FINAL REPORT – József Balla, OTKA-ID:83478

Title - Inhibition of vessel wall remodeling by catabolism of heme

Elevated inorganic phosphate (Pi) level along with reactive oxygen species, diabetes mellitus, hypertension, chronic kidney diseases (CKD) have long been recognized as a significant predictor of soft tissue mineralization. Such elevated Pi levels are commonly seen in patients with CKD in which the Pi homeostasis is deranged because of inability of the kidneys to excrete phosphate. Moreover, in healthy individuals with physiological serum Pi concentrations the higher Pi level leads to the higher cardiovascular mortality. Vascular calcification follows two distinct patterns: (i) intimal calcification, which occurs with atherosclerotic plaques, and (ii) medial calcification, which is characterized by diffuse calcification of the media, particularly at the level of the internal elastic lamina that is commonly seen in hemodialysis patients and is not always accompanied by atherosclerosis. Furthermore, development of calciphylaxis, which is a syndrome of diffuse arteriolar calcification and skin necrosis, is almost exclusively seen in patients with stage 5 CKD and correlates with extremely high fatal rates.

Previous studies indicated that elevated phosphate could induce vascular smooth muscle cell (VSMC) calcification as well as an osteochondrogenic phenotypic change. Evidence suggests that this transition is a highly regulated cellular process, involving upregulation of Cbfa1 (transcription factor involved in the regulation of osteoblast activity), 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 (OC), which is a very specific protein indicative of osteoblast activity.

We previously demonstrated that VSMC mineralization in response to Pi is regulated by intracellular ferritin. Therefore we tested if β-glycerolphosphate (BGP) and/or activated vitamin D3 (Calcitriol) induced calcification is prevented by ferritin. Our first goal was to assess the osteoblast-like transformation of VSMC by β-glycerolphosphate (BGP) and activated vitamin D3 (Calcitriol). We observed that both inducers have the ability to increase ALP in dose-dependent manner. Combined treatment resulted in additively higher enzyme activity. Direct staining for ALP showed increased numbers of positively stained cells following treatment with BGP or calcitriol alone or together. Expression of ALP can also be

estimated using immunfluorescence staining. Untreated cells were almost completely negative with this staining whereas cells experiencing BGP and/or calcitriol show increased expression. The cytoskeleton was counterstained for fibrillar actin. Western blots confirmed that the increased activity was due to enhanced expression of ALP. Ferritin blocks the induction of ALP activity. VSMC were treated with BGP and calcitriol alone or together to investigate the induction of ALP in the presence or absence of apo- and holo- forms of ferritin. Both forms of ferritin decreased the activity of ALP; there were no significant differences between the two forms. Up-regulation of heme oxygenase-1 in VSMC, or its products such as carbon monoxide, biliverdin, bilirubin did not alter the mineralization process.

In our previous studies we have demonstrated the role that H_2S play in the heme-mediated oxidative alteration in lipoproteins and atheroma lipids as well as the subsequent cell reactions. Hydrogen sulfide (H_2S) is now recognized as a gas with important functions in the cardiovascular system. In the vasculature, it is produced by VSMC by cystathionine γ-lyase (CSE) enzyme, and is involved in the regulation of vascular tone and myocardial contractility. H_2S deficiency was observed in various animal models of arterial and pulmonary hypertension. Exogenous H_2S ameliorates myocardial dysfunction associated with the ischemia/reperfusion injury. The potential inhibitory effect of H_2S on vascular calcification has recently emerged in calcific uremic arteriolopathy/calciphylaxis, a life-threatening complication of renal failure.

Drawing upon these previous observations, we examined the role that H_2S may have in VSMC calcification and transition of VSMC into osteoblast-like cells. We observed that H_2S suppressed deposition of calcium in the extracellular matrix of VSMC induced by Pi in a dose–responsive manner. More importantly, the inhibitory effect of H_2S was not limited to calcium deposition. In fact, H_2S suppressed the induction of genes involved in osteoblastic transformation of VSMC. H_2S inhibited Pi-mediated upregulation of ALP and OC in a dose-dependent manner. Cbfa1 is required for osteoblast differentiation, bone matrix gene expression, and, consequently, mineralization; therefore, we examined the effect of H_2S on Cbfa1 expression in HAoSMC. Similar to ALP and OC, the Pi-provoked upregulation of Cbfa1 was attenuated by H_2S.

Emerging evidence suggests that the effect of hyperphosphatemia on VSMC calcification is mediated through Pit-1 (sodium-dependent co-transporter-1), which facilitates entry of Pi into vascular cells. We therefore tested whether H_2S alters intracellular Pi level in VSMC. Importantly, the marked increase in the level of intracellular Pi due to phosphate exposure was substantially reversed by H_2S. We demonstrate that inhibition of entry of Pi into vascular cells provided by H_2S occurs through suppression of Pit-1 expression, thus decreasing the intracellular level of Pi that is fundamental to osteoblastic transformation of VSMC. Apoptosis of VSMC is also implicated in the pathogenesis of calcification in vessels, which is seen both in the intima in advanced plaques and in the media in CKD. Apoptotic smooth muscle cells may function as both a nidus for calcification and actively concentrate both calcium and phosphate to generate hydroxyapatite. H_2S was shown to induce apoptosis of VSMC. The concentration at which H_2S exhibits proapoptotic effect is ≥200 μmol/l. In our study, we did not observe alterations in viability of VSMC challenged with Pi or H_2S at concentrations studied.

H_2S is generated as an alternative product of the transsulfuration pathway, and in the vasculature it is produced mainly by VSMC via CSE-catalyzed reaction. Recently, it has been shown that deletion of CSE in mice results in hypertension. Our data confirmed that the plasma concentration of H_2S was decreased in stage 5 CKD patients. Moreover, the level of H_2S was further lowered by hemodialysis. We revealed that enzyme activity of CSE in monocytes derived from stage 5 CKD patients treated with hemodialysis was markedly reduced compared with healthy individuals, without changes in mRNA or protein expression. These results suggest potential post-translational modifications in CSE in CKD that remain to be determined. To prove the role of CSE in inhibiting Pi-induced VSMC mineralization, we used strategies to decrease endogenous production of H_2S via both pharmacological inhibition of CSE enzyme activity and silencing of CSE gene expression. By reducing CSE-mediated endogenous H_2S biogeneration, we observed a significant increase in both VSMC calcification and the expression of ALP and OC. Our findings further corroborate the imperative role of H_2S and CSE in the vasculature and suggest that reduced activity of CSE and subsequent decrease in the level of H_2S in stage 5 CKD patients could exacerbate the cardiovascular complications that commonly accompany this particular group of patients.

In conclusion, our study demonstrates a novel role of H_2S in the process of Pi-provoked mineralization and transition of VSMC into osteoblast-like cells. We provide evidence that H_2S, regardless of its exogenous or endogenous origin, inhibits the upregulation of osteoblast-specific genes such as ALP, OC, and Cbfa1. The inhibition of Pi uptake through Pit-1 is essential for providing beneficial effects against calcification and phenotypic modulation of VSMC by H_2S. Reduced CSE activity leading to decreased H_2S levels in stage 5 CKD patients might facilitate calcification of vasculature. These results offer a new strategy to prevent vascular calcification.

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. However, evidence in recent studies has been collected demonstrating that heavy alcohol consumption, in particular hard liquor, is associated with greater calcification in coronary arteries. 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, the addition of ethanol to VSMC promotes mineralization in a dose responsive manner. The elevated phosphate-induced calcification was further increased providing a significant additional extracellular calcium accumulation at dose of ≥60 mmol/l – such concentrations can be observed in heavy drinkers' blood. Because ALP is an important enzyme in the mineralization process and OC, a non-collagenous calcium binding protein, is specific for osteoblast phenotype, we also examined whether ethanol increases ALP activity and synthesis of OC in VSMC. Ethanol provoked a significant increase in the expression of ALP and OC. Moreover, in cells challenged with ethanol the expression of CBF-α1, a transcription factor involved in the regulation of osteoblastic transformation of VSMC, was also elevated. It has been established that osteoblastic differentiation induced by hyperphosphatemia is mediated via 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 is extensive in vitro evidence that apoptosis of VSMC 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. In fact, alcohol-induced apoptosis of vascular smooth muscle cells was recently demonstrated to occur although we did not observe significant decline in viability of VSMCs in our experiments. Transformation of vascular smooth muscle cells was elegantly demonstrated to be reversible. 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 VSMC was a possible contributing factor to the VSMC 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 VSMC 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.

We summarized the novel findings in Figure 1.

Figure 1

Scheme of H_2S biogenesis, ferritin, ethanol and their involvement in Pi-induced osteoblastic transformation of VSMC. H_2S is generated as an alternative product of the transsulfuration pathway. Intracellular ferritin is regulated by iron and heme. H_2S and ferritin inhibit various steps of osteoblast transition of VSMC. On the contrary, ethanol promotes such a phenotype change. Pi-induced phosphate uptake, Pit-1 upregulation, Cbfa1, ALP, osetocalcin (OC) expression, and Ca deposition are all inhibited by H2S. Blue arrows represent responses to elevated Pi, whereas red arrows represent the directions of effect. ALP, alkaline phosphatase; Cbfa1, core-binding factor alpha-1; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; H2S, hydrogen sulfide; VSMC, vascular smooth muscle cell.