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

Matrix mineralization and resorption in the vessel wall: effects of ferryl hemoglobin and hydrogen sulfide on the pathomechanism of vascular calcification

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The Examination takes place at 10:00 a.m, on 5th February, 2021, in online format.

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The online PhD Defense will be held at 11:30 a.m., on 5th of February, 2021.

Participation requires registration. For registration and for further information please email to karanyi.zsolt@med.unideb.hu. Deadline for registration is 12 p.m. 4th February, 2021. After deadline, for technical reasons, it is no longer possible to join in to the defense.

1. Introduction

1.1. Vascular calcification

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 morbidity and complications such as atherosclerotic plaque burden, myocardial infarction, coronary artery disease, and increased ischemic episodes in peripheral vascular disease. Coronary calcification may be predictive of increased propensity for sudden cardiac death. 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.

Vascular calcification follows two distinct patterns: (*1*) intimal calcification that occurs with atherosclerotic plaques and (*2*) medial calcification, which is characterized by diffuse calcification of the media, particularly at the level of the internal elastic lamina and is not always accompanied with atherosclerosis. The latter form of vascular calcification is commonly seen in hemodialysis patients. 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 (Chronic Kidney Disease) and correlates with extremely high fatal rates.

Elevated Pi levels are commonly seen in patients with end stage kidney disease where the Pi homeostasis is deranged due to inability of kidneys to excrete phosphate.

Evidence shows that vascular calcification results in increased cardiovascular mortality in hemodialysis patients.

1.2. Calcification and osteoblastic differentiation of vascular smooth muscle cells

Although the precise mechanisms of vascular calcification are not completely understood, abnormalities in mineral metabolism are considered important risk factors. High extracellular phosphate (Pi) has been widely established to induce vascular calcification. Previous studies indicated that elevated phosphate could induce VSMC (vascular smooth muscle cell) calcification as well as an osteochondrogenic phenotypic change. Pi uptake through a sodium-dependent phosphate co-transporter, Pit-1, is essential for VSMC calcification and phenotypic modulation in response to elevated Pi. Vascular calcification is a delicate and well regulated cellular process where VSMC gain an osteoblastic phenotype.

This is indicated by the increase in expression of Runt-related transcription factor 2 (Runx2) which is an osteoblast-specific transcription factor required for osteoblast differentiation, bone matrix gene expression, and consequently, bone mineralization. Upregulation of alkaline phosphatase (ALP) (an important enzyme in early osteogenesis) and osteocalcin (OC) (a major noncollagenous protein in bone matrix that regulates mineralization) was also shown to occur.

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 act as both a nidus for calcification, and actively concentrate both calcium and phosphate to generate hydroxyapatite.

1.3. Formation of osteoclasts and its role in atherosclerosis

1.3.1. Differentiation of osteoclasts

Osteoclasts (OCs) are multinucleated cells derived from monocyte/macrophage lineage specialized for bone resorption. Differentiation of macrophages to OCs requires macrophage/monocyte colony stimulating factor-1 (M-CSF) and the receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL interacts with receptor activator of nuclear factor kappa-B (RANK) activating downstream genes. Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) plays a pivotal role in OC maturation by regulating the expression of OC-specific genes, such as dendritic cell-specific transmembrane protein (DC-STAMP), tartrate-resistant acid phosphatase (TRAP), cathepsin K (CatK), and calcitonin receptor (CTR).

1.3.2. Osteoclasts in the vessel wall

The presence of osteoclast-like cells (OLCs) in the calcified area of atherosclerotic plaques is well demonstrated. The importance of functional OLCs in vascular calcification has been documented by several studies. Functional OLCs are essential for mineral resorption in the vasculature, and that lack of OLC activity promotes vascular calcification.

In atherosclerotic plaques, calcification and bone formation are common phenomena characterized by the presence of VSMCs, osteoblasts, and osteoclast-like cells (OLCs). OLCs differentiate from infiltrating macrophages and co-localize with cholesterol deposition and mineralization. The formation of OLCs in the vasculature is dependent on RANKL that is abundantly secreted by a number of cell types, such as VSMCs and

endothelial cells. Within the intramural compartment of the arteries, OLCs might degrade mineral deposits, thereby attenuating calcification and counterbalancing the activity of VSMCs-derived osteoblasts. The imbalance between bone formation by VSMC-derived osteoblasts and bone resorption by OLCs triggers pathological calcification process in the vessel walls.

1.3.3. Inhibition of osteoclast-differentiation

RANK and RANKL interaction is essential for OC formation, and RANK expression is vital for the differentiation of myeloid-derived OCs as evidenced by the lack of OC differentiation in $RANK^{(-/-)}$ mice. Osteoprotegerin (OPG) is a decoy receptor for RANKL and plays a regulatory role in bone resorption by inhibiting OC function. As a dimer, OPG competes with RANK for RANKL binding and effectively inhibits RANK-RANKL interaction. The pivotal function of NFATc1 is highlighted by significant impairment of osteoclastogenesis in OC-specific conditional NFATc1-deficient mice. In addition, NFATc1-deficient embryonic stem cells are unable to differentiate into OCs in response to RANKL. Previously, it was demonstrated by Zwerina *et al*. that heme inhibits osteoclastogenesis as well as the expression of OC marker genes such as TRAP, CTR and DC-STAMP via the induction of HO-1, a key enzyme of heme catabolism.

1.4. The presence of ferrylhemoglobin in the atherosclerotic plaques

Intraplaque hemorrhage frequently occurs in atherosclerotic plaques due to its disruption. Complicated plaques with hemorrhage are characterized by a highly oxidative scenario creating a “death zone” for red blood cells (RBCs). RBCs in these death zones are lysed, and free Hb is subjected to rapid oxidation forming met-hemoglobin (MetHb, Fe^{3+}) and ferryl hemoglobin (FHb, $Fe^{4+}=O^{2-}$). Importantly, oxidation of Hb also leads to the release of heme moieties. A significant body of evidence suggests that MetHb and FHb are present in hemorrhagic complicated plaques. Our research group previously reported that FHb is a potent pro-inflammatory agonist in endothelial cells that induces morphological changes, increases monolayer permeability, and enhances monocyte adhesion. These data suggest that oxidized Hb forms are involved in pathogenesis of atherosclerosis.

1.5. Formation of hydrogen sulfide and its biological functions in the vasculature

Hydrogen sulfide (H_2S) has traditionally been considered as a toxic gas; however, recently it has been recognized as the third endogenous gaseous transmitter besides carbon monoxide

and nitric oxide. In mammals H_2S is produced by three enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate-sulfurtransferase via the transsulfuration pathway using homocysteine, cystathionine and L-cysteine as substrates.

In the vasculature, H_2S is generated mainly by VSMCs by CSE. H_2S exerts a number of physiological actions in the cardiovascular system: (1) it dilates blood vessels mostly, if not exclusively, by a mechanism that involves opening of ATP-sensitive K^+ channels of smooth muscle cells, (2) it is cardioprotective against ischemic reperfusion damage and myocardial inflammation and (3) it preserves both mitochondrial structure and function after injury. Accumulating evidence suggests that it has direct inhibitory effects on the development of atherosclerosis. H_2S induces apoptosis, suppresses endothelin-induced proliferation of VSMC, and influences vascular inflammatory reactions. It has also been demonstrated that H_2S inhibits the oxidation of low-density lipoprotein and lipids from atheromatous plaques. In the context of CKD plasma level of H_2S was reported to be decreased in stage 5 CKD patients.

Further proof of the beneficial effect of H_2S in cardiovascular diseases is that calciphylaxis can be successfully treated with intravenous administration of sodium thiosulfate, a drug that – as discovered recently – increases H_2S biogenesis by inducing CSE expression. Additionally, H_2S was recently reported to ameliorate vascular calcification induced by vitamin D_3 plus nicotine in rats.

2. Aims of the study

Vascular calcification is defined by two processes: extracellular matrix minerilzation by osteoblast-like cells differentiated from VSMC and matrix resorption by OLCs differentiated from macrophages. Our aim was to determine factors that affect these processes.

1. The massive Hb content of hemorrhagic atheromas prompted us to examine whether the compensatory effect of OLCs in vascular calcification is influenced by products of Hb oxidation:

- Investigate the effects of different Hb forms on the differentiation and bone-resorption activity of OLCs
- Examine the potential role of HO-1 was investigated in HO-1-silenced cells and in HO-1 knockout mice
- Explore the effect of oxidized Hb on the direct RANK-RANKL interaction, which is essential for OC formation and signal pathways of osteoclastogenesis that may be affected by oxidized Hb
- Determine the presence of FHb in hemorrhaged atherosclerotic lesions whether alters the OC-differentiation

2. Our aim was to investigate the role that H_2S may play in the process of vascular calcification:

- Investigate the effect of exogen H_2S in HAoSMC mineralization and transition of smooth muscle cells into osteoblast like cells
- Examine the decline in the endogen H_2S levels by inhibition of H_2S-generating enzyme activity whether alters the HAoSMC osteoblastic differentiation
- Determine the enzyme by which low plasma level of H_2S occurs in stage 5 CKD patients

3. Materials and methods

3.1. Study approval

Carotid arteries were obtained from patients underwent carotid endarterectomies from the Department of Surgery at the University of Debrecen. The collection was approved by the Scientific and Research Ethics Committee of the Scientific Council of Health of the Hungarian Government under the registration number of DE OEC RKEB/IKEB 3712-2012. Blood samples were collected from healthy volunteers and from stage 5 CKD patients which was approved by the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center (Nr. DEOEC RKEB/IKEB 3287A-2010). Written informed consents were received from each participant. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

3.2. Patients

Controls were healthy subjects recruited among hospital staff, without any known diseases including hypertension, dyslipidemia, liver and kidney malfunctions (n=23, mean age 53 years, F/M 9/14). Stage 5 CKD patients treated with HD were selected (n=21, mean age 61 years, F/M 13/8) from our dialysis unit.

3.3. Cell culture and reagents

Human Aorta Smooth Muscle cells (HAoSMC) were obtained from Cambrex Bioscience (Wokingham, UK) and Murine macrophage RAW264.7 cells were obtained from ATCC (Manassas, VA, US). FBS from Invitrogen/GIBCO (Carlsbad, CA USA). Unless otherwise mentioned all other reagents were obtained from Sigma (St. Luis, MO USA). Cell cultures were maintained in DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Gibco, Thermo Scientific, Waltham, MA, US).

3.4. In vitro osteoclastogenesis

RAW264.7 cells were seeded onto 24 well plates and cultured in growth medium supplemented with RANKL(Miltenyi Biotec, Bergisch Gladbach, Germany) (50 ng/mL) (osteoclastogenic medium). Medium was changed every 2-3 days.

3.5. Isolation of bone marrow monocytes

Bone marrow derived monocytes (BMDMs) were isolated from wildtype and heme oxygenase-1 (HO-1) deficient mice (-/-) according to Madaan et al. BMDMs were treated

for 5 days with RANKL (100 ng/ml) and MCS-F (50 ng/ml) in the presence and absence of FHb (10 μmol/L).

3.6. Tartrate-resistant acid phosphatase (TRAP) staining

For TRAP staining, RAW264.7 cells were cultured in osteoclastogenic medium for 5 days. Osteoclastic differentiation was evaluated by TRAP staining using a leukocyte acid phosphatase kit according to the manufacturer's instructions. TRAP+ multinucleated (more than 3 nuclei/cell) cells were identified as OCs. The areas of OCs were measured by Image J software.

3.7. Bone resorption assay

Bone resorptive activity of formed OCs was measured by bone resorption assay using Corning OsteoAssay Surface plate according to the manufacturer's instructions. The areas of resorption pit were determined with Image J software.

3.8. HO activity assay

Microsome fractions of RAW264.7 cells grown on 6-well plates were used to measure HO activity as described previously by Balla et al. HO activity is expressed as pmol bilirubin formed/mg cell protein per 60 min.

3.9. Immunohistochemistry

Carotid artery specimens were fixed with PBS buffered formaldehyde (4%) solution at pH 7.4 for 1 to 3 days based on the size of the sample. After fixation, calcified samples were decalcified with 1.0 mol/L EDTA/Tris buffer. Paraffin-embedded 5-μm-sections were deparaffanized in xylenes, rehydrated in a series of ethanol rinses, then washed with distilled water. For immunohistochemistry, samples were incubated with Dako EnVision FLEX Peroxidase-Blocking Reagent (Dako, Glostrup, Denmark) for 5 min. Serial sections slides were incubated with antibodies against TRAcP (Roche, Mannheim, Germany, ready-to-use) or cathepsin K (Abcam, Cambridge, UK) or CD68 (Roche) or anti-FHb polyclonal antibody using ultraview universal DAB detection kit. The intensity and distribution of the immunostaining was assessed by light microscopy (Leica DM2500 microscope, DFC 420 camera).

3.10. Hemoglobin preparation

Hb was isolated from fresh blood drawn from healthy volunteers using ion-exchange chromatography on a DEAE Sepharose CL-6B column. To generate MetHb, of purified Hb was incubated with a 1.5-fold molar excess of $K_3Fe(CN)_6$ over heme for 30 min at 25°C. FHb was obtained by incubation of Hb for 1 h at 37°C with a 10:1 ratio of H_2O_2 to heme. Hb concentrations were calculated as described by Winterbourn.

3.11. Detection of crosslinked Hb by Western Blot

Detection of crosslinked Hb in healthy carotid arteries, atheromas and complicated carotid lesions with hemorrhage by Western blot was performed using HRP-conjugated goat anti-human Hb polyclonal antibody (ab19362-1 Abcam, Cambridge, UK) at a dilution of 1:15000.

3.12. Spectral scan of human carotid arteries

Healthy carotid arteries, calcified atheromas and calcified atheromas with hemorrhage were ground in liquid nitrogen, homogenized in phosphate buffered saline (PBS, pH 7.4) followed by sonication on ice 3 times for 5 sec. Samples were then spinned at 12000×*g* for 20 minutes at 4ºC, and the upper phase was measured by UV-visible spectra recorder (Beckman DU-800 spectrophotometer) from 500 nm to 700 nm wavelenghts.

3.13. Cell proliferation assay

RAW264.7 cells were cultured in 24-well plates in growth medium and osteoclastogenic medium alone or supplemented with FHb for 1-4 days. The amount of viable cells was assessed by MTT assay.

3.14. Immunofluorescence staining

After 24 hours of culturing, the fixed and blocked samples were incubated with primary antibody against NFATc1 (Novus Biologicals, Littleton, CO, US). The secondary antibody was a goat anti-mouse IgG conjugated to Alexa Fluor® 488 (Thermo Scientific). Nuclei were visualized with Hoechst. Nuclear translocation was investigated with TCS SP8 STED microscope using Leica Application Software X (Leica, Mannheim, Germany).

3.15. Cytoplasmic and nuclear protein extraction

Cells were lysed with Harvest buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 M Sucrose, 0.5% Triton X-100, and protease inhibitors). After 10 min of incubation on ice, samples

were spinned at 1000×*g* for 5 min and supernatants were collected as cytosolic fraction. Pellets containing the nuclear fraction were solubilized in nuclear protein extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% SDS, and protease inhibitors).

3.16. Expression of recombinant mouse RANKL with 6× His tag

Recombinant mouse RANKL with N-terminal 6×His tag was expressed in E. coli Rosetta 2. Total mRNA was isolated from the lung tissue of C57BL mice, reverse transcribed, cloned into pTriex-4 Neo and verified by sequencing. Protein expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were then lysed with cold lysis buffer pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, 1% TritonX-100, protease inhibitors and 1 mg/ml lysozyme). His-tagged RANKL was purified using Protino Ni-TED 150 packed columns according to the manufacturer's guide. Endotoxin contamination was removed using Pierce™ High Capacity Endotoxin Removal Resin (Thermo). The quality of recombinant RANKL was analyzed by Coomassie staining and immunoblot.

3.17. In vitro RANK-RANKL interaction assay

RAW264.7 cells were lysed with cold lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 2× protease inhibitor cocktail and 1% Triton X-100. Supernatants were then gently rocked at 4°C with 15 μg of RANK antibody overnight, then antigen-antibody complexes were co-incubated with pre-washed protein A/G magnetic beads (Thermo Scientific). Beads were then washed three times with cold wash buffer containing detergent (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Igepal CA630, and protease inhibitors), then three times with wash buffer without detergent (50 mM Tris pH 7.5, 150 mM NaCl) and co-incubated with 1 μg RANKL or 1 μg RANKL and 10 μM FHb at room temperature for 60 min. Beads were then washed three times with 50 mM Tris pH 7.5, 150 mM NaCl and samples were eluated with 2×SDS sample buffer without reducing agent at 50°C for 10 min, supplemented with 100 mM DTT and subjected to immunoblot analysis.

3.18. Induction of calcification

At confluence, HAoSMC were switched to calcification medium, which was prepared by adding 3 mmol/L of inorganic phosphate (Na_2HPO_4/NaH_2PO_4 pH 7.4) to the GM. Both GM and calcification medium were changed every 2 days.

3.19. Quantification of calcium deposition

3.19.1. Colorimetric method for the determination of calcium deposition

Cells grown on 48-well plates were washed twice with PBS and decalcified with 0.6 N HCl. Calcium content of the supernatants was determined by the QuantiChrome Calcium Assay Kit (Gentaur, Hayward, CA USA). After decalcification, cells were solubilized with a solution of NaOH 0.1 mol/L and SDS 0.1% and protein content of samples were measured with 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.

3.19.2. Alizarin Red staining

Cells grown on 48-well plates were washed twice with PBS and were fixed in 4% paraformaldehyde (PFA) solution. Cells were rinsed with PBS and were incubated with 2% Alizarin Red S solution for 5 min. Cells were washed three times with deionized water to remove the excess dye.

3.20. Alkaline phosphatase activity assay

HAoSMC grown on 6-well plates were washed with PBS twice, solubilized with 1% Triton X-100 in 0.9% NaCl. 130 μl of Alkaline Phosphatase Yellow Liquid Substrate (Sigma) was combined with 50 μg of protein samples and then the kinetics of p-nitrophenol formation was followed for 30 min at 405 nm at 37 C˚. Maximum slope of the kinetic curves was used for calculation.

3.21. Intracellular phosphate measurement

Pi content of cell lysate was determined by the QuantiChrome Phosphate Assay Kit (Gentaur). Cells were washed twice with PBS and solubilized with 1% Triton and the cell lysates were assayed for Pi. Phosphate content of the cells was normalized to protein content and expressed as μmol/L/mg cell protein.

3.22. Quantification of ostecalcin

To determine osteocalcin, the extracellular matrix of the cultured cells was dissolved in 0.5 mol/L EDTA (pH 6.9). Osteocalcin content of the samples were quantified by Osteocalcin Assay Kit (Bender MedSystems, Vienna, Austria) and were normalized to the protein content of the cell cultures.

3.23. Measurement of plasma H_2S concentration

Immediately after drawing, blood was centrifuged (3 min, 3000 x g) and 40 µl of plasma was rapidly added to the assay mixture that contained 50 µl of 1% zinc acetate, 40 µl of $FeCl_3$ (30 mmol/L in 1.2 mol/L HCl) and 50 µl of N,N-dimethyl-p-phenylenediamine dihydrochloride (20 mmol/L in 7.2 mol/L HCl). To deprotonize samples, 70 µl of 10% trichloracetic acid was added, and then the mixture was centrifuged at 3000 x g for 30 min at room temperature. The absorbance of the supernatant was read at 670 nm, and concentration was calculated using a calibration curve.

3.24. Separation of peripheral blood mononuclear cells (PBMC)

PBMC were separated from blood by density gradient centrifugation using Histopaque 1077. Cells were disrupted by ultrasonication in 100 mmol/L phosphate buffer pH 7.4 for immediate determination of CSE activity or in 100 mmol/L phosphate buffer containing 1% TritonX-100 and a mixture of protease inhibitors for measurment of CSE protein expression or in TriReagent for measurement of CSE mRNA levels. Cell lysate was kept at -70°C until use.

3.25. Cystathionine γ-lyase activity measurement

CSE activity was measured according to Stipanuk method. CSE activity from PBMC was assessed by cystathionine consumption and cysteine production. Lysed PBMC was incubated with cystathionine (2 mmol/L), pyridoxal 5'-phosphate (0.25 mmol/L) in Tris-HCl buffer (100 mmol/l, pH 8.3) for 60 minutes at 37 °C. Then acid ninhydrine reagent was added, samples were boiled for 5 minutes. After cooling the samples down, optical densities at 455 and 560 nm were determined and used for calculation of cystathionine and cysteine concentrations respectively.

3.26. Quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using TriReagent (Zymo Research, Irvine, CA, US), reverse transcribed with High Capacity cDNA kit (Applied Biosystems, Foster City, CA). Peripheral blood mononuclear cell mRNA derived from CKD patients and controls was isolated from whole blood with QIAamp® RNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real time polymerase chain reactions were performed using fluorescent TaqMan probes (Thermo Scientific, Waltham, MA, US). To measure the mRNA

levels, the 20 µl reaction mixture included 10 µl of reverse transcribed sample (4 ng/µl) and 10 µl Master Mix (Thermo Scientific) containing 1 µl TaqMan Assay (20 ×). Determination of Runx2, Pit-1 and CSE mRNA levels were performed using iQ SYBR Green Supermix (Bio-Rad). PCRs were carried out using the iCycler iQ Real Time PCR System (Bio-Rad Laboratories, Hercules, CA).

3.27. Western blot

Nuclear translocation of NFATc1 and NFκB were investigated using nuclear and cytosolic fractions, in case of the other proteins whole cell lysate was electrophoresed in 12% SDS-PAGE. To determine the protein expression level of osteocalcin extracellular matrix was dissolved in EDTA (0.5 mol/L, pH 6.9) and was electrophoresed in 16.5% Tris-Tricin/Peptide-PAGE (Bio-Rad Laboratories, Hercules, CA USA). Samples were blotted onto nitrocellulose membrane and were incubated with 5% non-fat-dry milk or 5% BSA (bovine serum albumin) for 1 hour, then were incubated with primary antibody overnight at 4ºC. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK). After detection, membranes were stripped and reprobed for GAPDH (Novus Biologicals) or HSP90 (Cell Signalling) or Lamin B1 (Proteintech). Quantification of chemiluminesce was done by using Image J software.

3.28. Short-interfering RNA (siRNA) transfection

Small interfering RNA (siRNA) specific to HO-1, CSE and negative control siRNA were obtained from Ambion (Austin, TX, US). Transfection of siRNA into cells was performed using the Oligofectamine Reagent (Invitrogen, Carlsbad, CA, US).

3.29. Statistical analysis

Statistical analysis was performed with GraphPad Prism 5 by one-way anova test followed by post hoc Bonferroni's Multiple Comparison test or *t*-test. A significant value of $p < 0.05$ was marked with *, $p < 0.01$ with ** and $p < 0.001$ was marked with ***. Non-significant (ns) differences were also marked. Data are shown as mean ± SEM.

4. Results

4.1. FHb inhibits osteoclastogenesis and OC bone resorption activity

Macrophages can transform into OCs in response to RANKL. To investigate the effect of Hbs on RANKL-induced osteoclastogenesis, murine macrophage RAW264.7 cells were cultured in osteoclastogenic medium containing 50 ng/mL RANKL in the presence or absence of Hb, MetHb or FHb. First, we analyzed the effect of the different Hb forms on RANKL-induced osteoclastogenesis using TRAP staining and bone resorption assay. Heme, a potent inhibitor of RANKL-induced osteoclastogenesis, was used as positive control for inhibition assays. RANKL-induced OC formation and bone-resorptive activity were significantly inhibited either by heme or FHb, but not by Hb or MetHb. Next, we examined whether a dose-response relationship exists between FHb concentration and inhibition of OC differentiation. We showed that FHb inhibited osteoclastogenesis in a dose-dependent manner, and as low as 2.5 μmol/L FHb significantly prevented OC formation as evidenced by TRAP staining and bone resorption assay.

To explore whether this inhibitory effect of FHb is associated with its potential cytotoxic effect, we cultured RAW264.7 cells for 4 days in the presence or absence of RANKL and FHb. Cell proliferation and viability were analyzed with MTT assay at various time points (one to four days). Our results showed the proliferation of RAW264.7 cells decreased in response to RANKL as well as to RANKL+FHb compared to untreated cells, however, FHb did not affect proliferation compared to RANKL-treated cells. Next, we explored whether such decreased proliferation was associated with apoptotic cell death. Accordingly, we analyzed caspase-3 cleavage as a marker of apoptosis by immunoblot which showed that apoptotic cell death did not occur during OC differentiation in response to RANKL and FHb.

4.2. FHb downregulates OC-specific gene expression in response to RANKL

Osteoclastic differentiation of macrophages requires changes in protein expression, therefore, we examined the effect of various Hb forms on the expression of osteoclast-specific markers (CTR, DC-STAMP, and NFATc1) using quantitative real-time PCR (qRT-PCR) and immunoblotting. As expected, RANKL significantly up-regulated CTR and DC-STAMP expression in RAW264.7 cells that was inhibited both by FHb and heme and, to a

lesser extent, by MetHb. Importantly, Hb did not prevent RANKL-induced CTR and DC-STAMP induction. Significantly, FHb and heme but not Hb and MetHb blunted NFATc1 expression induced by RANKL.

4.3. Inhibitory effect of FHb on osteoclastogenesis is independent of HO-1

We have previously described that FHb induces HO-1 in endothelial cells and it has been reported that up-regulation of HO-1 by heme inhibits OC formation and bone resorption *in vitro*. Therefore, we tested whether HO-1 mediates the inhibitory effect of FHb on OC formation. FHb, similar to heme, significantly induced HO-1 expression both at mRNA and protein levels and increased HO enzyme activity in RAW264.7 cells.

We knocked down HO-1 expression by HO-1-specific siRNA and analyzed osteoclastogenesis in response to RANKL and FHb. FHb prevented RANKL-induced OC formation in HO-1-silenced cells similar to the controls that was demonstrated by TRAP staining, bone resorption assay and CTR mRNA expressions.

We also assessed the ability of FHb to mitigate osteoclastogenesis in BMDMs obtained from wild type ($HO\text{-}1^{+/+}$) and HO-1 knock out ($HO\text{-}1^{-/-}$) mice (n=4). FHb inhibited RANKL-induced CTR expression in both $HO\text{-}1^{+/+}$ and in $HO\text{-}1^{-/-}$ BMDMs. These results support that inhibitory effect of FHb on osteoclastogenesis is independent of HO-1 expression.

4.4. FHb blocks RANK-RANKL interaction

Formation of OCs requires RANKL attachment to its receptor RANK. In addition, RANKL directly induces RANK expression. To decipher the molecular mechanism by which FHb inhibits OC formation, we tested whether FHb influences RANKL-induced RANK expression. We demonstrated that FHb significantly attenuated RANK expression in response to RANKL both at mRNA and protein level. To gain a more mechanistic insight into the inhibitory effect of FHb on OC formation, we analyzed whether FHb inhibits the RANK-RANKL interaction by measuring the amount of cell-associated RANKL by immunoblot. We showed that the association of exogenous RANKL to cells was markedly decreased when FHb was present in the experimental medium. To further verify this observation, we developed a recombinant His-tagged RANKL to study RANK-RANKL interaction in test tube experiments. In the test tube experiments, RANK was immunoprecipitated from RAW264.7 cell lysates and co-incubated with His-tagged recombinant RANKL (1 μg) in the presence or absence of FHb. The association of

recombinant RANKL with RANK was analyzed by immunoblot. Test tube experiments corroborated our findings in cell cultures that FHb inhibited RANK-RANKL interaction. These data suggest that the inhibitory effect of FHb on RANK expression and osteoclastic transformation of RAW264.7 cells was mediated by inhibition of the direct interaction between RANK and RANKL.

4.5. FHb inhibits RANKL-induced signaling involved in OC differentiation

RANK-RANKL interaction initiates a series of signaling events leading to OC formation from macrophages. Here, we showed that RANKL induced TRAF6 expression, p38 and JNK activation, c-Fos expression, nuclear translocation of NFκB and NFATc1. Importantly, exposure of cells to FHb prevented the induction of TRAF6 and c-Fos, phosphorylation of p38 and JNK, and nuclear translocation of NFκB and NFATc1. These results corroborate our hypothesis that FHb inhibits RANK-RANKL interaction and its subsequent signaling pathways, thereby preventing OC differentiation from macrophages.

4.6. Oxidation of Hb occurs in calcified lesions with hemorrhage

Our previous studies revealed that complicated lesions with hemorrhage contain oxidized forms of hemoglobin which is also corroborated by our current study. Spectrophotometric analysis of the human vessel samples showed that oxidized Hb was present in the calcified atheromas with hemorrhage, while healthy arteries and calcified lesions did not contain oxidized Hb. We observed marked accumulation of cross-linked Hb dimers, tetramers and multimers in hemorrhagic calcified plaques reflecting that Hb oxidation is extensive in these lesions compared to calcified atheromas without hemorrhage or healthy carotid arteries.

4.7. Lack of OLCs is associated with the presence of FHb in hemorrhagic calcified lesions in human vessels

To examine whether FHb inhibits OLC formation in patients who underwent carotid endarterectomy, the presence of OLCs was analyzed in healthy carotid arteries, calcified atheromas, and calcified atheromas with hemorrhage. Extracellular calcium deposits were present in calcified atheromas and in calcified atheromas with hemorrhage as evidenced by Von Kossa staining. Presence of FHb was prominent in calcified atheromas with hemorrhage while no positive staining pattern could be seen in carotid arteries from healthy individuals or calcified lesions without hemorrhage. Multinucleated giant cells showed the characteristics of OCs demonstrated by TRAP and CatK staining. A number of OLCs

positive to TRAP and CatK staining were identified and were evident around the calcified area in calcified atheromas suggesting the presence of OLCs in calcified vessels. In sharp contrast, the number of OLCs in calcified plaques with hemorrhage was scarce despite the extensive calcification. These data demonstrate that the presence of OLCs in calcified atheromas with hemorrhage is significantly limited when compared to calcified lesions without hemorrhage.

4.8. H_2S decreases HAoSMC mineralization in a dose responsive manner

To establish an in vitro model of human vascular calcification we cultured HAoSMC in calcification medium which was prepared by addition of 3 mmol/L Pi to the growth medium in the presence or absence of H_2S for 7 days. As expected, Pi provoked calcium deposition, which was inhibted by H_2S in a dose responsive manner, providing a significant inhibition at concentrations of $\geq$50 μmol/L. To confirm the effect of H_2S on calcium deposition we also performed alizarin red staining of HAoSMC. HAoSMC maintained in calcification medium showed development of granular calcium deposits throughout the cell culture. Supplementation of the calcification medium with H_2S prevented the accumulation of calcium in the extracellular matrix.

4.9. H_2S inhibits osteoblastic differentiation of HAoSMC

It has been shown that vascular calcification in vivo resembles bone mineralization, therefore we examined whether H_2S suppresses the phenotype transition of HAoSMC into osteoblast-like cells. Because upregulation of ALP and OCN are implicated in the pathogenesis of vascular calcification we measured the level of their expression in HAoSMC treated with H_2S. While HAoSMC maintained in calcification medium exhibited around a 10-fold increase in ALP activity compared to controls, addition of H_2S to calcification medium resulted in a dose dependent suppression. Similarly to ALP activity, the induction of osteocalcin was also abolished by H_2S.

Next we examined the level of Cbfa1, a transcription factor required for osteoblast differentiation, in our in vitro model. Pi increased Cbfa1 mRNA level compared to cells grown in control medium which was significantly suppressed by H_2S.

Evidence suggests that the effects of hyperphosphatemia are mediated via Pit-1 that facilitates entry of Pi into vascular cells. Intriguingly, addition of H_2S inhibited Pi uptake in a dose responsive manner. To explore the mechanism underlying the inhibition of Pi uptake,

we examined the expression of Pit-1. Maintaining HAoSMC in calcification medium we observed a significant elevation in Pit-1 mRNA level. Addition of H_2S into the calcification medium prevented the increase in Pit-1 expression.

4.10. Endogenous production of H_2S inhibits calcification and osteoblastic differentiation of HAoSMC

In the vasculature, H_2S is produced by VSMC expressing the pyridoxal-5'-phosphate-dependent enzyme CSE. Based on our observations, we thereby hypothesized that inhibition of CSE enzyme activity would lead to increased mineralization. Therefore first we inhibited CSE using DL-propargylglycine (PPG), a well known inhibitor of CSE activity. Cells treated with PPG showed a gradual decrease in CSE enzyme activity. Suppression of CSE by PPG almost doubled the deposition of calcium in the extracellular matrix of HAoSMC as compared to cells cultured in calcification medium without PPG. Accordingly, PPG provoked a significant additional increase in the activity of ALP and expression of OCN. We further confirmed the role of CSE-activity in the inhibition of calcification by using CSE-specific small interfering RNA (siRNA). Our results verified that decline in CSE enzyme activity by gene-silencing was accompanied by increased calcium deposition in HAoSMC.

4.11. Decreased plasma H_2S levels are associated with reduced CSE activity in end-stage chronic kidney disease patients

In agreement with previous observations of Perna et al., we found that plasma concentration of H_2S was decreased in stage 5 CKD patients and that H_2S level was further lowered by hemodialysis. Since the main enzyme responsible for H_2S biogenesis in the vasculature is CSE, we compared its expression and activity in mononuclear cells derived from stage 5 CKD patients treated with hemodialysis and healthy controls. Levels of CSE mRNA and protein expression were similar in CKD patients and controls. Importantly, CSE enzyme activity – that was measured by cystathionine consumption and cysteine production – was markedly decreased in mononuclear cells derived from stage 5 CKD patients treated with HD compared to healthy individuals.

5. Summary

The development of vascular calcification is the result of two opposite-sensed processes: the increased mineralization of the osteoblast-like cells and the decreased bone-resorption activity of the OCLs. In our study we investigated the possible factors that may affect these processes.

Intraplaque hemorrhage frequently occurs in atherosclerotic plaques resulting in cell-free hemoglobin, which is oxidized to ferryl hemoglobin (FHb) in the highly oxidative environment. We investigated whether oxidized hemoglobin alters osteoclast formation thereby affecting calcium removal from mineralized atherosclerotic lesions. FHb decreased bone resorption activity and inhibited osteoclast-specific gene expression induced by RANKL. In addition, FHb inhibited osteoclastogenic signaling pathways downstream of RANK (receptor activator of nuclear factor kappa-B). These effects were independent of heme oxygenase-1 demonstrated by knocking down HO-1 gene in RAW264.7 cells and in mice. Importantly, FHb competed with RANK for RANKL binding suggesting possible mechanisms by which FHb impairs osteoclastic differentiation. In diseased human carotid arteries, OLCs were abundantly present in calcified plaques and co-localized with regions of calcium deposition, while the number of these cells were lower in hemorrhagic lesions exhibiting accumulation of FHb despite calcium deposition. This effect of FHb suggests that the presence of FHb in hemorrhagic atheromas might create a unique microenvironment where OLC-mediated resorption of calcium deposits is impaired that blocks the endogenous calcium resorption capability in the vasculature.

In our study we investigated the role that H_2S may play in Pi-induced osteoblastic transformation and mineralization of VSMC. We provide evidence that H_2S regardless of its exogenous or endogenous origin inhibits the up-regulation of osteoblast specific genes such as ALP, OCN and Runx2. The inhibition of Pi uptake through Pit-1, is essential for providing beneficial effects against calcification and phenotypic modulation of HAoSMC 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 new strategies to prevent vascular calcification.

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List of publications related to the dissertation

1. **Zavaczki, E.**, Gáll, T., Zarjou, A., Hendrik, Z., Potor, L., Tóth, C., Méhes, G., Gyetvai, Á., Agarwal, A., Balla, G., Balla, J.: Ferryl Hemoglobin Inhibits Osteoclastic Differentiation of Macrophages in Hemorrhaged Atherosclerotic Plaques.
Oxidative Medicine and Cellular Longevity. 2020, 1-17, 2020.
DOI: http://dx.doi.org/10.1155/2020/3721383
IF: 5.076 (2019)

2. **Zavaczki, E.**, Jeney, V., Agarwal, A., Zarjou, A., Oros, M., Katkó, M., Varga, Z., Balla, G., Balla, J.: Hydrogen sulfide inhibits the calcification and osteoblastic differentation of vascular smooth muscle cells.
Kidney Int. 80 (7), 731-739, 2011.
DOI: http://dx.doi.org/10.1038/ki.2011.212
IF: 6.606

List of other publications

3. Nagy, A., Pethő, D., Gáll, T., **Zavaczki, E.**, Nyitrai, M., Posta, J., Zarjou, A., Agarwal, A., Balla, G., Balla, J.: Zinc Inhibits HIF-Prolyl Hydroxylase Inhibitor-Aggravated VSMC Calcification Induced by High Phosphate.
Front. Physiol. 10, 1-15, 2020.
DOI: http://dx.doi.org/10.3389/fphys.2019.01584
IF: 3.367 (2019)

4. 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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5. Katkó, M., **Zavaczki, E.**, Jeney, V., Paragh, G., Balla, J., Varga, Z.: Homocysteine metabolism in peripheral blood mononuclear cells: evidence for cystathionine beta-synthase activity in resting state.
   Amino Acids. 43 (1), 317-326, 2012.
   DOI: http://dx.doi.org/10.1007/s00726-011-1080-2
   IF: 3.914

6. Zarjou, A., Jeney, V., Arosio, P., Poli, M., **Zavaczki, E.**, Balla, G., Balla, J.: Ferritin ferroxidase activity: a potent inhibitor of osteogenesis.
   J. Bone Miner. Res. 25 (1), 164-172, 2010.
   DOI: http://dx.doi.org/10.1359/jbmr.091002
   IF: 7.059

7. Molnár, Z., Emri, T., **Zavaczki, E.**, Pusztahelyi, T., Pócsi, I.: Effects of mutations in the GanB/RgsA G protein mediated signalling on the autolysis ofAspergillus nidulans.
   J. Basic Microbiol. 46 (6), 495-503, 2006.
   DOI: http://dx.doi.org/10.1002/jobm.200610174
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