FINAL REPORT
THE ROLE OF HEME IN ATHEROSCLEROTIC LESION FORMATION

The central hypothesis of this proposal was that Fe-protoporphyrin IX (heme) that is a potent inducer of LDL oxidation *in vitro* is an important pathophysiological mediator of LDL oxidation *in vivo* and plays an important role in the progression of atherosclerosis.

The major source of heme in the vasculature is hemoglobin (Hb). We hypothesized that upon hemolysis cell free Hb oxidize and release heme that can target directly endothelial cells and macrophages causing endothelial cell and macrophage activation. Alternatively, heme can intercalate into low-density lipoprotein (LDL) and cause LDL oxidation, which in turn can also target endothelial cells and macrophages.

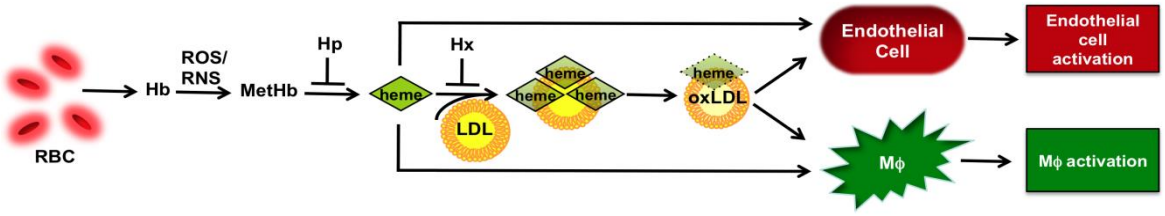


Figure 1. **Proposed mechanisms of heme-mediated LDL oxidation and proatherogenic effects of oxLDL.** Upon RBC lysis cell-free Hb can be oxidized by reactive oxygen/nitrogen species (ROS/RNS) to MetHb. MetHb readily releases its heme groups that can target LDL and cause its oxidative modification. OxLDL activates endothelial cells and Mφ. The deleterious effects of Hb and heme can be inhibited by Hp and Hx, respectively.

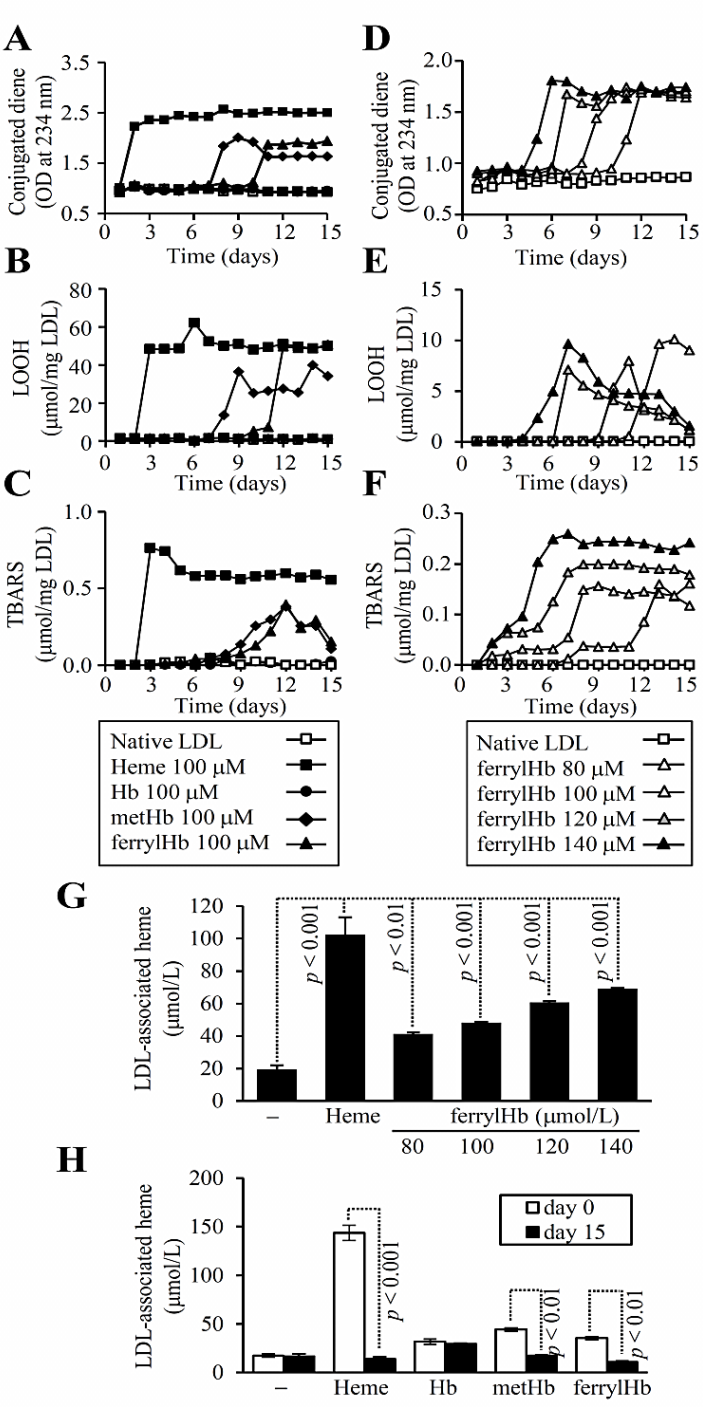
Several defense mechanisms evolved to cope with the deleterious effects of cell-free Hb as well as those of heme itself. Under homeostasis plasma cell-free Hb is bound by haptoglobin (Hp), forming Hb-Hp complexes that are cleared from circulation by hemophagocytic macrophages via the Hp receptor (CD163) that promotes heme catabolism by heme oxygenase-1 (HO-1). We hypothesized that Hp plays a central role in preventing deleterious effects of cell-free Hb and thus possesses an atheroprotective effect.

When the protective effect of Hp is overwhelmed, cell-free Hb accumulates in plasma and becomes oxidized, releasing its heme prosthetic groups. Hx is an acute-phase protein that binds free heme in plasma with the highest affinity of any protein described so far. The resulting heme-Hx complexes are removed via the Hx receptor (CD91), which promotes endocytosis of the heme-Hx complexes in macrophages. We hypothesized that Hx has a crucial effect in terms of preventing the deleterious effects of extracellular free heme in atherogenesis.

RESULTS

1. <u>Oxidized hemoglobin species induce oxidative modification of LDL.</u>

To model the possible interactions that could take place inside a complicated atherosclerotic lesion between lipids and different Hb species, we purified Hb from human blood and generated metHb and ferrylHb, that is not a homogenous chemical entity but is a mixture of globin- and porphyrin- centered radicals (which can be very short lived), and covalently cross-linked Hb multimers. Human plasma was incubated with heme and the three different Hb species, i.e.Hb, metHb and ferrylHb (100 μmol/L heme). After 1 hour of incubation at 37°C LDL was isolated by ultracentrifugation and oxidative modification of LDL was monitored by the formation of conjugated dienes, lipid hydroperoxides and TBARs in samples incubated at 4°C for 15 days.



Lipid peroxidation did not occur in LDL samples derived from non-treated or Hb-treated plasma samples (Fig. 2A-C). Starting on day 2 post-isolation, heme treatment caused extensive and rapid increase in conjugated dienes, LOOH and TBARs content of LDL (Fig. 2A-C). MetHb and ferrylHb also initiated oxidative modification of LDL and increased the levels of lipid peroxidation products at days 7-10 after isolation (Fig 2A-C). The kinetics of formation of lipid peroxidation products in the LDL was strictly dependent on the dose of ferrylHb (Fig. 2D-F).

Heme released from oxidized Hb in plasma preferentially associates with LDL and is degraded shortly thereafter in the course of lipid peroxidation. Therefore we assessed whether the heme moiety of ferrylHb is released and eventually taken up by LDL and degraded during lipid-peroxidation. Plasma was incubated with heme or ferrylHb for 1 hour at 37°C followed by LDL separation and measurement of LDL-associated heme. As shown in Figure 2, ferrylHb treatment dose-dependently increased the concentration of LDL-associated heme in the LDL (Fig. 2G).

In order to determine whether similar events occurred in whole plasma, we treated fresh plasma with heme, Hb, metHb or ferrylHb. Following the isolation of LDL, the concentration of LDL-associated heme was measured on the day of LDL isolation and 15 days after. We observed that LDL-associated heme underwent degradation when plasma was treated with heme, metHb or ferrylHb (Fig. 2H). In contrast the heme content of LDL derived from Hb-treated plasma did not change over a 15 day incubation period (Fig. 2H). These results suggest that ferrylHb, like metHb, readily releases heme, following which iron is released upon oxidative scission of heme and serves to catalyze the process of lipid peroxidation.

Figure 2. FerrylHb triggers oxidative modification of LDL.

Human plasma was incubated with heme, Hb, metHb and ferrylHb for 1 hour at 37°C, followed by separation of LDL. Concentrations are indicated and expressed as µmol/L heme groups. Following separation samples were kept at 4°C. Conjugated dienes (A and D), LOOH (B and E), and TBARs (C and F) in LDL samples were measured every day for 15 days. Results are representatives of 3 independent experiments. Heme contents of the same samples were measured on the day of separation (G) and 15 days later (H). Data represent mean ± S.D. of 3 independent experiments.

2. Oxidized Hb species trigger EC death.

We have shown previously that heme and metHb make EC more sensitive to oxidative stress by delivering redox active iron and thus amplifying the generation of reactive oxygen species. Here we tested whether ferrylHb, similar to metHb, can sensitize EC to oxidative stress. Confluent HUVECs were pretreated with heme, Hb, metHb or ferrylHb at a dose of 5 μmol/L heme. After 1 hour, heme containing solutions were removed and cells were challenged with H_2O_2 (75 μmol/L). Heme, different Hb species or H_2O_2 alone did not trigger EC death. Moreover, no cytotoxicity was observed when Hb-treated cells were exposed to H_2O_2. In contrast, when HUVECs were pretreated with heme, metHb or ferrylHb prior to H_2O_2 exposure, cell viability decreased (Fig. 3A). This shows that while not cytotoxic per se, heme, metHb and ferrylHb sensitize EC to H_2O_2 driven cytotoxicity.

In our previous work we demonstrated that heme and metHb can exert cytotoxic effects in EC via oxidative modification of LDL. To assess whether this is also the case with ferrylHb, LDL was incubated with heme, Hb, metHb or ferrylHb (10 μmol/L heme) overnight, and the resulting LDL was tested for cytotoxic effects. We observed that LDL samples treated with heme, metHb and ferrylHb became highly toxic to HUVECs (Fig 2B). In contrast, Hb does not generate cytotoxic LDL (Fig. 3B).

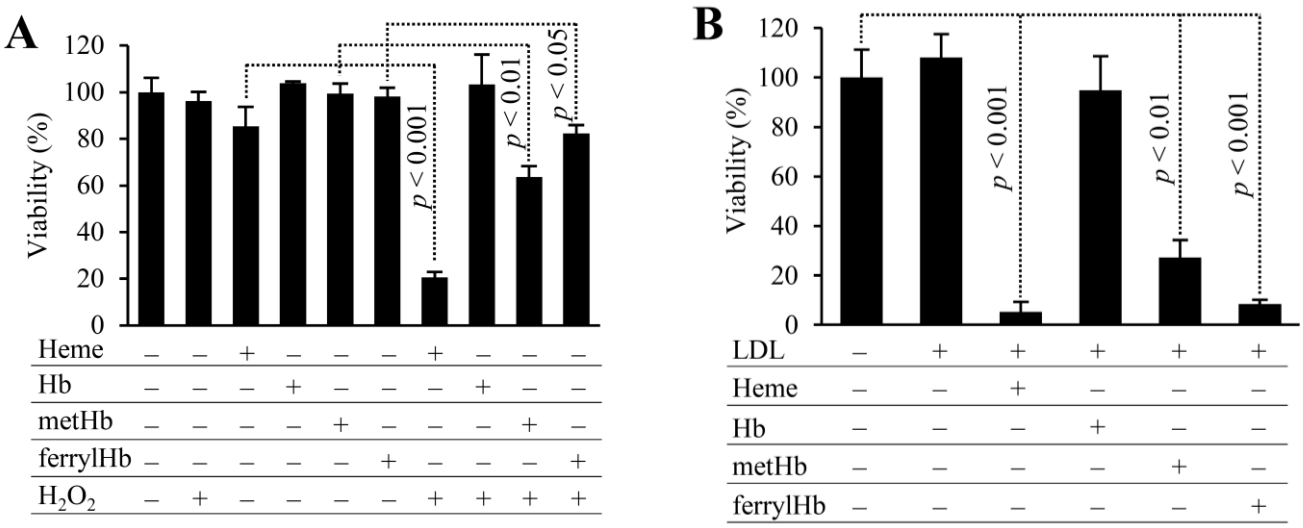


Figure 3. FerrylHb triggers EC death via different mechanisms.

(A) Confluent HUVECs grown on 96-well plates were exposed to heme, Hb, metHb and ferrylHb (5 μmol/L heme) in HBSS for 1 hour. After washing with HBSS, cells were challenged with H2O2 (75 μmol/L in HBSS) for 4 hours, followed by MTT assay to assess cell viability. (B) LDL was incubated with heme, Hb, metHb and ferrylHb (10 μmol/L heme) overnight. HUVECs were exposed to LDL samples at a dose of 250 μg protein/mL for 4 hours. MTT assay was performed to determine cell viability. Results are shown as mean ± S.D. (n=4) from one representative experiment of three.

3. Oxidized hemoglobin species induce HO-1 and ferritin expression in HUVECs.

Upon exposure to free heme, EC upregulate the expression of HO-1 and H-ferritin to assure degradation of heme and safe storage of liberated iron, respectively. We have previously demonstrated that native (oxy) Hb does not induce HO-1 and ferritin in EC, whereas metHb does, because it releases its heme moiety. We asked whether ferrylHb could transfer heme groups to the endothelium and thus upregulate HO-1 and ferritin synthesis. We observed that ferrylHb, similar to metHb, induces HO-1 mRNA and protein expression (Fig. 4A and B). As with HO-1 expression, ferritin level was also increased in ferrylHb-treated cells compared to vehicle-treated controls (Fig. 4C).

These effects occur in a dose dependent manner. However, when compared at a heme-molar ratio the effect of ferrylHb is lower to that of free heme (Fig. 4D and E).

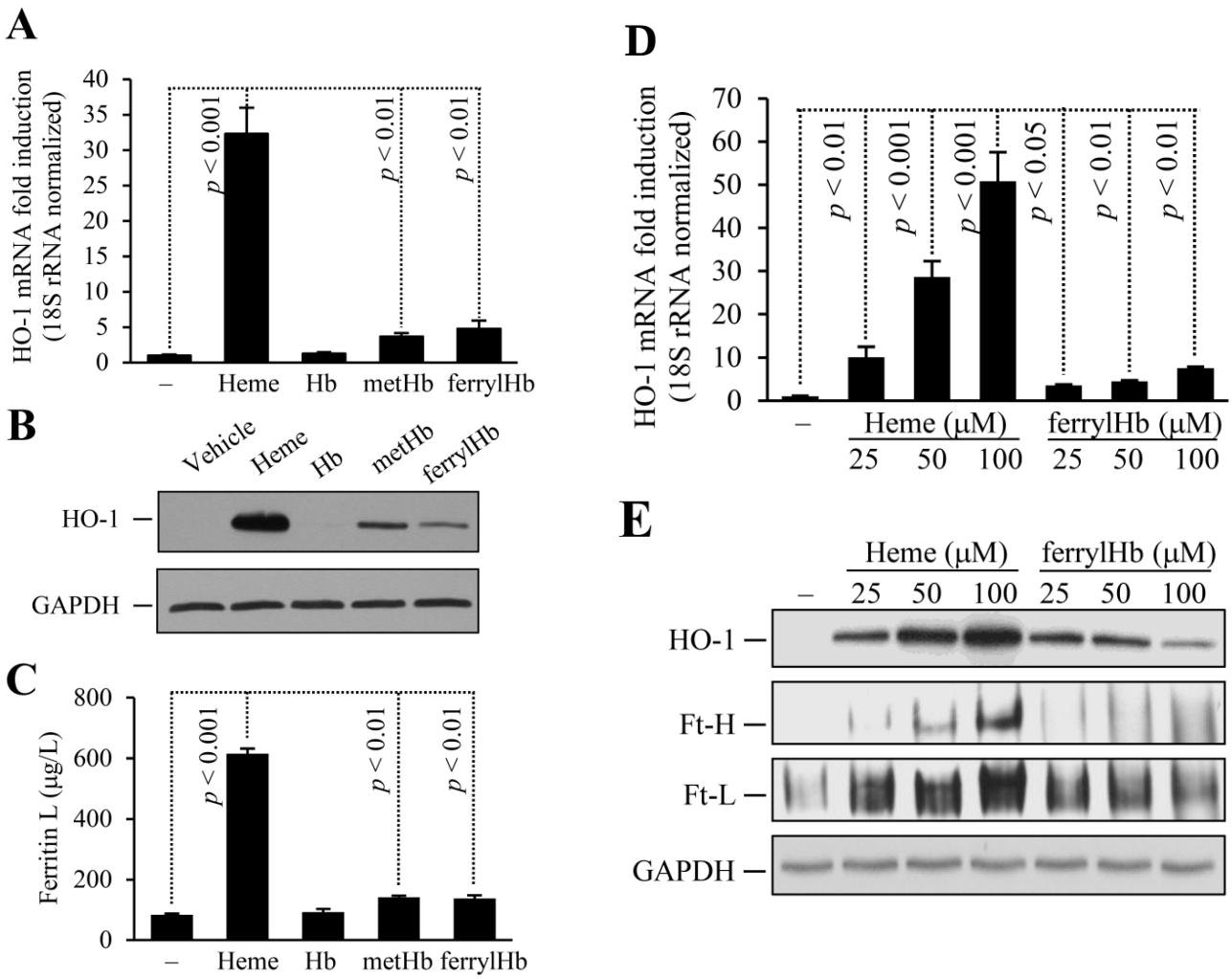


Figure 4. FerrylHb induces HO-1 and ferritin in EC.
Confluent HUVECs grown on 6-well plates were exposed to heme, Hb, metHb and ferrylHb (100 µmol/L heme or as indicated in complete medium containing 15% of FBS). After 4 hours of incubation total RNA was isolated and HO-1 mRNA level was measured by quantitative RT-PCR (panels A and D). For protein expression, HUVECs were solubilized after 8 hours of treatment. HO-1 and ferritin H and L expression was detected by Western blot (B and E) or with ELISA (C). Immunoblots were reprobed with GAPDH and are representative of three independent experiments. Results are shown as mean ± S.D. (n=3) from one representative experiment of three.

4. OxLDL and reactive lipid mediators derived from complicated atherosclerotic lesions initiate Hb oxidation and globin-globin crosslinking. Lipid hydroperoxides, such as those found in oxLDL as well as in lipids derived from atheromatous lesions, can initiate Hb oxidation resulting in metHb formation and subsequent heme release. Oxidized lipids and ferrylHb coexist in advanced atherosclerotic lesions but the role of reactive lipid mediators in the formation of ferrylHb and the subsequent crosslinking of Hb subunits has not been addressed. Therefore, we tested native and oxidized LDL, as well as lipids derived from human type IV atherosclerotic lesions, for their ability to induce Hb

crosslinking, a surrogate marker of ferrylHb formation. Hb (20 µmol/L heme) was treated with H_2O_2 (200 µmol/L), native or oxidized LDL (500 µg protein/ml) or lipids derived from human type IV atherosclerotic lesions (LP), (500 µg lipid/ml). After a 2-hour incubation at 37°C, samples (4 µg protein/lane) were subjected to SDS-PAGE followed by silver staining. Covalently cross-linked Hb dimer formation was observed in H_2O_2-treated as well as in oxLDL- and plaque lipid-treated Hb samples (Fig. 5A). In contrast, native LDL did not induce Hb crosslinking (Fig. 5A). Oxidation state of iron in the Hb modified by oxLDL was determined spectrophotometrically. OxLDL increased in a dose dependent manner the percentage of Fe3+ Hb as assessed by an increased absorbance at λ=630 nm accompanied by a decrease at λ=577 nm and λ=562 nm (Fig. 5B and C). With increasing doses of oxLDL, dimer formation became more prevalent and at higher doses tetrameric and multimeric ferrylHb formation occurred as well (Fig. 5D).

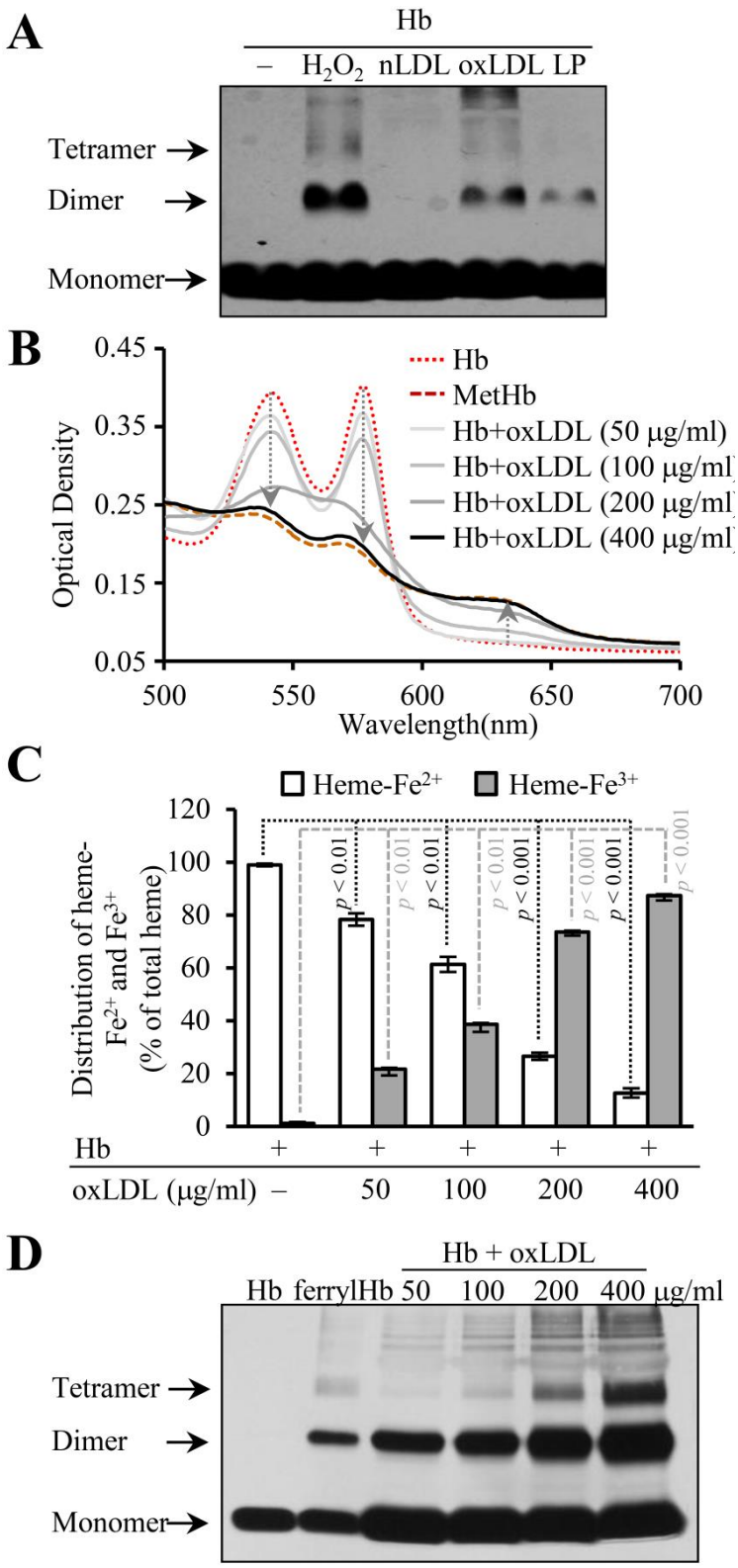
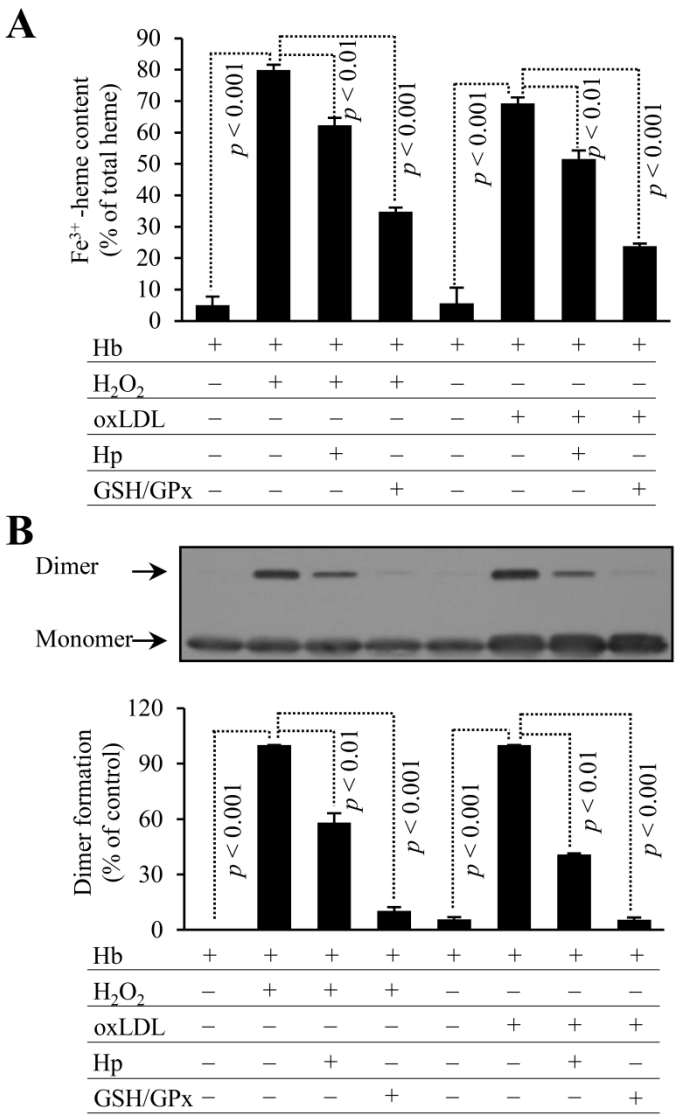


Figure 5. Oxidized LDL and atheroma lipids cause Hb oxidation and formation of ferrylHb. (A) Human Hb (20 µmol/L heme) was treated with H2O2 (200 µmol/L), native LDL (400 µg/mL), oxLDL (400 µg/mL), and lipid derived from human atherosclerostic plaque (LP), (400 µg/mL). After 90 minutes of incubation Hb samples (4 µg/lane) were subjected to SDS-PAGE followed by silver staining. (B, C and D) Human Hb was incubated with oxLDL (50-400 µg/ml) for 90 minutes. (B and C) Spectral scan of Hb samples were taken and concentrations of Hb and metHb were calculated based on the visible spectra. (D) Hb samples (4 µg/lane) were subjected to SDS-PAGE followed by silver staining. Silver staining images and spectral scan are representatives of three independent experiments. Results are shown as mean ± S.D. (n=3) from one representative experiment of three.

5. Hb oxidation and crosslinking induced by H_2O_2 and reactive lipid mediators can be inhibited by haptoglobin (Hp) or GSH/GPx.

Cell-free Hb binds to the acute-phase plasma protein Hp, promoting its endocytosis via the Hp receptor CD163, and thus preventing Hb accumulation in plasma. We tested whether binding of Hb to Hp inhibits ferrylHb formation. Hb oxidation was induced with H_2O_2 or oxLDL in the presence or absence of Hp. After 90 minutes Fe3+-heme content was determined spectrophotometrically (Fig. 6A), and covalently cross-linked Hb formation was assessed by western blotting (Fig 6B). Hp slightly inhibited H_2O_2-mediated metHb formation. A more pronounced inhibitory effect of Hp was seen on Hb crossliniking; Hp inhibited by 42% and 60% covalently cross-linked Hb dimer formation in response to H_2O_2 and oxLDL, respectively. These results suggest that Hp has a role in suppressing the loss of Hb heme/iron and preventing the formation of covalently cross-linked Hb species.



Lipid hydroperoxides in the oxLDL can trigger Hb oxidation, resulting in the formation of metHb, but the role of lipid hydroperoxides in Hb crosslinking has not been tested. To examine, whether lipid hydroperoxides in oxLDL are responsible for oxLDL-mediated Hb crosslinking, we used glutathione-glutathione peroxidase (GSH/GPx) to decompose H_2O_2 and lipid hydroperoxides and assessed their effect on Hb crosslinking. GSH/GPx reduced the formation of metHb by 93% when Hb was oxidized by H_2O_2, and by 70% when Hb was exposed to oxLDL (Fig. 6A). Exposing Hb to H_2O_2 or oxLDL in the presence of GSH/GPx led to 90-95% less dimer formation compared to Hb exposed to H_2O_2 or oxLDL in the absence of GSH/GPx (Fig. 6B). These data highlight the critical role of lipid hydroperoxides in mediating Hb oxidation and subsequent covalent crosslinking of the Hb subunits.

Figure 6. GSH/GPx and Hp inhibit H_2O_2 and oxLDL-mediated oxidation and crosslinking of Hb.
(A and B) Human Hb (20 μmol/L heme) was pretreated with Hp (50 μmol/L) for 10 minutes at 37°C followed by a 90-minute incubation with H_2O_2 (200 μmol/L) and oxLDL (400 μg/mL). Separately, H_2O_2 (200 μmol/L) and oxLDL (400 μg/mL) were pretreated with GSH/GPx for 10 minutes at 37°C followed by a 90-minutes of incubation with Hb (20 μmol/L heme). (A) MetHb was determined spectrophotometrically. (B) Oxidation-induced dimer formation was monitored by Western blot. Immunoblot is a representative of three independent experiments. Results are shown as mean ± S.D. (n=3) from one representative experiment of three.

6. FerrylHb increases EC monolayer permeability and enhances monocyte adhesion.

We have previously demonstrated that ferrylHb activates EC in vitro, leading to the formation of intercellular gaps disrupting the endothelial monolayer. Here we show that this effect is dose dependent (Fig 7A) and that intercellular gap formation is associated functionally with increased endothelial monolayer permeability (Fig 7B). This is a unique feature of ferrylHb as neither Hb nor metHb increased EC monolayer permeability. In our previous work we also showed that ferrylHb induces the expression of pro-inflammatory genes in EC. Therefore we asked whether ferrylHb-triggered induction of these proteins – known to play a role in cell adhesion – is accompanied by increased monocyte adhesion to vascular EC. We found that human monocytes readily adhered to ferrylHb-treated EC. In contrast, treatment of EC with heme, Hb or metHb did not promote monocyte adhesion (Fig. 7C). These data suggest that the formation of ferrylHb maybe a crucial event in the promotion of inflammatory responses.

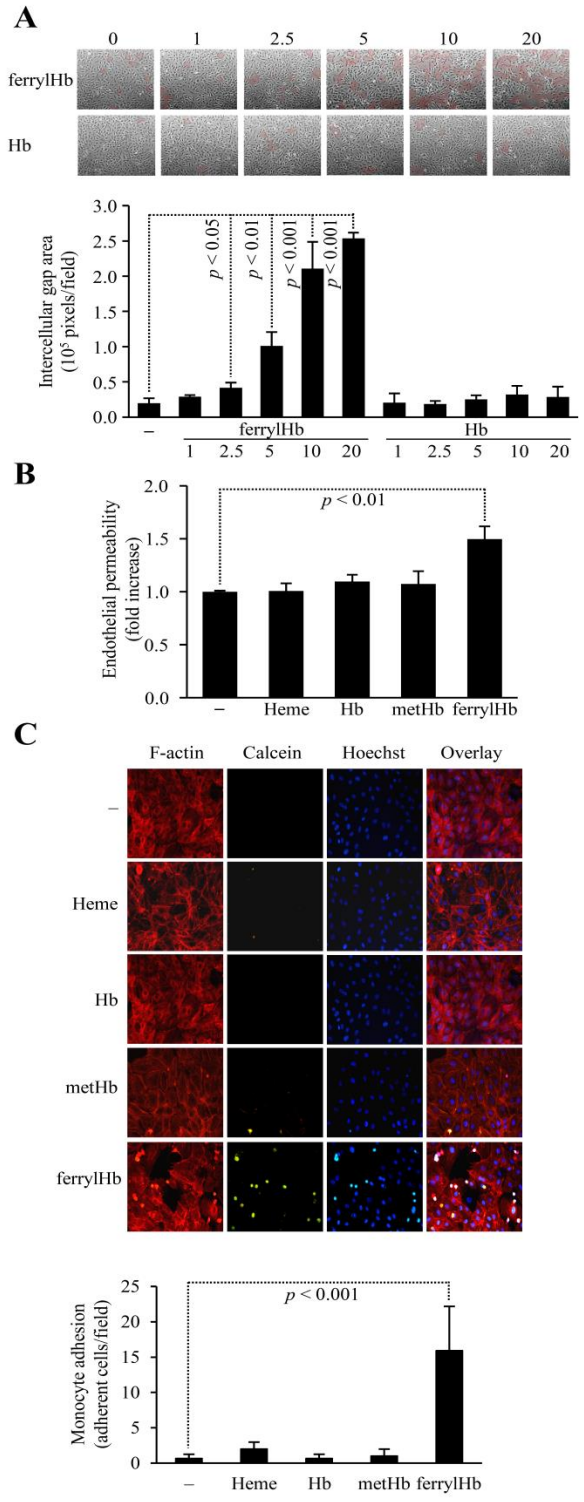


Figure 7. FerrylHb disrupts endothelial monolayer and induces leukocyte adhesion in HUVECs.

(A) Confluent HUVECs grown in 6-well plates were exposed to Hb and ferrylHb at a dose of 0-20 µg/ml over-night. Images are 100X, taken with an inverted microscope (Carl Zeiss 426126) and analyzed by ImageJ software. (B) Confluent HUVECs grown in hanging cell culture inserts were treated with heme, Hb, metHb and ferrylHb (10 µmol/L heme groups each) for 12 hours. Fluorescein (1 µmol/L) was added into the apical filter compartment and was detected in the lower compartment after a 60-minute incubation. Endothelial permeability is expressed as fold increase over non-treated cells. (C) Confluent HUVECs were treated with heme, Hb, metHb and ferrylHb (10 µmol/L heme) for 12 hours. Monocytes were labeled and added to HUVECs (105 cells/well) for 30 minutes at 37°C. Cells were stained with TRITC-conjugated phalloidin and with Hoechst. Images are 400X. Results are shown as mean ± S.D. (n=3) from one representative experiment of three. Images are representative of 3 independent experiments.

7. Chronic administration of Hb accelerate atherosclerosis in apoE deficient mice

After we proved that Hb oxidation can trigger deleterious events *in vitro* targeting LDL and endothelial cells, those are two crucial components of the atherosclerotic vessel wall, our next question was whether chronic administration of Hb can facilitate atherosclerosis in apoE deficient mice. We injected ApoE deficient mice with Hb and heme at a dose that provided 80 μmol/L of heme concentration in the plasma every other day for 8 weeks. Parallel with the Hb/heme injections mice were fed with high-cholesterol diet (HCD: 15.8% fat, 1.25% cholesterol). After 8 weeks of treatment aortas were dissected and cleaned from adventitial fat, and plaques were stained with oil o red and quantified (Fig 8). We found that administration of Hb increased plaque size in the aorta of ApoE-/- mice significantly, whereas heme did not have atherogenic effect (Fig. 8). We have two theories to explain this finding: (i) Hb is atherogenic independently of heme release, (ii) heme induces atheroprotective mechanisms more efficiently than Hb and it can compensate the deleterious effects of heme; those pathways should be tested in the future.

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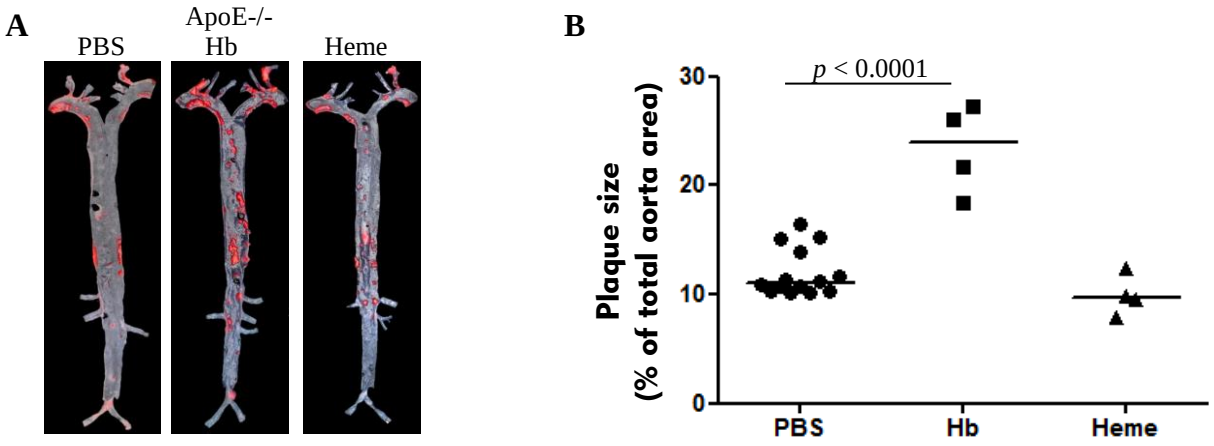


Figure 8. Chronic Hb administration accelerates atherosclerotic lesion progression in ApoE deficient mice.
(A) ApoE-/- mice were treated with Hb, heme or vehicle (PBS) every other day for 8 weeks. Parallel with Hb/heme/vehicle treatement all mice were fed with HCD. After 8 weeks of treatment aortas were dissected, cleaned and plaques were stained with oil O red. Representative images of aortas prepared for plaque size quantification are shown. Quantification was carried out by using ImageJ software. (B) Plaque size were determined in control mice (n=13), Hb-treated (n=4) and heme-treated (n=4) mice. Two-way Anova test was used for multiply comparisons.

8. Hemolysis accelerates atherosclerosis in apoE deficient mice.

Next we tested whether hemolysis can trigger atherosclerosis in apoE deficient mice. Hemolysis was induced by intraperitoneal injections of phenyl-hydrazine (30 mg/kg) in every other day for 8 weeks starting at the age of 8 weeks (n=5). This treatment induced about a 50-60 % of RBC lysis that could recover within two days. Parallel with the PHZ treatment ApoE mice received HCD to facilitate plaque formation. Control mice received PBS and HCD (n=14). After 8 weeks of treatment aortas were dissected and cleaned from adventitial fat, and plaques were stained with oil o red and quantified. Chronic hemolysis significantly increased plaque size (Fig. 9 A and B), and decreased collagen content (stained in blue) of the atherosclerotic vessel wall that was assessed by Trichrome staining of the aortic root (Fig. 9C).

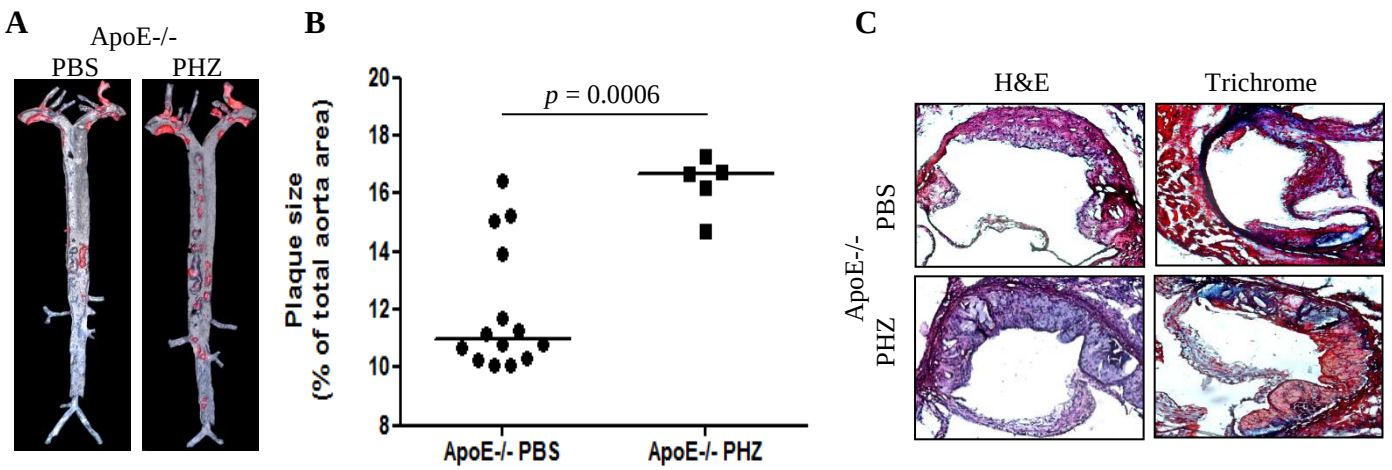


Figure 9. Hemolysis accelerates atherosclerotic lesion progression in ApoE deficient mice.
(A) ApoE-/- mice were treated with PHZ or vehicle every other day and parallel were fed with HCD for 8 weeks. Aortas were dissected, cleaned and plaques were stained with oil O red. Representative images of aortas prepared for plaque size quantification. Quantification was carried out by using ImageJ software. (B) Plaque size were determined in control mice (n=16) and PHZ-terated mice (n=5). Two-tailed unpaired T-test was used to calculate p value. (C) Aortic root samples were embedded in TissueTek and were cryosectioned into 6 μm thick slices. Representative images of hematoxylin-eosin (H&E) and Masson trichrome stainings (nuclei: black, cytoplasm: red, muscle fiber: red, collagen: blue) are shown. Images are 20X.

9. <u>Deficiency of haptoglobin and hemopexin provide atheroprotection in ApoE deficient mice.</u>
Hp and Hx play crucial roles in removing extracellular Hb and heme from circulation upon intravascular hemolysis. Therefore our next question was whether atherosclerosis is accelerated in ApoE-/-Hp-/- or ApoE-/-Hx-/- mice in comparison with ApoE-/- mice.

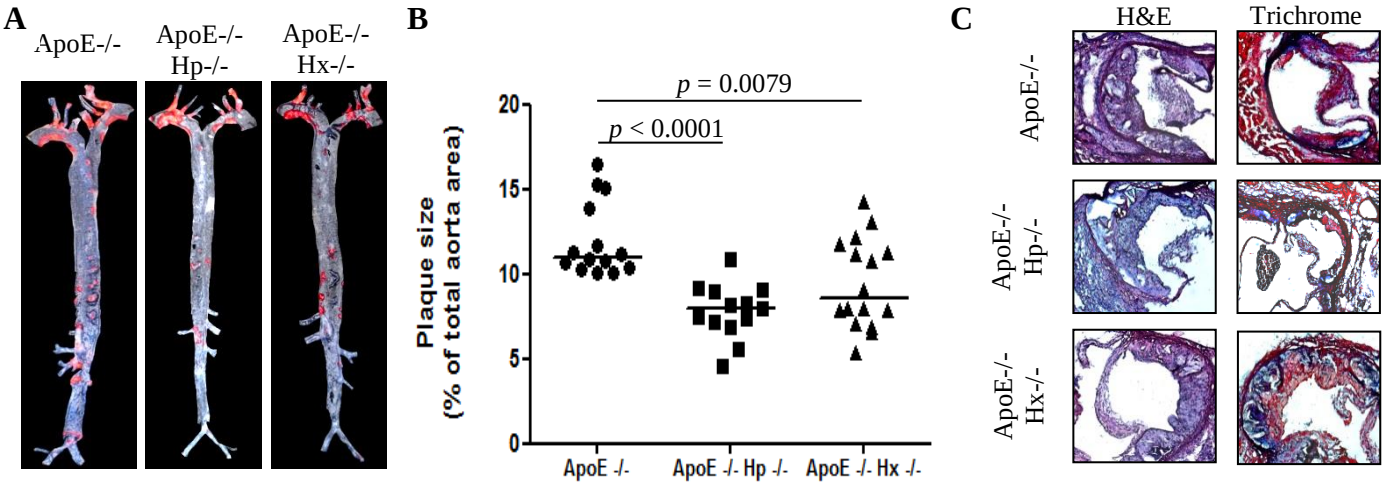


Figure 10. Hemolysis accelerates atherosclerotic lesion progression in ApoE deficient mice.
(A) ApoE-/- mice were treated with PHZ or vehicle every other day and parallel were fed with HCD for 8 weeks. Aortas were dissected, cleaned and plaques were stained with oil O red. Representative images of aortas prepared for plaque size quantification. Quantification was carried out by using ImageJ software. (B) Plaque size were determined in control mice (n=16) and PHZ-treated mice (n=5). Two-tailed unpaired T-test was used to calculate p value. (C) Aortic root samples were embedded in TissueTek and were cryosectioned into 6 μm thick slices. Representative images of hematoxylin-eosin (H&E) and Masson trichrome stainings are shown. Images are 20X.

Mice were on HCD to accelerate plaque formation for 8 weeks. After 8 weeks of treatment aortas were dissected and cleaned from adventitial fat, plaques were stained with oil o red and quantified. Unexpectedly we observed that both ApoE-/-Hp-/- and ApoE-/-Hx-/- mice were protected in HCD-induced atherosclerosis. Plaque sizes in these mice were significantly smaller when compared to ApoE-/- mice (Fig 10 A-C).

10. Chronic hemolysis accelerates atherosclerosis in ApoE-/-Hp-/- and ApoE-/-Hx-/- mice.

To further investigate the role of Hp and Hx in atherosclerosis in our next experiments we challenged the ApoE-/-Hp-/- and ApoE-/-Hx-/- mice with chronic hemolysis. Hemolysis was induced by injecting PHZ as before and all mice were fed with HCD to trigger plaque formation. As shown in figure 11 (A-B), plaque formation was accelerated in response to PHZ treatment in both double-deficient mice.

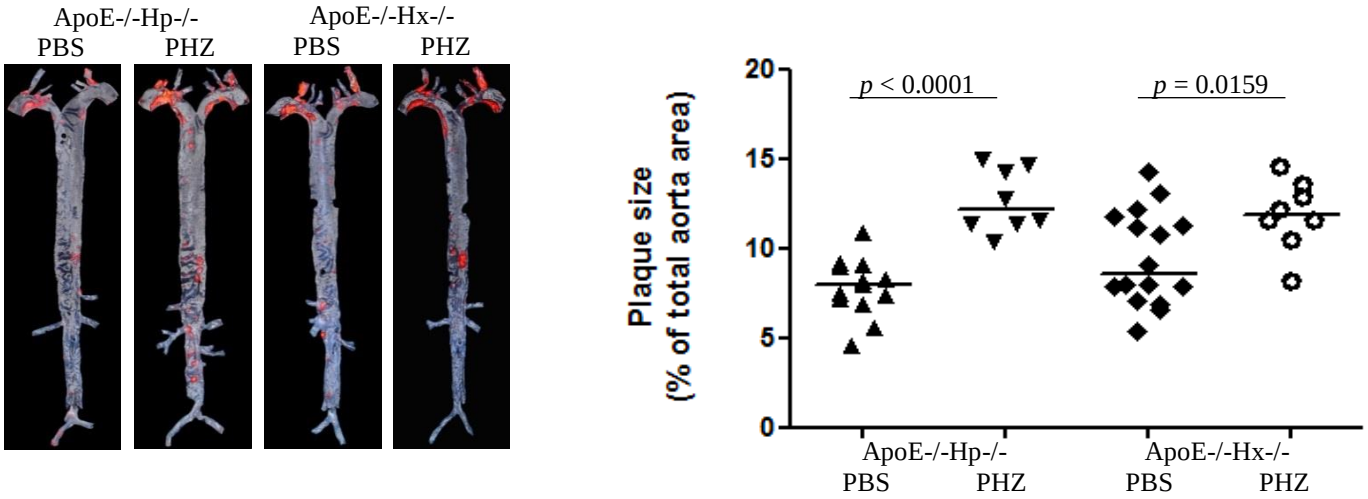


Figure 11. Hemolysis accelerates atherosclerotic lesion progression in ApoE-/-Hp-/- and ApoE-/-Hx-/- mice.
(A) ApoE-/-Hp-/- and ApoE-/-Hx-/- mice were treated with PHZ or vehicle every other day and parallel were fed with HCD for 8 weeks. Aortas were dissected, cleaned and plaques were stained with oil O red. Representative images of aortas prepared for plaque size quantification. Quantification was carried out by using ImageJ software. (B) Plaque size were determined in ApoE-/-Hp-/- vehicle-injected mice (n=11), ApoE-/-Hp-/- PHZ-terated mice (n=8), ApoE-/-Hx-/- vehicle-injected mice (n=16), ApoE-/-Hx-/- PHZ-terated mice (n=8). Two-tailed unpaired T-test was used to calculate p value.

CONCLUDING REMARKS

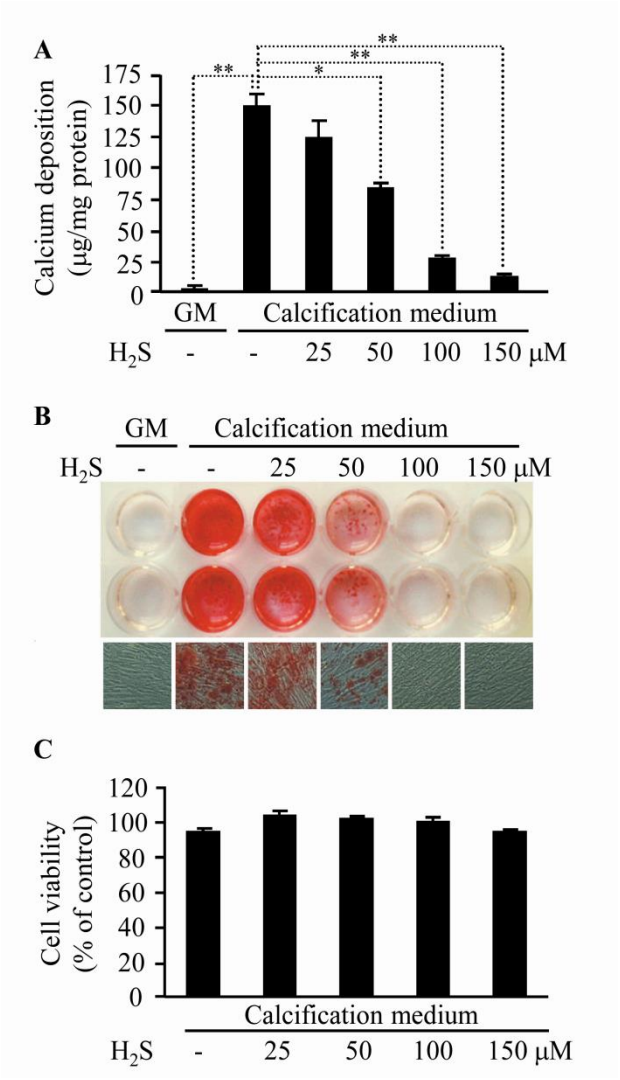
In this project our aim was to find out the pathophysiological role of Hb-derived heme in atherogenesis. We found that Hb and intravascular hemolysis can accelerate disease progression and that oxidized Hb species behave in a pro-inflammatory and pro-oxidant manner in the atherosclerotic vessel wall. Based on these results one can assume that deficiency of molecules responsible for clearing of these dangerous molecules from circulation could facilitate atheroma formation. Our results indicate that in fact, this is not the case. Mice deficient for Hp or Hx are somehow protected in this rodent model of atherosclerosis. The molecular mechanism behind this phenomenon remained to be elucidated, but certainly that is our next question. We assume that physiological level of hemolysis in the absence of Hp or Hx results in continuous low-level of oxidative stress that switch on stress-adaptation mechanisms in these mice. In our previous work we showed that such stress adaptation mechanism protects sickle cell anemic mice from severe forms of malaria.

OTHER RESULTS

During this grant period I was involved in other research projects those ended with interesting results. In the last 7 years we started to be interested in vascular calcification that can be focal, associated with atherosclerosis or can be generalized, affecting the whole vasculature. Vascular calcification has been long considered as a passive process, but nowadays accumulating evidence suggests that in fact this is not the case. Vascular calcification is an actively regulated process in which vascular smooth muscle cells trans-differentiate into osteoblast-like cells. This trans-differentiation is accompanied by phenotype changes: the loose of contractility and increase in extracellular matrix production.

We started to be interested in H_2S the lastly recognized endogenous gas molecule, that has direct inhibitory effects on the development of atherosclerosis. We investigated the role that H_2S may play in Pi-induced osteoblastic transformation and mineralization of VSMC.

1. <u>H_2S decreases HAoSMC mineralization in a dose responsive manner</u>



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 (GM). HAoSMC was cultured in calcification medium in the presence or absence of H_2S for 7 days followed by calcium measurement (Fig 12A). As expected, Pi provoked calcification, whereas in the control culture no deposits were formed during this period. Importantly, H_2S inhibited calcium deposition in a dose responsive manner, providing a significant inhibition at concentrations of ≥50 μmol/L. To confirm the effect of H_2S on calcium deposition we also performed alizarin red staining of HAoSMC (Fig 12B). 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. To test the viability of cells exposed to H_2S we carried out MTT assay (Fig 12C). We did not observe a decline in viability of HAoSMC challenged with H_2S in the concentration range of 25-150 μmol/L.

Figure 12. H_2S decreases HAoSMC mineralization in a dose responsive manner.

(A-C) HAoSMC were cultured in GM or in calcification medium alone or supplemented with 25, 50, 100 and 150 μmol/L of H_2S for 7 days. (A) Calcium content is shown as mean ± standard deviation of three independent experiments performed in duplicates. $^*P < 0.05$, $^{**}P < 0.01$. (B) Representative images of alizarin red staining of plates (upper) and microscopic views (100x, lower) from three independent experiments are shown. (C) MTT assay is shown as mean ± standard deviation of two independent experiments performed in triplicates.

2. 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, an important enzyme in osteogenesis and osteocalcin a major noncollagenous protein found in bone matrix, 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 for 7 days 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 providing a complete attenuation at dose of 100 μmol/L (Fig 13A). Similarly to ALP activity, the induction of osteocalcin was also abolished by H_2S. Maintaining HAoSMC in calcification medium for 7 days lead to a >10-fold increase in osteocalcin content in the extracellular matrix compared to control. H_2S decreased the expression of osteocalcin to the basal level observed in HAoSMC (Fig 13B and C) cultured in GM.

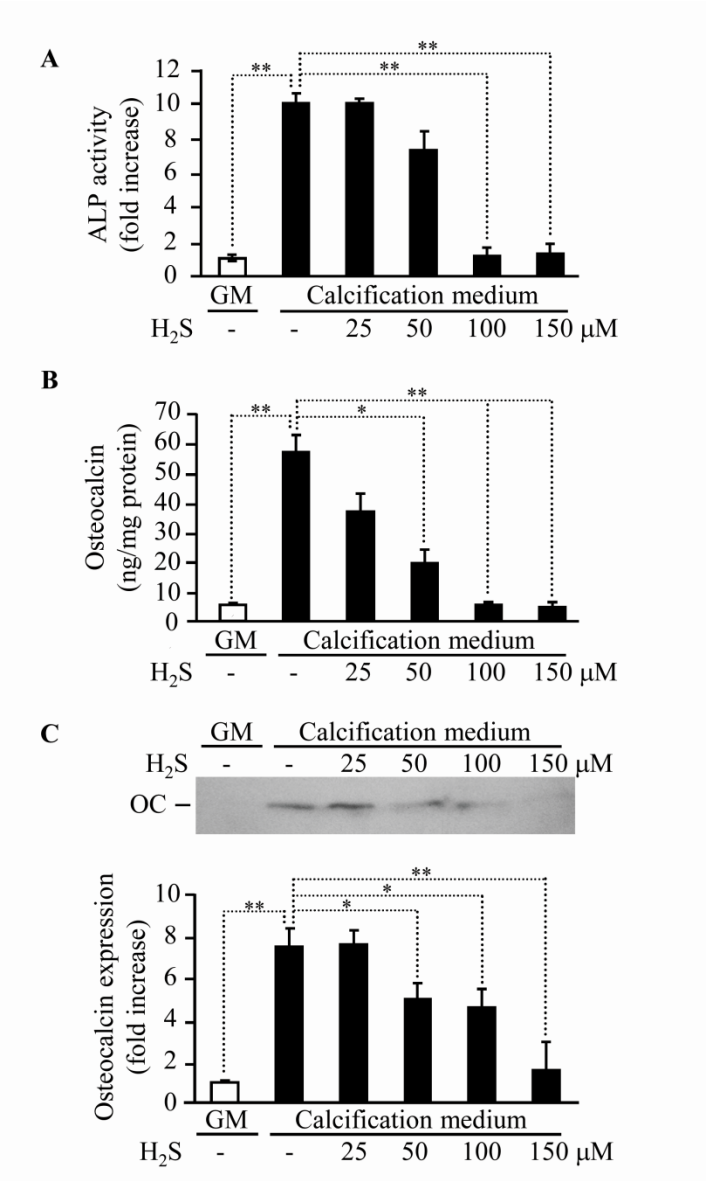


Figure 13. H_2S inhibits Pi-mediated up-regulation of osteoblast specific proteins in HAoSMC.

(A-C) HAoSMC were cultured in GM or in calcification medium in the absence or presence of different concentrations of H_2S for 7 days. (A) ALP activity is presented as means ± standard deviation of 3 independent experiments each performed in duplicates. $^{**}P < 0.01$. (B) Osteocalcin level in EDTA-solubilized extracellular matrix was determined by ELISA and shown as means ± standard deviation of 3 independent experiments each performed in duplicates.** $P < 0.01$. (C) Osteocalcin detected by western blot from EDTA-solubilized extracellular matrix. Blot is representative of 3 independent experiments. A graph of band intensity means ± standard deviation of 3 experiments is shown. $^{*}P < 0.05$, ** $P < 0.01$.

Evidence suggests that the effects of hyperphosphatemia are mediated via Pit-1 that facilitates entry of Pi into vascular cells. To further explore the mechanism via which H_2S inhibits vascular calcification, we measured Pi uptake of HAoSMC in the presence or absence of H_2S. Intriguingly, addition of H_2S inhibited Pi uptake in a dose responsive manner providing a significant and complete suppressions at concentrations of 50 and 100 μmol/L, respectively (Fig 14A). 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 1.6-fold elevation in Pit-1 mRNA level. Addition of H_2S into the calcification medium prevented the increase in Pit-1 expression (Fig 14B).

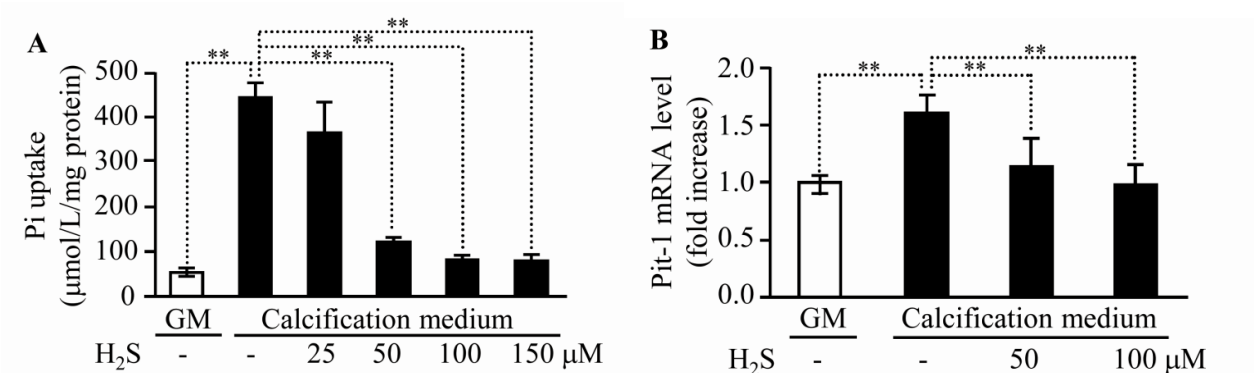


Figure 14. H_2S inhibits phosphate uptake by HAoSMC.

(A) HAoSMC were cultured in GM and calcification medium alone, or supplemented with H_2S (25, 50, 100, 150 μmol/L) for 4 days. Pi levels were determined from cell lysates and shown as means ± standard deviation of three independent experiments performed in duplicates.** $P < 0.01$. (B) HAoSMC were cultured in GM and calcification medium alone, or supplemented with H_2S (25, 50, 100, 150 μmol/L) for 48 hours and Pit-1 mRNA level was determined by quantitative RT-PCR. Results are presented as means ± standard deviation of 5 independent experiments performed in triplicates. ** $P < 0.01$.

3. 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.

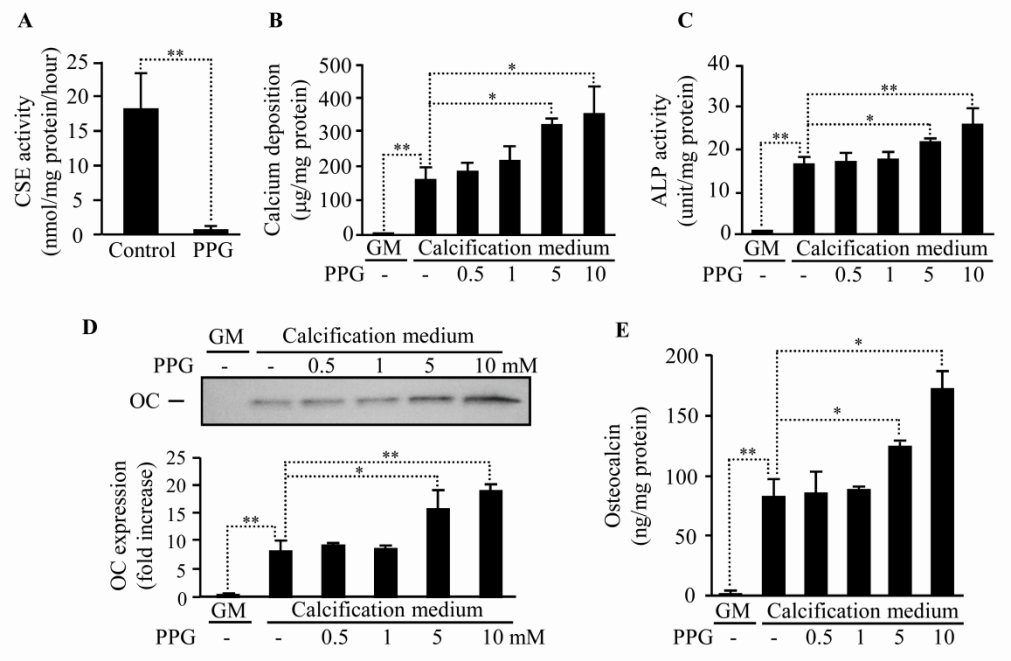


Figure 15. Inhibition of endogenous production of H_2S by PPG promotes osteoblastic differentiation of HAoSMC.

(A) CSE activity determined from HAoSMC in the absence or presence of PPG (10 mmol/L) is shown as means ± standard deviation of 3 independent experiments. **$P < 0.01$. (B-E) HAoSMC were cultured in GM or in calcification medium alone or in the presence of PPG (0.5, 1, 5 and 10 mmol/L) for 7 days. Extracellular calcium deposition (B), ALP activity (C), and osteocalcin expresssion (D-E) are shown. Data are expressed as means ± standard deviation of 3 independent experiments each performed in duplicates. *$P < 0.05$, ** $P < 0.01$.

Cells treated with PPG showed a gradual decrease in CSE enzyme activity (Fig 15A). Suppression of CSE by PPG almost doubled the deposition of calcium in the extracellular matrix of HAoSMC maintained in calcification medium for 7 days (Fig 15B) as compared to cells cultured in calcification medium without PPG. Accordingly, PPG provoked a significant additional increase in the activity of ALP (Fig 15C) and expression of osteocalcin (Fig 15D and E) by 73 and 120%, respectively. As a second approach to decrease CSE activity we transfected HAoSMC with CSE-specific small interfering RNA (siRNA). Transfection provided an approximately 70% decline in CSE enzyme activity, which was accompanied by increased calcium deposition in HAoSMC (Fig 16).

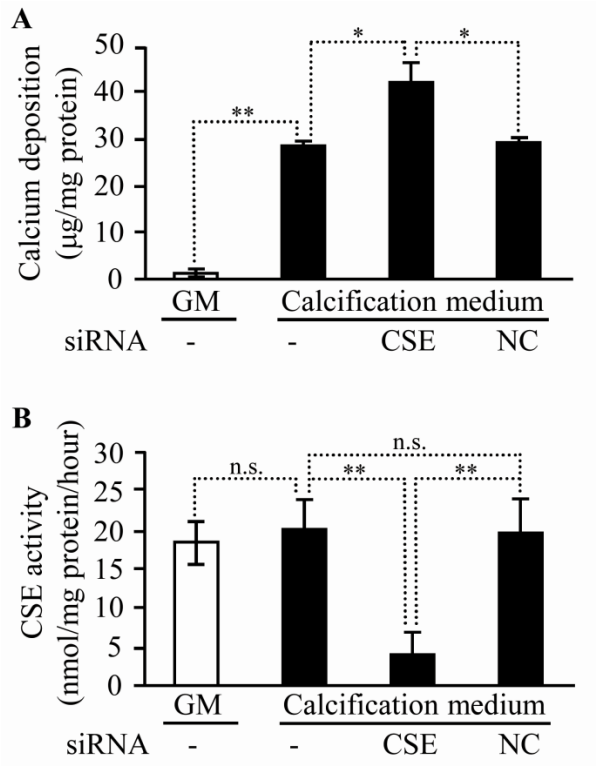


Figure 16. Silencing of cystathionine γ-lyase provokes mineralization of HAoSMC.
(A) Calcium deposition of HAoSMC transfected with siRNA for CSE or negative control siRNA (NC) after culturing cells in calcification medium for 4 days is shown as means ± standard deviation of 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. (B) CSE activity of transfected HAoSMC 48 hours post-transfection is shown as means ± standard deviation of 3 independent experiments. $^{**}P < 0.01$.

4. Decreased plasma H_2S levels are associated with reduced CSE activity in stage 5 CKD patients

In agreement with previous observations, 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 (Fig 17A). 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 HD and healthy controls. Levels of CSE mRNA and protein expression were similar in CKD patients and controls (Fig 17B, C, D). 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 (Fig 17 E, F).

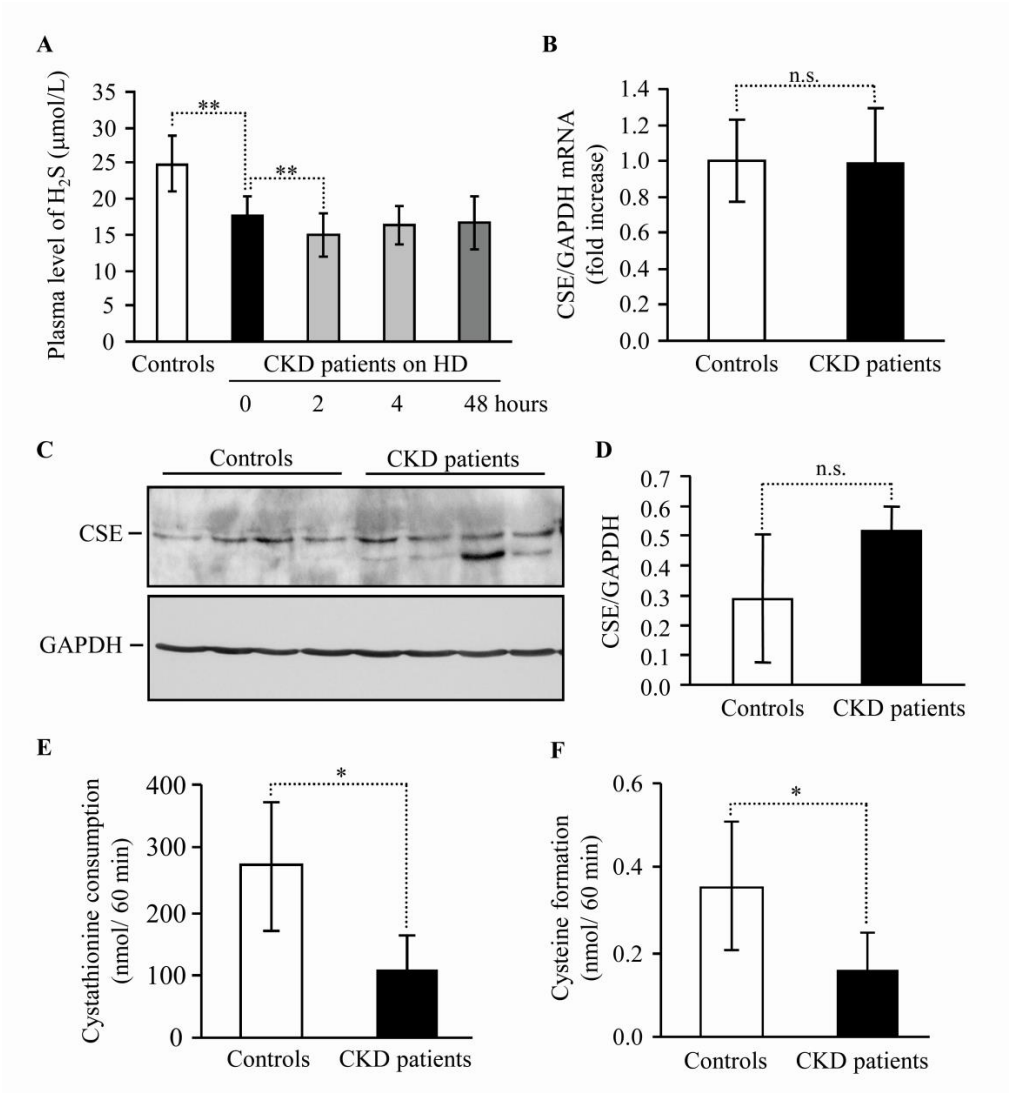


Figure 17. Decreased plasma level of H_2S and reduced CSE activity in stage 5 CKD patients.
(A) Plasma levels of hydrogen sulfide in healthy individuals (n=23) and in stage 5 CKD patients (n=21) on hemodialysis treatment at different time points is shown as means ± standard deviation ***P*<0.01. (B) CSE gene expression was measured by quantitative RT-PCR from PBMC mRNA isolated from controls (n=16) and stage 5 CKD patients (n=14). (C, D) CSE protein expression determined by western blot in PBMC isolated from controls (n=4) and stage 5 CKD patients (n=4). Levels of CSE were normalized to GAPDH. (E, F) CSE enzyme activities assessed by cystathionine consumption (E) and cysteine production (F) in PBMC isolated from controls (n=10) and stage 5 CKD patients (n=10) are shown as means ± standard deviation. **P*<0.05.

In conclusion, this study demonstrated a novel role of H_2S in the process of Pi provoked mineralization and transition of HAoSMC into osteoblast like cells (Fig 18). We provide evidence that H_2S regardless of its exogenous or endogenous origin inhibits the up-regulation of osteoblast specific genes such as ALP, osteocalcin and Cbfa1. 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 a new strategy to prevent vascular calcification.

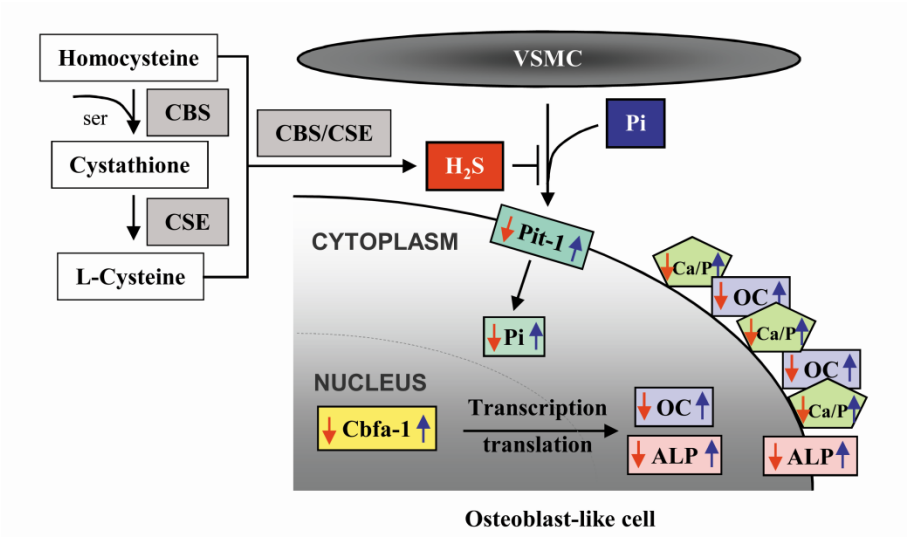


Figure 18. Scheme of H_2S biogenesis and its involvement in Pi-induced osteoblastic transformation of VSMC. H_2S is generated as an alternative product of the transsulfuration pathway. H_2S inhibits all the steps of osteoblast transition of VSMC. Pi-induced phosphate uptake, Pit-1 upregulation, Cbfa1, ALP, osetocalcin (OC) expression and Ca deposition are all inhibited by H_2S. Blue arrows represent responses to elevated Pi, whereas red arrows represent the effect of H_2S.

PUBLICATIONS

Sorszám	Közleményjegyzék	Dokumentum típusa	Impakt faktor	OTKA támogatás feltüntetve?	Támogató szervezetek
1.	Ferreira A, Marguti I, Bechmann I, Jeney V, Chora A, Palha NR, Rebelo S, Henri A, Beuzard Y, Soares MP: ***Sickle Hemoglobin Confers Tolerance to Plasmodium Infection***, CELL 145: (3) 398-409, 2011 *	folyóiratcikk	32.403	nem	
2.	Jeney V; Zarjou A; Arosio P; Poli M; Agarwal A; Balla G; Balla J: ***Ferritin/Ferroxidase Activity: A Potent Inhibitor of Vascular Calcification, Osteoblastic Transdifferentiation of Vascular Smooth Muscle Cells and Osteoblast Activity***, Ferritin: Funkctions, Biosynthesis and Regulation., 2011	könyvfejezet	-	igen	
3.	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 differentiation of vascular smooth muscle cells.***, Kidney Int. 2011 Oct;80(7):731-9., 2011	folyóiratcikk	6.105	igen	
4.	Oros M, Zavaczki E, Vadasz C, Jeney V, Tosaki A, 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, 2012	folyóiratcikk	4.125	igen	
5.	Jeney V, Eaton JW, Balla G, Balla J: ***Natural history of the bruise: formation, elimination, and biological effects of oxidized hemoglobin.***, OXID MED CELL LONGEV 2013: 703571, 2013 *	folyóiratcikk	3.393	igen	MTA, TÁMOP
6.	Potor L, Banyai E, Becs G, Soares MP, Balla G, Balla J, Jeney V: ***Atherogenesis may involve the prooxidant and proinflammatory effects of ferryl hemoglobin.***, OXID MED CELL LONGEV 2013: 676425, 2013 *	folyóiratcikk	3.393	igen	MTA, TÁMOP

SUBMITTED MANUSCRIPT

Besides the published works, I was invited to write a review article about the role of iron in atherosclerosis. The manuscript (see below) is under review at the special issue entitled The Importance Of Iron In Pathophysiologic Conditions.in Frontiers in Pharmacology. Because of size restrictions, I provide here the title page and the figures of the submitted manuscript.

Atherogenesis and iron: from epidemiology to cellular level

Running title: Atherogenesis and iron

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Abstract

Iron accumulates in human atherosclerotic lesions but whether it is a cause or simply a downstream consequence of the atheroma formation is still an open question. According to the so called “iron hypothesis”, iron is believed to be detrimental for the cardiovascular system, thus promoting atherosclerosis development and progression. Iron, in its catalytically active form can participate in the generation of reactive oxygen species and induce lipid-peroxidation, triggering endothelial activation, smooth muscle cell proliferation and macrophage activation; all of these processes are considered to be pro-atherogenic. On the other hand, the observation that hemochromatotic patients, affected by life-long iron overload, do not show any increased incidence of atherosclerosis is perceived as the most convincing evidence against the “iron hypothesis”.

Epidemiological data and animal models have provided conflicting data regarding the role of iron in atherogenesis, emerging the need for more careful studies in which issues like the source and the compartmentalization of iron would be addressed. This review article summarizes what we have learnt about iron and atherosclerosis from the epidemiological studies, animal models and cellular systems.

Keywords: atherosclerosis, iron, hemoglobin, heme, intraplaque hemorrhage

Figures

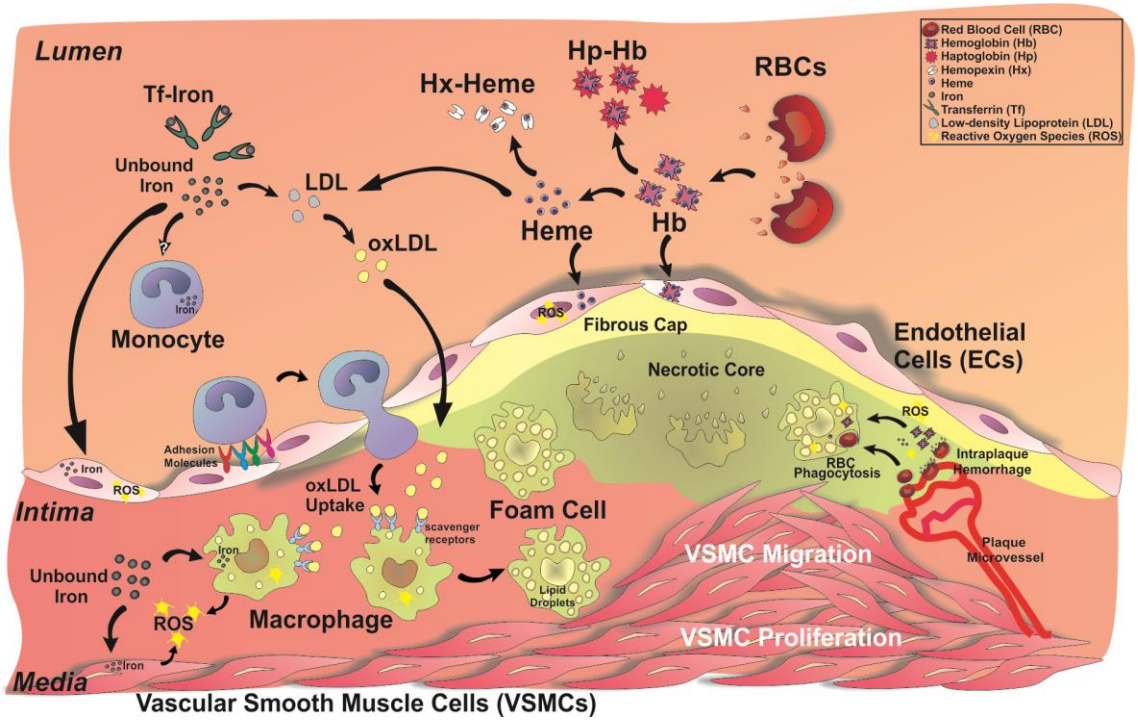

Figure 1.

How iron can access the lesions: a role for iron in atherosclerosis.

Iron can accumulate in the plaque as inorganic iron or hemoglobin-contained iron. Inorganic iron mainly derives from circulating iron, in the form of transferrin (Tf)-bound iron and non-transferrin-bound iron (NTBI). NTBI is generated upon increased iron absorption associated with pathological conditions, such as anemia and hemochromatosis. Circulating NTBI is thought to be accessible to many cell types in the context of an atherosclerotic plaque: endothelial cells, monocytes/macrophages and vascular smooth muscle cells (VSMCs). Hemoglobin- and heme-derived iron can access the plaque upon conditions of intravascular hemolysis and intraplaque hemorrhage, affecting endothelial cells and macrophages. Hemoglobin (Hb), heme and iron promote endothelial activation, by enhancing adhesion molecule expression. As a consequence, monocytes recruitment is expected to be increased. Circulating iron and Hb oxidize LDLs, thus enhancing subendothelial LDL retention and macrophage progression to foam cells. Iron also affects VSMC proliferation and migration into the lesion, favouring plaque progression.

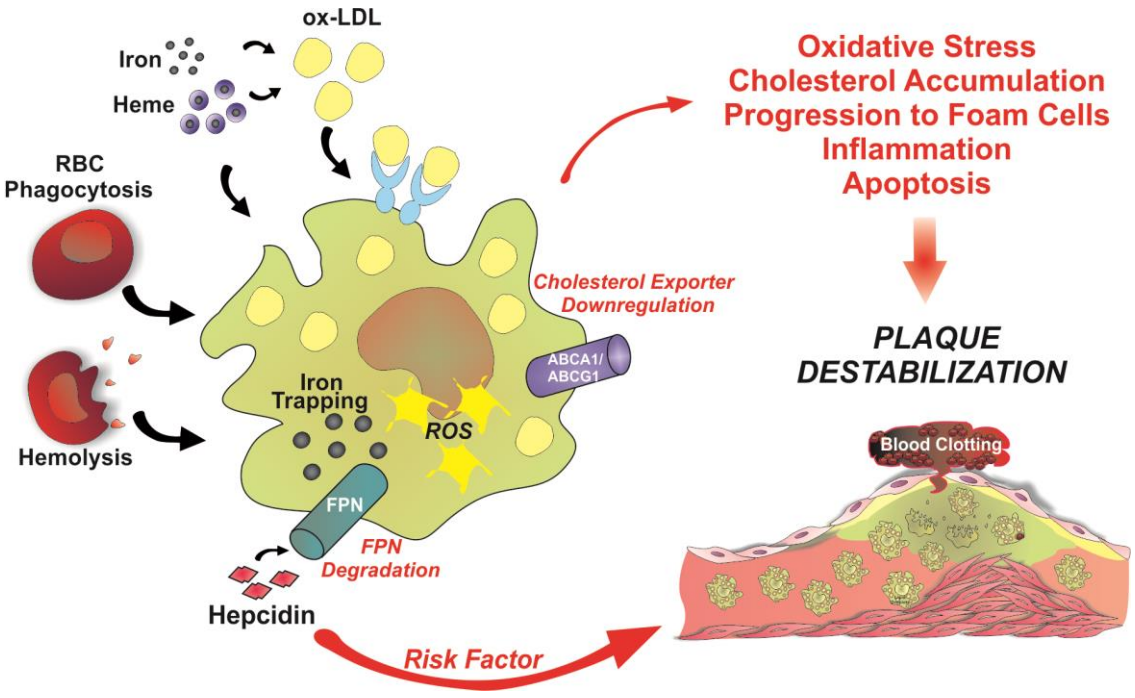

Figure 2.

Schematic overview of the refined iron hypothesis: a role for macrophage-retained iron in atherosclerosis.

Iron can be accumulated in the macrophage as inorganic iron and Hb-iron, upon erytrophagocytosis or hemolysis. Once stored into the cell, it can be recycled back via FPN-mediated export. According to the refined iron hypothesis, high hepcidin levels are considered a risk factor for plaque progression and destabilization. Hepcidin is known to bind FPN, thus promoting its degradation and blocking iron export. This is expected to increase intracellular ROS levels and to decrease cholesterol efflux. As a result, oxidative status alteration and LDL accumulation occur, promoting foam cell formation, inflammation and eventually plaque instability.

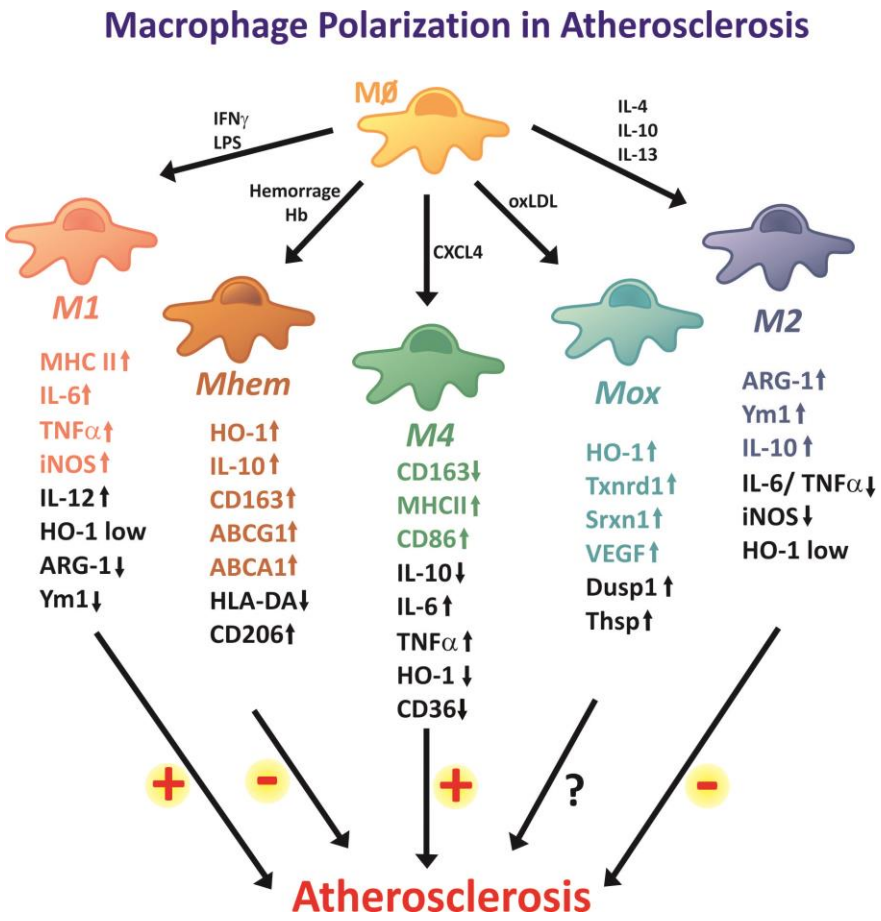

Figure 3.

Macrophage polarization in atherosclerosis.

In the atherosclerotic plaque macrophages could polarize towards different phenotypes, here depicted. The two most extreme phenotype are represented by M1 and M2 macrophages. M1 macrophages have strong pro-inflammatory properties, thus being potentially involved in lesion progression. M1 macrophages show high expression levels of iNOS, MHCII and inflammatory cytokines such as IL-6 and TNF-a. M2 macrophages are considered anti-inflammatory and involved in tissue repair and remodeling. M2 specific markers are Arginase 1, Ym1 and IL-10. The M2 phenotype is reported as anti-atherogenic. Between these two phenotypes, many others take place. Mhem macrophages originate upon intraplaque hemorrhage and are endowed with high Hb handling ability. These anti-atherogenic macrophages express high levels of the heme-degrading enzyme HO-1 and the Hp-Hb scavenger receptor CD163. Additionally, Mheme macrophages express the cholesterol exporters ABCA1 and ABCG1, thus efficiently activating reverse cholesterol efflux. Mox macrophages are generated upon oxidized phospholipid stimulation. They have anti-oxidant properties, since they express genes involved in the anti-oxidant response such as HO-1, Txnrd1 and Srxn1. Their potentially athero-protective effect still needs to be demonstrated. M4 macrophages generate in response to the chemo-attractant CXCL4, thus showing pro-inflammatory and pro-atherogenic effects. These macrophages express low levels of CD163 and high levels of MHCII and CD86. M1 and M4 macrophages promote, while M2 and Mhem macrophages counteract foam cell formation, thus having opposite effect on atherosclerosis progression.