Title: Oxidation of hemoglobin in the development of vascular diseases

Incidence and prevalence of vascular disorders such as atherosclerosis, arteriosclerosis, calcification of heart valves have paramount relevance in disease states including chronic kidney disease, diabetes, hypertension, and altered lipid metabolism.

I. Oxidation of hemoglobin and remodeling of vasculature

Vulnerable atherosclerotic early lesions, especially hemorrhaged lesions, contribute to a considerable extent to deaths (31%), according to the WHO. The complicated lesion is characterized by ruptures on the plaque surface and/or hemorrhage into the plaque. Intraplaque hemorrhage is also developed by the rupture of the neovascularized vessel, which is derived from the vasa vasorum. Although the pathology has been reported to occur in the affected arteries, its contribution to the pathophysiology and progression towards complicated atherosclerotic plaques has not been studied, including calcification of intima, media, and heart aortic valves, provoking inflammation, narrowing lumen of arteries, and hardening the vessel wall.

Our aim was to explore the contribution of red blood cell infiltration into vascular wall in the development of atherosclerosis, arteriosclerosis, calcification of heart valves. Furthermore, we revealed the cellular and molecular mechanisms by which hemoglobin triggers such pathologies.

In the research period our group revealed a remarkable lysis of red blood cells followed by a severe oxidation of hemoglobin to ferrylhemoglobin after the development of intraplaque hemorrhage due to minor ruptures within atherosclerotic early lesions, an oxidative changes of the globin moieties characterized by oxidative hotspots in hemoglobin (β1Cys93; β1Cys112; β2Cys112, α1Cys104). After producing a monoclonal anti-ferrylhemoglobin antibody, the oxidized hemoglobin was revealed to be localized extracellularly and also internalized by macrophages in the human hemorrhagic complicated lesions. We demonstrated that ferrylhemoglobin is taken up via phagocytosis as well as CD163 receptor-mediated endocytosis and then transported to lysosomes involving actin polymerization. Internalization of ferrylhemoglobin was accompanied by upregulation of heme oxygenase-1 and H-ferritin and accumulation of iron within lysosomes as result of heme/iron uptake. Importantly, macrophages exposed to ferrylhemoglobin in atherosclerotic plaques exhibited a proinflammatory phenotype, as reflected by elevated levels of tumor necrosis factor-α, and interleukin-1β (IL-1β and TNF-α). We performed RNA-seq analysis on biopsies from patients who underwent endarterectomies to find further signatures of ferrylhemoglobin in complicated lesions. RNA-seq analysis demonstrated that human complicated lesions had a unique transcriptomic profile different from arteries and atheromatous plaques. Pathways affected in complicated lesions included gene changes associated

with phosphoinositide 3-kinase (PI3K) signaling, lipid transport, tissue remodeling, and vascularization. Targeted analysis of gene expression associated with calcification, apoptosis, and hemolytic-specific clusters indicated an increase in the severity of complicated lesions compared with atheroma. A 39% overlap in the differential gene expression profiles of human macrophages exposed to ferrylhemoglobin and the complicated lesion profiles was uncovered. Among these 547 genes, we found inflammatory, angiogenesis, and iron metabolism gene clusters regulated in macrophages.

We hypothesize that 2 electron oxidation of hemoglobin generates fragmentation of globin moieties, therefore LC-MS/MS analysis was performed on complicated atherosclerotic plaques. Indeed, severe accumulation of hemoglobin-derived peptides was identified during hemoglobin oxidation both in complicated atherosclerotic lesions, triggering endothelial cell dysfunction. Oxidized hemoglobin and its products were followed with spectrophotometry, LC-MS/MS analysis. Cross-linking of globin chains in complicated atherosclerotic lesions of the human artery was detected. The vascular pathophysiologic role of oxidized hemoglobin and the resultant peptides was assessed by measuring endothelial integrity, the activation of endothelial cells and the induction of proinflammatory genes. Peptide fragments of hemoglobin (VNVDEVGGEALGRLLVVYPWTQR, LLVVYPWTQR, MFLSFPTTK, VGAHAGEYGAELERMFLSFPTTK, and FLASVSTVLTSKYR) were identified in ruptured atherosclerotic lesions. Fragments resulting from the oxidation of hemoglobin were accompanied by the accumulation of ferrylhemoglobin. Haptoglobin inhibited hemoglobin fragmentation provoked by peroxide. The resultant peptides failed to bind haptoglobin or albumin. Peptides derived from hemoglobin oxidation and ferrylhemoglobin induced intercellular gap formation, decreased junctional resistance in the endothelium, and enhanced monocyte adhesion to endothelial cells. Enhanced expression of TNF and the activation of NLRP3 and CASP1 followed by the increased generation of IL-1β and nuclear translocation of the NF-κβ transcription factor occurred in response to hemoglobin-derived peptides, and ferryl hemoglobin in endothelium was upregulated in both pathologies. We concluded that the oxidation of hemoglobin in complicated atherosclerotic lesions generates peptide fragments and ferrylhemoglobin with the potential to trigger endothelial cell dysfunction. To our surprise, similarly to complicated atherosclerotic lesions of the human arteries, a high level of oxidized and cross-linked hemoglobin and peptides derived from hemoglobin fragmentation were observed in the cerebrospinal fluid after intraventricular hemorrhage of the brain.

Osteoclast-like cells derived from macrophages signify a counterbalance mechanism for calcium deposition in vascular and valvular calcification. Therefore, was investigated whether oxidized hemoglobin alters osteoclast formation, affecting calcium removal from mineralized arteries. RANKL-(receptor activator of nuclear factor kappa-B ligand-) induced osteoclastogenic differentiation and osteoclast activity of RAW264.7 cells were studied in response to oxidized hemoglobin via assessing

bone resorption activity, expression of osteoclast-specific genes, and the activation of signalization pathways. Osteoclast-like cells in diseased human arteries were assessed by immunohistochemistry. ferrylhemoglobin, but not ferrohemoglobin, decreased bone resorption activity and inhibited osteoclast-specific gene expression (tartrate-resistant acid phosphatase, calcitonin receptor, and dendritic cell-specific transmembrane protein) induced by RANKL. In addition, ferrylhemoglobin was shown to inhibite osteoclastogenic signaling pathways downstream of RANK (receptor activator of nuclear factor kappa-B). It prevented the induction of TRAF6 (tumor necrosis factor (TNF) receptor-associated factor 6) and c-Fos, phosphorylation of p-38 and JNK (c-Jun N-terminal kinase), and nuclear translocation of NFκB (nuclear factor kappa-B) and NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1). These effects were independent of heme oxygenase-1 demonstrated by knocking down HO-1 gene in RAW264.7 cells and in mice. Importantly, ferrylhemoglobin competed with RANK for RANKL binding suggesting possible mechanisms by which oxidized hemoglobin impairs osteoclastic differentiation. Importantly, osteoclast-like cells were abundantly present in calcified plaques and colocalized with regions of calcium deposition, while the number of these cells were lower in hemorrhagic lesions exhibiting accumulation of ferrylhemoglobin despite calcium deposition. Our data indicates that ferrylhemoglobin inhibits RANKL-induced osteoclastic differentiation of macrophages and suggest that accumulation of ferrylhemogloibn in a calcified area of vessels retards the formation of osteoclast-like cell generation impairing calcium resorption.

II. Novel therapeutic targets for inhibiting vascular remodeling induced by hemoglobin oxidation in the vasculature

Hydrogen sulfide gas

Since interactions between hemoglobin/heme and lipids containing lipid hydroperoxide upon intraplaque hemorrhage result in the formation of reactive lipid mediators as well as oxidized hemoglobin species, we searched candidates for inhibiting oxidation of hemoglobin and heme moieties in such environment. We proposed hydrogen sulfide to exhibit such physiologic function within the vascular walls. Therefore, we investigated the kinetics of the reaction of ferrylhemoglobin species with sulfide in a cell-free system using stopped-flow spectrophotometry. Initially, ferrylHb was prepared in situ by the reaction of methemoglobin with 5 molar equivalents of peroxides in the first mixing cycle of a sequential stopped-flow experiment. Under our experimental conditions, the formation of ferrylhemoglobin was complete in 400 s and resulted in spectral transitions (see the 350–500 and 500–650 nm ranges that were characteristic to the formation of ferrylhemoglobin. Therefore, sulfide was reacted with ferrylhemoglobin in the second mixing cycle using a delay time of 400 s. Rapid changes in the UV-vis spectra predicted a favorable reaction between sulfide and ferryhemoglobin under these

conditions. The appearance of a new peak at 620 nm together with the shift of the Soret band at 400 nm and the absorbance increases at 530 and 580 nm were indicative of the formation of sulfhemoglobin. Kinetic traces were measured at a >10-fold excess of sulfide over ferrylhemoglobin to maintain pseudo-first-order conditions and recorded initially at 406, 425, and 570 nm. Under these conditions, they exhibited a biexponential character, which corresponded to separated reactions of the alpha and beta chain ferryl heme centers (faster and slower phase, resp.) as shown before. The obtained pseudo-first-order rate constants from the double exponential fits of the kinetic traces at 406, 425, and 570 nm show similar linear correlations with the applied sulfide concentrations, indicating first-order dependencies of the rate law on both ferrylhemoglobin and sulfide concentrations. Importantly, sulfide was show by our laboratory to inhibit the formation of lipid-peroxidation products in human hemorrhaged lesions. Thus, hydrogen sulfide has the potential to exhibit such beneficial physiologic function in the vasculature.

Atherosclerosis

Since hemoglobin oxidation and its derivative-related molecules trigger oxidative stress and cell activations and various cellular responses, we further investigated whether sulfide could attenuate these harmful reactions. We observed that sulfide donor molecules modulate endothelial responses via inhibiting ferrylhemoglobin formation. They inhibit the induction of expression of adhesion molecules, ICAM, VCAM-1, and production of the proinflammatory cytokines, IL-1β and TNF-α. Endothelial integrity disturbed by hemoglobin oxidation as reflected by gap formation between endothelial cells, increased permeability, altered F-actin polymerization was rescued. Intriguingly, ferrylhemoglobin, heme, lipidperoxidation products (oxidized low-density lipoprotein and lipids in the arterial wall), and cytokines (IL-1β and TNF-α) were found to enhance the expression of enzymatic system generating hydrogen sulfide gas to control and halt oxidation of proteins and lipids. This is also supported by the fact that inhibition of endogenous hydrogen sulfide gas generation facilitated maladaptation and remodeling of the vasculatures.

Importantly, hydrogen sulfide gas was demonstrated by our laboratory to retard atherosclerosis in an animal model employing apolipoprotein E deficient mice ($ApoE^{-/-}$ mice). In accordance, we observed that administration of hydrogen sulfide donors reduced, whereas inhibition of hydrogen sulfide generating cystathionine gamma-lyase enzyme (CSE) activity by DL-propargylglycine increased atherogenic diet-induced atherosclerotic plaque formation in ApoE−/− mice. Since heme oxygenase-1 (HO-1) was shown to be induced by lipid hydroperoxide as previously revealed in our laboratory, we also tested whether sulfide alters HO-1 expression in an atherosclerotic mouse model. HO-1 was strongly upregulated in the aorta of ApoE−/− mice fed with atherogenic diet and it was mainly

expressed by endothelial cell and macrophages. We found that HO-1 mRNA level was reduced in hydrogen sulfide-treated animals, due to the attenuated oxidative stress. Next, we examined whether sulfide releasing molecules could prevent lipid peroxidation of lipids derived from human atheromatous lesions. We provided evidence that lipid peroxidation provoked heme/hemoglobin after intraplaque hemorrhage was inhibited by sulfide-releasing molecules. On the contrary, neither polysulfide nor decomposed sulfide donors were able to block pluck development.

Calcification of vasculature – Aortic valve calcification and arteriosclerosis

In calcific aortic valve disease and arteriosclerosis, the valvular interstitial cells and vascular smooth muscle cells, as well as mesenchymal stem cells transdifferentiate into osteoblast-like cells contributing to the mineralization of tissue. Nuclear translocation of osteogenic transcription factors initiates the transition of cells towards an osteoblast phenotype in response to various pathological stimuli within the aortic valves and arteries. The phenotype switch is reflected by the induction and expression of osteoblast specific genes and proteins. Cystathionine gamma-lyase and cstathionine beta-synthase are the main cellular sources of generation hydrogen sulfide gas. We hypothesized that generation of hydrogen sulfide gas in the vasculature controls mineralization.

We observed that expression of pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) were increased in patients diagnosed with calcific aortic valve disease and in calcified aortic valve as well as aorta of ApoE-/- mice. Administration of hydrogen sulfide releasing donor (4-methoxyphenyl piperidinylphosphinodithioc acid (AP72)) exhibited inhibition on both calcification and inflammation in aortic valve and aortic wall of apolipoprotein E knockout mice is reflected by lowering IL-1β and TNF-α levels. Accordingly, AP72 prevented the accumulation of extracellular calcium deposition and decreased nuclear translocation of nuclear factor-κB (NF-κB) in human valvular interstitial cells and human vascular smooth muscle cells. This was also accompanied by reduced cytokine response. Double-silencing of endogenous hydrogen sulfude producing enzymes, Cystathionine gamma-lyase and Cystathionine beta-synthase in valvular interstitial cells and vascular smooth muscle cells exerted enhanced mineralization and higher levels of IL-1β and TNF-α. Importantly, silencing NF-κB gene or its pharmacological inhibition prevented nuclear translocation of runt-related transcription factor 2 (Runx2) and subsequently the calcification of human valvular interstitial cells and vascular smooth muscle cells. Increased levels of NF-κB and Runx2 and their nuclear accumulation occurred in ApoE-/- mice with a high-fat diet. Administration of AP72 decreased the expression of NF-κB and prevented its nuclear translocation in VIC of ApoE-/- mice on a high-fat diet, and that was accompanied by a lowered pro-inflammatory cytokine level. Similarly, activation of

Runx2 did not occur in VIC of ApoE-/- mice treated with hydrogen sulfude donor. Employing Stimulated Emission Depletion nanoscopy, a strong colocalization of NF-κB and Runx2 was detected during the progression of valvular calcification. We concluded that hydrogen sulfide inhibits inflammation and calcification of aortic valves. Furthermore, our study clearly demonstrated that the regulation of Runx2 by hydrogen sulfide generated by cystathionine gamma-lyase and cstathionine beta-synthase occurs via NF-κB establishing a link between inflammation and mineralization in vascular calcification. Therefor we proposed that endogenous hydrogen sulfide generation by cystathionine gamma-lyase/cstathionine beta-synthase system exerts anti-calcification function in heart valves.

Since endogenous production of hydrogen sulfide gas within the vasculature was found to exert a critical control on vascular and valvular mineralization our next aim was to assess the impact of exogenous hydrogen sources on aortic valve calcification. As we employed hydrogen sulfide donors, NaSH, Na2S, GYY4137, AP67, and AP72, significant dose-dependent inhibitions on mineralization and osteoblastic transdifferentiation were detected in apolipoprotein E deficient mice. Accumulation of calcium in the extracellular matrix and expression of osteocalcin and alkaline phosphatase was also inhibited. Runs2 was not translocated to the nucleus and phosphate uptake was decreased. Pyrophosphate generation was increased via up-regulating ENPP2 and ANK1, the pyrophosphate producing system in the extracellular space. Importantly, hydrogen sulfide releasing molecules were found to regulate cellular phosphate uptake through affecting the functions of phosphate channels. Lowering endogenous production of hydrogen sulfide by concomitant silencing of cystathionine γ-lyase and cystathionine β-synthase favored valvular interstitial cell calcification even in the presence of exogenous hydrogen sulfide. Analysis of human specimens revealed higher expression of cystathionine γ-lyase in aortic valves with calcification and stenosis compared to intact aortic valves. We concluded that exogenous hydrogen sulfide has the potential to provide a novel therapeutic approach to prevent hardening of valves.

The finding of high expression of cystathionine γ-lyase within the diseased aortic valves was very surprising to us, since it suggested that an increased generation of hydrogen sulfide gas occurred previously shown to prevent vascular mineralization by our laboratory. Therefore, we conducted a study to explore the metabolic control of hydrogen sulfide levels in human aortic valves. Importantly lower levels of bioavailable hydrogen sulfide were detected within the tissue of human aortic valves with calcification indicating that cystathionine γ-lyase was not functioning. Our metabolomic studies revealed increased production of lanthionine ruling out such scenario, in contrast, cystathionine γ-lyase activity was increased despite the fact, that hydrogen sulfide level dropped. Since elevated cystathionine γ-lyase expression and activity were associated with low levels of hydrogen sulfide level, we hypothesized that its increased catabolism was responsible for the depletion of gas. Indeed, the

expression of mitochondrial enzymes involved in hydrogen catabolism including sulfide quinone oxidoreductase, the key enzyme in mitochondrial hydrogen sulfide oxidation, persulfide dioxygenase, sulfite oxidase and thiosulfate sulfurtransferase were up-regulated in calcific aortic valve tissues, and a similar expression pattern was observed in response to high phosphate levels in valvular interstitial cells. Employing a mitochondria-targeting hydrogen sulfide donor AP39, rescued valvular interstitial cell from an osteoblastic phenotype switch and reduced the expression of IL-1β and TNF-α as well. Importantly, both pro-inflammatory cytokines aggravated calcification and osteoblastic differentiation of valvular interstitial cells derived from the calcific aortic valves and that was retarded by mitochondria-targeting hydrogen sulfide donor. We concluded that decreased levels of bioavailable hydrogen sulfide in human calcific aortic valves resulted from an increased hydrogen sulfude metabolism that facilitates the development of calcific aortic valve disease. cystathionine γ-lyase /hydrogen sulfude represent a pathway that reverses the action of calcifying stimuli.

Ferritin, H-ferritin, forroxidase, alpha-1-microglobulin,

Atherosclerosis

Infiltration of red blood cells into atheromatous plaques and oxidation of hemoglobin and lipoproteins are implicated in the pathogenesis of atherosclerosis as previously discussed. Alpha-1-microglobulin is a radical-scavenging and heme-binding protein. Therefore, we examined the origin and role of Alpha-1-microglobulin in human atherosclerotic lesions. Using immunohistochemistry, we observed a significant alpha-1-microglobulin immunoreactivity in atheromas and hemorrhaged plaques of carotid arteries in smooth muscle cells and macrophages. The most prominent expression was detected in macrophages of organized hemorrhage. To reveal a possible inducer of Alpha-1-microglobulin expression in ruptured lesions, we exposed aortic endothelial cells, smooth muscle cells and macrophages to heme, oxy- and ferrylhemoglobin. Both heme and ferrylhemoglobin, but not oxyhemoglobin, upregulated alpha-1-microglobulin mRNA expression in all cell types. Importantly, only ferrylhemoglobin induced alpha-1-microglobulin protein secretion in aortic endthelium, smooth muscle cells and macrophages. To assess the possible function of alpha-1-microglobulin in ruptured lesions, we analyzed Hb oxidation and heme-catalyzed lipid peroxidation in the presence of alpha-1-microglobulin. We showed that recombinant alpha-1-microglobulin markedly inhibited hemoglobin oxidation and heme-driven oxidative modification of low-density lipoproteins as well plaque lipids derived from atheromas. These results demonstrate the presence of alpha-1-microglobulin in atherosclerotic plaques and suggest its induction by heme and ferrylhemoglobin in the resident cells to control the progression of atherosclerosis.

Heme as well as iron is liberated during oxidation of hemoglobin in the progression of atherosclerosis. Therefore, we investigated whether the hydrophobic fungal iron chelator siderophore, desferricoprogen inhibits atherosclerosis. Desferricoprogen was found to reduce atherosclerotic plaque formation in ApoE-/- mice on an atherogenic diet. It lowered the plasma level of oxidized LDL and inhibited lipid peroxidation in aortic roots. The elevated collagen/elastin content and enhanced expression of adhesion molecule VCAM-1 were decreased. Desferricoprogen diminished oxidation of Low-density Lipoprotein and plaque lipids catalyzed by heme or hemoglobin. Formation of foam cells, uptake of oxLDL by macrophages, upregulation of CD36 and increased expression of TNF-α were reduced by desferricoprogen in macrophages. TNF-triggered endothelial cell activation (vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecules (ICAMs), E-selectin) and increased adhesion of monocytes to endothelium were attenuated. The increased endothelial permeability and intracellular gap formation provoked by TNF-α was also prevented by desferricoprogen. Siderophore acted as a cytoprotectant in endothelial cells and macrophages challenged with a lethal dose of oxLDL and lowered the expression of stress-responsive heme oxygenase-1 as sublethal dose was employed. Saturation of desferrisiderophore with iron led to the loss of the beneficial effects. We demonstrated that desferricoprogen accumulated within the atheromas of the aorta in ApoE-/- mice. We conclude that desferricoprogen represents a novel therapeutic approach to control the progression of atherosclerosis.

Vascular calcification

We investigated the potential role of iron metabolism in the pathogenesis of calcific aortic valve disease. Cultured valvular interstitial cells of stenotic aortic valve with calcification from patients undergoing valve replacement exhibited significant susceptibility to mineralization/osteoblastic transdifferentiation in response to phosphate. This process was abrogated by iron via induction of H-ferritin as reflected by lowering ALP and osteocalcin secretion and preventing extracellular calcium deposition. Cellular phosphate uptake and accumulation of lysosomal phosphate were decreased. Accordingly, expression of phosphate transporters Pit1 and Pit2 were repressed. Translocation of ferritin into lysosomes occurred with high phosphate-binding capacity. Importantly, ferritin reduced nuclear accumulation of RUNX2 (Runt-related transcription factor 2), and as a reciprocal effect, it enhanced nuclear localization of transcription factor Sox9 (SRY [sex-determining region Y]-box 9). Pyrophosphate generation was also increased via upregulation of ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase-2). 3H-1, 2-dithiole-3-thione mimicked these beneficial effects in valvular interstitial cell via induction of H-ferritin. Ferroxidase activity of H-ferritin was essential for this function, as ceruloplasmin exhibited similar inhibitory functions. Histological analysis of stenotic aortic valve revealed high expression of H-ferritin without iron accumulation and its relative dominance over

ALP in noncalcified regions. Increased expression of H-ferritin accompanied by elevation of TNF-α (tumor necrosis factor-α) and IL-1β (interleukin-1β) levels, inducers of H-ferritin, corroborates the essential role of ferritin/ferroxidase via attenuating inflammation in calcific aortic valve disease. Our results indicated that H-ferritin is a stratagem in mitigating valvular mineralization/osteoblastic differentiation. Utilization of 3H-1, 2-dithiole-3-thione to induce ferritin expression may prove a novel therapeutic potential in valvular mineralization.