Erythrocytes, hemoglobin and iron are present in advanced atherosclerotic lesions. We have investigated whether red cell infiltration of atheromatous lesions promotes the later stages of atherogenesis.

We find that oxidation of ferro (FeII) hemoglobin in ruptured advanced lesions intensively occurs generating ferri (FeIII) hemoglobin and via more extensive oxidation (FeIII/FeIV=O) ferrylhemoglobin. Protein oxidation marker, dityrosine, is accumulated in complicated lesions accompanied by the formation of crosslinked hemoglobin a hallmark of ferrylhemoglobin. Exposure of normal red cells to lipids derived from atheromatous lesions causes hemolysis and oxidation of liberated hemoglobin to ferri- and ferrylhemoglobin. In these interactions between hemoglobin and atheroma lipids hemoglobin and heme promote further lipid oxidation and subsequently endothelial reactions such as upregulation of heme oxygenase-1 or cytotoxicity to endothelium. Oxidative scission of heme leads to release of iron and a feed-forward process of iron-driven plaque lipid oxidation**.** The inhibition of heme release from globin by haptoglobin and sequestration of heme by hemopexin suppress hemoglobin-mediated oxidation of lipids of atheromatous lesions and attenuate endothelial cytotoxicity.

Interior of advanced atheromatous lesions is a pro-oxidant environment in which erythrocytes are lysed, hemoglobin is readily oxidized to ferri- and ferrylhemoglobin, and released heme and iron promote further oxidation of lipids. These events amplify the endothelial cell cytotoxicity of plaque components.

Several pathologic conditions are associated with hemolysis, *i.e.* release of ferro (FeII) hemoglobin from red blood cells including atherosclerosis. Oxidation of cell-free hemoglobin produces (FeIII) ferrihemoglobin. More extensive oxidation produces (FeIII/FeIV=O) ferrylhemoglobin. Both cell-free ferrihemoglobin and ferrylhemoglobin are thought to contribute to the pathogenesis of hemolytic disorders including atherosclerosis.
We revealed that ferrylhemoglobin, but not hemoglobin or ferrihemoglobin, acts as a potent proinflammatory agonist that induces vascular endothelial cells *in vitro* to rearrange the actin cytoskeleton, forming intercellular gaps and disrupting the integrity of the endothelial cell monolayer. Furthermore, ferrylhemoglobin induces the expression of proinflammatory genes in endothelial cells *in vitro*, *e.g. E-selectin*, *Icam-1*, and *Vcam-1*, through the activation of the nuclear factor B family of transcription factors. This proinflammatory effect, which requires actin polymerization, involves the activation of the c-Jun N-terminal kinase and the p38 mitogen-activated protein kinase signal transduction pathways. When administered to naive mice, ferrylhemoglobin induces the recruitment of polymorphonuclear cells, demonstrating that it acts as a proinflammatory agonist *in vivo*. We conclude that oxidized hemoglobin, *i.e.* ferrylhemoglobin, acts as a proinflammatory agonist that targets vascular endothelial cells.

Interaction of hemoglobin with vasculature is remarkable in malaria. Plasmodium, the causative agent of malaria, causes extensive hemolysis, and cerebral malaria claims more than 1 million lives per year. Therefore we chose a model of malaria to study the interaction of hemoglobin with the constituent of vasculature. We hypothesized that carbon monoxide might counter the pathogenesis of cerebral malaria, an inflammatory syndrome that can develop in the course of malaria infection and lead to neurological disturbances revealed clinically by abnormal behavior, impairment of consciousness, seizures and irreversible coma, ultimately leading to death. We report that heme oxygenase-1 (encoded by Hmox1) prevents the development of experimental cerebral malaria. BALB/c mice infected with Plasmodium berghei ANKA upregulated heme oxygenase-1 expression and activity and did not develop cerebral malaria. Deletion of Hmox1 and inhibition of heme oxygenase activity increased cerebral malaria incidence to 83% and 78%, respectively. Heme oxygenase-1 upregulation was lower in infected C57BL/6 compared to BALB/c mice, and all infected C57BL/6 mice developed cerebral malaria (100% incidence). Pharmacological induction of heme oxygenase-

1 and exposure to the end-product of heme oxygenase-1 activity, carbon monoxide, reduced cerebral malaria incidence in C57BL/6 mice to 10% and 0%, respectively. Whereas neither heme oxygenase-1 nor carbon monoxide affected parasitemia, both prevented blood-brain barrier disruption, brain microvasculature congestion and neuroinflammation, including CD8+ T-cell brain sequestration. These effects were mediated by the binding of carbon monoxide to hemoglobin, preventing hemoglobin oxidation and the generation of free heme, a molecule that triggers cerebral malaria pathogenesis.

Heme-mediated oxidation of low-density lipoprotein has been implicated in the pathogenesis of vascular disorders such as atherosclerosis. We performed investigations to test whether hydrophobic fungal siderophores – hexadentate trihydroxamates desferricoprogen, desferrichrome, desferrirubin, and desferrichrysin – might suppress heme-catalyzed LDL oxidation and the toxic effects of heme-treated LDL on vascular endothelium. Indeed, two of these – desferricoprogen and desferrichrome – markedly increased the resistance of LDL to heme-catalyzed oxidation. In similar dose–response fashion, these siderophores also inhibited the generation of LDL products cytotoxic to human vascular endothelium. When iron-free fungal siderophores were added to LDL/heme oxidation reactions, the product failed to induce heme oxygenase-1, a surrogate marker for the noncytocidal effects of oxidized LDL (not in the case of desferrichrysin). Desferricoprogen also hindered the ironmediated peroxidation of lipids from human atherosclerotic soft plaques in vitro, and was taken up in the gastrointestinal tract of rat. The absorbed siderophore was accumulated in the liver and was secreted in its iron-complexed form in the feces and urine. The consumption of mold-ripened food products such as aged cheeses and the introduction of functional foods and food additives rich in fungal iron chelators in diets may lower the risk of cardiovascular diseases.

Since heme interacts with vascular wall, we wondered if it plays a role in the pathogenesis of calcification in atherosclerosis, diabetes, and chronic kidney disease. Human aortic smooth muscle cells undergo mineralization in response to elevated levels of inorganic phosphate in an active and well-regulated process. This process involves increased activity of alkaline phosphatase and increased expression of core binding factor-1, a bone-specific transcription factor, with the subsequent induction of osteocalcin. Mounting evidence suggests an essential role for the heme oxygenase-1/ferritin system to maintain homeostasis of vascular function. We examined whether induction of heme oxygenase-1 and ferritin provoked by heme alters mineralization of human aortic smooth muscle cells provoked by high phosphate. Upregulation of the heme oxygenase-1/ferritin system inhibited human aortic smooth muscle cells calcification and osteoblastic differentiation. Of the products of the system induced by heme, only ferritin and, to a lesser extent, biliverdin were responsible for the inhibition. Ferritin heavy chain and ceruloplasmin, which both possess ferroxidase activity, inhibited calcification; a site-directed mutant of ferritin heavy chain, which lacked ferroxidase activity, failed to inhibit calcification. In addition, osteoblastic transformation of human aortic smooth muscle cells provoked by elevated phosphate (assessed by upregulation of core binding factor -1, osteocalcin, and alkaline phosphatase activity) was diminished by ferritin/ferroxidase activity. We conclude that induction of the heme oxygenase-1/ferritin system in response to heme prevents phosphate -mediated calcification and osteoblastic differentiation of human smooth muscle cells mainly *via* the ferroxidase activity of ferritin.

Calcification of intima or media of vascular wall is often associated with calcium depletion of bone therefore we examined its remodeling capacity in response to heme stress. It has been suggested that mobilization of calcium from bone results in translocation of mineral into the vasculature. Bone is an active tissue with constant remodeling capacity. Osteoblast development and maturation are under the influence of core binding factor a-1, which induces expression of osteoblast specific genes, including alkaline phosphatase, an important enzyme

in early osteogenesis, and osteocalcin, a noncollagenous protein deposited within the osteoid. We investigated the mechanism by which heme affects human osteoblast activity, which in vivo may lead to decreased mineralization, osteopenia, and osteoporosis along with vascular calcification. We demonstrated that heme inhibited osteoblast activity and the effect on osteoblast is mediated by ferritin and its ferroxidase activity. We confirmed this notion by using purified ferritin H-chain and ceruloplasmin, both known to possess ferroxidase activity that inhibited calcification, whereas a sitedirected mutant of ferritin H-chain lacking ferroxidase activity failed to provide any inhibition. Furthermore, we are reporting that such suppression is not restricted to inhibition of calcification, but osteoblast-specific genes such as alkaline phosphatase, osteocalcin, and core binding factor a-1 are all downregulated by heme induced ferritin in a dose-responsive manner. This study provided evidence that heme decreases mineralization and demonstrates that this suppression is provided by heme-induced upregulation of ferritin. In addition, we conclude that inhibition of osteoblast activity, mineralization, and specific gene expression is attributed to the ferroxidase activity of ferritin.