least significant difference at 0.05 probability level) for alcohol consumption data in RQI strains were analyzed using GLM Univariate ANOVA procedure of SPSS software ver.13.

3.16 Bioinformatics

Chromosome segments carrying significant and suggestive QTL peaks were investigated for relevant QTLs and genes using <u>http://informatics.jax.org</u> and <u>http://omicspace.riken.jp</u>. Range of QTL peak was approximated by inspecting marker genotype patterns in all strains, identifying all donor segments which contained the peak position (cM), and recording rough approximation gives an estimate of minimum range of donor segments because positions of segment-limiting donor-type markers were used.

4 **RESULTS**

4.1 Ethanol fosters human vascular smooth muscle cell calcification in a dose-response fashion enhanced by increased inorganic phosphate

Since studies have shown the paramount importance of high extracellular inorganic phosphate to induce vascular calcification in humans, we established an *in vitro* model for vascular calcification by culturing human vascular smooth muscle cells in growth medium containing increased concentration of P_i (hereafter designated calcification medium). Calcification medium was prepared using 1 to 4 mmol/L of P_i growth medium (GM) consisting of DMEM containing 15% FBS, 60 U/mL penicillin, 60 µg/mL streptomycin and 120 µg/mL neomycin, and supplemented with 1 mM of sodium pyruvate. We maintained human vascular smooth muscle cells for 7 days and measured the calcium accumulation in the extracellular matrix in the presence or absence of ethanol.

As expected, phosphate induced mineralization in a dose-response fashion. Significant calcium accumulation was observed at $\geq 3 \text{ mmol/L}$ of P_i. To our surprise, treatment of human vascular smooth muscle cells with ethanol promoted deposition of calcium provoked by P_i. Exposure of cells to 60 mmol/L of ethanol (a concentration occasionally observed in very heavy drinkers' blood) resulted in a further enhancement in calcium accumulation (100%). We suspected that ethanol alone might alter the calcification of human vascular smooth muscle cells over a longer period of time. However, cells maintained for up to 14 days in growth medium supplemented with ethanol failed to develop mineralization.

In order to confirm the additional accumulation of calcium provoked by ethanol in the extracellular matrix of human vascular smooth muscle cells we performed Alizarin Red staining of cells cultured in calcification medium in the presence or absence of ethanol. Importantly, after exposure of vascular smooth muscle cells to ethanol for 7 days, more granular deposits developed throughout the cell cultures as compared to vascular smooth muscle cells maintained in ethanol free calcification medium. On the contrary, in cells cultured in growth medium without P_i and ethanol, no calcification occurred.

We next tested whether ethanol dose-dependently enhanced phosphate-provoked mineralization. Calcium content of extracellular matrix was measured in smooth muscle cell monolayers after exposing them to increasing concentrations of ethanol (20 to 80 mmol/L) for 7 days. Phosphate induced calcification at concentrations of 3 mmol/L and above, whereas cells maintained in growth medium failed to accumulate calcium. Ethanol further increased calcium accumulation, reaching significance at ≥ 60 mmol/L concentration.

Next we performed the time-course experiment of ethanol enhancing phosphateprovoked mineralization. Calcium content of extracellular matrix was measured in smooth muscle cell monolayers after exposing them to increasing concentrations of ethanol (20 to 80 mmol/L) for 5, 7, 10 and 12 days. Phosphate induced calcification at concentrations of 3 mmol/L and above, whereas cells maintained in growth medium failed to accumulate calcium. Intriguingly, ethanol further increased calcium accumulation, reaching significance at ≥ 60 mmol/L concentration.

4.2 Ethanol promotes the increase in alkaline phosphatase activity in human vascular smooth muscle cells cultured in calcification medium

We sought to determine whether the enhancing effects of ethanol on calcium deposition were just limited to calcium deposition or whether this involved up-regulation of genes responsible for osteoblastic transformation of vascular smooth muscle cells. Expression of alkaline phosphatase is important in early osteogenesis. This enzyme liberates P_i for mineralization and is a characteristic feature of vascular calcification *in vivo* that parallels bone mineralization. We therefore tested whether ethanol increases alkaline phosphatase activity in human vascular smooth muscle cells. While cells maintained in medium supplemented with 3 mM P_i resulted in a 1.61-fold increase in alkaline phosphatase activity, exposure of human vascular smooth muscle cells this concentrations of P_i and 60 and 80 mmol/L of ethanol led to a significant further enhancement of 2.17- and 2.12-fold, respectively. In contrast, ethanol failed to alter the increase in alkaline phosphatase activity in human vascular smooth muscle cells cultured in normal growth medium.

4.3 Ethanol increases the synthesis of osteocalcin, a calcium binding protein in calcifying human vascular smooth muscle cells

Osteocalcin is the major noncollagenous protein in bone matrix that regulates mineralization via binding calcium in the extracellular matrix. Accumulation of osteocalcin in vessels was shown to occur during vascular calcification in humans. Therefore we assessed the expression of osteocalcin in human vascular smooth muscle cells exposed to ethanol for 7 days.

Osteocalcin was not detectable in the solubilized extracellular matrix derived from monolayers of cells maintained in normal growth medium. Extracellular matrix of human vascular smooth muscle cells cultured in high phosphate medium exhibited a marked accumulation of osteocalcin. Importantly, exposure of cells to ethanol at concentrations of \geq 60 mmol/L resulted in a more pronounced increase in osteocalcin content of extracellular

matrix. In order to confirm that ethanol fosters phosphate-induced transition of human vascular smooth muscle cells into osteoblast-like cells we performed Western blot analysis for osteocalcin. The expression of osteocalcin was significantly higher after vascular smooth muscle cells were maintained in ethanol containing calcification medium compared to cells cultured in ethanol free calcification medium. Treatment of vascular smooth muscle cells with ethanol in normal growth medium had no effect on osteocalcin synthesis.

4.4 Ethanol enhances the expression of osteoblast specific transcription factor Cbfa-1 provoked by high inorganic phosphate

The essential regulator of osteoblast differentiation is the core binding factor alpha-1 (*Cbfa-1*), a transcription factor that controls the expression of matrix genes and, consequently, bone mineralization. Because transcription factor *Cbfa-1* has been also implicated in the transition of vascular smooth muscle cells into osteoblast-like cells provoked by high phosphate, we examined whether ethanol might affect the expression of *Cbfa-1* in human vascular smooth muscle cells cultured in calcification medium possibly providing an explanation for the phenotypic transition. Maintaining human vascular smooth muscle cells in high phosphate containing medium for 24 hours led to a 1.62-fold increase in *Cbfa-1* mRNA level compared to cells cultured in a normal growth medium. While ethanol in a normal growth medium at concentrations of 60 mmol/L and above further enhanced the *Cbfa-1* mRNA level compared to those grown in calcification medium alone.

4.5 Ethanol does not alter intracellular phosphate levels in human vascular smooth muscle cells exposed to elevated extracellular phosphate concentrations

Sodium-dependent phosphate co-transporter (Pit-1) facilitates entry of P_i into vascular smooth muscle cells to render a signal for *Cbfa-1*. Because evidence suggests that vascular calcification caused by hyperphosphatemia might be mediated in part by the function of Pit-1 we investigated whether ethanol could alter intracellular phosphate levels in human vascular smooth muscle cells cultured in calcification medium which might further promote the transition of smooth muscle cells into osteoblast-like cells. After 24 hours incubation we measured the phosphate content of smooth muscle cells maintained in calcification medium in the presence or absence of ethanol. Ethanol did not affect the intracellular phosphate levels in human vascular smooth muscle cells exposed to high extracellular phosphate concentrations.

4.6 The effect of ethanol in calcification medium on the viability of human vascular smooth muscle cells

Studies suggest that apoptosis of vascular smooth muscle cells may contribute to calcification in vessels in atherosclerosis and medial sclerosis in patients diagnosed with chronic kidney disease. Apoptotic cells might serve as a nidus for mineralization and generation of hydroxyapatite. Hence, by performing MTT assay we assessed the viability of cells challenged with ethanol in calcification medium. The viability of human vascular smooth muscle cells cultured in calcification medium for 7 days dropped significantly only at 80 mmol/L. Importantly, vascular smooth muscle cells challenged with 20 to 60 mmol/L of ethanol in a calcification medium for 7 days did not exhibit a decline in cell viability.

4.7 Alcohol dehydrogenase 1 is not expressed in human vascular smooth muscle cells

Ethanol is catabolized by alcohol dehydrogenase 1 in hepatocytes, producing acetaldehyde. If this was also to occur in vascular smooth muscle cells, acetaldehyde might be a contributing factor to mineralization and osteoblastic phenotype transition. We therefore measured the expression of alcohol dehydrogenase 1 in human vascular smooth muscle cells using Western blot analysis. There was a marked protein expression in a human-derived hepatoma cell line (HepG2 cells). On the contrary, we found no expression of this enzyme whether the HUVECs were exposed to ethanol or not. Similar to human endothelium, alcohol dehydrogenase 1 was not detectable by Western blot analysis in human vascular smooth muscle cells.

In our next studies 3 mmol/L P_i or 60 mmol/L ethanol alone did not enhance ROS production in vascular smooth muscle cells. In contrast, in smooth muscle cells exposed to ethanol in the presence of P_i ROS production was significantly enhanced.

4.8 QTL mapping

QTLs are designated as *Eac1-6* (ethanol consumption 1-6). CIM with 43 B6.Cb₅i₇ RQI strains gave significant peaks on chr.6 at 36.5 cM (p<0.001), 42.5 cM (p<0.001) 62.2 cM (p<0.01), and 73.5 cM (p<0.001), and on chr. 12 at 51 cM (p<0.01). Peaks were also found on chrs. 1, 5, 15, although these peaks did not reach the significant level. Only data on the chr. 15 peak are shown, since this QTL was included in the MIM model. In the B6.Ib₅i₇ set of 35 RQI strains two QTLs reached the significant level at LOD=2.0 (chr. 12: 21 cM, and chr. 19: 38 cM), but not at empirical threshold determined by 1000 permutations. CIM found several other nonsignificant peaks on chrs. 1, 3, 4, 12, 13, and 16 with LOD<2. Since this QTL was included in the chr. 12 peak were presented. In all examined

QTLs the B6 alleles enhanced alcohol consumption. CIM detected several QTLs where the donor allele was associated with increaser effect, but none of these reached the significant level as determined empirically by 1000 permutations. Markers next to the QTL peak were identified, and the number of strains containing the donor allele in homozygous condition were counted.

Analysis of B6.Cb₅i₇ RQI strains with MIM employing the BIC-M0 relative criterion of QTL Cartographer, resulted in a model of three QTLs on chrs. 6, 12, and 15 (genetic $R^2=0.47$). According to tests for epistasis found no significant additive-additive interactions. MIM with the B6.Ib₅i₇ set of strains resulted in a model of three QTLs positioned on chrs. 8, 12, and 4 (genetic $R^2=0.5$). Similarly to B6.Cb₅i₇ strains, no epistatic effect was found in the B6.Ib₅i₇ set. Testing the MIM identified QTLs demonstrating that all QTLs were significant.

CIM data, and range of peaks were evaluated with comparison of microsatellite marker genotype patterns across quasi-congenic RQI strains. In the 43 B6.Cb₅i₇ RQI strains validity of QTLs on chrs. 1, 5, 6, 12, and 15 were judged. The nonsignificant chr.1 QTL (7.5 cM) was excluded since it was associated with D1Mit167, which also mapped to chr. 5 and chr. 14. As in our earlier studies demonstrated, in the RQI strains D1Mit167 does not co-segregate with chr. 1 markers, however it does with proximal markers of chr. 5 (Saito et al., 2003). The nonsignificant chr. 5 QTL (59.01 cM) showed small negative additive effect (-1.29) in the B6.Cb₅i₇ population, therefore it was also excluded from further examination.

In all of the examined QTLs on chrs. 6, 15, and 12 were retained for bioinformatic analysis. In the 35 B6.Ib₅i₇ RQI strains only QTLs on chrs. 12 and 19 were further studied (p<0.2). The chr. 12 QTL was retained for further evaluation since the donor allele of the peak marker was present in several strains (N=4) and the average donor genome content of these strains was low (1.86%), approaching congenic conditions.

4.9 Alcohol consumption in a congenic strain

Using microsatellite marker-assisted selection and repeated backcrosses to the alcoholpreferring B6By background strain, we developed a new congenic strain, B6By.C.6.132.54 (B6By.C6). This congenic strain carries a BALB/cJ (C) donor chromosome segment on Chr. 6 with proximal and distal background markers D6Mit275 (25.5 cM, 51.1 Mb, from UniSTS annotation of NCBI build 36) and D6Mit134 (57.5 cM, 125.3 Mb), respectively. The congenic strain in a two-bottle free-choice alcohol preference test demonstrated significantly lower consumption of 12% (v/v) alcohol in comparison to its background partner B6By, conforming a significant decreasing effect of the genetic factor(s) residing on the donor segment.

In an additional independent test, using a two-bottle free choice paradigm with intermittent access to alcohol, we found that consumption of 12% alcohol was significantly higher among B6By males (12.70+-1.03, n=13) in comparison with B6By.C6 males [8.37+-0.96 (g/kg/day, mean+-SE), n=19, average of four 3-day trials, t30=3.00, p=0.005, two-tailed]. To test the hypothesis that other undetected chromosome segments are responsible for this effect, a genome scan with a mouse single-nucleotide polymorfism (SNP) panel of 402 markers was carried out. No additional donor segments were detected.

Because the genome scan showed flanking background markers at 75.9 Mb (rs4226008; NCBI mouse build 36/db SNP build 126) and 122.3 Mb (rs3023093), and limiting donor markers at 81.8 (rs4226024) and at 91.8 Mb (rs3712161), we concluded the segment size must be between 9.9 and 46.4 Mb.

An integrated genome and transcriptome analysis of the *Eac2* region on chr.6 suggests that a gene coding for metabotropic glutamate receptor subtype-7 (*Grm7*) is a candidate QTG for *Eac2*.

5 DISCUSSION

Epidemiologic studies pointed to a dichotomous association between alcohol consumption and cardiovascular disease. With few exceptions, studies from several countries have revealed a 20–40% lower cardiovascular disease incidence among drinkers of alcoholic beverages compared with non-drinkers. Although it has been confirmed that moderate alcohol use has an apparent protective effect on the development of coronary heart disease, a recent studies found no evidence of such a protective association of alcohol consumption and calcification of vessels. What is more, firm evidence that heavy alcohol consumption, in particular hard liquors, is associated with greater calcification in coronary arteries was recently reported. This finding has also been confirmed by Kim W. et al. demonstrating that intracoronary administration of ethanol provokes vascular calcification. Moreover, alcohol consumption was found to dramatically increase the risk of calciphylaxis in individuals who have normal renal function and calcium-phosphate metabolism.

These studies prompted us to investigate whether ethanol promotes vascular smooth muscle cell mineralization and transition of smooth muscle cells into osteoblast like cells *in vitro*. Since phosphate levels have long been recognized to strongly correlate with vascular

calcification both in patients with chronic kidney disease particularly above the physiological range and in healthy individuals whose phosphate concentration is within the physiological range and have no sign of altered renal function and mineral metabolism. We therefore employed elevations in exogenous phosphate to induce mineralization in our model.

Importantly, in our studies, exposure of cells to ethanol in calcification medium enhanced mineralization of the extracellular matrix of human vascular smooth muscle cells in a dose-response manner. Phosphate-induced calcification was further augmented providing significant additional extracellular calcium and phosphate deposition at concentrations of 60 mmol/L or above. Such concentrations can be found in heavy drinkers' blood. Since alkaline phosphatase is an important enzyme in the mineralization process and osteocalcin, a noncollagenous calcium binding protein, is specific for osteoblast phenotype, we also examined whether ethanol increases alkaline phosphatase activity and synthesis of osteocalcin in vascular smooth muscle cells. Ethanol caused a significant increase in the expression of alkaline phosphatase and osteocalcin. Furthermore, in cells challenged with ethanol the expression of *Cbfa-1*, a transcription factor involved in the regulation of osteoblastic transformation of smooth muscle cells, was also elevated.

Osteoblastic differentiation induced by hyperphosphatemia is mediated via a sodiumdependent co-transporter Pit-1 that facilitates entry of phosphate into vascular cells. Therefore, we measured phosphate uptake. Our results demonstrate that the observed effects of ethanol are not due to alterations of phosphate uptake.

There are *in vitro* studies suggesting that apoptosis of human vascular smooth muscle cells can contribute to calcification of vessels. Apoptosis was reported to occur both in the intima of advanced lesions and in the media of arteries in chronic kidney disease. In fact, alcohol-induced apoptosis of vascular smooth muscle cells was recently demonstrated. Apoptotic smooth muscle cells might act as a nidus for calcification, and thereby actively concentrate both calcium and phosphate to generate hydroxyapatite. In our study we did not observe any decline in the viability of smooth muscle cells challenged with ethanol up to 60 mmol/L concentration. Thus, apoptosis was not involved in the augmented mineralization provoked by ethanol.

Ethanol is catabolized mainly by the action of alcohol dehydrogenase 1 resulting in the generation of acetaldehyde in the liver. If acetaldehyde were produced by alcohol dehydrogenase 1 in smooth muscle cells, it might be a possible contributing factor to the mineralization in our model. Hence we measured the expression of alcohol dehydrogenase 1 in vascular smooth muscle cells by Western blot. Alcohol dehydrogenase 1 was not detectable

in human vascular smooth muscle cells indicating that acetaldehyde did not promote the mineralization induced by ethanol.

Our results strongly suggest that mineralization of human vascular smooth muscle cells and their transition into osteoblast-like cells induced by ethanol may contribute to the augmented vascular calcification observed in heavy alcohol consumption. It also offers an alternative mechanism by which calciphylaxis develops in heavy drinkers without kidney diseases or alterations in calcium-phosphate metabolism. This study may have relevance in chronic kidney diseases in which high alcohol consumption might promote vascular calcification.

In our studies we found that ethanol alone did not enhance ROS production in vascular smooth muscle cells. In contrast, in smooth muscle cells exposed to ethanol in the presence of P_i ROS production was significantly enhanced.

The association of heavy alcohol consumption, alcoholism with hard liquor use, and arterial calcification prompted us to study the genetic background that might be connected to vascular diseases. Metabotropic glutamate receptor type 7 gene was shown to serve a promising candidate that might be implicated in altered alcohol preference. In the central nervous system, the excitatory amino acid glutamate serves as a potent neurotransmitter exerting its effects via various membrane glutamate receptors. Nerves containing glutamate located close to bone cells exhibit functional glutamate receptor. It was revealed that glutamate stimulates bone resorption *in vitro* via a mechanically sensitive glutamate/aspartate transporter protein suggesting a function for glutamate in mechanical load and bone remodeling.

For mapping the genes related to alcoholism we employed Quantitative Trait Loci (QTL) mapping of 43 B6.C and 35 B6.I quasi-congenic RQI mouse strains of the b₅i₇ series. During the congenic breeding we introgressed BALB/cJ and CXBI/ByJ transgenes or targeted gene disruptions into C57BL/6ByJ genetic background. In these strains less than 3% of the donor genome was shown to be present on C57BL/6ByJ background. The B6.C genome carries BALB/cJ donor genes, while the B6.I strains carry CXBI/ByJ donor genes. C57BL/6ByJ, BALB/cJ and CXBI/ByJ animals exhibit different drinking behavior. Namely, BALB/cJ and CXBI/ByJ strains consume less alcohol compared to C57BL/6ByJ. We take advantage such a difference during our experiments. Here in this work we identified 6 *Eac* loci that play a role in alcohol preference and drinking behavior, one cis-regulated candidate gene of alcoholism, *Grm*7.

The 33.5-48.2 cM region is also rich in candidate genes. Employing bioinformatic tools, such as the Genome-Phenome Superbrain computational system (GPS; http://omicspace.riken.jp/gps/full.jsp), genes located in the interval were assessed. Syntenic vomeronasal receptor genes are potential candidates since olfaction may influence alcohol preference. Other genes may also influence neurotransmission. Importantly, the region contains *Grm7*, the metabotropic glutamate receptor type 7 gene. Since disturbances in glutamate function have been implicated in the pathophysiology of behavioral disorders, which may underlie some types of addiction, *Grm7* is a promising candidate to be studied.

This region was subjected to genetic, transcriptome and bioinformatics analysis to identify the quantitative trait genes (QTGs). According to our results the *Grm7* gene,-which encodes the group III metabotropic glutamate receptor 7 (mGluR7) protein-, influences alcohol consumption. Mice carrying the *Grm7* gene in the brain coding for lower expression of *Grm7* consume more alcohol in the preference drinking behavioral paradigm. Molecular, cellular and biological function of *Grm7*/mGluR7 strongly support this suggestion.

6 SUMMARY

The epidemiological consensus currently is that moderate consumption of alcohol is associated with the lowest risk for coronary artery disease and mortality. Although moderate alcohol use has an apparent protective association with coronary heart disease, heavy alcohol consumption, in particular hard liquor, is associated with greater calcification of arteries. Moreover, calciphylaxis, known to almost exclusively develop in patients with Stage 5 chronic kidney disease, was also reported to occur in heavy drinkers with physiological renal function. While we are gradually deciphering how alcohol might exert beneficial effects on atherosclerosis leading to prevention of cardiovascular and cerebrovascular disease, the molecular basis of the dichotomous effects of alcohol on vascular calcification have not been explored.

Abnormalities in mineral metabolism are considered important risk factors of vascular calcification. It has been well established that it is an actively well regulated multistep process in which interchangeable cellular phenotypes under certain pathological conditions are the driving force. One of the main contributors is the trans-differentiation of smooth muscle cells into osteoblast-like cells. After osteoblastic differentiation these cells lack characteristics of smooth muscle cells, and develop osteoblast features. This process results in mineralization and involves increased activity of alkaline phosphatase, increased expression of core binding factor α -1 with the subsequent induction of osteocalcin.

We provide evidence that exposure of vascular human smooth muscle cells to ethanol at high concentration might contribute to vascular calcification. Accumulations of calcium and phosphate occurs forming hydroxyapatite in the extracellular matrix of smooth muscle cells as a response to exposure to ethanol. Phenotype change of the cells towards osteoblast is the main characteristic of such a transition. Mineralization is accompanied with enhancement of osteoblast specific gene products including alkaline phosphatase and a significant increase in the synthesis of osteocalcin occurs. Moreover, ethanol enhances the expression of *Cbfa-1*, a transcription factor involved in the regulation of osteoblastic transformation of human vascular smooth muscle cells. Our results may provoke clinical studies and preventive measures in the field of high alcohol consumption and vascular calcification.

Glutamate receptors are implicated in bone remodeling and are demonstrated to serve as candidates in altered alcohol preference. We present results of our animal study, in which we expanded genome scanning of 5 chromosomes (1, 2, 3, 9 and 15) to include all autosomal chromosomes, and increased sample sizes in alcohol preference tests of b_5i_7 RQI strains. The combined data were analyzed by composite interval mapping (CIM) which shows a better performance than interval mapping in the case of multiple linked QTLs, and by multiple interval mapping (MIM). In our studies we identified new QTLs for alcohol consumption with genome-wide significance on chrs. 6 and 12. *Eac1* and *Eac6* overlap previously reported QTLs for alcohol preference and alcohol acceptance, respectively. Metabotropic glutamate receptor type 7 gene was shown to serve a promising candidate that might be implicated in altered alcohol preference.

7 NOVEL FINDINGS

Ethanol enhances mineralization of human vascular smooth muscle cells provoked by inorganic phosphate

Ethanol fosters phenotype change and transition of human vascular smooth muscle cells into osteoblast like cells triggered by inorganic phosphate.

Ethanol promotes the activity of alkaline phosphatase in human vascular smooth muscle cells.

Ethanol increases the synthesis of osteocalcin, a calcium binding protein in human vascular smooth muscle cells.

Ethanol enhances the expression of osteoblast specific transcription factor *Cbfa-1* in human vascular smooth muscle cells.

Ethanol does not alter intracellular phosphate levels in human vascular smooth muscle cells.

Alcohol dehydrogenase 1 is not expressed in human vascular smooth muscle cells, and acetaldehyde is not a contributing factor to mineralization and osteoblastic phenotype transition.

We identified new QTLs for alcohol consumption with genome-wide significance on chrs. 6 and 12. Eac1 and Eac6 overlap previously reported QTLs for alcohol preference.

Metabotropic glutamate receptor type 7 gene was shown to serve a promising candidate that might be implicated in altered alcohol preference.

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8 LIST OF PUBLICATIONS



Register Number: DEENKÉTK/285/2013. Item Number: Subject: Ph.D. List of Publications

Candidate: Melinda Oros Neptun ID: N00W7Y Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

 Oros, M., Zavaczki, E., Vadász, C., Jeney, V., Tósaki, Á., Lekli, I., Balla, G., Nagy, L., Balla, J.: Ethanol increases phosphate-mediated mineralization and osteoblastic transformation of vascular smooth muscle cells. *J. Cell. Mol. Med.* 16 (9), 2219-2226, 2012. DOI: http://dx.doi.org/10.1111/j.1582-4934.2012.01533.x IF:4.753

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