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Ethanol increases phosphate-mediated mineralization and osteoblastic transformation of vascular smooth muscle cells

Melinda Oros [a], Erzsebet Zavaczki [b], Csaba Vadasz [c, d], Viktoria Jeney [e], Arpad Tosaki [f], Istvan Lekli [f], Gyorgy Balla [b, g], Laszlo Nagy [a, h], Jozsef Balla [b, e, *]

[a] Department of Biochemistry and Molecular Biology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary
[b] Hemostasis, Thrombosis and Vascular Biology Research Group, Hungarian Academy of Sciences, Debrecen, Hungary
[c] Department of Psychiatry, New York University School of Medicine, New York, NY, USA
[d] Laboratory of Neurobehavioral Genetics, Nathan S. Kline Institute for Psychiatric Research, Orangeburg, NY, USA
[e] Department of Internal Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary
[f] Department of Pharmacodynamics, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary
[g] Department of Pediatrics, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary
[h] Apoptosis and Genomics Research Group of the Hungarian Academy of Sciences, Research Center for Molecular Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

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Abstract

Vascular calcification is implicated in the pathogenesis of atherosclerosis, diabetes and chronic kidney disease. Human vascular smooth muscle cells (HSMCs) undergo mineralization in response to elevated levels of inorganic phosphate (Pi) in an active and well-regulated process. This process involves increased activity of alkaline phosphatase and increased expression of core binding factor α-1 (CBF-α1), a bone-specific transcription factor, with the subsequent induction of osteocalcin. It has been shown that heavy alcohol consumption is associated with greater calcification in coronary arteries. The goal of our study was to examine whether ethanol alters mineralization of HSMCs provoked by high Pi. Exposure of HSMCs to ethanol increased extracellular matrix calcification in a dose responsive manner, providing a significant additional calcium deposition at concentrations of $\geq$60 mmol/l. HSMC calcification was accompanied by further enhancement in alkaline phosphatase activity. Ethanol also provoked a significant increase in the synthesis of osteocalcin. Moreover, in cells challenged with ethanol the expression of CBF-α1, a transcription factor involved in the regulation of osteoblastic transformation of HSMCs, was elevated. The observed effects of ethanol were not due to alterations of phosphate uptake by HSMCs. We conclude that ethanol enhances Pi-mediated human vascular smooth muscle calcification and transition of these cells into osteoblast-like cells.

Keywords: ethanol • smooth muscle cells • vascular calcification

Introduction

Vascular calcification is implicated in the pathogenesis of various vascular diseases and can result in devastating clinical consequences. It is related to an increased risk of cardiovascular morbidities and complications such as atherosclerotic plaque burden [1–3], myocardial infarction [4, 5], coronary artery disease [6, 7], postangioplasty dissection [8], and increased ischemic episodes in peripheral vascular disease [9]. It has been shown to be a powerful independent marker of coronary heart disease events in patients with diabetes [6]. Studies also indicated that coronary calcification may be predictive of or associated with sudden cardiac death [10, 11]. Recently it has been found that cardioprotective effect of moderate alcohol consumption against ischemic heart disease disappears when light to moderate drinking is mixed with irregular heavy drinking occasions [12]. Moreover, there is evidence that heavy consumption of hard liquor is associated with greater accumulation of calcium in coronary artery [13]. Calciphylaxis known to almost exclusively develop in patients with Stage 5 chronic kidney disease (CKD) was also reported to occur in heavy drinkers with physiological renal function [14–17].

*Correspondence to: Jozsef BALLA, M.D., D.Sc.
Pf. 19. Nagyerdei krt. 98. 4012 Debrecen, Hungary.
Tel./Fax: +36 52 413653
E-mail: balla@internal.med.unideb.hu

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Although the mechanism of vascular calcification is not completely understood, abnormalities in mineral metabolism are considered important risk factors. Indeed, 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 [11]. Strong associations between arterial calcification and stiffness, pulse pressure, or mortality in dialysis patients have also been revealed contributing to the high rates of cardiac and peripheral ischemic disease and left ventricular hypertrophy in this population [18–20]. Many studies have demonstrated the role of high extracellular Pi to induce vascular calcification [21–24]. It has been revealed that phosphate uptake through a sodium-dependent phosphate co-transporter, Pit-1 is essential for vascular smooth muscle cell calcification and phenotypic modulation in response to elevated phosphate [25]. Recently novel functions of Pit-1 related to cell signalling, cell proliferation and apoptosis were emerged [26]. Transition of smooth muscle cells into osteoblast-like cells is a delicate and well-regulated cellular process where cells gain an osteoblastic phenotype. This is indicated by the increase in expression of CBF-α1 which is an osteoblast-specific transcription factor required for osteoblast differentiation, bone matrix gene expression, and consequently, bone mineralization [27]. Upregulation of alkaline phosphatase, an important enzyme in early osteogenesis and osteocalcin, a major noncollagenous protein in bone matrix demonstrated to regulate mineralization, was also shown to occur [28].

These previous studies prompted us to investigate the role that ethanol may play in vascular smooth muscle cell mineralization and transition of smooth muscle cells into osteoblast-like cells *in vitro*. We observed that ethanol at high concentration found in blood during heavy alcohol consumption enhances Pi-provoked HSMC calcification leading to formation of granular calcium deposits in extracellular matrix. Osteoblastic transformation of HSMCs is promoted by ethanol as assessed by upregulation of CBF-α1, osteocalcin, and alkaline phosphatase.

Materials and methods

Cell culture and reagents

Human aortic smooth muscle cells were purchased from Cell Applications Inc. (San Diego, CA, USA) and 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 μg/ml streptomycin and 120 μg/ml neomycin and 1 mM of sodium pyruvate. Cells were grown to confluence and used from passages 3 to 7. HepG2 cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Induction of calcification

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

Quantification of calcium deposition

Cells grown on 48 well plates were washed twice with PBS and decalcified with 0.6 N HCl for 30 mins. 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), SDS (0.1%) and protein content of samples were 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 μg/mg protein. Mineral deposition in the extracellular matrix was also assessed by Alizarin Red staining [29]. 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 hr, followed by measuring the absorbance of the dissolved dye at 570 nm.

Phosphate measurement

Pi content of cell lysate was determined by the QuantiChrome Phosphate Assay Kit (Gentaur, Paris, France). After the ethanol treatment cells were washed twice with PBS and solubilized with 1% Triton X-100 and the cell lysates were assayed for Pi. Phosphate content of the cells was normalized to protein content and expressed as mmol/mg cell protein [29].

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 μl of Alkaline Phosphatase Yellow Liquid Substrate (Sigma-Aldrich) was combined with 50 μg of protein samples. Kinetics of *p*-nitrophenol formation was followed for 30 min. at 405 nm during incubation at 37°C. Maximum slope of the kinetic curves was used for calculation [29].

Western blot and osteocalcin assay

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

alcohol dehydrogenase 1 cell lysate was electrophoresed on a 12.5% SDS-PAGE and blotted onto 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 labelled anti-rabbit IgG antibody. After detection, membrane was reprobed for glyceraldehyde-3-phosphate dehydrogenase. Antigen-antibody complex was visualized with the horseradish peroxidase chemiluminescence system according to the manufacturer's instructions (Amersham Biosciences). Quantification of proteins was performed with computer-assisted videodensitometry (Alpha DigiDoc RT, Alpha Innotech, San Leandro, CA, USA). Osteocalcin content of the same EDTA solubilized extracellular matrix sample was quantified by an enzyme-linked immunoabsorbent assay (Bender MedSystems, Vienna, Austria), according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated, reverse transcribed and CBF-α1 mRNA was determined as described previously [29, 30]. Briefly, for CBF-α1 mRNA levels the 25 μl reaction mixture contained 5 μl of reverse transcribed sample, 0.3 nmol/l of forward (5′-ATGGCGGGTAACGATGAAAAT-3′) and reverse primers (5′-ACGGCGGGGAAGACTGTGC-3′) and 12.5 μl of iQ SYBR Green Supermix (Bio-Rad). PCRs were carried out using the iCycler iQ Real Time PCR System (Bio-Rad). Results were normalized by Cyclophilin mRNA levels.

Cell viability assay

After treatment of cells with 20–80 mmol/l of ethanol in the presence or absence of calcification medium for seven days we assessed cell viability using PrestoBlue cytototoxicity assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Statistics

Data are shown as mean ± S.D. Statistical analysis was performed by one-way ANOVA test followed by a Newmann-Keuls test for multiple comparison and two-way ANOVA test for Figure 1. A value of $P < 0.05$ was considered significant and marked with *, and $P < 0.01$ was considered highly significant and marked with **.

Results

Ethanol increases HSMC calcification in a dose responsive manner

To develop an *in vitro* model of human vascular calcification, we cultured HSMCs in calcification medium, which was prepared by the addition of 1–4 mmol/l of Pi to the growth medium. After culturing HSMC in calcification medium for 7 days, extracellular calcium measurements were performed in the presence or absence of ethanol (Fig. 1). As expected, phosphate provoked calcification in a dose dependent manner, providing a significant induction at a dose of 3 mmol/l and above. Importantly, exposure of cells to 60 mmol/l of ethanol further enhanced calcium deposition in HSMC culture. In contrast, ethanol alone did not alter HSMC calcification for 14 days of incubation time (data not shown). To confirm the additional enhancement in extracellular calcium deposition provided by ethanol we performed Alizarin Red staining of HSMCs cultured in calcification medium (Fig. 2). After exposure of HSMCs from 20 to 80 mmol/l of ethanol for seven days, more granular deposits developed throughout the cell culture (Fig. 2A) compared to HSMCs cultured in ethanol free calcification medium, whereas in the control culture, no deposits were found during this period. Extracellular calcium measurements were carried out to prove that phosphate-induced calcification is dose dependently further increased by ethanol. As shown in Figure 2B and C, the facilitating effect of ethanol on extracellular calcification is dose dependent, providing a significant additional increase in calcium deposition at a concentration of $\geq$ 60 mmol/l.

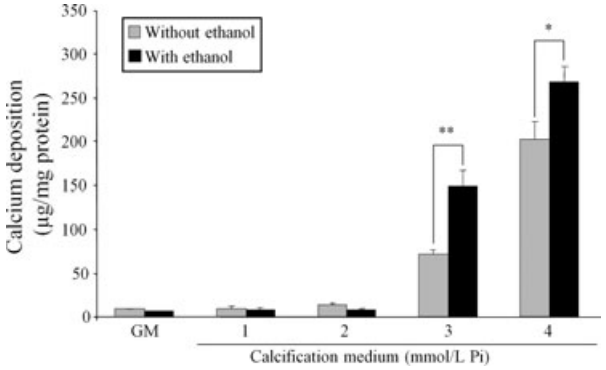

Fig. 1 Ethanol promotes human vascular smooth muscle cell calcification provoked by phosphate. Human vascular smooth muscle cells were cultured in GM alone or in calcification medium containing 1–4 mmol/l of phosphate. Media were supplemented with 60 mmol/l of ethanol. Calcium content of cells was measured after 7 days of culture as described in Materials and Methods, and was normalized by protein content. Data are presented as the mean ± S.D. of three independent experiments performed in duplicates. *$P < 0.05$, **$P < 0.01$.

Ethanol promotes the expression of alkaline phosphatase activity in HSMCs

It has been demonstrated that vascular calcification *in vivo* shares similarities with bone mineralization; therefore we asked the question of whether ethanol fosters the transition of HSMCs into osteoblast-like cells. Alkaline phosphatase (ALP) is an important enzyme in early mineralization of bone and teeth. We therefore examined whether ethanol increases ALP activity in HSMCs maintained in calcification medium (Fig. 3). While HSMCs cultured in calcification medium exhibited a 1.61-fold increase in ALP activity, addition of 60 and 80 mmol/l ethanol to calcification medium resulted in further enhancement up to 2.17- and 2.12-fold, respectively. On the contrary, exposure of cells

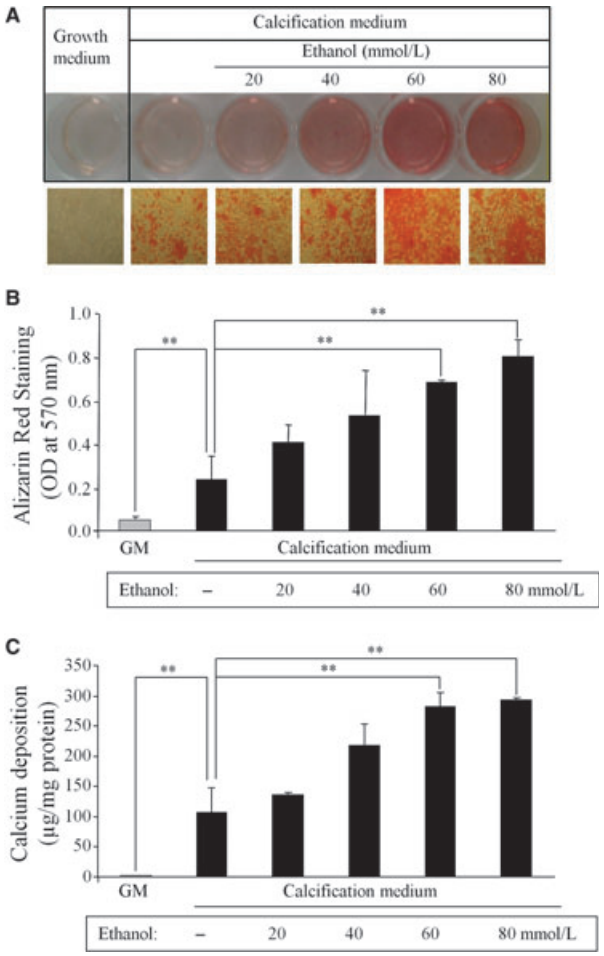

Fig. 2 Dose dependent effect of ethanol on human smooth muscle cell calcification. (**A**) HSMCs were cultured in GM or in calcification medium in the absence or presence of increasing concentration of ethanol for 7 days then Alizarin Red staining was performed. Representative images of stained plates (upper) and microscopic views (×100, lower) from three independent experiments are shown. (**B**) Quantification of Alizarin Red staining after solubilization of granular deposits with cetylpyridinium chloride as described in Materials and Methods. (**C**) In the same experiments extracellular calcium measurements were carried out using QuantiChrome Calcium Assay. Data are presented as mean ± S.D. of three independent experiments performed in duplicates. $*P < 0.05$, $**P < 0.01$.

to ethanol failed to alter the expression of ALP in HSMCs maintained in normal growth medium (data not shown).

Ethanol increases the synthesis of osteocalcin in HSMCs

Next we investigated expression of another bone-specific gene, osteocalcin. As demonstrated by the immunoblot in Figure 4B, higher amounts of osteocalcin were present in the extracellular matrix of ethanol-treated HSMCs cultured in calcification medium as compared to cells maintained in ethanol free calcification medium. Osteocalcin was not detectable in the matrix of HSMCs cultured in normal growth medium. Extracellular osteocalcin measurements were performed to confirm that phosphate-induced transition of HSMCs into osteoblast-like cells is dose-dependently promoted by ethanol. As shown in Figure 4A and B, ethanol treatment of HSMCs maintained in a calcification medium led to a more pronounced increase in osteocalcin synthesis at a concentration of $\geq$60 mmol/l compared to HSMCs cultured in ethanol free calcification medium. In contrast, treatment of cells with ethanol did not increase the synthesis of osteocalcin in HSMCs maintained in normal growth medium (data not shown).

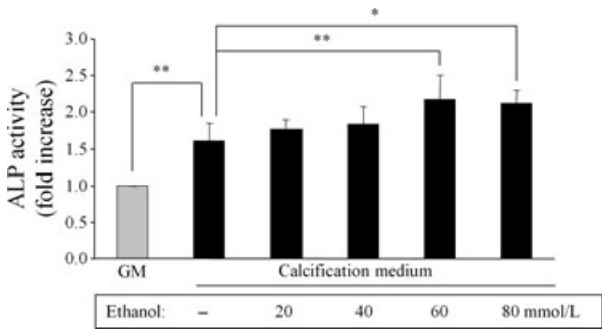

Fig. 3 Ethanol enhances the expression of alkaline phosphatase activity in human vascular smooth muscle cells. HSMCs were cultured in GM or in calcification medium in the absence or presence of increasing concentration of ethanol for seven days. Alkaline phosphatase activity of cells was measured as described in Materials and Methods. Data are expressed as the means ± S.D. of six independent experiments each performed in duplicates. $*P < 0.05$, $**P < 0.01$.

Ethanol enhances the expression of osteoblast-specific transcription factor CBF-α1

Transcription factor CBF-α1 is implicated in the osteoblastic differentiation of vascular smooth muscle cells induced by high phosphate level. Hence, we investigated if ethanol could alter the expression of CBF-α1 in HSMCs maintained in calcification medium (Fig. 5), and therefore put forward a possible explanation for the observed promoting effects of ethanol on transition of HSMCs into osteoblast-like cells. Culturing HSMCs in a calcification medium for 24 hrs resulted in a 1.62-fold increase of CBF-α1 mRNA level compared to cells maintained in a normal growth medium. The addition of $\geq$60 mmol/l ethanol to the calcification medium further enhanced the CBF-α1 mRNA level in HSMCs, although ethanol in a normal growth medium failed to alter CBF-α1 expression.

The effect of ethanol on intracellular phosphate level and cell viability

Because evidence suggests the effects of hyperphosphatemia are mediated through a sodium-dependent phosphate co-transporter that

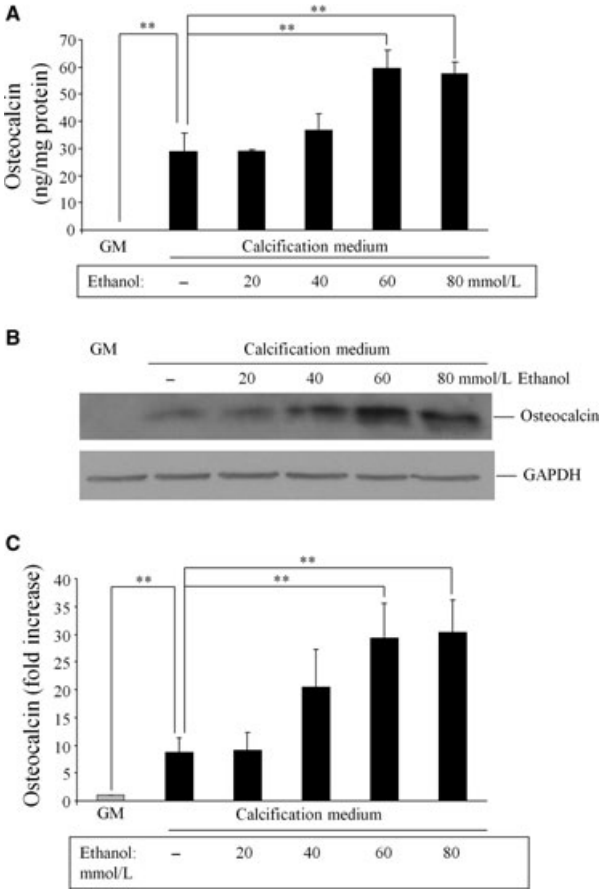

Fig. 4 Ethanol increases the synthesis of osteocalcin in human vascular smooth muscle cells. (**A**) After seven days culturing of HSMCs in GM or calcification medium alone or supplemented with 20, 40, 60 and 80 mmol/l ethanol, the extracellular matrix was dissolved and osteocalcin deposition was quantified as described in Materials and Methods. (**B**) Western blot analysis was performed and densitometric measurement of the band intensities for osteocalcin and the corresponding GAPDH from cell monolayer were determined as described in Materials and Methods. Data are expressed as the means ± S.D. of four independent experiments. $*P < 0.05$, $**P < 0.01$.

facilitates entry of phosphate into vascular cells, we measured the phosphate uptake of HSMCs after 24 hrs of incubation in a calcification medium in the presence and absence of ethanol. Our results indicate (Fig. 6A) that ethanol did not alter the elevation of intracellular phosphate level in HSMCs that resulted from high extracellular phosphate concentration. To test the viability of cells challenged with ethanol in calcification medium, we performed a PrestoBlue assay. As shown in Figure 6B, the calcification medium did not decrease the viability of HSMCs. Furthermore, HSMCs exposed to 20–80 mmol/l of ethanol in a calcification medium for seven days did not exhibit a decline in cell viability.

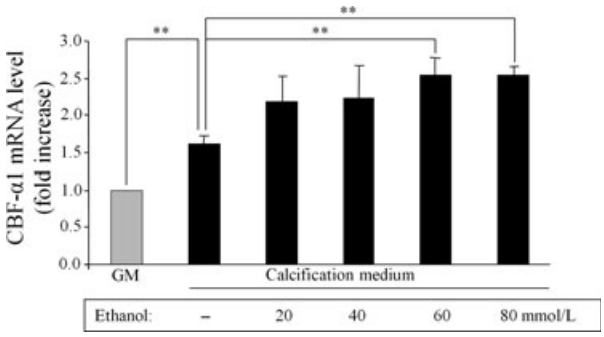

Fig. 5 Ethanol enhances the expression of osteoblast-specific transcription factor CBF-α1 in human vascular smooth muscle cells. HSMCs were cultured in GM or in calcification medium alone or in the presence of ethanol (20, 40, 60 and 100 mmol/l) for 24 hrs. CBF-α1 mRNA levels were determined by quantitative RT-PCR as described in Materials and Methods. Results are presented as the mean ± S.D. of three independent experiments performed in triplicates. $*P < 0.05$, $**P < 0.01$.

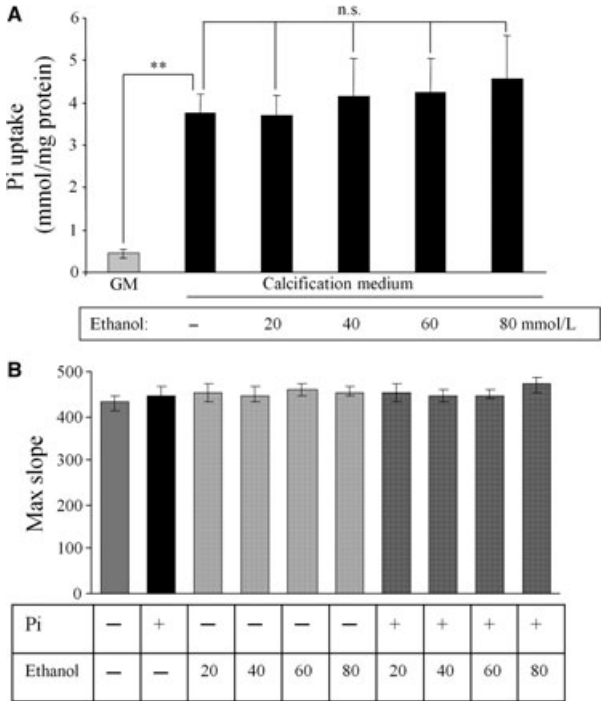

Fig. 6 Ethanol does not alter phosphate entry into human vascular smooth muscle cells. (**A**) HSMCs were cultured in GM or calcification medium alone or supplemented with ethanol (20, 40, 60, 80 mmol/l) for 24 hrs. Cell lysate was used to measure Pi levels as described in Materials and Methods. Results are presented as the mean ± S.D. of six independent experiments performed in duplicates. (**B**) For investigation of whether ethanol causes significant toxicity a PrestoBlue assay was performed after seven day of incubation. Data are the means ± S.D. of four separate experiments in duplicates. $*P < 0.05$, $**P < 0.01$.

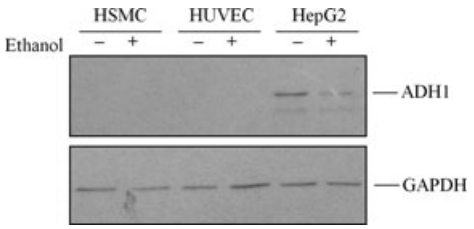

Fig. 7 Alcohol dehydrogenase 1 is not detectable in human vascular smooth muscle cells. After seven days culturing of HSMCs, human umbilical vein endothelial cells (HUVEC) and HepG2 cells in medium alone or supplemented with 60 mmol/l ethanol, Western blot analysis was performed for alcohol dehydrogenase 1 (ADH1). Membrane was reprobed for GAPDH as described in Materials and methods.

Alcohol dehydrogenase 1 is not detectable in HSMCs

Because metabolism of ethanol by alcohol dehydrogenase occurs in cells including hepatocytes generating acetaldehyde, we assessed its expression in HSMCs. Employing Western blot analysis alcohol dehydrogenase 1 was not detectable in HSMCs regardless their exposure to ethanol (Fig. 7). As shown in Figure 7, there was a marked protein expression in a human-derived hepatoma line (HepG2 cells).

Discussion

Mineralization in soft tissues develops under pathological conditions and has detrimental consequences, particularly when it occurs within vessel walls and heart valves. Elevated phosphate level has long been recognized to highly correlate with vascular calcification in patients with Stage 5 CKD [31–33]. In fact, the major cause of mortality in patients requiring haemodialysis is cardiovascular events. In particular, the development of calciphylaxis, which is a syndrome of vascular calcification and skin necrosis, is almost exclusively seen in patients with Stage 5 CKD and correlates with extremely high fatality rates. Previous studies indicated that elevated phosphate could induce smooth muscle cell calcification, as well as an osteochondrogenic phenotypic change *in vitro* [34]. Evidence suggests a highly regulated cellular process where many different inducers and inhibitors of osteoblast differentiation have been recognized [35]. These studies have demonstrated an *in vitro* model where HSMCs cultured with calcification medium containing high levels of phosphate not only undergo mineralization, but also manifest upregulation of osteoblast markers [24, 25, 27, 28]. These include CBF-α1, a key regulatory transcription factor critical for the differentiation of osteoblasts, and its downstream transcript proteins such as ALP, a crucial enzyme in the context of bone and teeth formation, and osteocalcin, which is a very specific protein indicative of osteoblast activity. CBF-α1 knockout mice fail to form mineralized bone [36] and exhibit low ALP activity and osteocalcin expression.

Alcohol consumption has been consistently found to have a J-shaped association with coronary heart disease – moderate drinkers have a lower risk than both heavy and non-drinkers. This relationship has not been studied extensively across races or ethnicities, but evidence to date consistently shows a protective effect [37–39]. A recent study confirmed that moderate alcohol use has an apparent protective association with coronary heart disease [13]. However, the authors found no evidence of such a protective association of alcohol consumption and coronary artery calcification. In fact, they reported evidence that heavy alcohol consumption, in particular hard liquor, is associated with greater calcification in coronary arteries.

Calciphylaxis was reported to occur in patients diagnosed with alcoholic liver disease who did have physiological renal function and calcium-phosphate metabolism [14–16]. Possible contributing factors were suggested to be protein C and protein S deficiencies due to the liver disease. Calciphylaxis was also reported in alcoholic cardiomyopathy in patients who did not develop liver and kidney disease and any alteration in calcium-phosphate metabolism [17].

These studies prompted us to investigate whether or not ethanol promotes vascular smooth muscle cell mineralization and its transition into osteoblast-like cells *in vitro*. Importantly, in the present study, the addition of ethanol to the calcification medium enhanced HSMC mineralization in a dose responsive manner. The elevated phosphate-induced calcification was further increased providing a significant additional extracellular calcium accumulation at dose of $\geq$60 mmol/l – such concentrations can be observed in heavy drinkers' blood. Because ALP is an important enzyme in the mineralization process and osteocalcin, a non-collagenous calcium binding protein, is specific for osteoblast phenotype, we also examined whether ethanol increases ALP activity and synthesis of osteocalcin in HSMCs. Ethanol provoked a significant increase in the expression of ALP and osteocalcin. Moreover, in cells challenged with ethanol the expression of CBF-α1, a transcription factor involved in the regulation of osteoblastic transformation of HSMCs, was also elevated. It has been established that osteoblastic differentiation induced by hyperphosphatemia is mediated *via* a sodium-dependent co-transporter (Pit-1) that facilitates entry of phosphate into vascular cells [25]; therefore, we measured phosphate uptake. Our results demonstrate that the observed effects of ethanol are not due to alterations of phosphate uptake.

There is extensive *in vitro* evidence that apoptosis of HSMCs can promote calcification in vessels, which is seen both in the intima in advanced plaques and in the media in CKD. Apoptotic smooth muscle cells may act as both a nidus for calcification, and actively concentrate both calcium and phosphate to generate hydroxyapatite [40–42]. In fact, alcohol-induced apoptosis of vascular smooth muscle cells was recently demonstrated to occur [43] although we did not observe significant decline in viability of HSMCs in our experiments.

In a study by Giachelli's group osteoblastic transformation of vascular smooth muscle cells was elegantly demonstrated to be reversible [44]. They found that vascular cells with osteochondrogenic phenotype regain smooth muscle cell properties and down-regulated osteochondrogenic gene expression in environment that favours vascular smooth muscle cells. Runx2/CBF-α1 was shown to be a decisive factor in the smooth muscle cell reprogramming. Therefore, we also need to answer in future whether or not mineralization of HSMCs promoted by ethanol is reversible.

Ethanol is metabolized in the liver mainly by the action of alcohol dehydrogenase 1 leading to the generation of acetaldehyde. Thus, acetaldehyde if produced by alcohol dehydrogenase 1 in HSMCs was a possible contributing factor to the HSMC mineralization in our model. Therefore we measured the expression of alcohol dehydrogenase 1 in vascular smooth muscle cells. Using Western blot analysis alcohol dehydrogenase 1 was not detectable in HSMCs indicating that acetaldehyde did not act as a promoter in mineralization induced by ethanol.

In conclusion, our results suggest that HSMC mineralization and transition into osteoblast-like cells induced by ethanol may contribute to greater vascular calcification observed in heavy alcohol consumption. It also offers an alternative explanation as to why calciphylaxis occurs in heavy drinkers without kidney diseases and any alterations in calcium-phosphate metabolism. This study may have relevance in CKDs in which high alcohol consumption might assist vascular calcification.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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