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Review

Heme, heme oxygenase and ferritin in vascular endothelial cell injury

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Iron-derived reactive oxygen species are implicated in the pathogenesis of numerous vascular disorders including atherosclerosis, microangiopathic hemolytic anemia, vasculitis, and reperfusion injury. One abundant source of redox active iron is heme, which is inherently dangerous when released from intracellular heme proteins. The present review concerns the involvement of heme in vascular endothelial cell damage and the strategies used by endothelium to minimize such damage. Exposure of endothelium to heme greatly potentiates cell killing mediated by polymorphonuclear leukocytes and other sources of reactive oxygen. Free heme also promotes the conversion of low-density lipoprotein (LDL) into cytotoxic oxidized products. Only because of its abundance, hemoglobin probably represents the most important potential source of heme within the vascular endothelium; hemoglobin in plasma, when oxidized, transfers heme to endothelium and LDL, thereby enhancing cellular susceptibility to oxidant-mediated injury. As a defense against such toxicity, upon exposure to heme or hemoglobin, endothelial cells up-regulate heme oxygenase-1 and ferritin. Heme oxygenase-1 is a heme-degrading enzyme that opens the porphyrin ring, producing biliverdin, carbon monoxide, and the most dangerous product – free redox active iron. The latter can be effectively controlled by ferritin *via* sequestration and ferroxidase activity. Ferritin serves as a protective gene by virtue of antioxidant, antiapoptotic, and antiproliferative actions. These homeostatic adjustments have been shown effective in the protection of endothelium against the damaging effects of exogenous heme and oxidants. The central importance of this protective system was recently highlighted by a child diagnosed with heme oxygenase-1 deficiency, who exhibited extensive endothelial damage.

Keywords: Atherosclerosis / Endothelial cell injury / Ferritin / Heme / Heme oxygenase

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1 Introduction

Heme is absolutely required for aerobic life. However, free heme can be quite cytotoxic, particularly in the presence of oxidants or activated phagocytes. 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 mmol/L and are vulnerable to unexpected lysis. The extracellular hemoglobin is easily oxidized to ferrihemoglobin 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. Here, we present a brief review of the nature of heme-mediated cytotoxicity and of the strategies by which normal endothelium manages to protect itself from this clear and present danger (Fig. 1).

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2 Direct heme and hemoglobin toxicity to vascular endothelial cells

Damage caused by reactive oxygen species can be greatly amplified by "free" redox active iron [1]. For example, iron-rich *Staphylococcus aureus* are three orders of magnitude more susceptible to killing by hydrogen peroxide than are iron-poor staphylococci [2]. Conversely, depletion of

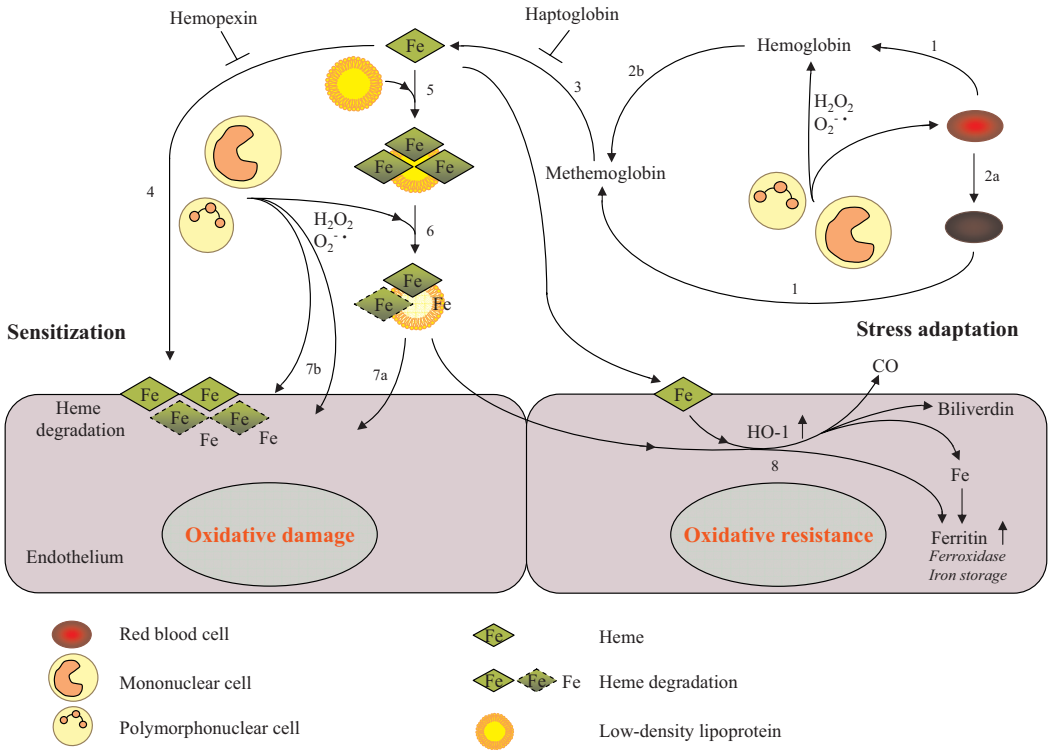

Figure 1. Oxidative stress and adaptation induced by heme and hemoglobin. 1: Leakage of hemoglobin from red blood cells; 2a and b: oxidation of hemoglobin; 3: heme release from methemoglobin; 4: heme uptake and sensitization of endothelium to oxidative stress; 5: heme uptake by LDL; 6: oxidative modification of LDL; 7a and b: oxidative stress induced by oxidized LDL and oxidants derived from leukocytes; and 8: induction of adaptation to oxidative stress *via* up-regulating heme oxygenase-1 and ferritin.

cellular iron powerfully protects eukaryotic and prokaryotic cells against oxidant challenge [3]. We have shown that one critical feature of highly damaging iron to endothelium is due to permeation 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 8-hydroxyquinoline-iron chelate was exquisitely sensitive to both endogenous and exogenous oxidant stress [4].

One abundant source of potentially toxic iron is iron protoporphyrin IX or heme with its hydrophobic nature. Heme, a ubiquitous iron-containing compound, is present in large amounts in many cells [5] and is also inherently dangerous, particularly when it escapes from intracellular sites [6–9]. Heme greatly amplifies cellular damage arising from activated oxygen [6–8].

The potential toxicity of free heme derives from the ease with which this highly hydrophobic compound can enter and cross cell membranes, and therefore readily concentrates within the hydrophobic milieu of intact cells [6, 7]. Both *in vitro* and *in vivo*, cells will accumulate exogenous heme and synergistically amplify the cytotoxic effects of oxidants of reagent, enzymatic, or cellular origin. Heme uptake by endothelial cells can exacerbate their damage by polymorphonuclear leukocytes (PMNs) – cells that tend to marginate along endothelial surfaces in the presence of diverse inflammatory mediators [6, 8]. Intriguingly, heme was shown by Graca-Souza *et al.* [10] to induce PMN activation as well. Moreover, Wagener *et al.* [11, 12] revealed that heme can enhance endothelial cell adhesion molecule expression, which regulates PMN adhesion and provokes 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 hemopexin [6, 7]. The plasma heme-binding protein, hemopexin, can block catalytic activity of heme [13, 14]. 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 and its pro-oxidant effects. Once within the cell, heme can promote oxidative damage either directly or, perhaps more importantly, *via* the release of

iron which can occur through either nonenzymatic oxidative degradation of heme [6, 7] 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 [6].

We asked: Could heme sensitize endothelial cells to oxidative challenge in the presence of plasma [8]? After all, plasma is enriched with binding proteins, such as albumin and hemopexin, known to inhibit heme-mediated cell damage. Exposure of endothelium to heme in the presence of whole human plasma synergizes cellular oxidant damage for added oxidants, with an optimal heme-exposure duration of 60 min. Intriguingly, cytotoxicity studies showed little added toxicity to endothelium if water solubility of heme is conferred associatively with the arginate counterion (heme arginate). Even with efficient permeation heme arginate does not amplify oxidant-induced cytotoxicity. In support, 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 can be obtained in the presence of human plasma although at two orders of magnitude greater concentration for both heme arginate and heme.

The hydrophobicity of various heme-analogs, 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; accordingly, hypersusceptibility is similarly provoked. On the contrary, if water solubility of heme is conferred associatively with the arginate counterion 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 failed to sensitize cells to oxidants or activated PMNs.

Although free heme is rapidly incorporated into hydrophobic domains of cells and serves as a source of highly damaging iron, the question remains as to 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 [15, 16]. This is because ferrihemoglobin readily releases its heme moieties as first demonstrated by Bunn and Jandl [17]. Released heme from ferrihemoglobin can indeed be rapidly incorporated into hydrophobic domains of cultured endothelium and serve 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 that it can readily be oxidized to heme-releasing methemoglobin in the presence of inflammatory-cell-derived oxidants [15, 18, 19]. For instance, PMNs, when activated with the phorbol ester PMA, markedly oxidize ferrohemoglobin to ferrihemoglobin within 30 min [15]. Accordingly, ferrohemoglobin in the presence of activated PMNs can provide heme to endothelium, which greatly enhances cellular susceptibility to oxidant-mediated cell injury [15, 16]. The oxidation of ferrohemoglobin to ferrihemoglobin is essential for this deleterious effect. Another candidate for generating methemoglobin is nitric oxide. Reaction of nitric oxide with free hemoglobin produces methemoglobin and leads to decreased nitric oxide bioavailability, causing pulmonary hypertension, vascular damage, and end-organ injury as reviewed by Gladwin *et al.* [20].

The initial release of heme from ferrihemoglobin can be inhibited by complexing with the hemoglobin-binding protein, haptoglobin [17]. If metheme binding to globin is strengthened by haptoglobin or if released heme is religanded to hemopexin, ferrihemoglobin loses much of its capacity to sensitize endothelium to reactive oxygen [15]. Hemoglobin : haptoglobin complex is eliminated from the circulation through the recently characterized CD163 receptor [21], 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, all of which avidly bind heme [22], do not alter endothelial integrity. At higher concentrations of free methemoglobin 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 those that revealed that hemoglobin behaves as a biologic Fenton reagent [23, 24] made us wonder whether hemoglobin in plasma could provide heme-iron to endothelium *in vivo*. We demonstrated 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 moieties to the vessel wall and aggravates endothelial cell damage in the short term. Ferrihemoglobin present in plasma increases the level of endothelial cell associated heme in lung [25], indicating that protective effects of haptoglobin [26], hemopexin [6, 13, 14], and albumin can be overwhelmed and the delivery of heme-iron to the endothelium occurs *in vivo* [25].

3 Indirect heme and hemoglobin toxicity to endothelial cells: LDL oxidation

Heme can also threaten vascular endothelial cell integrity indirectly by its ability to mediate the oxidative modification of low-density lipoprotein (LDL) [27]. The process of heme-mediated LDL oxidation involves coupled oxidative interactions between LDL, heme, oxidants, and antioxidants. The initial step of 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, 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 (TBARS).

Heme will oxidatively modify both the lipid moiety of LDL as well as the apoprotein, which latter can be detected through increased anodal electrophoretic mobility. This increased mobility suggests a loss of net positive charge, which can also be assayed independently by measurement of free amino group on the LDL particles. Fluorescamine-titratable free amino groups of apolipoprotein B-100 progressively fall during exposure of LDL to heme. During these oxidative reactions between heme, LDL, and peroxides, the heme ring (protoporphyrin IX) is degraded, with the resultant release of free iron. Both the destruction of 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 405 nm. 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 since desferrioxamine attenuates both the oxidative modification of LDL and the degradation of heme.

The requirement for intimate association between LDL and heme in LDL oxidation is supported by experiments employing hemopexin. This serum protein, present in remarkably high plasma concentration (≈1 g/L), binds heme with extraordinary avidity [28] and will prevent insertion of heme into LDL [27]. Not surprisingly, hemopexin, in stoichiometric amounts with heme, inhibits oxidative modification of LDL. As would also be expected, given the exception 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.

If water solubility of heme is conferred associatively with the arginate counterion, LDL lipid peroxidation is characterized by a longer lag phase and ΔT at V_{max} as well as a slower propagation phase compared to heme-mediated lipid peroxidation of LDL as judged by conjugated diene formation [8]. 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. Since the number of heme molecules associated with LDL particles was the same in LDL exposed to heme in serum as in LDL exposed to heme arginate, the more efficient free radical catalysis in LDL by heme could not be attributed to quantitative characteristics. In diluted serum (20%), heme was confirmed by Camejo [29] to bind to LDL leading to 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, since in heme-catalyzed oxidation of lipoprotein heme degradation occurs inversely with formation of lipid oxidation products including conjugated dienes and lipid hydroperoxide; thus, heme degradation functions as a probe for lipid peroxidation process [27]. Based on the kinetics of heme-catalyzed lipid peroxidation, we developed an assay for clinical laboratory to judge the susceptibility of LDL to oxidative modification [30], a risk factor of atherosclerosis. The oxidative resistance of LDL was characterized by ΔT at maximum velocity (V_{max}) in seconds, the time period until the maximal velocity of heme degradation as defined by the maximum change in absorbance of heme in the propagation phase of lipid peroxidation. The shortening of ΔT at V_{max} indicates the decrease in oxidative resistance of LDL. This novel assay is suitable for testing large number of LDL samples on an automated microplate reader. The advantages of our method over existing measurements [31 – 33] 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.

4 Hemoglobin-derived heme and LDL oxidation

Oxidative modification of LDL is implicated in the pathogenesis of atherosclerosis [27, 34 – 38]. LDL particles entering subendothelial "sanctuaries" of 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 is also directly cytotoxic, particularly to vascular endothelial cells. Such damage would presumably exacerbate atheroma formation both by allowing LDL to freely enter the artery wall and by promoting platelet adherence and growth factor liberation.

Although beyond heme [27, 39] a number of heme proteins – such as hemoglobin [40], myoglobin [41], horseradish peroxidase [42], myeloperoxidase [43], and lipoxygenase [44, 45] – have been reported to act as oxidants of LDL, 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 [46]. The authors of that study concluded that negligible heme transfer from hemoglobin to LDL, or none at all, occurred under the oxidative conditions they employed. Oxidation of hemoglobin to the ferryl state by peroxides has been reported to be accompanied by tyrosyl radical formation [47, 48]. In end-stage renal failure patients on chronic hemodialysis therapy, a high degree of apolipoprotein B-100 modification resulting from covalent association of hemoglobin with LDL was observed [49]. Authors postulated that tyrosyl radical species of hemoglobin that forms by oxidation of methemoglobin with hydrogen peroxide to ferryl hemoglobin 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 [50] generally support such a mechanism insofar as maneuvers which 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 using various strategies to stabilize the heme moiety. Haptoglobin or cyanide was shown to strengthen heme–globin liganding, preventing heme release from ferrihemoglobin. Preincubation of ferrihemoglobin with sodium cyanide or stoichiometric amounts of haptoglobin prevented the generation of oxidized LDL. Our findings might explain why haptoglobin polymorphisms were found in clinical studies to be a risk factor in the pathogenesis of atherosclerosis [51].

In elegant studies Shaklai's group [52] recently revealed that haptoglobin phenotypes differ in their ability to inhibit heme transfer from hemoglobin to LDL. Heme transfer from methemoglobin to LDL was demonstrated to be almost completely omitted by haptoglobin 1-1 and only partially by haptoglobin 2-2. Accordingly, haptoglobin 1-1 was shown to inhibit hemoglobin-induced oxidation of lipoprotein more vigorously compared to haptoglobin 2-2. These findings might explain why individuals with haptoglobin 2-2 have more atherosclerotic incidences as compared to those with haptoglobin 1-1 [51].

Although ferrohemoglobin in plasma does not itself provoke oxidation of LDL, hemoglobin can readily be oxidized to heme-releasing methemoglobin in the presence of inflammatory-cell-derived oxidants [8, 15, 18]. Concordantly, if endothelial cells are exposed to LDL isolated from plasma containing ferrohemoglobin and activated PMNs, oxidative endothelial damage develops [50]. 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; concomitantly, LDL isolated from plasma containing ferrohemoglobin, activated PMNs, and catalase leads to reduced endothelial cell cytotoxicity.

In a recent study, we have shown that LDL-associated lipid hydroperoxides convert ferrohemoglobin to methemoglobin in a dose-dependent manner as well [53]. Reduction of lipid hydroperoxide content of LDL with GSH peroxidase prevents the formation of methemoglobin. Interestingly, haptoglobin, a hemoglobin-binding protein, could not inhibit this oxidation, but it can prevent heme release from the resultant methemoglobin.

The results of several independent assays for LDL lipid peroxidation support the conclusion that ferrihemoglobin-derived heme promotes LDL oxidation [50]. Shortening 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 TBARS. In contrast, ferrihemoglobin complexed with haptoglobin or cyanomethemoglobin did 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. Finally, ferrohemoglobin in plasma does not have the capacity to increase the susceptibility of LDL to oxidative modification.

The release of free heme from ferrihemoglobin is an important precedent event in generating toxic LDL. Once heme is lodged within the LDL, spontaneous oxidative reactions involving small amounts of lipid hydroperoxides or other oxidizing equivalent will lead to oxidative lysis of heme group and release of heme-iron within the LDL particle. Most likely, it is the hemoglobin derived heme-iron that catalyzes the further breakdown of heme as well as the oxidation of polyunsaturated fatty acids and other components of the LDL.

These observations raised the question of the nature of the toxic substance(s), which 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 [54] and 7-oxysterols [55] have been reported to be highly cytotoxic. Moreover, ebselen, a seleno organic compound, which has hydroperoxide reducing activity, protects against oxidized LDL-induced cell death in human fibroblast cells [56]. Reaction of LDL with heme derives a markedly toxic LDL in less than 2 h [27]. Our results suggest that an accumulation of LOOH is the predominant toxic species within oxidized LDL catalyzed by heme because specific enzymatic reduction of LOOH to LOH yields LDL with minimal toxic effects [50]. Furthermore, we find that, on an equimolar basis, LOOH within oxidized LDL and an organic hydroperoxide, cumene hydroperoxide, have very similar toxic effects on endothelial cells.

5 Adaptation to oxidative stress in vascular endothelial cells: heme oxygenase-1 and ferritin

First correlative support for the notion of heme oxygenase and ferritin being an antioxidant cytoprotective stratagem of endothelium derived from the time-dependent dichotomous effects of heme exposure on endothelial cells [57]. Within cells, heme can directly mediate damaging oxidation reactions, and undergo oxidative breakdown, releasing free iron which is well known to catalyze oxidant degradation of a large variety of biologic substances such as fatty acids, proteins, and nucleic acids. The foregoing considerations prompted us to hypothesize that endothelial cells might synthesize a natural iron "chelator" to limit the reactivity of heme-derived, intracellular iron, although this notion was triggered by our observation that endothelial cells exposed to heme for a longer period of time convert from hypersusceptibility to hyperresistance to oxidative challenge [57]. Namely (1) heme after rapid incorporation into endothelial cells, as little as 1 h, markedly aggravates cytotoxicity engendered by PMN oxidants or various forms of reactive oxygen; (2) intriguingly, if vascular endothelial cells are briefly pulsed with heme and then allowed to incubate for a more prolonged period (12 – 72 h), the cells become highly resistant to oxidant-mediated injury and to the accumulation of endothelial lipid peroxidation products. As in our studies with heme itself, the effects of ferrihemoglobin on endothelial susceptibility to oxidant damage were found to be also dichotomous [15, 16]. Brief exposure to ferrihemoglobin produced an endothelium hypersusceptible to oxidant damage, more prolonged exposure rendered it highly resistant.

We were extremely curious about the molecular basis of this cellular protection against oxidant damage. Since heme and various stimuli were shown to cause the induction of both heme oxygenase-1 [58 – 62] and ferritin [63 – 65] in various cell types, we wondered whether one or both of these constituents might be expressed in endothelial cells in response to heme. Endothelial cells exposed to heme were shown to be also rapidly induced to increase heme oxygenase-1 mRNA level and enzyme activity as well as both H and L ferritin synthesis [57]. Heme oxygenase is thought to serve as a provider of intracellular iron from heme; this iron, in turn, drives the synthesis of ferritin. Alternatively, heme itself might enhance ferritin synthesis directly by increasing RNA translation [65]. Indeed, exposure of endothelial cells to the combination of heme and tin mesoporphyrin IX causes substantial increases in intracellular ferritin with significant decrement in heme oxygenase activity, or subcutaneous injection of Sn-protoporphyrin IX slightly but insignificantly decreased ferrihemoglobin-induced ferritin accumulation in rat lungs [25]. Although we demonstrated that free heme can be rapidly incorporated into hydrophobic domains of endothelium and serve as an inducer for both heme oxygenase-1 and ferritin, the question remained as to whether intact hemoglobin would also be similarly assimilated. Hemoglobin was demonstrated to up-regulate vascular endothelial cell heme oxygenase-1 and ferritin genes [15, 16]. Oxidation of ferrohemoglobin to ferrihemoglobin is essential for this effect [15], presumably because ferrihemoglobin readily releases its heme moieties; ferrohemoglobin or other heme proteins, such as metmyoglobin and cytochrome c, all of which avidly bind heme, do not alter the expression of heme oxygenase-1 and ferritin. Furthermore, if heme binding to globin is strengthened by haptoglobin or cyanide [17], or if released heme is liganded to hemopexin [28], 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* [25] in spite of the fact that two plasma proteins, haptoglobin and hemopexin, may act to depress endothelial heme-iron loading by the mechanism through their ability to tightly bind free hemoglobin and heme, respectively. We note that this depression is not absolute however, since slight but significant increases in ferritin content continue to occur in ferri-

hemoglobin-treated endothelium, despite the addition 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 heme or heme-proteins bound to hemopexin or haptoglobin [66, 67]. However, the blunted response of endothelium suggests 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 [25]; methemoglobin, but not ferrohemoglobin, increases the expression of total lung heme oxygenase-1 mRNA that is accompanied by a marked enhancement of total lung heme oxygenase enzyme activity in rats. *In situ* hybridization for heme oxygenase-1 mRNA revealed endothelial cell-associated accumulation of heme oxygenase-1 mRNA. The heme-iron uptake in endothelium was also supported by detection of immunoreactive ferritin. We have reasoned that heme might derive from damaged circulating red cells in close contact with vascular lining cells and since oxidation of hemoglobin to ferrihemoglobin is essential for endothelial perturbation, we sought to model oxidant conditions which might be relevant to vascular pathophysiology [15, 16]. Activated inflammatory cells (PMNs and monocytes) can efficiently oxidize hemoglobin contained in red cells to ferrihemoglobin. We demonstrated that soluble hemoglobin in plasma is more rapidly oxidized to ferrihemoglobin when exposed to activated PMNs; moreover, endothelial cells are induced to increase the expression of heme oxygenase-1 and ferritin. Although the released heme is the critical component in these inductions, 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 oxidant species, such as superoxide anion and hydrogen peroxide, since heme oxygenase-1 has been shown to be induced in other cells by reagent oxidants [62], or instead might be due to other heme-containing PMN constituents, such as myeloperoxidase.

Heme or hemoglobin amplifies oxidant-mediated endothelial cytotoxicity during brief exposure, yet markedly induces adaptation and protects oxidant-exposed target cells if provided for longer duration. This resistance parallels with the synthesis and accumulation of large amounts of heme oxygenase-1 and ferritin [15, 57]. In order to identify the ultimate cytoprotectant we developed experimental conditions [57] 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 causes substantial increases in intracellular ferritin without any increment in heme oxygenase activity. In both these cases, an associated marked protection against subsequent oxidant challenge was noted. In order to further examine the consequences of elevated intracellular ferritin, we directly loaded preformed apoferritin into cultured endothelial cells. Ferritin-loaded cells become resistant, in a dose-responsive fashion, to oxidant stress imposed by various forms of reactive oxygen. Accompanying this resistance there was a parallel reduction in the peroxidation products.

In our studies of endothelial cells, the protection provided by ferritin is evidently attributable to either iron storage and/or the intrinsic ferroxidase activity of the heavy (H) subunit [57]. Ferroxidase activity of ferritin is located only on the H but not the light subunit [68 – 70]. This activity catalyzes the oxidation of ferrous iron under aerobic conditions to ferric iron to allow intracellular iron storage in biological systems. Ferroxidase activity in human H-chain ferritin has been widely studied by Arosio's group with the aid of site-directed mutagenesis [68]. A site discovered by his group employing 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 [57].

Furthermore, we found that the recombinant ferritin, mutant 222 [70], which lacks ferroxidase activity and is unable to take up iron is an ineffectual protectant [57]. Ferritin is unlikely to protect cells by indiscriminant iron chelation. Ferritin's protective function is possibly linked to its affinity for ferrous (Fe^{2+}) iron formed by superoxide or endogenous reductants, and although its ferroxidase activity stores and inactivates iron in a ferric form, it is the reduced form of iron that can fuel production of the toxic hydroxyl radical. In effect, increased cellular ferritin may compete for ferrous iron, thus reducing the pool of catalytically active iron species.

There is disagreement concerning the ability of ferritin to maintain iron in a safe state incapable of catalyzing oxidative injury. All of these studies were performed in cell-free systems and suggested that ferritin can amplify oxidative phenomena [71 – 73]. For example, *in vitro* superoxide [72] and nitric oxide [74] have been shown to displace iron from ferritin in the presence of strong chelators. These conflicting observations likely relate to the iron content of the ferritin used in the cell-free experiments; excess apoferritin kinetically would be expected to bind excess iron whereas iron-laden ferritin would be expected to release it under harsh experimental conditions. Endothelial cells induced to

produce ferritin by iron compounds were protected despite the increased cellular iron 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 ferroxidase activity and storing iron [57].

To examine the relevance of our *in vitro* data on endothelial cells, we collected specimens of coronary arteries of cardiac explants from patients with atherosclerotic or idiopathic cardiomyopathy and determined whether there was ferritin present at sites of atherosclerotic disease [75]. Immunoperoxidase studies revealed abundant immunoreactive ferritin specifically in coronary atherosclerotic lesions with active inflammation in all specimens with virtually no detectable staining seen in normal coronary arteries of the dilated cardiomyopathy hearts. Of note, no ferritin was seen extracellularly or in coronary artery atherosclerotic lesions where there was little cellular infiltrate or inflammation. The identity of the cell types within the lesion was supported by their immunoreactive characteristics on serial sections. 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 SMA-positive myofibroblasts in the neointima of the fibrous cap and in foamy macrophages. Similarly to ferritin, up-regulation of heme oxygenase-1 also occurs in the atherosclerotic plaques [76, 77]. 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.

Since oxidative modification of LDL has been suggested to be a key event in atherosclerosis [27, 34 – 38] and heme oxygenase-1 was revealed to be inducible by oxidants [62], we tested whether LDL oxidation alters the expression of endothelial heme oxygenase-1. Indeed, we demonstrated that oxidative modification of LDL, catalyzed by either heme or copper, elicits massive induction of heme oxygenase-1 in endothelial cells accompanied by significant increments in ferritin content [78]. Such induction correlated with the oxidative insult imposed by LDLox, since preconditioning of LDL or the endothelium by selected antioxidants markedly diminished the expression of both heme oxygenase-1 and ferritin. Our results suggest that an accumulation of LOOH within oxidized LDL 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 yields LDL with minimal effects [50]. Agarwal *et al.* recently investigated the mechanism by which oxidized LDL regulates the expression of heme oxygenase-1. They found that among the components of oxLDL, the most potent inducer of heme oxygenase-1 is a lipid-hydroperoxide, the 13-hydroperoxy-octadecadienoic acid (13-HPODE) which transcriptionally regulates the heme oxygenase-1 through a 13-HPODE-specific regulatory element in the human heme oxygenase-1 promoter [79].

Pretreatment of endothelial cells by the iron chelator desferrioxamine B also prevented such induction, thereby uncovering the fundamental role of intracellular iron in the response to LDL oxidation [78]. We observed an intracellular GSH depletion in endothelium [50] known to enhance heme oxygenase-1 expression [80]. In experiments in which oxidation of LDL is catalyzed by heme, heme *per se* cannot be ascribed as the cause for the induction of heme oxygenase-1 and ferritin, since in the course of catalyzing the oxidation of LDL, heme itself undergoes degradation. Furthermore, treatment of LDL with antioxidants prior to its exposure to heme prevents the oxidation of lipoproteins and the induction of both heme oxygenase-1 and ferritin, in spite of the fact that heme content of LDL remains high.

As in our studies with heme and hemoglobin, the effects of LDL oxidation on endothelial susceptibility to oxidant damage were found to be also dichotomous. Exposure to LDLox produced substantial cytotoxicity; a prolonged exposure at sublethal concentration rendered endothelium resistant to oxidant damage. This prompted us to hypothesize that ferritin might provide resistance to LDLox in the vasculature. Apoferritin loading of endothelium provides a means by which intracellular ferritin can be increased without an inducing stimulus [57]. Indeed, cells incubated with apoferritin are protected in a dose-dependent fashion from LDLox-mediated toxicity [75]. To test whether the ferroxidase activity of ferritin is necessary for its protective role against LDLox, we employed a recombinant human H ferritin and a mutant H ferritin, 222, formed by site-directed mutagenesis. Mutant 222, containing two amino acid substitutions, lacks ferroxidase activity and is devoid of iron-chelating capability [70] but is taken up by endothelial cells shown by ELISA [57]. Apoferritin or recombinant H ferritin efficiently protects endothelial cells from LDLox whereas similar loading with mutant 222 is without significant effect. That induction of heme oxygenase and ferritin results in adaptation to LDL-mediated oxidative injury is supported by the finding that endothelial cells treated with heme or other cell-permeant iron chelate (iron-pyridoxal isonicotinoyl hydrazone) are also protected. Importantly, it has been shown by Van Lenten *et al.* [81] that intracellular ferritin abolishes iron-induced LDL modification.

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 [82 – 84]. This has led to the speculation that such receptor up-regulation may be advantageous for

tumor proliferation by supplying iron for DNA synthesis [85]. Indeed, neoplastic cells have an increased tendency to incorporate iron into metabolically active compounds [86]. Chelation of cellular iron by desferri-exochelin was shown to induce death by apoptosis in human breast cancer cells [87]. Since diverse chemotherapeutic agents and tumor-engaging immune cells act *via* oxidant-mediated toxicity, it seems reasonable to question whether the quantity and quality of cellular iron in tumor cells might modulate their susceptibility to oxidants, or to prototype oxidant chemotherapeutic agents, for instance, bleomycin.

Similarly to endothelial cells, colon and breast cancer lines (Caco-2 and BT-20 tumor cells) undergo dose-related lysis when challenged by heme followed by various forms of oxidants, but the former cells are generally less sensitive to oxidant injury [88]. To assess the relevance of cellular iron on oxidant-dependent chemotherapeutic efficacy, we examined the effect of pretreating BT-20 cells with heme on 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.

In contrast to endothelial cells – keeping with a post-transcriptional control of ferritin synthesis, ferritin light and heavy chain mRNA levels are not affected by cellular labile iron pool in endothelium – in both BT-20 and Caco-2 tumor cell lines induced by heme or $FeSO_4$ there is a significant increase in the level of H ferritin mRNA [57, 88]. An increased synthesis of H ferritin in different tumor cells has also been described by others [89] who, like us, have speculated that it might protect rapidly proliferating cells from toxicity of free ferrous iron [90] otherwise necessary for new cell metabolism. Beaumont *et al.* [91] described a five-fold increase in H ferritin mRNA in DMSO-induced Friend erythroleukemia cells. Gurner *et al.* [92] demonstrated that advanced breast cancer cells (stage II or III) had five times higher cytosolic ferritin levels than stage I cancers, implying that cellular ferritin may confer protection against immune cell 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 using bleomycin validate this suggestion; *i.e.*, this chemotherapeutic agent, known to oxidatively degrade cellular DNA, requires free Fe^{2+} and O_2 for its toxic effects. The ultimate agent of DNA damage is probably a form of reduced oxygen produced during tertiary complex formation between DNA, bleomycin, Fe, and O_2 [93, 94]. Sequestration of available cellular reactive iron by its incorporation into newly synthesized ferritin endows reduced sensitivity of the 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 Fe^{2+} complexer, 1,10-phenanthroline [94].

Catabolism of heme by heme oxygenase may rid the cells of a membrane-permeant form of iron, but the resultant nonheme iron would represent a potential hazard unless sequestered by ferritin [57]. The cytoprotective nature of heme oxygenase and ferritin has been confirmed in various models. Keyse and Tyrell [62] discovered that heme oxygenase-1 is the major 32-kDa stress protein inducible by oxidative stress such as UVA radiation and hydrogen peroxide. This very important observation prompted us to study the role of heme oxygenase in heme-catalyzed oxidative damage of endothelial cells. In their subsequent work Vile *et al.* [95] demonstrated that heme oxygenase-1 induction mediates an adaptive response to oxidative stress *via* ferritin synthesis in human skin fibroblast. Maines's group [96] raised the notion that increases in heme oxygenase-1 transcript and protein reflect a means to elevate levels of antioxidants in cells with compromised defense mechanisms caused by stress. Abraham *et al.* [97] described 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 by Lee *et al.* [98] to result in cell growth arrest and increased resistance to hyperoxia. In a series of studies Lin and Girotti [99 – 101] revealed that heme-enhanced resistance to oxidative killing is mediated by H ferritin chain in human leukemia cells confirming the antioxidant role of ferritin. Increased ferroxidase activity *via* overexpression of wild-type ferritin H-chain was demonstrated by Cozzi *et al.* [102] and Broxmeyer *et al.* [70] to reduce cell growth and increase resistance to hydrogen peroxide toxicity in HeLa cells [102]. Furthermore, heavy chain ferritin was nicely shown by Berberat *et al.* [103] to act as an antiapoptotic gene that protects livers from ischemia/reperfusion injury.

In a cell culture oxidative stress model carried out by Rothfuss and Speit [104], where lymphocytes were treated by hyperbaric oxygen, the cells became resistant to oxygen toxicity by increasing their own cellular ferritin levels. In the same experiments exogenous CO did not protect cells from hyperbaric oxygen-induced oxidative DNA damage. The interaction of H ferritin with DNA explored by Surguladze *et al.* [105] may be the responsible step to modulate the iron-mediated gene damage. 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 *in vitro* cell culture system. The cytoprotective ferritin counteracts the toxic effect of the intracellular labile iron pool as nicely shown by Fonseca *et al.* [106] in these experiments. The importance of the labile iron pool and H ferritin interaction is underlined by Epsztejn's observation where murine erythroleukemia cells transfected with H ferritin subunits show lower production of reactive oxygen species, and the long-term cell damage induced by free radicals was significantly lower in the H ferritin over-expressing clones [107].

Brain is especially rich in lipoproteins, which can be the target of heme-catalyzed free radical toxicity. In an *in vitro* cortical astrocyte model the induced intracellular ferritin was explored by Regan *et al.* [108] to provide defense against heme-mediated injury. In cases of human intracerebral and subarachnoid hemorrhages, Wu *et al.* [109] and Suzuki *et al.* [110] suggested that ferritin plays a protective role against heme-iron-mediated pathological reactions. Hemoglobin was shown by Taylor *et al.* [111] to induce protection against hyperoxia-mediated lung injury in rats. The surface of human body can suffer injuries from radiations. Employing *in vitro* tissue culture experiments Goralska *et al.* [112] and Applegate *et al.* [113] revealed that lens epithelial and skin cells are protected by endogenous ferritin against photooxidative stress induced by UV and infrared radiation. Furthermore, the aspirin-induced endothelial cytoprotection against hydrogen peroxide toxicity was revealed by Oberle *et al.* [114] to depend upon the up-regulation of ferritin synthesis. Importantly, it has been found that intracellular ferritin attenuates iron-catalyzed LDL oxidation [81]. These studies emphasize the paramount role of ferroxidase activity and iron sequestration by ferritin to serve as a protective gene by virtue of antioxidant, anti-apoptotic, and antiproliferative actions.

Increased coexpression of heme oxygenase and ferritin can be observed in humans and in animal models. Nath *et al.* [115] provided the first *in vivo* evidence in an animal model that induction of heme oxygenase-1 coupled to ferritin synthesis is a rapid protective antioxidant response; induction of heme oxygenase-1 and ferritin protects rats against rhabdomyolysis-induced renal failure. Otterbein *et al.* discovered that hemoglobin-induced protection against lethal endotoxemia in rats [116] is mediated by a pathway independent from ferritin [117]. Furthermore, they also demonstrated that heme oxygenase-1 can provide protection against hyperoxia-induced lung injury *in vivo* by modulation of neutrophil inflammation and lung apoptosis [118]. Dennery [119] described that heme oxygenase-2 is also essential for protection against hyperoxia-induced lung injury since heme oxygenase-2-deficient mice were shown to be sensitive to hyperoxia-induced oxidative lung injury with the absence of ferritin induction. Poss and Tonegawa provided further *in vivo* evidence – employing mice lacking functional heme oxygenase-1 [120] – that up-regulation of heme oxygenase-1 serves as an adaptive mechanism to protect cells from oxidative damage during stress [121]. Heme oxygenase-1 was explored by Soares *et al.* [122] to rescue cardiac xenograft from rejection, as transplanted hearts from heme oxygenase-1 knockout mice were rapidly rejected. Bak *et al.* [123] provided evidence that up-regulation of heme oxygenase-1 in myocardium prevents arrhythmia; hearts from heme oxygenase-1 knockout mice exposed to ischemia/reperfusion rapidly develop ventricular fibrillation.

By demonstrating the protective effect of carbon monoxide in a model of hyperoxic lung injury [124] and mouse cardiac xenotransplantation model [122, 125], Otterbein *et al.* and Soares *et al.* opened a new chapter in heme oxygenase-1 research. It has been suggested by Motterlini's group [126] that transition metal carbonyls should be utilized for therapeutic delivery of carbon monoxide. Conversion of heme by heme oxygenase to biliverdin and bilirubin was demonstrated by Dore *et al.* and Clark *et al.* to protect neurons against oxidative stress [127] and to ameliorate postischemic myocardial dysfunction [128]. In fact, Stocker [129] demonstrated that bilirubin is an important antioxidant. Description of a cycle by Baranano *et al.* [130], in which bilirubin, acting as an antioxidant, is itself oxidized to biliverdin and then recycled by biliverdin reductase back to bilirubin, suggests a mechanism to amplify the antioxidant effect. It was suggested by Ferris *et al.* [131] that heme oxygenase-1 prevents cell death by regulating cellular iron; Fe^{2+} up-regulates an iron-transporter pump that might remove Fe^{2+} from cells. Carbon monoxide and bilirubin produced during the degradation of heme have attracted a great deal of interest on its potential function in regulating vascular tone and hemostasis, as well as antiinflammatory, anti-apoptotic, and antiproliferative responses.

Since the main interest of our group has been the pathological roles of heme, heme proteins in human diseases, and the endogenous protective mechanisms of different cells and organs, the last 10 years provided us a great satisfaction seeing the scientific effort to study the details of the heme-heme oxygenase-ferritin system. The presence and the increased levels of heme oxygenase and ferritin alone are not enough for cell and organ protection; the availability of heme as substrate is also necessary to produce carbon monoxide and bilirubin. During these processes a decrease in heme concentration, "labile heme pool", also occurs, together with the consumption of reducing substances and oxygen. This complexity is the reason why in various models and pathological states ferritin, heme oxygenase, or both together are required for the protective phenomenon.

6 Heme oxygenase-1 deficiency

The toxic effects of heme may be important in a number of pathologies. These include not only acute conditions such as intravascular hemolysis, which can lead to renal failure, but also more insidious processes such as atherogenesis. Intriguingly, intralesional deposits of iron [132, 133] perhaps derived from heme of erythrocytes have been observed. Studies from our laboratory have revealed that ferritin is highly expressed in human atherosclerotic lesions [75] coupled to heme oxygenase-1 [76] while Wang *et al.* [77] have shown that there is increased expression of heme oxygenase-1 in atherosclerotic plaques. The up-regulation of heme oxygenase-1 and ferritin genes in endothelium in the early phase of progression of atherosclerosis possibly reflects a cellular response to heme- or iron-generated lipid peroxidation products.

The central importance of heme oxygenase-1 in vascular biology was highlighted by the discovery of a child with heme oxygenase-1 deficiency diagnosed by Yachie *et al.* [134]. In the heme oxygenase-1 deficient child, both intravascular hemolysis and endothelial cell injury were revealed. Lymphoblastoid cell line derived from the patient was demonstrated to have increased sensitivity to heme toxicity. Fatty streaks and fibrous plaques in the aorta reported by Kawashima *et al.* [135], as sign of severe atherosclerosis, as well as mesangioproliferative glomerular changes in the kidney, were also prominent features [134, 135]. Similar damage to endothelium, as well as hepatic and renal tubular cytotoxicity, has been observed in transgenic knockout mice deficient in heme oxygenase-1 [120].

We hypothesized that oxidation of free hemoglobin in plasma could threaten vascular endothelial cell integrity *via* oxidative modification of LDL *in vivo* and that oxidized LDL might also induce cytoprotectants such as heme oxygenase-1 and ferritin. Evidence that toxic species of LDL accumulate *in vivo* derived from experiments involving LDL isolated from the plasma of the heme oxygenase-1 deficient child reported earlier [50]. Whereas heme, or ferrihemoglobin, may be directly cytotoxic to vessel walls, investigations presented earlier [50] determined that hemoglobin in plasma in heme oxygenase-1 deficiency might, at least in part, represent an indirect process. Specifically, extensive hemoglobin/heme-iron-mediated oxidation of LDL produces oxidized forms of LDL with appreciable cytotoxicity.

Importantly, spectral analysis of the heme oxygenase-1 deficient child's plasma revealed that oxidation of hemoglobin to ferrihemoglobin occurred in his plasma. Hemoglobin was predominantly ferrihemoglobin, the proportion of total hemoglobin present as ferrihemoglobin was around 80% (~60 μM).

Intriguingly, LDL of the heme oxygenase-1 deficient child had increased electrophoretic mobility suggesting the loss of net positive charge, which was confirmed by measurement of fluorescamine-titratable free amino groups on the LDL particle; fluorescamine-reactive amino group content fell to 732 mol/mol apolipoprotein B-100 compared to control (978 mol/mol apolipoprotein B-100). 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 mol/mol 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 while the iron content of LDL is increasing to 2.9 ± 0.3 mol/mol apolipoprotein B-100. 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 and there was 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. Conjugated dienes, LOOHs, and TBARS accumulated in his lipoprotein.

One of the biological features of the heme oxygenase-1 deficient child's LDL was shown to induce endothelial cell cytotoxicity. These results raised the question of what kinds of cytotoxic materials might be present in the child's LDL. LDL is a complex mixture which includes triglycerides, cholesterol esters, phospholipids, unesterified cholesterols, lysophosphatidylcholine, phosphatidylethanolamine, diacylglycerol, ceramide, and some phosphatidylinositol. Oxidation leads to formation of a wide range of biologically active products, and some of these, such as 7-oxysterols, have been reported to be highly cytotoxic [55]. However, 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. If endothelial cells were exposed to either heme oxygenase-1 deficient child's LDL or cumene hydroperoxide, we observed a precipitous decline in intracellular GSH content.

Finally, we hypothesized that cells of the heme oxygenase-1 deficient child were 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 [52].

A vicious circle could be formed by heme-iron catalysis of LDL peroxidation in the heme oxygenase-1 deficient child. These complex reactions include (1) oxidation of ferrohemoglobin to methemoglobin, (2) spontaneous insertion of the heme released from methemoglobin into LDL, (3) subsequent oxidative scission of the porphyrin ring, (4) release of free iron from the porphyrin ring, (5) iron catalysis of oxidation of further heme molecules, LDL fatty acids, and proteins, and (6) LDL-associated lipid hydroperoxides further convert ferrohemoglobin to methemoglobin. Certainly, Fe^{2+} reacts very readily with lipid hydroperoxides at a rate several orders of magnitude greater than the reaction between Fe^{2+} and hydrogen peroxide (1.5×10^3 *vs.* 76/ $M \times s$, respectively) yielding alkoxyl radicals ($R-O^{\bullet}$) and Fe^{3+}. Fe^{3+}, although less reactive, can catalyze the formation of both alkoxyl and peroxy ($RO_2^{\bullet}$) radicals and the coupled formation of Fe^{2+}. The continued reduction of iron may be important because maximal rates of lipid peroxidation require the presence of both ferrous and ferric species.

Since endothelial cytolysis was induced by the heme oxygenase-1 deficient child's LDL, we wondered if it is also capable of enhancing the expression of heme oxygenase-1 and ferritin in endothelial cells of healthy subjects. Exposure of endothelial cells derived from healthy subjects to sublethal stress of the child's LDL led to marked increase in enzyme activity for heme oxygenase and doubled ferritin content. Inhibition of heme oxygenase enzyme activity in 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 of heme oxygenase-1 deficient child by glutathione/glutathione peroxidase prevented the up-regulation 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 human, represents a hazard to vascular endothelial cells by not only sensitizing endothelium to oxidant damage but also catalyzing the oxidation of LDL. Rise of adaptation against such an insult relies on the response 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 adaptation. The up-regulation of heme oxygenase-1 and ferritin genes in atherosclerotic lesions possibly reflects a response to heme and heme-iron-generated lipid peroxidation products.

7 References

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