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Forum Review

Heme, Heme Oxygenase, and Ferritin: How the Vascular Endothelium Survives (and Dies) in an Iron-Rich Environment

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ABSTRACT

Iron-derived reactive oxygen species are involved in the pathogenesis of numerous vascular disorders. One abundant source of redox active iron is heme, which is inherently dangerous when it escapes from its physiologic sites. Here, we present a review of the nature of heme-mediated cytotoxicity and of the strategies by which endothelium manages to protect itself from this clear and present danger. Of all sites in the body, the endothelium may be at greatest risk of exposure to heme. Heme greatly potentiates endothelial cell killing mediated by leukocytes and other sources of reactive oxygen. Heme also promotes the conversion of low-density lipoprotein to cytotoxic oxidized products. Hemoglobin in plasma, when oxidized, transfers heme to endothelium and lipoprotein, thereby enhancing susceptibility to oxidant-mediated injury. As a defense against such stress, endothelial cells upregulate heme oxygenase-1 and ferritin. Heme oxygenase opens the porphyrin ring, producing biliverdin, carbon monoxide, and a most dangerous product—redox active iron. The latter can be effectively controlled by ferritin *via* sequestration and ferroxidase activity. These homeostatic adjustments have been shown to be effective in the protection of endothelium against the damaging effects of heme and oxidants; lack of adaptation in an iron-rich environment led to extensive endothelial damage in humans. *Antioxid. Redox Signal.* 9, 2119–2137.

INTRODUCTION

HEME IS THE MOST IMPORTANT IRON COMPLEX in the human body and is responsible for oxygen and electron transport, among other functions. Unfortunately, in certain situations, heme may be released from heme proteins, and both the heme and iron released from it may subsequently catalyze free radical reactions. Of all sites in the body, the vasculature—and in particular, the endothelial lining—may be at greatest risk of exposure to free heme. This is because erythrocytes contain heme in a concentration of 20 m*M* and are vulnerable to unexpected lysis. Extracellular hemoglobin is easily oxidized from ferro- to ferri-hemoglobin (methemoglobin), which, in turn, will readily release heme. Given the hydrophobic nature of heme, it is no surprise that it easily crosses cell membranes and can synergistically enhance cellular oxidant damage. The present review concerns the involvement of heme in vascular endothelial cell injury and the strategies used by endothelium to minimize such damage.

FREE HEME AND HEME IRON ARE TOXIC TO ENDOTHELIUM

Cell and organ damage provoked by reactive oxygen species can be greatly amplified by "free" redox active iron (56). For example, iron-rich *Staphylococcus aureus* is three orders of magnitude more susceptible to killing by hydrogen peroxide

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than are iron-poor staphylococci (105). Conversely, depletion of cellular iron powerfully protects eukaryotic and prokaryotic cells against oxidant challenge (48). We have shown that one critical feature required for iron-mediated damage to endothelium is intrusion of the metal into cells. Chelation of iron by certain lipophilic chelators, such as 8-hydroxyquinoline, results in the accumulation of catalytically active lipophilic iron chelates in endothelial lipid compartments; endothelium pretreated with the 8-hydroxyquinoline-iron chelate is exquisitely sensitive to both endogenous and exogenous oxidant stress (10).

One abundant source of potentially toxic iron is iron protoporphyrin-IX or heme, which is also hydrophobic. Heme, a ubiquitous iron-containing compound, is present in large amounts in many cells (98) and is also inherently dangerous, particularly when it escapes from intracellular sites (9, 11, 12, 97). Heme greatly amplifies cellular damage arising from activated oxygen (Fig. 1A) (9, 11, 12).

The potential toxicity of free heme derives from the ease with which this highly hydrophobic compound can enter and cross cell membranes; heme readily concentrates within the hydrophobic milieu of intact cells (9, 11). Nitric oxide was shown by Foresti *et al.* (47) to augment the incorporation of heme in endothelium. This effect may result from formation of heme–nitrosyl complexes and the altered permeability of membranes to heme (46). Heme uptake by endothelial cells can exacerbate

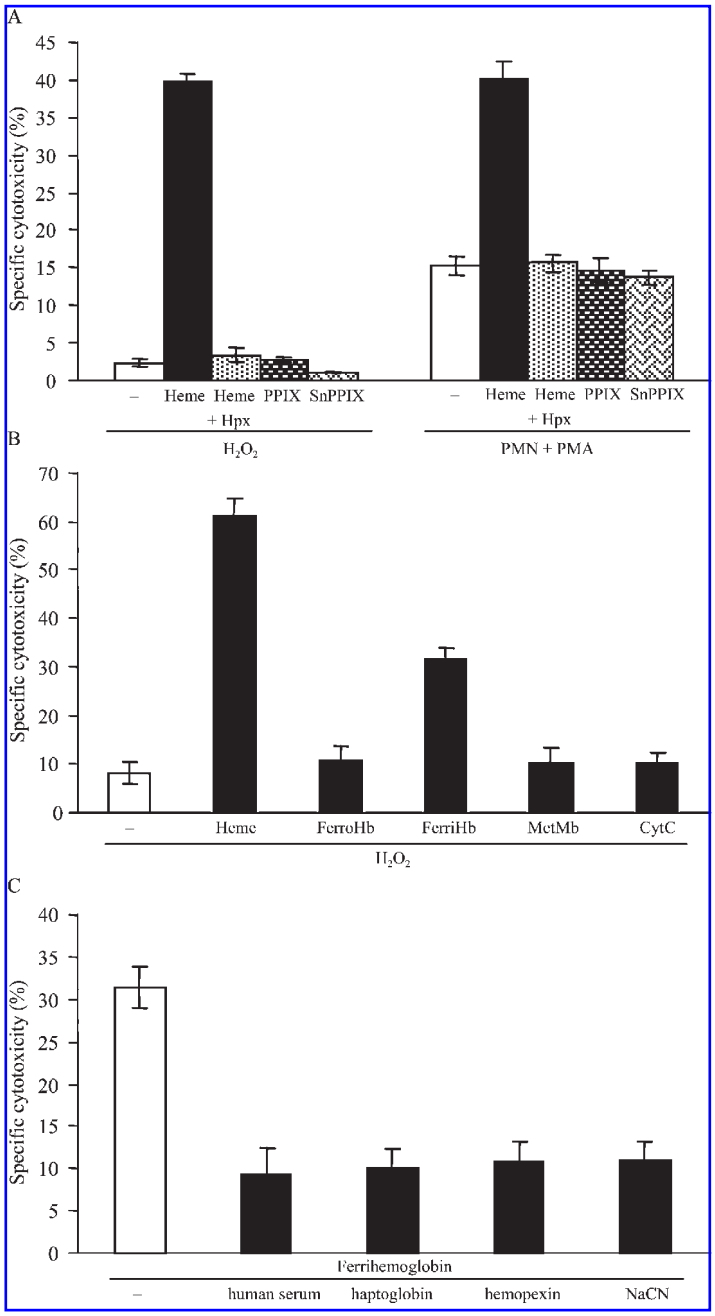

FIG. 1. Direct cytotoxic effects of free heme or heme released from ferrihemoglobin on endothelial cells. (**A**) Confluent porcine aortic endothelial cells (PAECs) were loaded with 5 μM heme alone, heme precomplexed with hemopexin, protoporphyrin IX (PPIX), or tin protoporphyrin (SnPPIX) for 1 h. After washing, cells were exposed to H_2O_2 (100 μM) or PMA-stimulated PMNs for 2 h. From Balla G, Vercellotti GM, Muller-Eberhard U, Eaton J, and Jacob HS. Exposure of endothelial cells to free heme potentiates damage mediated by granulocyte and toxic oxygen species. *Lab Invest* 64: 648–655, 1991, with permission). (**B**) Confluent PAECs were pretreated with heme (5 μM), ferrohemoglobin (10 μM), ferrihemoglobin (10 μM), metmyoglobin (40 μM), or oxidized cytochrome *c* (40 μM) for 1 h, and then exposed to H_2O_2 (100 μM) for 2 h. (**C**) PAECs were pretreated with ferrihemoglobin (10 μM) alone or in the presence of 15% human serum, or stoichiometric amounts of haptoglobin or hemopexin, or cyanomethemoglobin (10 μM) for 1 h. Cytotoxicity was provoked by H_2O_2 (100 μM for 2 h). In all experiments, specific cytotoxicity was determined by ^{51}Cr-release assay. Expressed as mean ± SEM of three independent experiments performed in duplicate. From ref. 15, with permission.

their damage by activated polymorphonuclear leukocytes (PMNs) (see Fig. 1A)—cells that tend to marginate along endothelial surfaces in the presence of diverse inflammatory mediators (9, 11, 12). Intriguingly, heme was shown by Graca-Souza and colleagues (52) to induce PMN activation as well. Moreover, Wagener *et al*. (131, 132) reported that heme can enhance endothelial cell adhesion molecule expression, increasing PMN adhesion and provoking inflammation.

The uptake of heme is required for this synergistic toxicity, and the hydrophobicity of heme is critical for entry into endothelial cells. The spontaneous uptake of heme and the associated amplification of cellular oxidant sensitivity are both inhibited by the heme-binding protein, hemopexin (see Fig. 1A) (9, 11), which blocks the catalytic (*i.e*., peroxidase) activity of heme and promotes its clearance (9, 11, 40, 54). Hemopexin is certainly not the sole factor in plasma that protects against heme-amplified oxidant damage to endothelium. Albumin may also limit the intrusion of extracellular heme into cells and lessen its prooxidant effects. Heme bound to hemopexin or albumin is in ferric form.

Once within the cell, heme can promote oxidative damage either directly or, perhaps more important, through the release of iron, which can occur through either nonenzymatic oxidative degradation of heme (9, 11) or enzymatic, heme oxygenase–catalyzed heme cleavage. In either case, the iron may initially lodge within the hydrophobic interstices of the phospholipid bilayer. Within this highly oxidizable matrix, iron acts as an especially active catalyst of oxidation of cell membrane constituents (11).

Given the protective effects of plasma constituents such as hemopexin and albumin, we wondered whether heme could sensitize endothelial cells to oxidative challenge in the presence of plasma (12). Exposure of endothelium to heme in the presence of whole human plasma also synergizes cellular damage caused by exogenous oxidants, with an optimal heme-exposure duration of 60 min (12). Interestingly, cytotoxicity studies showed little toxicity to endothelium if water-soluble heme arginate is added instead of heme (12). This is so although exposure of endothelium to heme arginate in plasma-free medium increases endothelial cell heme content to an extent similar to what is observed after heme treatment. Comparable heme uptake is also observed in the presence of human plasma, although at 2 orders of magnitude greater concentration for both heme arginate and heme.

The hydrophobicity of various heme analogues (ferriporphyrins) is critical for entry into cells and required for the synergistic oxidative toxicity. Substitution of vinyl side chains of heme with hydrogen does not alter the hydrophobicity of the resultant ferriporphyrin (iron deuteroporphyrin IX), and cytotoxicity remains high. In contrast, if water solubility of heme is increased with the arginate counter ion or the vinyl side chains of heme are substituted by sulfonate, propionate, or glycol, leading to hydrophilic ferriporphyrins (iron deuteroporphyrin IX,2,4-bis-sulfonate, iron coproporphyrin III, and iron deuteroporphyrin IX,2,4-bis-glycol), these ferriporphyrins fail to sensitize cells to oxidants or activated polymorphonuclear leukocytes (12). Although free heme is rapidly incorporated into hydrophobic domains of cells and serves as a source of highly damaging iron, the question remains whether intact heme liganded to proteins, as in hemoglobin, might also transfer heme to vascular endothelium. Whereas reduced (ferro- or oxy-) hemoglobin is relatively innocuous to endothelial cells, oxidized (ferri- or met-) hemoglobin greatly amplifies oxidant-mediated endothelial injury (see Fig. 1B) (14, 15). This is because ferrihemoglobin readily releases its heme moieties, as first demonstrated by Bunn and Jandl (23). Released heme from ferrihemoglobin is rapidly incorporated into hydrophobic domains of cultured endothelium and serves as a source of highly damaging iron. Although ferrohemoglobin itself is not capable of sensitizing vascular endothelial cells to oxidant injury, we and others have shown it can readily be oxidized to heme-releasing ferrihemoglobin in the presence of inflammatory cell–derived oxidants (15, 32, 135). For instance, polymorphonuclear leukocytes, when activated with phorbol ester, rapidly oxidize ferrohemoglobin to ferrihemoglobin (15). Accordingly, ferrohemoglobin oxidized to ferrihemoglobin by activated PMNs can provide heme to endothelium, which greatly enhances cellular susceptibility to oxidant-mediated cell injury (14, 15). Another candidate for generating ferrihemoglobin is nitric oxide. Reaction of nitric oxide with free hemoglobin produces ferrihemoglobin and leads to decreased nitric oxide bioavailability, causing pulmonary hypertension, vascular damage, and end-organ injury, as reviewed by Gladwin *et al.* (50). Recent articles by Foresti and colleagues (46, 47) demonstrated that heme derived from hemoglobin can interact with nitric oxide to enhance heme uptake.

The initial release of heme from ferrihemoglobin can be inhibited by complexing with the hemoglobin-binding protein, haptoglobin (23). If metheme binding to globin is strengthened by haptoglobin, cyanide, or the released heme is religanded to hemopexin, ferrihemoglobin loses much of its capacity to sensitize endothelium to reactive oxygen (see Fig. 1C) (15). The hemoglobin:haptoglobin complex is eliminated from the circulation, at least in part through the recently characterized CD163 receptor (70), which is expressed exclusively by cells of the monocyte–macrophage lineage.

The importance of heme release from ferrihemoglobin in such toxicity is emphasized by the fact that ferrohemoglobin or other heme proteins, such as metmyoglobin and cytochrome *c*, none of which readily release heme (115), do not alter endothelial integrity (see Fig. 1B). At higher concentrations of free ferrihemoglobin in plasma (such as might occur in certain hemolytic diseases, atherosclerosis, and malaria infections), the normal mechanisms for control of hemoglobin (haptoglobin/hemopexin) can be overwhelmed, and released heme will enter the endothelial cells.

These previous studies and others indicating that hemoglobin might behave as a "biologic Fenton reagent" (108, 109) made us wonder whether hemoglobin in plasma could provide heme-iron to endothelium *in vivo*. As was the case *in vitro*, we found *in vivo* that oxyhemoglobin does not serve as a source of damaging heme-iron to endothelium. In contrast, oxidation of hemoglobin to ferrihemoglobin by phagocyte-mediated oxidation fosters transfer of heme to the vessel wall and aggravates endothelial cell damage in the short term. Ferrihemoglobin present in plasma also increases the level of endothelial cell–associated heme in lung (16), indicating that protective effects of haptoglobin (55), hemopexin (11, 40, 54), and albumin can be overwhelmed and the delivery of heme-iron to the endothelium can occur *in vivo* (16).

HEMOGLOBIN-, HEME- AND IRON-MEDIATED OXIDATION OF LOW-DENSITY LIPOPROTEIN (LDL)

It appears that endothelium may not be the only target of heme-mediated oxidation reactions (Fig. 2). Oxidative modification of LDL is implicated in the pathogenesis of atherosclerosis (8, 41, 59, 106, 118, 126, 138). LDL particles entering subendothelial "sanctuaries" within the artery wall can become trapped and exposed to oxidative stresses. LDL oxidation has been shown to foster recruitment of macrophages and, by binding to scavenger receptors on the surface of macrophages, oxidized LDL can ultimately generate foam cells. Oxidized LDL (hereafter, LDLox) is also directly cytotoxic, particularly to vascular endothelial cells. Such damage would presumably exacerbate atheroma formation both by allowing LDL to penetrate the endothelial barrier and promoting platelet adherence and smooth muscle growth factor production.

We earlier proposed that heme might be a possible physiologic mediator of LDL oxidation and subsequent endothelial cell injury (8). The process of heme-mediated LDL oxidation involves coupled interactions between LDL, heme, oxidants, and antioxidants. The initial step in these complex reactions is the spontaneous insertion of heme into LDL particles. The inserted heme directly promotes extensive oxidative modification of LDL; such modification can be amplified by trace amounts of hydrogen peroxide (Fig. 3), PMN-derived oxidants, or preformed lipid hydroperoxides within the LDL. Depletion of α-tocopherol in LDL is followed by the formation of conjugated dienes, lipid hydroperoxides, and thiobarbituric acid–reactive substances.

Heme will oxidatively modify both the lipid moiety of LDL and the apoprotein; the latter can be detected through increased anodal electrophoretic mobility. This increased mobility reflects a loss of net positive charge, arising from oxidative destruction of amine groups, which can also be assayed independently by measurement of free amino groups on the LDL particles. Fluorescamine-titratable free amino groups of apolipoprotein B-100 progressively decrease during exposure of LDL to heme. During these oxidative reactions between heme, LDL, and peroxides, the heme ring (protoporphyrin IX) is degraded, with resultant release of free iron. Both the destruction of the porphyrin ring and the release of ferrozine-trappable free iron are evidently involved in LDL oxidation. The oxidative scission of the porphyrin ring, presumably *via* reaction with lipid hydroperoxides, can be detected spectrophotometrically by the decrease in heme absorption at 412 nm (see Fig. 3). The subsequent release of free iron results in iron catalysis of oxidation of further heme, fatty acids, cholesterol, and apolipoprotein B-100 in LDL particles. The importance of this degradation is emphasized by the fact that conjugated diene formation occurs in parallel with the release of iron. The released iron-driven component in heme-mediated LDL oxidation is critical because desferrioxamine attenuates both the oxidative modification of LDL and the degradation of heme (8).

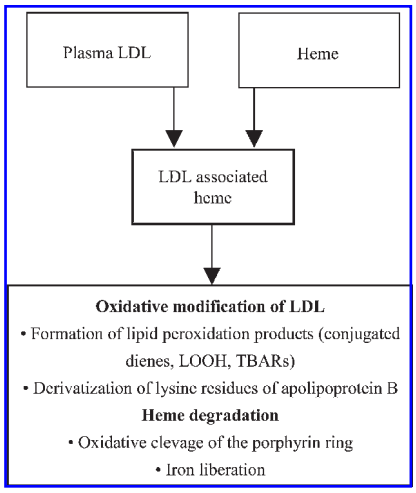

FIG. 2. Low-density lipoprotein is a target of heme-mediated oxidation reactions.

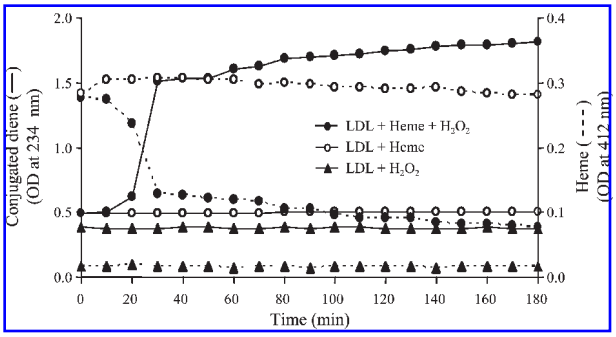

FIG. 3. Heme promotes oxidative modification of low-density lipoprotein. LDL (100 μg/ml protein) was incubated with heme alone (5 μM, *open circles*), H_2O_2 alone (100 μM, *solid triangles*), or both heme and H_2O_2 (*solid circles*) for 3 h at 37°C. Concentrations of conjugated dienes (*solid lines*) and heme (*dashed lines*) were determined spectrophotometrically in every 10 min. These results are representative of three experiments. This research was originally published in *Arterioscler Thromb.* From ref. 8, with permission.

The requirement for intimate association between LDL and heme in LDL oxidation is supported by experiments using hemopexin. This serum protein, present at remarkably high concentrations in plasma (≈1 g/L), binds heme with extraordinary avidity (113) and will prevent insertion of heme into LDL (8). Not surprisingly, hemopexin, in stoichiometric amounts with heme, inhibits oxidative modification of LDL and subsequent endothelial cell damage (Fig. 4). As would also be expected, given the exceptional affinity of hemopexin for heme, other proteins such as haptoglobin and albumin at equimolar concentration do not protect LDL from heme-catalyzed oxidation. Potentially relevant to *in vivo* vascular damage are studies demonstrating that activated PMNs potentiate oxidation of LDL catalyzed by heme-iron. That such heme-induced LDL oxidation may be involved in vascular damage is supported by the finding that LDL oxidized by heme is extremely cytotoxic to endothelial cells (see Fig. 4A). Lipid-soluble butylated hydroxytoluene, probucol, and the 21-amino steroid lazaroid, U74500A, are all potent inhibitors of heme-catalyzed LDL oxidation and subsequent cytotoxicity (see Fig. 4B). Although the reaction of LDL with heme produces a markedly toxic oxidized

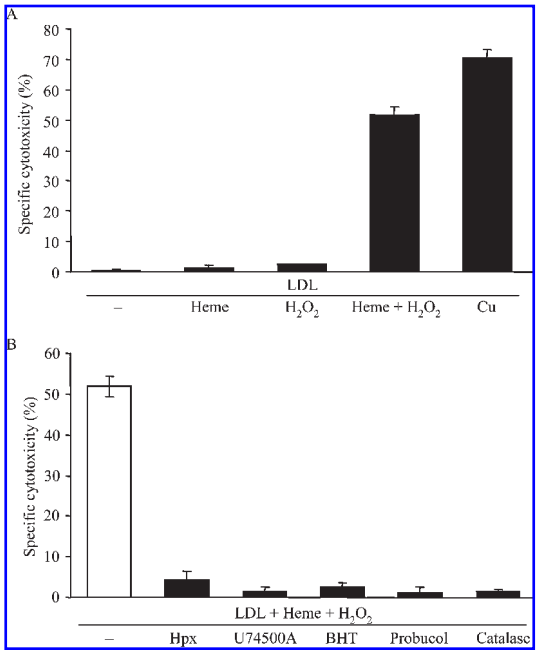

FIG. 4. Heme is indirectly cytotoxic to endothelial cells through the induction of oxidative modification of LDL. (**A**) LDL (200 μg/ml) was incubated with heme (5 μM), or H_2O_2 (25 μM), or both heme and H_2O_2 or $CuSO_4$ (5 μM) for 3 h at 37°C. (**B**) Inhibitors used during the 3-h LDL oxidation were hemopexin (5 μM), U74500A (a "lazaroid" that, like vitamin E, suppresses lipid peroxidation) (25 μM), butylated hydroxytoluene (BHT) (25 μM), probucol (25 μM), and catalase (20 μg/ml). (**A, B**) Confluent ^{51}Cr-loaded PAECs were treated with LDL samples for 8 h, and specific cytotoxicity was determined by ^{51}Cr-release assay. Results represent mean ± SEM of three experiments performed in duplicate. This research was originally published in *Arterioscler Thromb.* From ref. 8, with permission.

LDL in <2 h, more prolonged incubation actually reduces the LDL toxicity (Fig. 5A). Measurement of lipid peroxidation products led us to the conclusion that a strong connection exists between the lipid hydroperoxide content and the toxicity of the LDL oxidized by heme (see Fig. 5B). This is in agreement with other experiments wherein specific enzymatic reduction of LOOH to LOH yields LDL with minimal toxic effect (*vide infra*).

If water-soluble heme (heme arginate) is added to LDL, lipid peroxidation is characterized by a longer lag phase and ΔT at V_{max} as well as a slower propagation phase compared with heme-mediated lipid peroxidation of LDL, as judged by conjugated diene formation (12). The results of several independent assays for LDL oxidation stimulated by heme or heme arginate all support the conclusion that heme arginate promotes LDL oxidation less efficiently. Accordingly, the cytotoxicity of heme arginate–conditioned LDL to endothelial cells was significantly less than endothelial cell cytotoxicity evoked by LDL conditioned with heme. However, the number of heme molecules associated with LDL particles was the same in LDL exposed to heme *versus* LDL exposed to heme arginate (both in serum), suggesting that the more efficient oxidation of LDL by heme *versus* heme arginate is partially a function of increased hydrophobicity. In diluted serum (20%), Camejo (25) also observed heme binding to LDL leading as well as its oxidation in the presence of hydrogen peroxide.

Continuous monitoring of the process of oxidative modification of LDL is possible by measuring the decreasing absorbance of heme at 405–412 nm, because in heme-catalyzed oxidation of lipoprotein, heme degradation occurs concurrent with formation of lipid oxidation products, including conjugated dienes and lipid hydroperoxide. Thus, heme degradation functions as a probe for lipid peroxidation processes (8). Based on the kinetics of heme-catalyzed lipid peroxidation, we developed an assay for the clinical laboratory to judge the susceptibility of LDL to oxidative modification (127), a risk factor of atherosclerosis. The oxidative resistance of LDL was characterized by ΔT at maximum velocity (V_{max}) in seconds; the time point of maximal velocity of heme degradation as defined by the maximal change in absorbance of heme in the propagation phase of lipid peroxidation. The shortening of ΔT at V_{max} indicates a decrease in the oxidative resistance of LDL. This novel assay is suitable for testing large numbers of LDL samples on an automated microplate reader. The advantages of

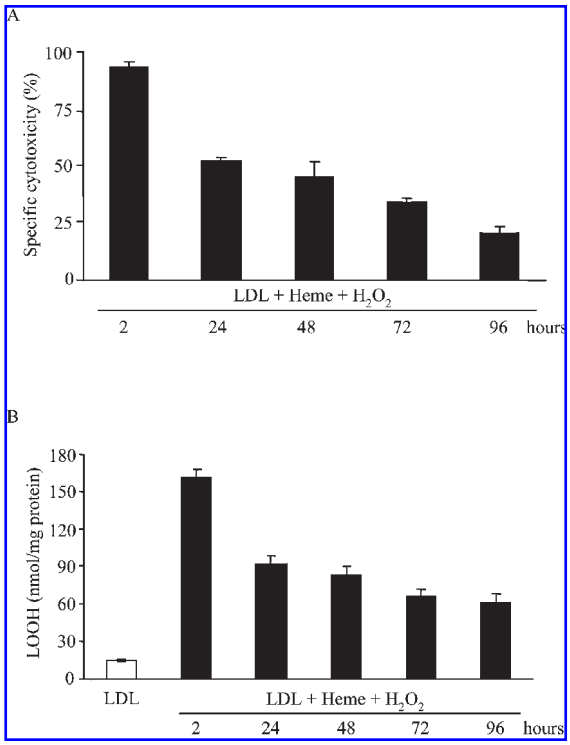

FIG. 5. Cytotoxicity of oxidized LDL is strongly dependent on its lipid hydroperoxide content. LDL (200 μg/ml) was oxidized with heme (5 μM) and H_2O_2 (75 μM) for 2, 24, 48, 72, and 96 h at 37°C. (**A**) Confluent HUVECs were treated with LDL samples for 3 h, and specific cytotoxicity was measured by MTT assay. (**B**) Lipid hydroperoxide content of LDL samples was determined by the FOX assay. All parameters were measured by using four different LDL samples in triplicate. The results are presented as mean ± SEM. This research was originally published in *Cell Mol Biol (Noisy-le-grand)*. From ref. 87, with permission.

our method over existing measurements (38, 42, 102) are the ability to follow the kinetics of LDL lipid peroxidation at a visible wavelength and to use Na_2EDTA during isolation and analysis of LDL.

In addition to free heme (8, 83), a number of heme proteins—such as hemoglobin (95), myoglobin (61), horseradish peroxidase (137), myeloperoxidase (111), and lipoxygenase (71, 117)—have been reported to act as oxidants of LDL. However, the mechanisms involved are by no means clear. In a plasma-free model, hemoglobin reacting with hydrogen peroxide was shown to induce lipid peroxidation of LDL accompanied by oxidative cross-linking of apolipoprotein B-100 *via* the formation of ferryl hemoglobin and the subsequent generation of radicals on the globin surface (82). The authors of that study concluded that negligible heme transfer from hemoglobin to LDL, or none at all, occurred under the oxidative conditions they used. Oxidation of hemoglobin to the ferryl state by peroxides has been reported to be accompanied by tyrosyl radical formation (49, 81). In end-stage renal failure patients receiving prolonged hemodialysis therapy, a high degree of apolipoprotein B-100 modification resulting from covalent association of hemoglobin with LDL was observed (141). The authors postulated that a tyrosyl radical species of hemoglobin that forms by oxidation of ferrihemoglobin to ferrylhemoglobin with hydrogen peroxide induces cross-linking of LDL, accompanied by an increase in dityrosine formation, and the modification of lipoprotein occurs through a mechanism independent of lipid peroxidation.

Our studies offer an alternative pathway for modification of LDL by hemoglobin in plasma involving heme release from ferrihemoglobin. The results reported (64) generally support such a mechanism insofar as maneuvers that restrict heme transfer to LDL uniformly diminish or block LDL oxidation. We hypothesized that oxidation of free hemoglobin in plasma could threaten vascular endothelial cell integrity *via* oxidative modification of LDL by heme. Indeed, LDL isolated from plasma incubated with either ferrihemoglobin or heme was found to be markedly cytotoxic. In contrast, LDL isolated from plasma incubated with ferrohemoglobin or other heme proteins such as metmyoglobin or cytochrome *c*, all of which avidly bind heme, failed to harm endothelial cell monolayers. These results suggest that the release of heme from ferrihemoglobin is an important precedent event in generating toxic (presumably oxidized) LDL. Therefore, we conducted similar experiments by using various strategies to stabilize the heme moiety. Preincubation of ferrihemoglobin with sodium cyanide or stoichiometric amounts of haptoglobin stabilized the heme–globin complex and prevented the generation of LDLox. These findings might explain why haptoglobin polymorphisms were found in clinical studies to be a risk factor in the pathogenesis of atherosclerosis (33).

In studies, Shaklai's group (18) recently revealed that haptoglobin phenotypes differ in their ability to inhibit heme transfer from hemoglobin to LDL. Heme transfer from ferrihemoglobin to LDL was demonstrated to be almost completely blocked by haptoglobin 1-1 but only partially by haptoglobin 2-2. Accordingly, haptoglobin 1-1 was shown to inhibit hemoglobin-induced oxidation of lipoprotein more vigorously than haptoglobin 2-2. This difference in "antioxidant" capacity of different haptoglobin types may help explain why individuals with haptoglobin 2-2 have more severe atherosclerotic disease compared with those with haptoglobin 1-1 (33).

Although ferrohemoglobin in plasma does not itself provoke oxidation of LDL, as mentioned earlier, hemoglobin can readily be oxidized to heme-releasing ferrihemoglobin in the presence of inflammatory cell–derived oxidants (12, 15, 135). Accordingly, if endothelial cells are exposed to LDL isolated from plasma containing ferrohemoglobin and activated PMNs, oxidative endothelial damage develops (64). Importantly, neither activated PMNs alone nor ferrohemoglobin alone causes the generation of cytotoxic LDL. Oxidation of ferrohemoglobin by activated PMNs in plasma can be inhibited by catalase. Accordingly, LDL isolated from plasma containing ferrohemoglobin, activated PMNs, and catalase has reduced endothelial cell cytotoxicity.

In a recent study, we showed that LDL-associated lipid hydroperoxides convert ferrohemoglobin to ferrihemoglobin in a dose-dependent manner as well (Fig. 6) (87). Reduction of the lipid hydroperoxide content of LDL with GSH/glutathione peroxidase prevents the formation of ferrihemoglobin. Interestingly, haptoglobin does not inhibit this oxidation but does prevent heme release from the resultant ferrihemoglobin.

The results of several independent assays for LDL lipid peroxidation support the conclusion that ferrihemoglobin-derived heme promotes LDL oxidation (Table 1) (64). Shorten-

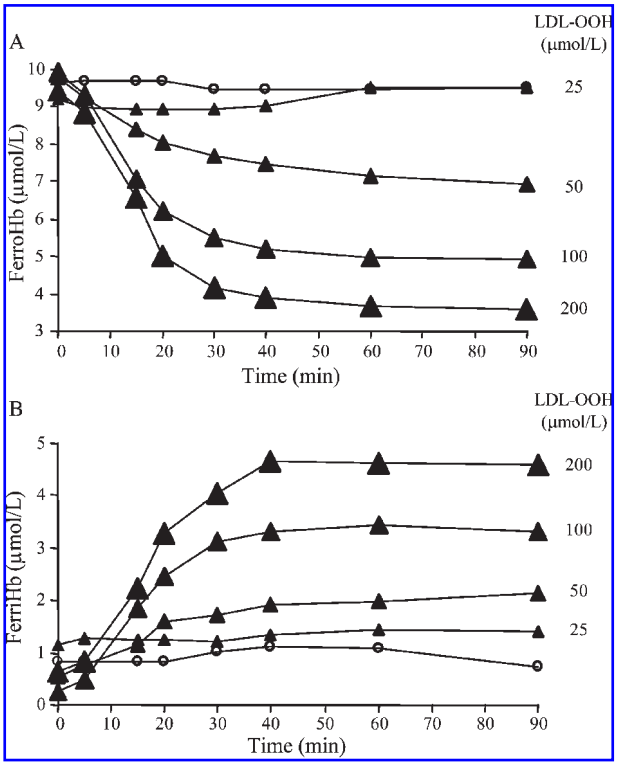

FIG. 6. LDL-associated lipid hydroperoxides provoke oxidation of ferrohemoglobin to ferrihemoglobin. Ferrohemoglobin (10 μM) was treated with LDL samples containing 25, 50, 100, and 200 μM LDL-associated lipid hydroperoxide (*solid triangles*). *Open circles*, Autooxidation of ferrohemoglobin. Concentration of ferrohemoglobin (**A**) and formation of ferrihemoglobin (**B**) were monitored for 90 min. Figures are representatives of three experiments. This research was originally published in *Cell Mol Biol (Noisy-le-grand)*. From ref. 87, with permission.

TABLE 1. OXIDATIVE RESISTANCE, CONJUGATED DIENES, THIOBARBITURIC ACID—REACTIVE SUBSTANCES, TOTAL LIPID HYDROPEROXIDE, AND α-TOCOPHEROL CONTENT OF LOW-DENSITY LIPOPROTEIN FROM NORMAL PLASMA TREATED WITH HEME OR HEMOGLOBIN

Treatment	*ΔT at V_{max} (sec)*	*Conj. Dien‡ (OD at 234)*	*TBARSs (nmol/mg LDL)*	*Total LOOHs (nmol/mg LDL)*	*α-Tocopherol (mol/mol ApoB100)*
PI	3,120 ± 272	0.175 ± 0.011	0.32 ± 0.09	5.34 ± 2.28	7.71 ± 2.26
Pl + heme (80 μM)	150 ± 118	0.483 ± 0.099	14.22 ± 1.46	142.82 ± 25.93	0.8 ± 0.86
Pl + ferroHb (20 μM)	3,190 ± 346	0.17 ± 0.05	0.34 ± 0.21	7.5 ± 4.49	7.59 ± 0.93
Pl + metHb (20 μM)	300 ± 189	0.321 ± 0.026	6.65 ± 0.52	101.5 ± 35.02	0.71 ± 0.78
Pl + metHb + Hp (20 μM)	3,721 ± 762	0.189 ± 0.027	0.21 ± 0.12	5.04 ± 1.29	6.88 ± 0.39
Pl + cyanometHb (20 μM)	3,480 ± 722	0.166 ± 0.005	0.31 ± 0.14	4.72 ± 3.17	7.29 ± 1.09

Pl, plasma; Hp, haptoglobin; Conj dien, conjugated dien; TBARSs, thiobarbituric acid–reactive substances; LOOH, lipid hydroperoxide.

This research was originally published in *Blood*. Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, Eaton JW, Balla G. Pro-oxidant and cytotoxic effects of circulating heme. *Blood* 100: 879–887, 2002 (with permission).

ing of ΔT at V_{max} by ferrihemoglobin is paralleled by a rapid decrease in the α-tocopherol content of LDL, which is followed by the formation of conjugated dienes, lipid hydroperoxides (LOOHs), and thiobarbituric acid–reactive substances (TBARS). In contrast, ferrihemoglobin complexed with haptoglobin or cyanomethemoglobin does not alter either ΔT at V_{max} or the α-tocopherol content of LDL. This also prevents the generation of conjugated dienes, lipid hydroperoxides, and TBARs in LDL.

These observations raised the question of the nature of the toxic substance(s) that might arise from hemoglobin/heme iron-mediated LDL oxidation. Oxidation of LDL leads to formation of a wide range of biologically active products, and some of these, such as 7β-hydroperoxycholesterol (63) and 7-oxysterols (75), have been reported to be highly cytotoxic. In this regard, ebselen, a seleno organic compound that has hydroperoxide reducing activity, protects against LDLox-induced cell death in human fibroblast cells (30). It appears that accumulated LOOH is the predominant toxic species within LDLox catalyzed by heme because specific enzymatic reduction of LOOH to LOH yields LDL with minimal toxic effects (64). Furthermore, we find that, on an equimolar basis, LOOH within LDLox and an organic hydroperoxide, cumene hydroperoxide, have very similar toxic effects on endothelial cells.

HOW ENDOTHELIUM ADAPTS AND SURVIVES IN AN IRON-RICH ENVIRONMENT: HEME OXYGENASE-1 AND FERRITIN

Early support for the notion of heme oxygenase and ferritin being an antioxidant cytoprotective stratagem of endothelium derived from the strange time-dependent effects of heme exposure on the oxidant sensitivity of endothelial cells (Fig. 7) (7). After a brief incubation with exogenous heme (as little as 1 h), endothelial cells become extraordinarily sensitive to oxidant challenge (*e.g.*, reagent hydrogen peroxide or activated PMNs). However, endothelial cells briefly exposed to heme in the same way and then allowed to incubate in the absence of heme for more prolonged periods (12–72 h) become highly resistant to oxidant-mediated injury (see Fig. 7A) (7). Exposure of cells to ferrihemoglobin yielded similar time-dependent effects on oxidant susceptibility (14, 15).

Because heme and various other stimuli were known to cause the induction of both heme oxygenase-1 (2, 67, 80, 112, 123) and ferritin (35, 79, 136) in other cell types, we wondered whether one or both of these proteins might be expressed in endothelial cells previously exposed to heme and then incubated for longer periods in response to heme. Indeed, we found that both heme oxygenase-1 mRNA level and enzyme activity as well as both H and L ferritin synthesis were upregulated in endothelial cells a few hours after initial exposure to heme (Fig. 8) (7). Heme oxygenase, through cleavage of the heme ring, will release intracellular iron, which, in turn, can promote the synthesis of ferritin. Alternatively, heme itself might enhance ferritin synthesis directly by increasing RNA translation (79).

As might be expected, ferrihemoglobin was also found to upregulate vascular endothelial cell heme oxygenase-1 and ferritin genes (14, 15). However, as was true in other systems, if heme binding to ferrihemoglobin is strengthened by haptoglobin or addition of cyanide (23), or if released heme is liganded to hemopexin (113), ferrihemoglobin loses much of its capacity to induce both heme oxygenase-1 and ferritin in endothelial cells (15). Endothelium can successfully compete for heme derived from ferrihemoglobin *in vivo* (16) despite the fact that two plasma proteins, haptoglobin and hemopexin, may act to inhibit endothelial heme-iron loading by binding free hemoglobin and heme, respectively. We note that this inhibition is not absolute, however, because slight but significant increases in ferritin content do occur in ferrihemoglobin-treated endothelium, despite the presence of these binding proteins. This might reflect the previously reported capacity, shown with other mammalian tissues, of cells to incorporate and metabolize iron derived from

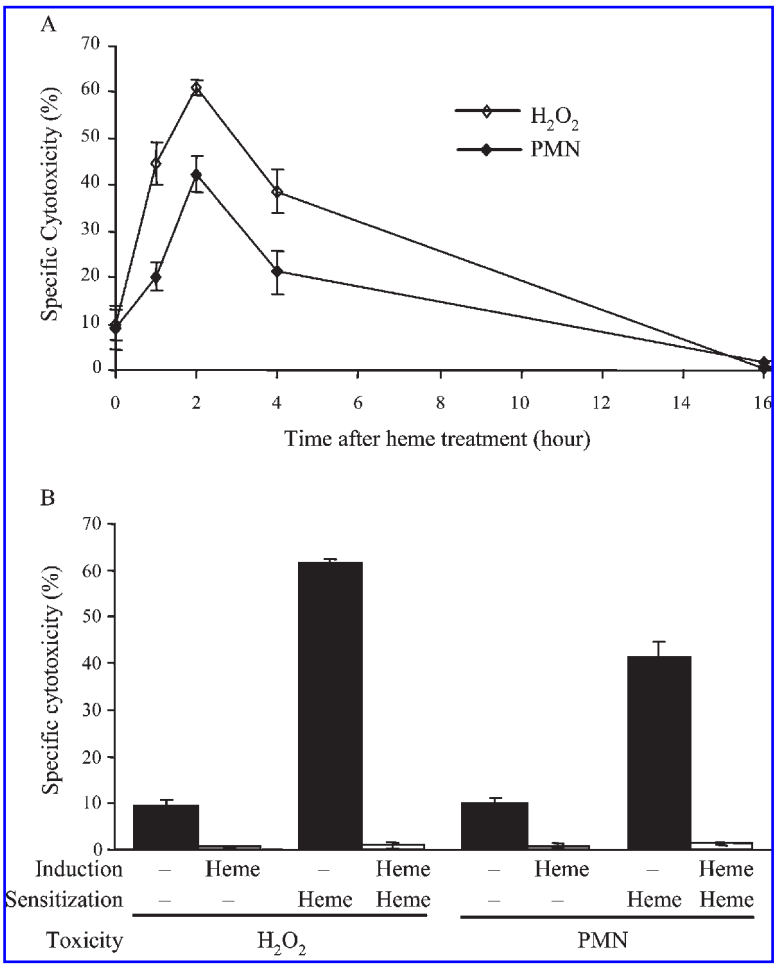

FIG. 7. The effect of heme pretreatment on endothelial cytotoxicity mediated by reactive oxygen is time dependent. (**A**) Confluent PAECs were pretreated with heme for 1 h. After the indicated time periods, cells were challenged by either H_2O_2 (100 μM, *open diamonds*), or PMA-activated PMNs (2:1 PMN/endothelial cell ratio, *solid diamonds*). After 2 h, specific cytotoxicity was determined. (**B**) Endothelial cells were untreated or pretreated with heme (10 μM, 1 h), followed by an induction period of 15 h. Then cells were either untreated or sensitized with heme (5 μM for 1 h). Two hours later, cytotoxicity was provoked by either H_2O_2 or activated neutrophils, as described earlier. Results represent mean percentage specific cytotoxicity ± SEM of at least five experiments performed in duplicate. This research was originally published in *J Biol Chem.* From ref. 7, with permission.

heme or heme-proteins bound to hemopexin or haptoglobin (4, 69). However, the blunted response of endothelium suggests that endothelial cells have relatively few haptoglobin or hemopexin receptors (15).

Endothelial cells were shown to exhibit increased heme oxygenase-1 and ferritin synthesis in lungs in an *in vivo* model in which hemoglobin was present in plasma (16). In this regard, ferrihemoglobin, but not ferrohemoglobin, increased the expression of total lung heme oxygenase-1 mRNA and heme oxygenase enzyme activity in rats. *In situ* hybridization revealed endothelial cell–associated accumulation of heme oxygenase-1 mRNA and enhanced total lung immunoreactive ferritin.

We reasoned that, in certain pathologic conditions, heme might derive from damaged circulating red cells in close contact with vascular lining cells. Because oxidation of hemoglobin to ferrihemoglobin is essential for endothelial perturbation, we sought to model oxidant conditions that might be relevant to vascular pathophysiology (14, 15). We found that free hemoglobin in plasma is rapidly oxidized to ferrihemoglobin when exposed to activated PMNs, and, as expected, this ferrihemoglobin leads to increased endothelial cell expression of heme

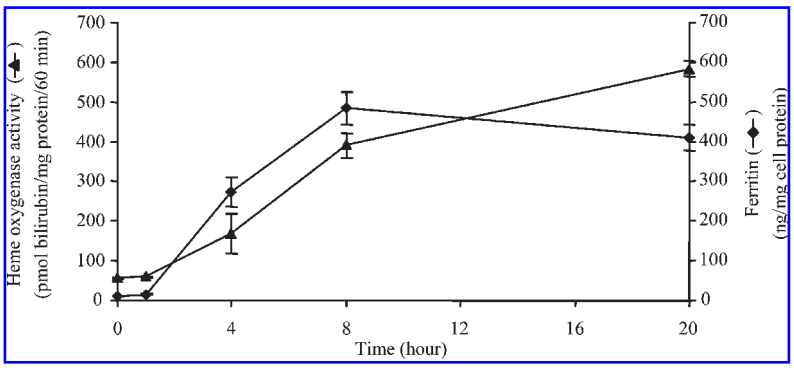

FIG. 8. Heme induces heme oxygenase-1 and ferritin in endothelial cells. PAECs were treated with heme (10 μM) for 1 h, and the culture medium was then replaced. At the indicated time, heme oxygenase activity (*solid triangles*) and ferritin protein content (*solid circles*) were determined. Results represent mean ± SEM of three experiments done in duplicate. This research was originally published in *J Biol Chem.* From ref. 7, with permission.

oxygenase-1 and ferritin. Although released heme appears to be the critical mediator in the heme oxygenase and ferritin induction, some caveats are acknowledged: for instance, activated PMNs can directly release inorganic iron from heme, so that free iron may itself contribute to the demonstrated ferritin accumulation. In addition, activated PMNs alone (with no hemoglobin present) were also shown to induce endothelial heme oxygenase-1 (15). This might reflect the production of reactive oxygen species, such as superoxide anion and hydrogen peroxide, because heme oxygenase-1 has been shown to be induced in other cells by oxidants (67).

Heme or hemoglobin amplifies oxidant-mediated endothelial cytotoxicity during brief exposure, yet markedly protects oxidant-exposed target cells, if incubated for a longer time after this brief exposure. This resistance parallels the synthesis and accumulation of large amounts of heme oxygenase-1 and ferritin (7, 15). To identify the ultimate cytoprotectant, we developed experimental conditions (7) that increase endothelial ferritin level, but not heme oxygenase-1 activity. Preincubation of endothelial cells with a cell-permeant iron-pyridoxal isonicotinoyl hydrazone chelate, or with a combination of heme and tin mesoporphyrin IX (an inhibitor of heme oxygenase activity) causes substantial increases in intracellular ferritin without any increment in heme oxygenase activity. In both these cases, marked protection of endothelium against subsequent oxidant challenge was noted. Direct loading of purified apoferritin into cultured endothelial cells causes the cells to become resistant, in a dose-dependent fashion, to oxidant stress imposed by various forms of reactive oxygen. Accompanying this resistance was a parallel reduction in the peroxidation products arising from oxidant challenge.

The induction of ferritin by heme in endothelium in the presence of tin mesoporphyrin IX was an unexpected finding (7). In these experiments, cells were exposed to 10 μM heme and 25 μM tin mesoporphyrin IX. Degradation of added heme by heme oxygenase provides intracellular iron to drive the synthesis of ferritin. Alternatively, heme itself enhances ferritin synthesis directly by increasing RNA translation (79). Therefore, induction of ferritin by heme in the presence of tin mesoporphyrin IX indicates the importance of the alternative pathway in the regulation of endothelial ferritin expression at a high concentration of heme.

In our studies of endothelial cells, the protection provided by ferritin is evidently attributable to either iron storage or the intrinsic ferroxidase activity of the heavy (H) subunit or both (Table 2) (7). Ferroxidase activity of ferritin is associated only with the H subunit (22, 58, 73). This activity catalyzes the oxidation of ferrous to ferric iron under aerobic conditions and facilitates iron insertion into ferritin. The ferroxidase activity of human H-chain ferritin has been widely studied by Arosio's group with the aid of site-directed mutagenesis (73). A site discovered by his group by using x-ray crystallography was identified as the ferroxidase center. The critical role of ferroxidase activity in ferritin-mediated cytoprotection against oxidant challenge is supported by the fact that human recombinant wild-type H ferritin loaded into endothelium also prevented endothelial damage provoked by oxidants (7). Furthermore, we found that a recombinant ferritin H chain mutant (22), which lacks ferroxidase activity and is unable to incorporate iron into the ferritin core, has no protective effect (7).

Earlier studies, performed in cell-free systems and with ferritin of questionable purity, suggested that ferritin might release iron and, thereby, amplify oxidative damage (104, 124, 125). For example, *in vitro* superoxide (125) and nitric oxide (103) were shown to displace a small fraction of total iron from iron-loaded ferritin. However, later investigations cast doubt on the assertion that significant amounts of iron might be released from ferritin during *in vivo* oxidative or inflammatory reactions (58). The fact is that endothelial cells induced to produce ferritin by iron compounds are protected despite the increased cellular iron

TABLE 2. FERRITIN INDUCTION AND FERRITIN LOADING OF PORCINE AORTIC ENDOTHELIAL CELLS INFLUENCE ENDOTHELIAL CELL SENSITIVITY TO HEMIN CATALYZED HYDROGEN PEROXIDE TOXICITY

Endothelial cell treatment	*Ferritin content (mg/mg cell protein)*	*Hemin + H_2O_2 cytotoxicity (% of ^{51}Cr release)*
Buffer	58.3 ± 8.0	61.9 ± 1.5
Hemin (μM)		
1.0	146.7 ± 35.8	51.1 ± 4.6
2.5	282.0 ± 56.4	37.7 ± 5.0
5.0	400.0 ± 18.0	19.0 ± 3.6
10.0	574.4 ± 18.4	0.54 ± 0.9
Horse apoferritin (mg/ml)		
0.1	180.0 ± 58.8	65.4 ± 3.7
0.5	499.4 ± 64.7	46.8 ± 3.0
1.0	1,139.0 ± 129.0	22.8 ± 2.3
2.0	2,879.0 ± 352.0	0.45 ± 1.0
Recombinant human heavy-chain ferritin		
Wild type 1 mg/ml	1,193.0 ± 154.0	23.1 ± 2.9
Mutant (222) 1 mg/ml	1,012.0 ± 112.5	68.3 ± 2.4

This research was originally published in *J Biol Chem.* Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM. Ferritin: a cytoprotective antioxidant stratagem of endothelium. *J Biol Chem* 267: 18148–18153, 1992 (with permission).

content, suggesting that susceptibility to oxidative injury is not dependent on cellular iron content *per se*, but rather on the presence of apoferritin capable of storing iron and making it relatively unreactive (7).

HEMOGLOBIN, HEME AND IRON: POSSIBLE IMPORTANCE IN LDL OXIDATION AND ATHEROGENESIS

These *in vitro* observations showing the induction of heme oxygenase-1 and ferritin by heme and iron compounds led us to examine the possibility that delocalized heme and iron might play a part in the process of atherogenesis. As a partial test of this, we collected specimens of coronary arteries of cardiac explants from patients with atherosclerotic disease or idiopathic cardiomyopathy and determined whether ferritin was present at sites of atherosclerotic disease (65). Immunoperoxidase studies revealed abundant immunoreactive ferritin in all coronary atherosclerotic lesions with active inflammation, with virtually no detectable staining seen in normal coronary arteries of dilated cardiomyopathy hearts. Of note, no ferritin was seen in coronary artery atherosclerotic lesions where little cellular infiltrate or inflammation was present. Pronounced ferritin reactivity was present in large amounts in Ulex and factor VIII–positive endothelial cells, mainly in the inflamed shoulder areas bordering the fibrous caps. Ferritin was also found within the cytoplasm of α-smooth muscle actin–positive myofibroblasts in the neointima of the fibrous cap and in foamy macrophages. Upregulation of heme oxygenase-1 in these lesions generally appears to coincide with ferritin induction (13, 133). The increased expression of ferritin and heme oxygenase-1 in the atherosclerotic lesions might reflect cellular response to heme or heme-iron–generated lipid peroxidation products.

Because oxidative modification of LDL has been suggested to be a key event in atherosclerosis and heme oxygenase-1 is induced by oxidants (67), we tested whether LDLox might alter the expression of endothelial heme oxygenase-1. We found that LDL oxidized by either heme or copper causes massive induction of heme oxygenase-1 in endothelial cells, accompanied by significant increments in ferritin content (3). Such induction correlates with the oxidative insult imposed by LDLox, and pretreatment of the oxidized LDL or the endothelium with selected antioxidants markedly diminishes the expression of both heme oxygenase-1 and ferritin. Our results further suggest that an accumulation of LOOH within LDLox is mainly responsible for the induction of heme oxygenase-1 and ferritin in endothelium exposed to LDLox because specific enzymatic reduction of LOOH to LOH (with reduced glutathione/glutathione peroxidase) yields LDL with minimal effects (64). Agarwal and coworkers (60) recently investigated the mechanism by which LDLox regulates the expression of heme oxygenase-1. They found that among the components of LDLox, the most potent inducer of heme oxygenase-1 is a lipid-hydroperoxide; 13-hydroperoxyoctadecadienoic acid (13-HPODE), which transcriptionally regulates the heme oxygenase-1 through a 13-HPODE–specific regulatory element in the human heme oxygenase-1 promoter.

Pretreatment of endothelial cells with the iron chelator desferrioxamine B also prevented heme oxygenase-1 induction by LDLox, suggesting that redox active intracellular iron is involved (3). We also observed an intracellular GSH depletion in endothelium exposed to LDLox (64); such GSH depletion is also known to enhance heme oxygenase-1 expression (89).

As in our studies with heme and hemoglobin, the effects of LDL oxidation on endothelial susceptibility to oxidant damage were found to be time dependent. Whereas short-term exposure to LDLox produced substantial cytotoxicity, a prolonged exposure at sublethal concentration rendered endothelium resistant to oxidant damage. This suggested that ferritin *per se* might provide resistance to LDLox toxicity. Indeed, endothelium loaded with exogenous apoferritin is protected in a dose-dependent fashion from LDLox-mediated toxicity (65). To test whether the ferroxidase activity of ferritin is necessary for its protective role against LDLox, we used recombinant human H ferritin and a mutant H ferritin ("222") (which lacks ferroxidase and iron-storage functions) (22). Despite being functionally defective, mutant 222, containing two amino acid substitutions, is taken up as readily by endothelial cells as is wild-type ferritin (shown by enzyme-linked immunosorbent assay) (7). Whereas apoferritin or recombinant H ferritin efficiently protects endothelial cells from LDLox, similar loading of endothelial cells with mutant 222 is without significant effect.

IMPORTANCE OF FERRITIN IN CYTOPROTECTION AND CELL PROLIFERATION

The role of iron in cell proliferation is thought to represent an important factor in the clonal expansion of cancer cells; thus, in a variety of tumors, including breast cancer and colon cancer, transferrin receptors are increased relative to their minimal expression on surfaces of nonmalignant cells in the same tissue (44, 57, 129). This has led to the speculation that such receptor upregulation may be advantageous for tumor proliferation by supplying iron for DNA synthesis and for heme- and iron-dependent protein synthesis (72). Indeed, neoplastic cells tend to show increased incorporation of iron into various products (134). Chelation of cellular iron by desferri-exochelin was shown to induce death by apoptosis in human breast cancer cells (96). Because diverse chemotherapeutic agents and tumor-engaging phagocytic cells act *via* oxidant-mediated toxicity, it seems reasonable that changes in the availability of cellular iron in tumor cells might modulate their susceptibility to oxidants, or to oxidant chemotherapeutic agents such as bleomycin.

As was true of endothelial cells, colon and breast cancer cell lines (Caco-2 and BT-20 tumor cells) undergo dose-dependent lysis when challenged by heme followed by various forms of oxidants, but the former cells are generally less sensitive to oxidant injury (27). To assess the relevance of cellular iron to oxidant-dependent chemotherapeutic effects, we pretreated BT-20 cells with nonlethal amounts of heme and measured cytotoxicity induced by bleomycin, an agent known to damage DNA through iron-driven oxygen radicals. Augmented cytotoxicity and cellular DNA strand scission were noted in cells treated with heme added a few hours before bleomycin exposure (27).

In endothelial cells, a posttranscriptional control of ferritin synthesis exists; ferritin L- and H-chain mRNA levels are not affected by increased intracellular iron, but ferritin synthesis (translation) is upregulated. In contrast, in both BT-20 and Caco-2 tumor cell lines exposed to heme or $FeSO_4$, a significant increase in the level of H ferritin mRNA accompanies ferritin induction (7, 27). An increased synthesis of H-ferritin in other types of tumor cells has also been described by others (29) who, like us, have speculated that it might protect rapidly proliferating cells from toxicity of free ferrous iron (26) as well as serving as an iron depot to support cell growth. Beaumont *et al*. (20) described a fivefold increase in H-ferritin mRNA in dimethyl sulfoxide–induced Friend erythroleukemia cells. Guner *et al.* (53) demonstrated that advanced breast cancer cells (stage II or III) had 5 times higher cytosolic ferritin levels than did stage I cancers, implying that increased cellular ferritin may be necessary both for rapid cell proliferation and to confer protection against phagocyte or chemotherapy-derived oxidants.

Regardless of the mechanism by which tumor cell ferritin is increased, once synthesized, its ability to modulate oxidant-cytotoxic susceptibility may have important ramifications for chemotherapy efficacy. Our results with bleomycin validate this suggestion (*i.e.,* this chemotherapeutic agent, known oxidatively to degrade cellular DNA, requires chelatable iron and O_2 for its toxic effects). The ultimate agent of DNA damage is probably an *in situ* iron-mediated reaction produced by a complex of DNA, bleomycin, Fe and O_2 (24, 121). Sequestration of available cellular reactive iron by its incorporation into newly synthesized ferritin reduces the sensitivity of cancer cells to bleomycin, as measured by both cytotoxicity and DNA-scission assays. The latter results are reminiscent of data of others demonstrating inhibition of bleomycin-induced cellular DNA strand scission in bleomycin-treated Ehrlich ascites tumor cells that were simultaneously incubated with the iron chelator, 1,10-phenanthroline (24).

Catabolism of heme by heme oxygenase may rid cells of a membrane-permeant form of iron, but the resultant nonheme iron represents a potential hazard unless sequestered by ferritin (7). The cytoprotective nature of heme oxygenase and ferritin has been confirmed in various models. Keyse and Tyrell (67) discovered that heme oxygenase-1 is the major 32-kDa stress protein induced by oxidative stress such as UVA radiation and hydrogen peroxide (67). In subsequent work, Vile *et al.* (130) demonstrated that heme oxygenase-1 induction mediates an adaptive response to oxidative stress *via* ferritin synthesis in human skin fibroblasts. Maines's group (43) raised the notion that increases in heme oxygenase-1 transcript and protein reflect a means to elevate levels of other antioxidants in cells with compromised defense mechanisms caused by stress. Abraham's group (1) showed that increased expression of heme oxygenase-1 in endothelium—*via* transfection of the human heme oxygenase-1 gene into rabbit coronary microvessel endothelial cells—provides protection against heme and hemoglobin toxicity. Overexpression of heme oxygenase-1 in human pulmonary epithelial cells was shown to result in cell-growth arrest and increased resistance to hyperoxia (74). In a series of studies, Lin and Girotti (76–78) found that heme-enhanced resistance to oxidative killing is mediated by an H ferritin chain in human leukemia cells, confirming the antioxidant role of ferritin. In-

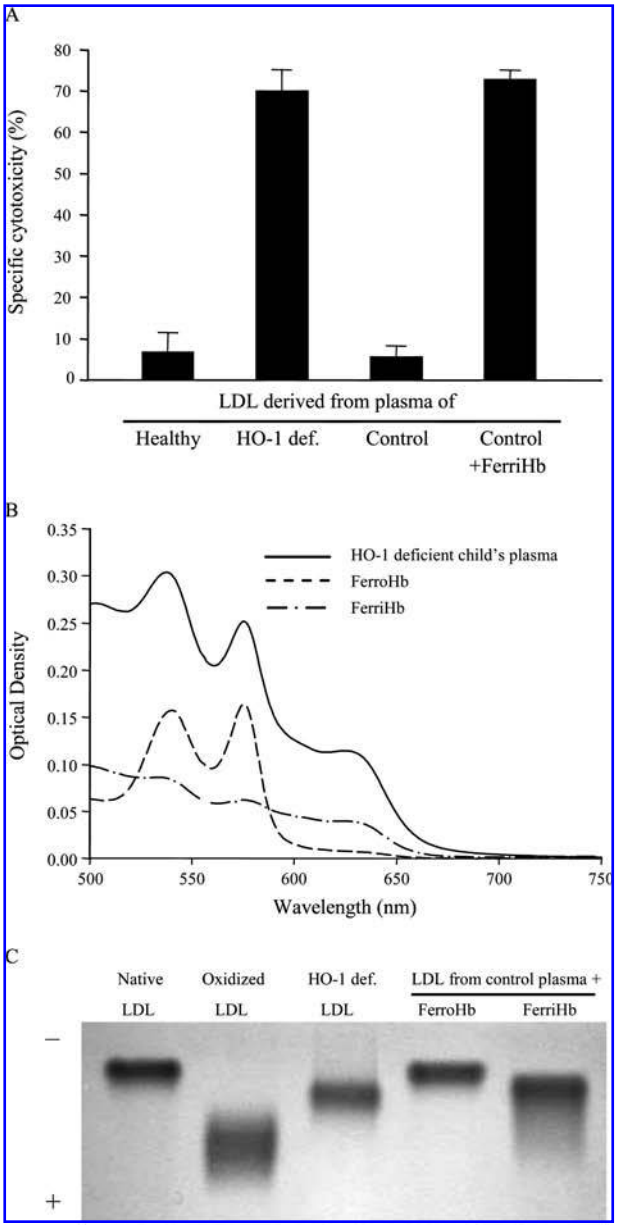

FIG. 9. LDL derived from the HO-1–deficient child is cytotoxic to endothelial cells and has increased electrophoretic mobility accompanied by oxidation of plasma Hb. (**A**) HUVECs were treated with LDL samples (200 μg/ml protein) derived from a healthy subject, LDL from HO-1–deficient patient, or LDL from healthy individuals' plasma samples handled similarly to those from the patient or LDL from plasma pretreated with ferriHb (80 μM). After a 4-h incubation with LDL samples, MTT assay was performed. Results represent mean ± SEM of two experiments done in triplicate. (**B**) *Solid line*, Wavelength scan of the HO-1–deficient child's plasma; also represented are wavelength scans of diluted plasma from healthy subjects containing 2.5 μM ferroHb (*long dash*) or 2.5 μM ferriHb (*dash-dot*). (**C**) Native LDL, oxidized LDL, HO-1–deficient child's LDL, and LDL derived from plasma containing 20 μM ferroHb or 20 μM ferriHb were electrophoresed on agarose gel. This research was originally published in *Blood*. From ref. 64, with permission.

creased expression of wild-type ferritin H-chain was reported to reduce cell growth (22, 31) and increase resistance to hydrogen peroxide toxicity in HeLa cells (31). Furthermore, heavy-chain ferritin was shown to act as an antiapoptotic gene that protects liver from ischemia/reperfusion injury (21).

In a cell-culture oxidative stress model, when lymphocytes were treated with hyperbaric oxygen, the cells became resistant to oxidative stress, concordant with increased cellular ferritin levels (107). Ferritin alone or together with heme oxygenase reduces oxidative stress, providing cytoprotection for dividing T cells, and may set a favorable environment for T-cell growth and survival in a red blood cell–T cell co-culture system (45). The importance of intracellular iron/ferritin interactions is emphasized by Epsztejn's report (39) that murine erythroleukemia cells transfected with H ferritin subunits show lower production of reactive oxygen species and resist long-term cell damage induced by free radicals (39).

Brain is especially rich in lipoproteins, which can be the target of heme-catalyzed free radical toxicity. In an *in vitro* cortical astrocyte model, increased intracellular ferritin was reported to provide defense against heme-mediated injury (101). In cases of human intracerebral and subarachnoid hemorrhages, it has been suggested that ferritin plays a protective role against heme-iron–mediated pathologic reactions (120, 139). Ferritin was also shown by Taylor *et al.* (122) to induce protection against hyperoxia-mediated lung injury in rats. In *in vitro* tissue-culture experiments, it was reported that lens epithelium and skin fibroblasts are protected by endogenous ferritin against UV and infrared radiation (5, 51). Furthermore, aspirin-induced endothelial cytoprotection against hydrogen peroxide toxicity was reported to depend on the upregulation of ferritin synthesis (90). Importantly, it was found that intracellular ferritin attenuates iron-catalyzed LDL oxidation (128). These studies emphasize the paramount role of ferroxidase activity and iron sequestration by ferritin to serve as a protective gene by virtue of antioxidant, antiapoptotic, and antiproliferative actions.

Nath and his colleagues (88) provided the first *in vivo* evidence in an animal model that induction of heme oxygenase-1 coupled with increased ferritin synthesis is a rapid protective antioxidant response. In this work, induction of heme oxygenase-1 and ferritin protected rats against rhabdomyolysis-induced renal failure.

CYTOPROTECTIVE EFFECTS OF HEME OXYGENASE

Otterbein *et al*. (92) discovered that hemoglobin-induced protection against lethal endotoxemia in rats is mediated by a pathway dependent on heme oxygenase-1 (91). Furthermore, they demonstrated that heme oxygenase-1 can provide protection against hyperoxia-induced lung injury *in vivo* by modulation of neutrophil inflammation and lung apoptosis (93). Heme oxygenase-2 also appears essential for protection against hyperoxia-induced lung injury because heme oxygenase-2–deficient mice were shown to be sensitive to hyperoxia-induced oxidative lung injury with the absence of ferritin induction (34). Catalytically inactive mutant heme oxygenase-2 was also found to be cytoprotective (68). Poss and Tonegawa (99) provided further *in vivo* evidence—by using mice lacking functional heme oxygenase-1—that upregulation of heme oxygenase-1 serves as an adaptive mechanism to protect cells from oxidative damage during stress (100). In elegent studies, Soares *et al*. (116) found that heme oxygenase-1 overexpression would rescue cardiac xenografts from rejection (116). In this model system, transplanted hearts from heme oxygenase-1 knockout mice were rapidly rejected. Upregulation of heme oxygenase-1 in myocardium was also reported to suppress cardiac arrhythmia (6), and hearts from heme oxygenase-1 knockout mice exposed to ischemia/reperfusion were very susceptible to ventricular fibrillation. Dulak *et al*. (37) explored that heme oxygenase-1 modulates vascular endothelial growth factor synthesis in vascular smooth muscle cells. Heme oxygenase-1 also may be part of a protective system used by cells to counteract nitrosative stress derived from excessive production of nitric oxide (86). Impor-

Table 3. Low-density Lipoprotein from 62 Healthy Subjects and a Heme Oxygenase-1–deficient Child Compared for Oxidative Resistance, Conjugated Dienes, Thiobarbituric Acid—Reactive Substances, Total Lipid Hydroperoxide, and α-Tocopherol Content

Parameter deficient child	*LDL from healthy subjects (n = 62)*			*LDL from HO-1*
	Mean ± SD	*Min*	*Max*	*Mean ± SD*
$\triangle$T at V_{max} (sec)	3,040.6 ± 515.2	2220	4740	120 ± 57
Conj. dien (OD) at 2.34	0.1794 ± 0.0156	0.1471	0.2203	0.436 ± 0.055
TBARSs (nmol/mg LDL)	0.316 ± 0.094	0.128	0.577	1.75 ± 0.31
Total LOOHs (nmol/mg LDL)	4.689 ± 1.417	2.170	8.140	32.6 ± 2.53
α-Tocopherol (mol/molApoB100)	7.91 ± 1.85	4.32	14.56	0.2 ± 0.11

Hp, haptoglobin; Conj dien, conjugated dien; TBARSs, thiobarbituric acid reactive substances; LOOH, lipid hydroperoxide. This research was originally published in *Blood*. Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, Eaton JW, Balla G. Pro-oxidant and cytotoxic effects of circulating heme. *Blood* 100: 879–887, 2002 (with permission).

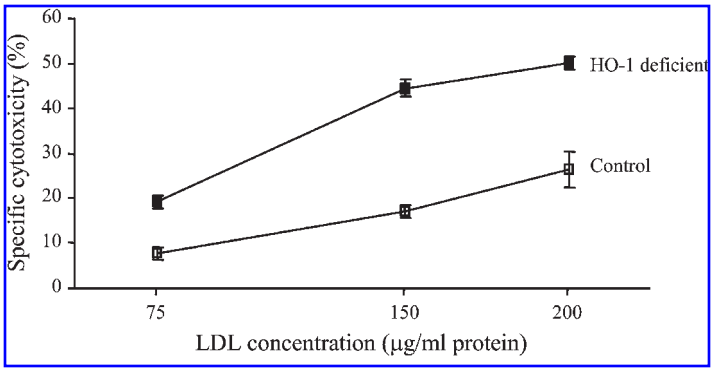

FIG. 10. HO-1–deficient cells are more sensitive to oxidized LDL than are control cells. LDL (200 μg/ml) was oxidized with heme (5 μM) and H_2O_2 (75 μM) for 2 h. HO-1–deficient and control lymphoblastoid cell suspensions were treated with the indicated concentrations of oxidized LDL overnight. Cytotoxicity was determined by MTT assay. Figure is representative of three experiments done in triplicate. This research was originally published in *Cell Mol Biol (Noisy-le-grand)*. From ref. 87, with permission.

tantly, nitric oxide was revealed by Motterlini *et al.* (85) to be a powerful inducer of heme oxygenase-1. In a number of cell systems, heme oxygenase induction has been shown to have antiinflammatory, antiapoptotic, antiproliferative, antithrombotic, and vasodilatory effects (6, 19, 28, 36, 37, 84, 94, 110, 119). It is not, however, clear how this panoply of protective effects is mediated by heme oxygenase upregulation. Products of heme degradation by heme oxygenase include carbon monoxide, biliverdin, bilirubin, and iron. The first three have attracted a great deal of interest, inasmuch as carbon monoxide has effects on the vasculature, and bilirubin is an effective antioxidant. In attempting to connect these products to the observed effects, Choi's group (94) and Soares and Bach (110, 116) have shown that carbon monoxide provides protection against hyperoxic lung injury and graft rejection in a mouse cardiac xenotransplantation model. It has even been suggested by Motterlini's group (84) that transition metal carbonyls might be used for therapeutic delivery of carbon monoxide. Yet it is far from certain that the effects of heme oxygenase upregulation are exerted through the same mechanisms involved in carbon monoxide exposure because (a) only miniscule amounts of carbon monoxide would be generated by heme oxygenase activity, especially considering (b) that most cells have almost no available substrate (heme), and (c) carbon monoxide is a gas and would be expected to diffuse from sites of production by heme oxygenase very quickly.

Mystery also shrouds the suggestions that two other products of heme catabolism by heme oxygenase—biliverdin and bilirubin—might explain the salubrious effects of heme oxygenase upregulation. However, reports that such upregulation does protect neurons against oxidative stress (36) and ameliorates postischemic myocardial dysfunction do exist (28). Furthermore, bilirubin was demonstrated by Stocker (119) to be an effective antioxidant and might act through being oxidized to biliverdin and then recycled by biliverdin reductase back to bilirubin, a mechanism that would amplify the antioxidant effect as shown by Baranano *et al.* (19).

Nonetheless, the same questions pertain to these suggestions as to those regarding carbon monoxide as an effector: (a) In any normal cell, would free heme be abundant enough to generate significant amounts of biliverdin/bilirubin? (b) Would the latter remain very long at the site of generation or (more likely) be rapidly cleared? Thus, although little doubt exists regarding the amazing protective effects of heme oxygenase upregulation, substantial doubt regards how these effects are achieved. It is possible that heme degradation with the simultaneous production of effectors molecules (*e.g.*, iron, carbon monoxide, and biliverdin) is part of a complex, concerted, and dynamic mechanism by which cells amplify their ability to adapt and protect themselves against oxidative stress. Further studies on how the products of heme oxygenase interact directly with specific biologic targets and transduce their effect into cellular functions will enable to us better to address this conundrum.

AN UNFORTUNATE EXPERIMENT OF NATURE: CONGENITAL DEFICIENCY OF HEME OXYGENASE-1

Subsequent to most of the experimental work cited earlier, the central importance of heme oxygenase-1 in vascular biol-

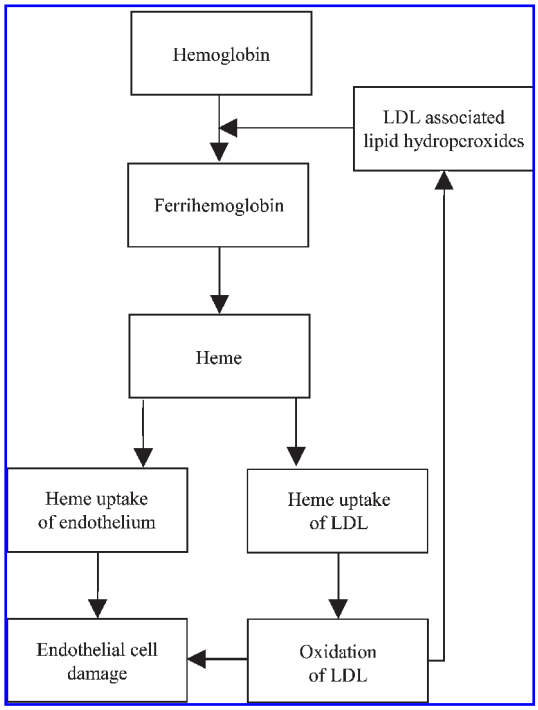

FIG. 11. A vicious cycle arises from increased susceptibility of endothelium to oxidative damage and oxidation of low-density lipoprotein induced by hemoglobin-derived heme in the heme oxygenase-1–deficient child.

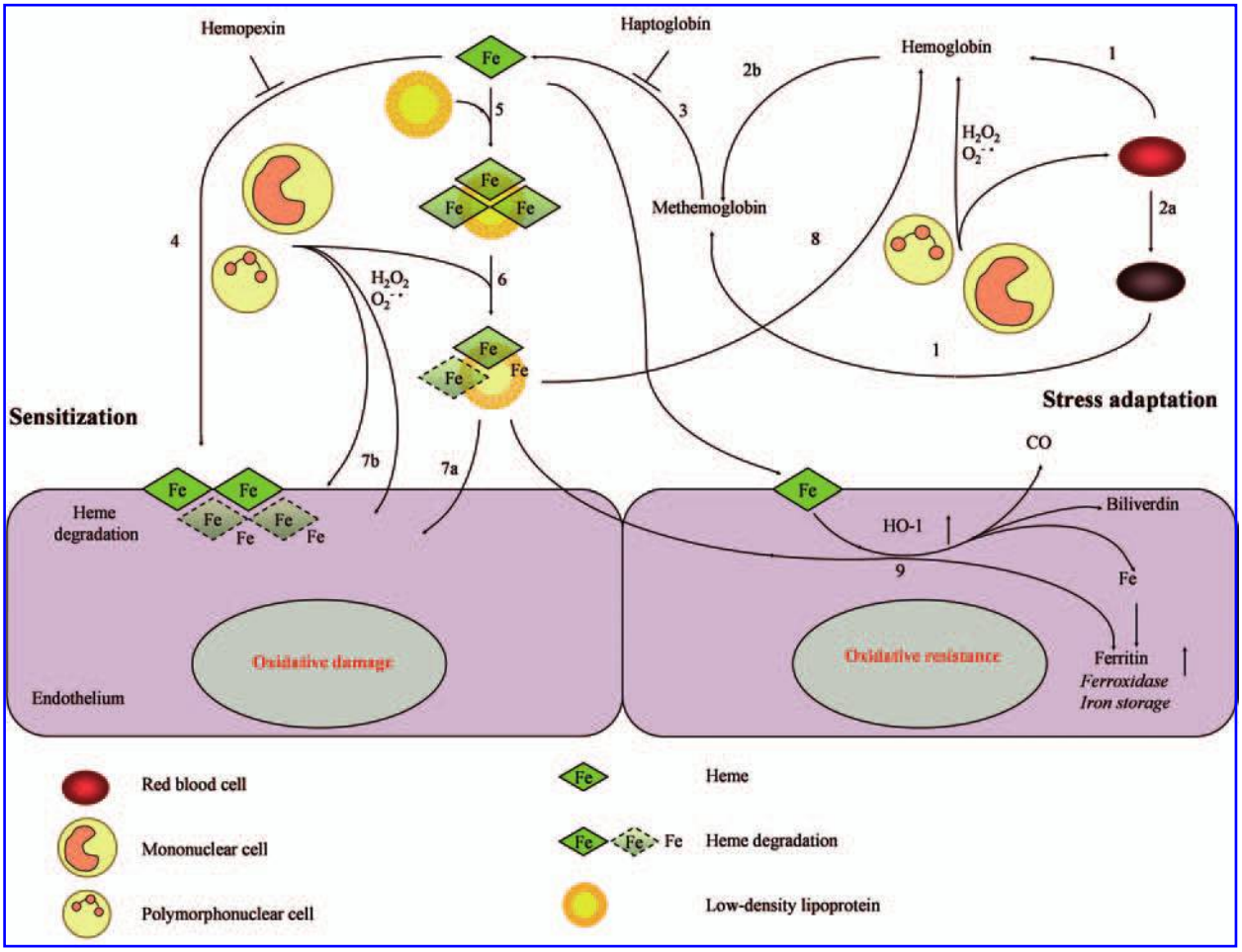

FIG. 12. Oxidative stress and stress adaptation induced by heme and hemoglobin. (1) Leakage of hemoglobin from red blood cells. (2a and b) Oxidation of hemoglobin. (3) Heme release from methemoglobin. (4) Heme uptake and sensitization of endothelium to oxidative stress. (5) Heme uptake by LDL. (6) Oxidative modification of LDL (7a, b) Oxidative stress induced by oxidized LDL and oxidants derived from leukocytes. (8) Oxidation of ferroHb to ferriHb induced by oxidized LDL. (9) Induction of adaptation to oxidative stress *via* upregulating heme oxygenase-1 and ferritin. This modified scheme was originally published in *Mol Nutr Food Res*. From ref. 17, with permission.

ogy was highlighted by the discovery of a child with heme oxygenase-1 deficiency, diagnosed by Yachie *et al.* (140). The phenotype exhibited by this child was in excellent agreement with those features that might have been predicted from the *in vitro* experiments. In the heme oxygenase-1–deficient child, both intravascular hemolysis (of unexplained etiology) and endothelial cell injury were prominent. A lymphoblastoid cell line derived from the patient was shown to have increased sensitivity to heme toxicity. Fatty streaks and fibrous plaques in the aorta were present (66), suggesting severe atherosclerosis. Similar damage to endothelium, as well as hepatic and renal tubular cytotoxicity, has been observed in transgenic knockout mice deficient in heme oxygenase-1 (99).

With particular regard to the accelerated atherosclerosis observed in this child, in "normal" atherosclerotic lesions, deposits of iron, perhaps derived from heme released by erythrocytes, have been observed (62, 114). Studies from our laboratory have revealed that both ferritin and heme oxygenase-1 are highly expressed in human atherosclerotic lesions (13, 65). Wang *et al.* (133) found increased expression of heme oxygenase-1 in atherosclerotic plaques. The upregulation of heme oxygenase-1 and ferritin genes in endothelium early in atherogenesis may reflect a cellular response to heme or iron and associated lipid-peroxidation products. Failure of such upregulation may help explain the occurrence of what appears to be greatly accelerated atherogenesis in this child.

As suggested earlier, free hemoglobin in plasma might threaten vascular endothelial cell integrity *via* oxidative modification of LDL *in vivo*. Evidence from our laboratory that toxic species of LDL were present in LDL isolated from the plasma of the heme oxygenase-1–deficient child was reported earlier (Fig. 9A) (64). Importantly, spectral analysis of the heme oxygenase-1–deficient child's plasma revealed that oxidation of hemoglobin to ferrihemoglobin occurred in his plasma (Fig. 9B), and the proportion of total hemoglobin present as ferrihemoglobin was ~80% (~60 μM). Furthermore, LDL of the heme oxygenase-1–deficient child had increased electrophoretic mobility (Fig. 9C), suggesting the loss of net positive charge (as

also seen after *in vitro* LDL oxidation by metals or heme). This was confirmed by measurement of fluorescamine-titratable free amino groups on the LDL particle. Fluorescamine-reactive amino groups were diminished (732 moles per mole apolipoprotein B-100 compared with 978 mol/mole apolipoprotein B-100 for controls). Similar alterations in the anodal mobility of LDL occur when normal plasma is exposed to ferrihemoglobin *in vitro,* whereas ferrohemoglobin has no effect.

Iron accumulated in the heme oxygenase-1–deficient child's LDL (8 moles per mole apolipoprotein B-100). A comparable amount of heme is taken up by LDL if plasma samples from healthy subjects are exposed to ferrihemoglobin for a few hours (3.2 ± 0.2 heme molecules/LDL particle), and within 2–3 days, the heme in LDL particles is degraded, and the iron content of LDL is increasing to 2.9 ± 0.3 moles per mole apolipoprotein B-100. Interestingly, heme was not detectable in the child's LDL. The oxidative resistance of the heme oxygenase-1–deficient boy's LDL was found to be virtually zero, with only tiny amount of α-tocopherol in his LDL particles. The oxidative modification of the child's LDL was demonstrated by several independent assays for lipid peroxidation (Table 3). Conjugated dienes, LOOHs, and TBARS accumulated in his lipoprotein.

As might be expected, the heme oxygenase-1–deficient child's LDL was shown to induce endothelial cell cytotoxicity. We suspected that the majority of the toxicity of child's LDL might derive from the high concentrations of LOOH, which is chemically very similar to organic hydroperoxides. In support, preincubation of the heme oxygenase-1–deficient child's LDL with reduced glutathione/glutathione peroxidase (which will relatively specifically reduce the LOOH to the alcohol) abolished almost 100% of the cytotoxic effects. In further support of the toxicity of the LOOH *per se*, when endothelial cells were exposed to a concentration of cumene hydroperoxide approximately equal to the LOOH content of the toxic LDL, almost identical cytotoxicity was observed. Furthermore, in endothelial cells exposed to either heme oxygenase-1–deficient child's LDL or cumene hydroperoxide, we observed a precipitate decline in intracellular GSH content.

Finally, we hypothesized that cells of the heme oxygenase-1–deficient child would be prone to oxidative damage arising from heme-mediated oxidation of LDL. Indeed, we found elevated cytotoxicity induced by heme-catalyzed oxidation of LDL in lymphoblastoid cells derived from the heme oxygenase-1–deficient patient (Fig. 10) (87).

A vicious cycle could arise from heme-iron catalysis of LDL peroxidation in the heme oxygenase-1–deficient child. These reactions may include (a) oxidation of ferrohemoglobin to ferrihemoglobin; (b) spontaneous insertion of heme released from ferrihemoglobin into LDL; (c) subsequent oxidative scission of the porphyrin ring; (d) release of free iron from the porphyrin ring; (e) iron catalysis of further oxidative heme scission, LDL, and protein oxidation; and (f) further oxidation of ferro- to ferrihemoglobin by LDL-associated lipid hydroperoxides and concomitant LDLox-mediated injury to endothelium (Fig. 11).

Because endothelial cell damage and death were induced by the heme oxygenase-1–deficient child's LDL, we tested whether it would also be capable of enhancing the expression of heme oxygenase-1 and ferritin in endothelial cells of healthy subjects. Exposure of normal endothelial cells to sublethal amounts of the child's LDL led to marked increases in heme oxygenase activity and doubled ferritin content. Inhibition of heme oxygenase enzyme activity in the endothelium blunted the rapid ferritin response to the child's LDL, suggesting that the induction of ferritin synthesis was in part due to iron liberated from endogenous heme. Reduction of LDL-associated lipid hydroperoxide by GSH/glutathione peroxidase prevented the upregulation of both heme oxygenase-1 and ferritin, indicating that this induction probably involves LDL-associated hydroperoxides or secondary oxidation events caused by these peroxides.

Oxidation of hemoglobin in plasma leading to heme release, as it occurs in heme oxygenase-1 deficiency in humans, represents a hazard to vascular endothelial cells by not only sensitizing endothelium to oxidant damage but also catalyzing the oxidation of LDL. Protection against such toxicity seems to involve, most important, upregulation of heme oxygenase and ferritin. Endothelial cell damage and progression of atherosclerosis in the heme oxygenase-1–deficient child might be explained, at least in part, by heme-catalyzed oxidation of LDL and the lack of these normal homeostatic responses. The upregulation of heme oxygenase-1 and ferritin genes in atherosclerotic lesions possibly reflects a response to heme and heme-iron–generated lipid peroxidation products (Fig. 12) (17).

Overall, our observations on material from the heme oxygenase-1–deficient child lend further support to the results of earlier *in vitro* and *in vivo* experiments regarding the toxic effects of heme and the importance of ferritin and heme oxygenase-1 induction as protective devices. It seems clear that induction of heme oxygenase—and, as important, ferritin—is critical to the survival and function of the vascular endothelium and its adaptation to life in an extraordinarily iron-rich environment.

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ABBREVIATIONS

Fe, iron; Fe^{2+}, ferrous ion; Fe^{3+}, ferric ion; GSH, reduced glutathione; HeLa, human epithelial cell line; 13-HPODE, 13-hydroperoxyoctadecadienoic acid; LDL, low-density lipoprotein; LDLox, oxidative modified low-density lipoprotein; LOH, lipid hydroxide; LOOH, lipid hydroperoxide; mRNA, messenger ribonucleic acid; Na_2EDTA, ethylendiaminetetracetic acid disodium salt; PMNs, polymorphonuclear leukocytes; $R\text{-}O^{\cdot}$, alkoxyl radicals; $RO_2^{\cdot}$, peroxy radicals; ΔT at V_{max}, the time point of maximal velocity of heme degradation defined as the maximal change in absorbance of heme in the propagation phase of lipid peroxidation; TBARS, thiobarbituric acid–reactive substances; UVA, ultraviolet A radiation.

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