Ferritin: A Cytoprotective Antioxidant Strategem of Endothelium*

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Phagocyte-mediated oxidant damage to vascular endothelium is likely involved in various vasculopathies including atherosclerosis and pulmonary leak syndromes such as adult respiratory distress syndrome. We have shown that heme, a hydrophobic iron chelate, is rapidly incorporated into endothelial cells whereas, after as little as 1 h, it markedly aggravates cytotoxicity engendered by polymorphonuclear leukocyte oxidants or hydrogen peroxide (H₂O₂). In contrast, however, if cultured endothelial cells are briefly pulsed with heme and then allowed to incubate for a prolonged period (16 h), the cells become highly resistant to oxidant-mediated injury and to the accumulation of endothelial lipid peroxidation products. This protection is associated with the induction within 4 h of mRNAs for both heme oxygenase and ferritin. After 16 h heme oxygenase and ferritin have increased approximately 50-fold and 10-fold, respectively. Differential induction of these proteins determined that ferritin is probably the ultimate cytoprotector. Ferritin inhibits oxidant-mediated cytolysis in direct relation to its intracellular concentration. Apoferritin, when added to cultured endothelial cells, is taken up in a dose-responsive manner and appears as cytoplasmic granules by immunofluorescence; in a similar dose-responsive manner, added apoferritin protects endothelial cells from oxidant-mediated cytolysis. Conversely, a site-directed mutant of ferritin (heavy chain Glu² → Lys; His⁶⁰ → Gly) which lacks ferroxidase activity and is deficient in iron sequestering capacity, is completely ineffectual as a cytoprotectant. We conclude that endothelium and perhaps other cell types may be protected from oxidant damage through the iron sequestrant, ferritin.

Aerobic organisms are well endowed with enzymatic oxidant defense systems which protect against direct assault by activated oxygen species such as superoxide and hydrogen peroxide (H₂O₂). However, much of the cellular damage caused by activated oxygen involves the collaboration of intracellular iron. For example, the amount of H₂O₂ required to kill Staphylococcus aureus decreases 1,000-fold if the bacteria are raised in iron-rich media (1). Conversely, iron chelators such as deferoxamine and dipyridyl protect eukaryotic and prokaryotic cells against challenge by oxidants such as H₂O₂ (2, 3). This implies that oxidative reactions, when catalyzed by intracellular iron, are especially potent and important damaging events and, further, that endogenous iron sequestrants might be critical in antioxidant strategy.

Heme, a ubiquitous iron-containing compound, is present in large amounts in many cells and is also inherently dangerous, particularly when it escapes from intracellular sites (4). Its toxicity may reflect the fact that, unlike "free" iron, heme readily enters the hydrophobic domain of biologic membranes. The vascular endothelium, because of its continuous contact with circulating red blood cells, might be at risk from exogenous heme exposure. In previous studies we showed that hemin rapidly intercalates into cultured endothelial cells resulting in their marked hypersusceptibility to subsequent oxidant-mediated cytolysis by H₂O₂ or adherent polymorphonuclear leukocytes (PMNs) (5).

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. Within most cells the major depot of nonmetabolic iron is ferritin, a high molecular mass (450 kDa), multimeric (24-subunit) protein (heavy or H chain M, 21,000, light or L chain M, 20,700) with a very high capacity for storing iron (4,500 mol of iron/mol of ferritin). In the ferritin shell the proportion of H and L subunits depends on the iron status of the cell or tissue and varies between organs and species (6). Despite earlier reports that under certain chemical circumstances ferritin can release catalytically active iron (7) which can actually foster peroxidation of lipids (8), recent studies suggest that such release is slight under more physiologic circumstances, less than 2 of 4,500 potential iron atoms released per ferritin molecule (9). Alternatively, ferritin might beneficially sequester intracellular iron, limiting the pro-oxidant hazard posed by this reactive metal; moreover, the fact that the H chain of ferritin manifests ferroxidase activity (10, 11) implies that ferritin-stored iron might resist cyclical reduction/oxidation reactions which tend to propagate and amplify oxidative damage. The following studies, which have been presented in preliminary form elsewhere (12), demonstrate that endothelial exposure to heme induces sequential synthesis of heme oxygenase followed by ferritin; the latter whether endogenously produced or added directly as the apoprotein, protects endo-

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1 The abbreviations used are: PMN(s), polymorphonuclear leukocyte(s); H, heavy; L, light; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; PAEC, porcine aortic endothelial cells; Fe-PH, iron-pyridoxal isonicotinoyl hydrazone.
themelium from oxidant-mediated cytolysis and does so in a dose-responsive manner. Using an oligonucleotide site-directed mutant of ferritin H chain, we further validate the critical role of the ferroxidase and iron reoxidation properties of ferritin in this cytoprotection.

**Experimental Procedures**

**Reagents**—Dubelco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and Hank's balanced salt solution (HBSS) were obtained from Gibco, and minimal essential medium was from Hazleton (Lenexa, KS). Collagenase type I was purchased from Worthington. Soybean trypsin inhibitor (obtained from Miles, Inc., Kankakee, IL); hydrogen peroxide (H2O2, 30%), from Fisher Scientific; 6% hydroxethyl starch, from Du Pont; deferoxamine mesylate, from Ciba-Geigy; and 11CrO4 (as the sodium salt), from Amersham Corp. Bovine hemin type I and tmsnaporphyrin IX were obtained from Porphyrin Products (Logan, UT). All other reagents utilized were obtained from Sigma unless otherwise specified.

**Endothelial Cell Isolation and Culture**—As described previously (5), porcine aortic endothelial cells (PAEC) were isolated from porcine aorta using type I collagenase (0.25% for 15 min at 37 °C). Endothelial cells were grown in DMEM containing 10% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml), and supplemented with L-glutamine to a concentration and used from passages 5 to 15. Human umbilical vein endothelial cells and human aortic endothelial cells were grown as described previously and utilized (13, 14).

**Human Neutrophil Preparation**—As in our previous studies (15), neutrophils (PMNs) were isolated from human volunteers after informed consent (following guidelines of the Committee on the Use of Humans Subjects in Research of the University of Minnesota).

**Endothelial Cytotoxicity Assays**—Confluent endothelial cells (usually PAEC) grown in 24-well (2 cm2/well) tissue culture plates were radiolabeled with 2 μCi/well Na21CrO4 in DMEM-FCS overnight. The cells were washed three times with HBSS and H2O2 (100 μM) or phorbol myristate acetate (PMA) was added (100 ng/ml) in supplemented PME (2:1 PMN/endothelial cell ratio) added for 2 h. Specific cytotoxicity values were calculated as described previously (15). Sequel 11Cr release was below 10% in all experiments.

**Endothelial Cell Treatments**—We have shown previously (5) that pretreatment of PAEC with hemin sensitized these cells to free radical-induced cytolysis. In a single-step condition, 1 μm hemin was prepared in 10 mM NaOH and was diluted to the desired final concentration in DMEM. After washing, the confluent PAEC were incubated with hemin (5 μM) for 1 h, and at the end of this sensitization period, the cells were washed with HBSS prior to the cytotoxicity assay. For induction of free radical killing, PAEC were pretreated with hemin for prolonged time periods (up to 16 h). This induction phase was accomplished by pulsing the cells with hemin (10 μM) for 60 min; the culture medium was then replaced with heme-free solution for 15 h (or less as indicated). In some experiments, the reagents were added during the endothelial cell induction phase. Iron-proxidoxal isonicotinyl hydrazone (Fe-PH) cholate (10 μM iron content) synthesized from ferric ammonium citrate, isonicotinic acid hydrazide, and pyridoxal hydrochloride (16, 17) was added to PAEC in DMEM-FCS for 16 h prior to the cytotoxicity assay. Sodium arsenite (50 μM) was added to DMEM for 60 min, the cells washed, and the culture medium replaced for 15 h prior to the killing assay.

**Ferritin Assays**—Endothelial cell ferritin content was measured in cells grown in 3.5-cm tissue culture dishes treated with control media, hemin, or other reagents as indicated for various time periods up to 20 h. At the indicated time points, the cells were solubilized with Triton X-100. Nondet P-40-containing Tris-HCl buffer, pH 7.2, with protease inhibitors (24), centrifuged at 10,000 X g for 10 min at 4 °C, and the supernatant was then analyzed for ferritin content using a radioimmunoassay with 125I-labeled goat anti-human ferritin serum (a kind gift from Dr. J. E. Smith, Kansas State University) as a primary antibody. The anti-goat IgG peroxidase conjugate was utilized as the secondary antibody. The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). Ferritin was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18). The anti-goat IgG was visualized by enhanced chemiluminescence using an enhanced chemiluminescence detection system (18).

**Results**

As shown in Fig. 1A (and reported previously in another context (5)), brief (1–4 h) but not prolonged (16 h) exposure of porcine endothelial cells to 5 μM hemin synergizes cellular oxidant damage by added H2O2 (open symbols) or phorbol-stimulated PMNs (solid symbols) with an optimal hem exposure duration of 2 h. However, if endothelial cells were preincubated with hemin for longer periods, cells become completely resistant to subsequent oxidant killing. In further studies which amplify this phenomenon (shown in Fig. 1B), endothelial cells then resist the usually very damaging combinations of briefly heme or hemin (10 μM) and then washed and incubated for a further 15 h; these cells then resist the usually very damaging combinations of newly added hemin (5 μM) plus H2O2 (compare third and fourth bars) or stimulated PMNs (compare seventh and eighth bars). Similar results were observed when human umbilical vein or human aortic endothelial cells were used instead of porcine aortic cells (data not shown).
Since incubation of hepatocytes and fibroblasts with hemin is known to cause induction of both heme oxygenase (28, 32) and ferritin (28, 33), we wondered whether one or both of these constituents might provide oxidation resistance. As shown in Fig. 2A, porcine aortic endothelial cells, when incubated with 10 μM hemin, are rapidly induced to synthesize heme oxygenase mRNA. In addition, as shown in Fig. 2B, light chain ferritin mRNA increases, albeit gradually. Conversely, endothelial ferritin H chain mRNA did not increase following exposure to hemin (data not shown); this coincides with previous studies demonstrating that iron-mediated regulation of ferritin synthesis occurs primarily by post-transcriptional/translational modulation (6, 29). An associated increased synthesis of the subsequent intact heme oxygenase and ferritin proteins eventuates (Fig. 2C), consistent with the hypothesis that the protective effect of hemin preincubation might reflect induction of one or both of these substances.

That ferritin, rather than heme oxygenase, is the ultimate protectant is implied by data in Table I. We developed two experimental conditions that induced endothelial ferritin but not heme oxygenase; both were cytoprotective. Thus, preincubation of endothelial cells with a cell permeant Fe-PH chelate or with a combination of hemin and tin mesoporphyrin IX (an inhibitor of heme oxygenase) causes substantial increases in intracellular ferritin without any increment in heme oxygenase activity. In both of these cases, an associated marked protection against subsequent hemin + H2O2 challenge is noted. However, under converse circumstances (if cells were incubated with hemin + deferoxamine or with sodium arsenite) high levels of heme oxygenase, but not ferritin, accrue; no associated protection against later challenge by hemin and H2O2 is afforded. Not surprisingly, inhibition of all new protein synthesis with cycloheximide also ablates induction of this protective mechanism. In studies not shown, addition of the potent heme-binding protein, hemo- pexin, to our system prevented both heme uptake by endothelial cells and subsequent ferritin synthesis; no cytoprotection was induced under these circumstances.

Evidence favoring a critical antioxidant role for intracellular ferritin remains, nonetheless, circumstantial. Cells preincubated with heme and other protective compounds might be making numerous, subtle and unsuspected adjustments involving neither heme oxygenase nor ferritin synthesis. Therefore, to examine the consequences of elevated intracellular ferritin per se, we directly loaded preformed ferritin into...
thiobarbiturate-reactive tolysis, 0.1 nmol/mg of cell protein); conversely, in cytolysis-resistant, endothelial cells of lipid peroxidation products, measured as H2O2 cytotoxicity assays endothelial cell ferritin contents were also determined except at recombinant human ferritin treatments incubation of the cells with increasing concentrations of exogenous apoferritin. Diverse cells have been shown to pinoocytose extracellular ferritin and also accumulate substantial ferritin during such incubations (see above). As shown in the middle portion of Table II, these ferritin-loaded cells become resistant, in a dose-responsive fashion, to the oxidant stress imposed by the combination of H2O2 + heme or to H2O2 alone (not shown). Accompanying this resistance was a parallel reduction in the incubated endothelial cells of lipid peroxidation products, measured as thiobarbiturate-reactive material (5). For instance, in control heme/H2O2-exposed cells undergoing approximately 60% cytolyis, thiobarbiturate-reactive material accumulates (2.3 ± 0.1 nmol/mg of cell protein); conversely, in cytolsis-resistant, ferritin-enriched cells thiobarbiturate-reactive material is negligible following heme/H2O2 exposure (0.2 ± 0.1 nmol/mg of cell protein). Protection from heme/H2O2 assualt is afforded specifically by apoferritin, and endothelial cells preincubated with other proteins (including apotransferrin, apolactoferrin, and albumin) fail to exhibit any increase in resistance to H2O2 or heme + H2O2 (results not shown).

Indeed, under various experimental conditions a close inverse linear correlation (r ~ 0.97) between induced intracellular ferritin content and the susceptibility of endothelial cells to oxidant challenge can be appreciated (Fig. 3). Ferritin has ferroxidase activity located on the H but not the L subunit (18). This activity catalyzes the oxidation of ferrous iron under aerobic conditions to ferric iron to allow intracellular iron storage in biological systems. In our studies of endothelial cells, the protection provided by ferritin is evidently attributable either to iron storage and/or to the intrinsic ferroxidase activity of the H chain. This is supported by experiments shown in the bottom portion of Table II in which human recombinant wild type or mutant H chain ferritin (18) was loaded into cultured endothelium. As expected, the wild type ferritin H chain protected as well as intact ferritin. However, the mutant H chain, which lacks both ferroxidase activity and iron storage capability, fails to provide any protection. By immunofluorescent assay ferritin induced in human umbilical vein endothelial cells by exposure to hemin is found

### Table I

| Endothelial cell induction | Heme oxygenase enzyme activity | Ferritin content | Hemin + H2O2 cytotoxicity |
|---------------------------|-------------------------------|-----------------|--------------------------|
|                           | µmol bilirubin formed/ mg cell protein/60 min | ng/mg cell protein | % 51Cr release |
| Buffer                    | 13.7 ± 1.6                    | 58.3 ± 8.0      | 61.90 ± 1.5              |
| Hemin (10 µM)            | 275.0 ± 28.6                  | 574.4 ± 18.4    | 0.54 ± 0.9               |
| Fe-PH 100-200 μM        | 15.0 ± 1.6                    | 431.5 ± 27.3    | 10.20 ± 4.3              |
| Hemin + Sn-meso-P (25 µM)| 6.7 ± 2.9                     | 522.6 ± 17.6    | 0.88 ± 1.1               |
| Hemin + DF (1 mM)       | 343.3 ± 41.2                  | 36.2 ± 2.4      | 58.50 ± 3.9              |
| Sodium arsenite (50 µM) | 77.5 ± 5.2                     | 68.9 ± 6.3      | 59.50 ± 1.3              |
| Hemin + CHX (10 µg/ml)  | 10.0 ± 1.36                   | 28.4 ± 2.3      | 58.89 ± 2.3              |

Endothelial cells were induced with heme or other reagents as described under “Experimental Procedures.” At the time points of the H2O2-mediated cytotoxicity assays endothelial cell heme oxygenase activities and ferritin contents were also determined.

### Table II

| Endothelial cell treatment | Ferritin content | Hemin + H2O2 cytotoxicity |
|---------------------------|-----------------|--------------------------|
|                           | ng/mg cell protein | % 51Cr 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                       | 1,879.0 ± 352.0  | 0.45 ± 1.0              |
| Recombinant human heavy chain ferritin | | |
| Wild type 1.0 mg/ml      | 1,193.0 ± 154.0  | 23.4 ± 2.9              |
| Mutant (222) 1.0 mg/ml   | 1,012.0 ± 112.5  | 68.3 ± 2.4              |

* Endothelial cells were induced with hemin or apoferritins as described under “Experimental Procedures.” At the time points of the H2O2-mediated cytotoxicity assays endothelial cell heme oxygenase activities and ferritin contents were also determined.

* For PAEC cytotoxicity, 100 µM H2O2 was used after the 60-min 5 µM hemin sensitization period as described in Fig. 1B.

* The results represent mean ± S.E. of at least three experiments done in duplicate.
widely distributed in the cytoplasm in a finely granular fashion in almost every cell (Fig. 4B); this contrasts with the sparse and sporadic distribution noted in control endothelial cells (Fig. 4A). When exogenous apoferritin is added, nearly every cell contains ferritin but in a coarser granular pattern (Fig. 4C). Fig. 4D demonstrates morphologically that hemin-induced ferritin synthesis is inhibited by deferoxamine, corroborating the fluorometric enzyme immunoassay results and cytotoxicity data presented in Table I. In nonpermeabilized (3.6% paraformaldehyde-fixed) endothelial cells, no immunoreactive ferritin was noted on the cell surface of heme-treated cells, whereas there was slight and variable surface staining of endothelial cells exposed to apoferritin (data not shown). This suggests that the ferritin complex is primarily localized intracellularly.

**DISCUSSION**

Ferritin has generally been thought to function as a “housekeeper” storage protein (6) which can release iron required for cellular proliferation (e.g. for ribonucleotide reductase) and metabolic renewal (e.g. for cytochrome synthesis). Although some studies have suggested that ferritin can amplify oxidative phenomena (7, 8), evidence can also be marshalled to suggest that ferritin may play a protective role against toxic effects of iron overload in cells. For instance, rat liver nuclei from iron-intoxicated animals excessively transcribe genes for cytochrome synthesis. Allo- 

The protective effects of elevated intracellular ferritin reported herein presumably add to those of antioxidant enzymes such as catalase, glutathione-related enzymes, and superoxide dismutase. Induction of these enzymes has been linked by others to subsequent protection of cells from oxidant challenge. In that regard, in our hands hemin-exposed endothelial cells do not increase its catalase or glutathione peroxidase levels over 18 h (data not shown).

New H chain recombinants will soon be available which should allow further dissection of molecular characteristics critical for cytoprotection. Thus, endothelial protection studies are planned which will utilize a mutant that manifests ferroxidase activity but which cannot incorporate iron because of deletion of 22 amino acids forming the hydrophobic channel of the molecule (40).

It is becoming increasingly clear that the susceptibility of eukaryotic and prokaryotic cells to oxidants is powerfully influenced (if not dictated) by levels of reactive intracellular iron. Relatively iron-poor cells (and many inherently iron-poor organisms) are highly resistant to oxidants and oxidant drugs (41). By contrast, cells containing excess iron (for instance, bacteria grown in iron-rich conditions or malaria parasites which generate ferruginous deposits from digestion of host red cell hemoglobin) are easily destroyed by oxidants (1, 42). Germane to the potential role of endothelial oxidant damage in atherogenesis are recent observations: namely, that vessel wall cells from atherosclerotic lesions are remarkably iron-rich as compared with neighboring unaffected vascular tissue from the same individuals (43).

We suggest from our studies that under iron-loading con-
Iron Sequestration

In conditions the facile synthesis of ferritin, an intracellular protein present in species ranging from bacteria to humans, may be an important mechanism to control reactive iron and thereby suppress oxidant damage. Studies in press (44) further support this notion. We have shown that oxidant-mediated and universally fatal renal failure results from rhabdomyolysis in glycercol-injected rats but not if animals are preinfused with hemoglobin. Protection parallels the induction of high levels of renal ferritin; conversely, ablating this response with a competitive inhibitor of heme oxygenase, tin protoporphyrin, markedly accelerates kidney dysfunction in these animals (44).

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