THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 267, No. 25, Issue of September 5, pp. 18148-18153, 1992
Printed in U.S.A.

Ferritin: A Cytoprotective Antioxidant Strategem of Endothelium*

(Received for publication, April 3, 1992)

György Balla, Harry S. Jacob, Jozsef Balla, Mark Rosenberg, Karl Nath, Fred Apple, John W. Eaton‡, and Gregory M. Vercellotti§

From the Departments of Medicine, and Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455 and the ‡Department of Pathology and Laboratory Medicine, Albany Medical College, Albany, New York 12208

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 where, after as little as 1 h, it markedly aggravates cytotoxicity engendered by polymorphonuclear leukocyte oxidants or hydrogen peroxide (H_2O_2). 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 cytoprotectant. 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 $Glu^{62} \rightarrow Lys$; $His^{65} \rightarrow Gly$) 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 (H_2O_2). However, much of the cellular damage caused by activated oxygen involves the collaboration of intracellular iron. For example, the amount of H_2O_2 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 H_2O_2 (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 H_2O_2 or adherent polymorphonuclear leukocytes (PMNs)[1] (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_r 21,000, light or L chain M_r 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 catalyticallyactive 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-

* This work was supported by National Institutes of Health Grants RO1 HL33793, R32, HL28935, and RO1 AI25625. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Division of Hematology, Dept. of Medicine, Box 480, University of Minnesota Hospital, Harvard St. at East River Rd., Minneapolis, MN 55455. Tel.: 612-624-0123; Fax: 612-625-6919.

[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-PIH, iron-pyridoxal isonicotinoyl hydrazone.

thelium 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 sequestering properties of ferritin in this cytoprotection.

EXPERIMENTAL PROCEDURES

Reagents—Dulbecco'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. Heparin was obtained from Lypho-Med (Melrose Park, IL); hydrogen peroxide (H_2O_2, 30%), from Fisher Scientific; 6% hydroxyethyl starch, from Du Pont; deferoxamine mesylate, from CIBA-Geigy; and $^{51}CrO_4$ (as the sodium salt), from Amersham Corp. Bovine hemin type I and tin mesoporphyrin 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 (0.2% for 15 min at 37 °C). Endothelial cells were grown in DMEM containing 10% FCS, penicillin (100 units/ml), streptomycin (100 units/ml), and supplemented with L-glutamine to confluence 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 Human Subjects in Research of the University of Minnesota).

Endothelial Cytotoxicity Assays—Confluent endothelial cells (usually PAEC) grown in 24-well (2 cm^2/well) tissue culture plates were radiolabeled with 2 μCi/well $Na_2{}^{51}CrO_4$ in DMEM-FCS overnight. The wells were washed three times with HBSS and H_2O_2 (100 μM) or phorbol myristate acetate- (100 ng/ml) activated PMNs (2:1 PMN:endothelial cell ratio) added for 2 h. Specific cytoxicity values were calculated as described previously (15). Spontaneous ^{51}Cr 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-mediated injury. In brief, a stock solution of 1 mM 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 (see above). For inducing cytoprotection against 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) before oxidant exposure. In some experiments, other reagents were utilized during the endothelial cell induction phase. Iron-pyridoxal isonicotinoyl hydrazone (Fe-PIH) chelate (100 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 in DMEM for 60 min, the cells washed, and the culture medium replaced for 15 h prior to the killing assay. Tin mesoporphyrin IX (25 μM), or deferoxamine (1 mM), or cycloheximide (10 μg/ml) was each added with hemin (10 μM) during the endothelial cell induction phase and kept in place until the hemin sensitization phase. In control experiments these treatments did not cause cell damage except for cycloheximide, which caused approximately 10% specific cytotoxicity. In some experiments, endothelial cells were loaded with horse apoferritin (0.1–2.0 mg/ml) or human recombinant wild type or mutant (222) H chain ferritin (1 mg/ml) (a gift from Drs. Hal Broxmeyer, Indiana University, and Paolo Arosio, Milano, Italy) for 16 h prior to the cytotoxicity assay. The mutant 222, prepared by oligonucleotide site-directed mutagenesis and whose properties have been described in detail (18), has a substitution of 2 amino acids in the H subunit. The mutation abolishes ferroxidase activity of the otherwise intact ferritin molecule and also differs from the native molecule in its poor-to-absent iron sequestrant capacity: thus, it fails to react with iron at acidic pH and does not facilitate incorporation of iron by transfected *Escherichia coli*.

Heme Oxygenase Enzyme Activity—Heme oxygenase activity in endothelial cell microsomes was measured by bilirubin generation (19–23). PAEC grown in 10-cm-diameter tissue culture dishes were treated with control media, hemin (10 μM), or other reagents as indicated for various time periods up to 20 h. The cells were washed, scraped with a rubber policeman, centrifuged at 1,000 × *g* for 10 min at 4 °C. The cell pellet was suspended in $MgCl_2$ (2 mM) phosphate (100 mM) buffer (pH 7.4), frozen to −70 °C, and thawed three times and sonicated on ice prior to centrifugation at 18,800 × *g* for 10 min at 4 °C. The supernatant was added to the reaction mixture (400 μl) containing rat liver cytosol (2 mg), hemin (20 μM), glucose 6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 units), and NADPH (0.8 mM) for 1 h at 37 °C in the dark. The formed bilirubin was extracted with chloroform and Δ O.D. 464–530 nm was measured (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). Heme oxygenase activity is expressed as pmol of bilirubin formed/mg of endothelial cell protein/60 min.

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, Nonidet P-40-containing Tris-HCl buffer, pH 7.2, with protease inhibitors (24), centrifuged at 10,000 × *g* for 10 min at 4 °C, and the supernatant analyzed for ferritin content using the Stratus fluorometric enzyme immunoassay system (25). Human, horse, and porcine apoferritin (a kind gift from Dr. J. E. Smith, Kansas State University) were used as standards. The results are expressed as ng of ferritin/mg of endothelial cell protein. Endothelial cell ferritin was also demonstrated by immunofluorescent techniques in methanol-permeabilized and fixed human umbilical vein endothelial cells using a goat anti-human ferritin serum as primary antibody (ATAB, Stillwater, MN) and a rabbit anti-goat IgG fluorescein isothiocyanate-labeled secondary antibody. Nonspecific goat serum or rabbit IgG alone served as a control.

Heme Oxygenase and Ferritin mRNA Analysis—Heme oxygenase and H and L chain ferritin mRNA content were analyzed in PAEC grown in 10-cm tissue culture dishes after treatment with control media, hemin, or other reagents for various time periods. Endothelial cell RNA was isolated by the RNAzol method (TEL-TEST, Inc., Friendswood, TX). Aliquots (20 μg) of total RNA were electrophoresed in a 1% agarose gel and transferred to nitrocellulose membranes (26). In each gel the position of the 28 and 18 S ribosomal RNA was determined in a separate lane by ethidium bromide staining. The size estimates for the signals were checked by the use of a ^{32}P-labeled DNA ladder. The membranes were then hybridized at 42 °C with nick-translated ^{32}P-labeled cDNA probes for human heme oxygenase (27) (obtained from Dr. R. Tyrrell, Swiss Institute for Experimental Research, Epalinges, Switzerland) and rat H ferritin and L ferritin (28, 29) (obtained from Dr. H. N. Munro, Tufts University, Boston, MA). Autoradiographs were obtained and quantified by computer-assisted videodensitometry (30).

Protein and Lipid Peroxidation Assay—The protein content of the endothelial cell monolayers was determined using bovine serum albumin as a standard (31). Endothelial cell lipid peroxidation was measured using the thiobarbituric acid colorimetric assay as described previously (5).

RESULTS

As shown in Fig. 1*A* (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 H_2O_2 (*open symbols*) or phorbol-stimulated PMNs (*solid symbols*) with an optimal heme-exposure duration of 2 h. However, if endothelial cells are preincubated with hemin for longer periods, cells become completely resistant to subsequent oxidant killing. In further studies which amplify this phenomenon (shown in Fig. 1*B*), the cells were pulsed with a brief preincubation of 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 H_2O_2 (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).

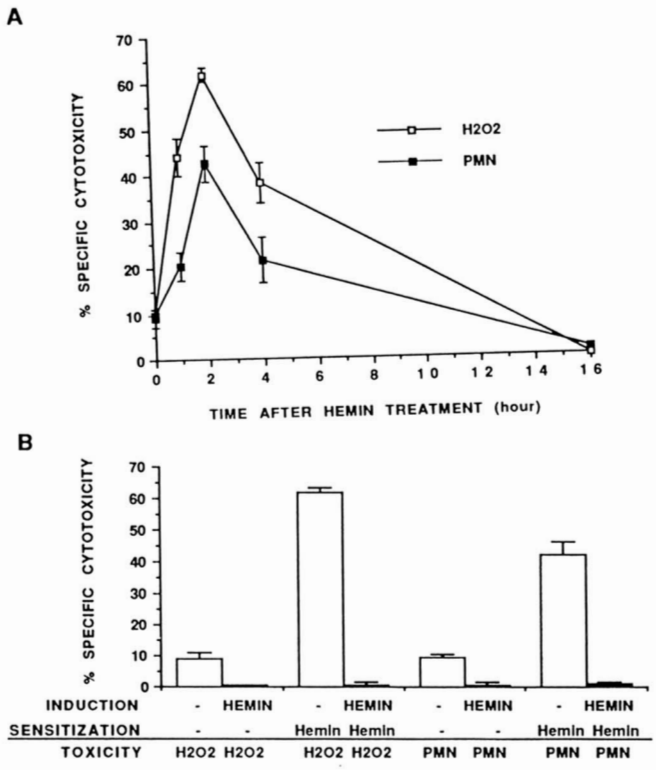

FIG. 1. *A*, time-dependent effect of hemin treatment on hydrogen peroxide and activated neutrophil-mediated endothelial cell lysis. Confluent ^{51}Cr-labeled porcine aortic endothelial cells grown in 24-well (2 cm^2/well) tissue culture plates were incubated with hemin (5 μM) in 500-μl DMEM for 60 min (5). After removal of the hemin solution the cells were cultured for the indicated time periods. Before the killing phase, the endothelial cells were washed twice with HBSS; H_2O_2 (100 μM) (*open squares*) or PMA- (100 ng/ml) activated neutrophils (PMNs, 2:1 PMN:endothelial cell ratio) (*closed squares*) were added to the endothelial cells for 2 h, and the percent specific cytotoxicity values were calculated. Results represent mean ± S.E. of five experiments performed in duplicate. *B*, cytoprotection against hemin-catalyzed injury of endothelial cells induced by hemin pretreatment. Treatment of endothelial cells was as described under "Experimental Procedures" with the indicated exception: namely that endothelial cells were exposed to hemin at two separate times. The induction phase was accomplished by 10 μM hemin treatment for 60 min, the culture medium replaced, and the cells incubated a further 15 h before the sensitization phase. Endothelial cell sensitization was provided by the addition of 5 μM hemin for 60 min 2 h before the cytotoxicity phase (addition of H_2O_2 or PMNs) as described under "Experimental Procedures." Results represent mean percent specific cytotoxicity ± S.E. of at least seven experiments performed in duplicate.

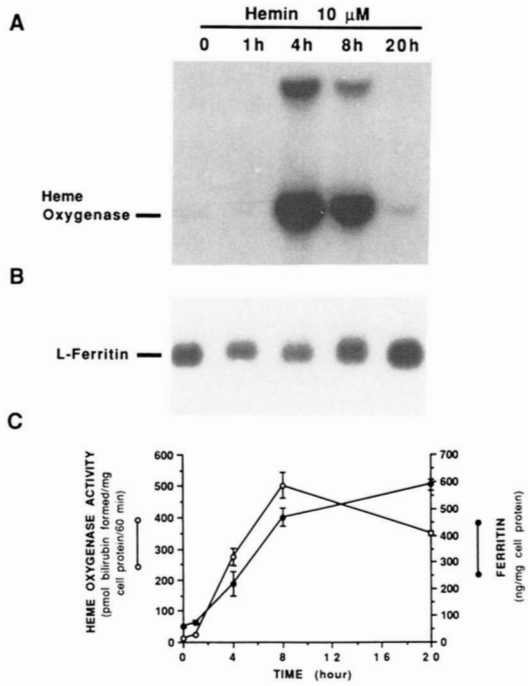

FIG. 2. **Hemin induction of PAEC heme oxygenase and ferritin.** *A*, for heme oxygenase mRNA analysis, endothelial cells were treated with hemin (10 μM) in 10 ml of DMEM for 60 min, and 10 ml of culture medium was then replaced. At the indicated time, RNA was isolated, electrophoresed, blotted, and hybridized to a ^{32}P-labeled heme oxygenase cDNA probe. *B*, L ferritin mRNA analysis was performed after the same treatment of endothelial cells using a ferritin cDNA probe. *C*, heme oxygenase activity (*open circles*) and ferritin protein (*closed circles*) were measured in PAEC treated as in *panel A* and measured as described under "Experimental Procedures." Results represent mean ± S.E. of at least three experiments done in duplicate. New synthesis of both H and L ferritin subunits was induced by hemin as measured by ferritin immunoprecipitation using [^{35}S]methionine-labeled cells (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. 2*A*, porcine aortic endothelial cells, when incubated with 10 μM hemin, are rapidly induced to synthesize heme oxygenase mRNA. In addition, as shown in Fig. 2*B*, 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. 2*C*), 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-PIH 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 + H_2O_2 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 H_2O_2 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, hemopexin, 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

TABLE I

Heme oxygenase and ferritin inductions in porcine aortic endothelial cells by hemin: effect on hemin plus hydrogen peroxide-mediated free radical injury

Sn-*meso*-P, tin mesoporphyrin IX; CHX, cycloheximide, DF, deferoxamine mesylate.

Endothelial cell induction[a]	Heme oxygenase enzyme activity	Ferritin content	Hemin + H_2O_2[b] cytotoxicity
	pmol bilirubin formed/ mg cell protein/60 min	*ng/mg cell protein*	*% ^{51}Cr release*
Buffer	13.7 ± 1.6[c]	58.3 ± 8.0	61.90 ± 1.5
Hemin (10 μM)	275.0 ± 28.6	574.4 ± 18.4	0.54 ± 0.9
Fe-PIH (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

[a] Endothelial cells were induced with hemin or other reagents as described under "Experimental Procedures." At the time points of the H_2O_2-mediated cytotoxicity assays endothelial cell heme oxygenase activities and ferritin contents were also determined.
[b] For PAEC cytotoxicity, 100 μM H_2O_2 was used after the 60-min 5 μM hemin sensitization period as described in Fig. 1*B*.
[c] The results represent mean ± S.E. of at least three experiments done in duplicate.

TABLE II

Ferritin induction and ferritin loading of porcine aortic endothelial cells influence endothelial cell sensitivity to hemin catalyzed hydrogen peroxide toxicity

Endothelial cell treatment[a]	Ferritin content	Hemin + H_2O_2[b] cytotoxicity
	ng/mg cell protein	*% ^{51}Cr release*
Buffer	58.3 ± 8.0[c]	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

[a] PAECs were treated with hemin or apoferritins as described under "Experimental Procedures." At the time points of the H_2O_2-mediated cytotoxicity assays endothelial cell ferritin contents were also determined.
[b] 5 μM Hemin sensitization and 100 μM H_2O_2 injury of PAECs were carried out as described in Fig. 1*B*.
[c] The results represent mean ± S.E. of at least three experiments done in duplicate except at recombinant human ferritin treatments which were done two times in duplicate.

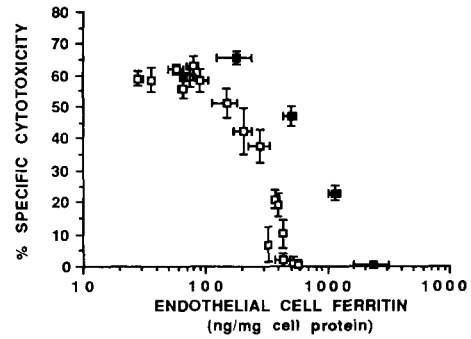

FIG. 3. **Endothelial cell ferritin inversely correlates with H_2O_2/hemin-mediated cytotoxicity.** Ferritin was determined as described under "Experimental Procedures" in PAEC whose levels were either increased by exposure to hemin, Fe-PIH, or apoferritin at varying concentrations (Table II) or decreased with cycloheximide or deferoxamine (Table I). H_2O_2 endothelial cytotoxicity was measured after sensitization with hemin as in Fig. 1*B*. Each point represents at least two experiments done in duplicate. The *closed squares* represent cells loaded with exogenous apoferritin.

cultured endothelial cells. This was accomplished via simple incubation of the cells with increasing concentrations of exogenous apoferritin. Diverse cells have been shown to pinocytose extracellular ferritin (34), and as validated by chemical and immunofluorescent assays, our cultured endothelial cells also accumulate substantial ferritin during such incubations (see below). 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 H_2O_2 + heme or to H_2O_2 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/H_2O_2-exposed cells undergoing approximately 60% cytolysis, thiobarbiturate-reactive material accumulates (2.3 ± 0.1 nmol/mg of cell protein); conversely, in cytolysis-resistant, ferritin-enriched cells thiobarbiturate-reactive material is negligible following heme/H_2O_2 exposure (0.2 ± 0.1 nmol/mg of cell protein). Protection from heme/H_2O_2 assault 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 H_2O_2 or heme + H_2O_2 (results not shown).

Indeed, under various experimental conditions a close inverse linear correlation ($r \sim -0.97$) 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

widely distributed in the cytoplasm in a finely granular fashion in almost every cell (Fig. 4*B*); this contrasts with the sparse and sporadic distribution noted in control endothelial cells (Fig. 4*A*). When exogenous apoferritin is added, nearly every cell contains ferritin but in a coarser granular pattern (Fig. 4*C*). Fig. 4*D* 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 ferritin (L subunit) mRNA as compared with control animal nuclei (29). We believe the present studies more directly support this latter notion by demonstrating a dose-responsive cytoprotective, antioxidant effect of ferritin in iron-loaded cells (Fig. 3). Further correlative support may derive from the time-dependent dichotomous effects of heme exposure on endothelial cells. Thus, heme amplifies oxidant-mediated cytotoxicity during brief (2 h) exposure, yet markedly protects oxidant-exposed target cells if provided for longer durations. These results correlate with the 1–2-h time lag that is then followed by rapid synthesis of ferritin in endothelium exposed to heme (Fig. 2*C*). Finally, the likelihood that ferritin *per se* is the critical cytoprotectant in our study is buttressed by its significant (albeit less potent) efficacy even when exogenously added to cultured cells (compare *solid* and *open symbols*, Fig. 3).

Usually cells take up iron in a highly regulated fashion dependent on transferrin binding and cellular transferrin receptor modulation; however, cells can evidently also incorporate iron by transferrin receptor-independent pathways with potential deleterious outcome. For example, studies by others which seem particularly relevant to the present work have demonstrated that the uptake of heme with resultant iron loading can occur in cultured cell lines via specific non-transferrin receptors (35). Perhaps as a countermeasure, diverse species possess avid heme-binding proteins, such as hemopexin, which efficiently inhibit cellular heme uptake and heme-catalyzed oxidation reactions (36, 37). Moreover, many uni- and multicellular organisms also appear purposefully to destroy free intracellular heme by enzymatic means. For instance, heme oxygenase, a ubiquitous enzyme which opens the porphyrin ring and releases heme iron, is induced in several tissues by oxidant stress (and by free heme) (27, 38). In fact, it has been suggested that the other product of heme oxygenase activity, bilirubin, may itself be an important antioxidant. Nevertheless, the salutary effects of enhanced heme oxygenase activity are not intuitively obvious. Although catabolism of heme may rid the cell of a membrane-permeant form of iron, the resultant non-heme iron would seem to represent a potential hazard unless sequestered in some way. Indeed, the present studies demonstrate that heme oxygenase activity, unlike ferritin, does not correlate with cytoprotection. Instead, we suggest that heme oxygenase serves mainly to provide free intracellular iron from added heme; this iron, in turn (unless chelated by deferoxamine; Table I), drives the synthesis of ferritin, which is likely the proximate protectant against oxidant damage. Alternatively, heme itself might enhance ferritin synthesis directly by increasing RNA translation as recently shown by Lin and co-workers (39).

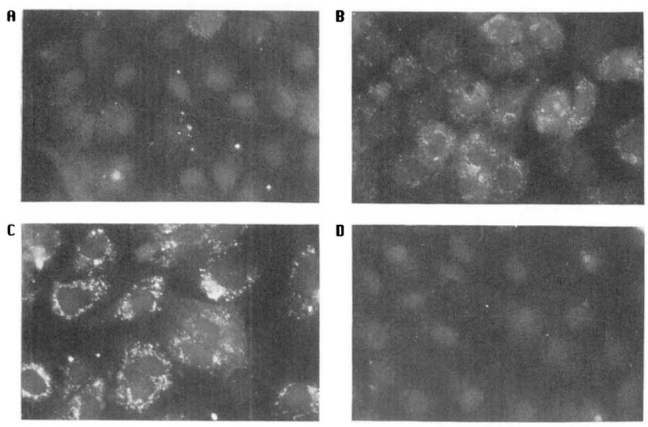

FIG. 4. **Ferritin immunofluorescence in human umbilical vein endothelial cells.** *A*, control; *B*, hemin (10 μM) induction for 16 h; *C*, exogenous apoferritin-loaded for 16 h; and *D*, hemin (10 μM) plus deferoxamine (1 mM) induction for 16 h, as described under "Experimental Procedures" (magnification for all *panels*: 400 ×).

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 endothelium does not increase its catalase or glutathione peroxidase levels over 16 h (data not shown).

Our results using the recombinant mutant 222 demonstrate that ferritin's potent antioxidation efficiency probably depends on one or both of its unique molecular characteristics: namely, its high sequestering capacity for inorganic iron and the ferroxidase activity of its H chain, both lacking in the mutant. Presumably the latter prevents significant accumulation of cellular Fe^{2+}, the ultimate catalyst of oxidative damage. 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-

ditions 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 glycerol-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).

Acknowledgments—We thank Dr. Ursula Muller-Eberhard (Cornell University) for the gift of hemopexin, Dr. Alexander Antonov (U. S. S. R. Cardiology Research Center, Moscow) for human aortic endothelial cells and immunofluorescence expertise, Dr. Hal Broxmeyer (University of Indiana) for the gift of ferritin mutant 222 and helpful advice, Dong Tuong and David Chmielewski for technical assistance, and Carol Taubert for manuscript preparation.

REFERENCES

1. Repine, J. E., Fox, R. B., and Berger, E. M. (1981) *J. Biol. Chem.* **256,** 7094–7096
2. Gannon, D. E., Varani, J., Phan, S. H., Ward, J. H., Kaplan, J., Till, G. O., Simon, R. H., Ryan, U. S., and Ward, P. A. (1987) *Lab. Invest.* **57,** 37–44
3. Schraufstatter, I. U., Hyslop, P. A., Jackson, J. H., and Cochrane, C. G. (1988) *J. Clin. Invest.* **82,** 1040–1050
4. Sadrzadeh, S. M. H., and Eaton, J. W. (1988) *J. Clin. Invest.* **82,** 1510–1515
5. Balla, G., Vercellotti, G. M., Muller-Eberhard, U., Eaton, J., and Jacob, H. S. (1991) *Lab. Invest.* **64,** 648–655
6. Theil, E. C. (1990) *Adv. Enzymol. Relat. Areas Mol. Biol.* **63,** 421–449
7. Thomas, C. E., and Aust, S. D. (1986) *J. Biol. Chem.* **261,** 13064–13070
8. Thomas, C. E., Morehouse, L. A., and Aust, S. D. (1985) *J. Biol. Chem.* **260,** 3275–3280
9. Bolann, B. J., and Ulvik, R. J. (1990) *Eur. J. Biochem.* **193,** 899–904
10. Bakker, G. R., and Boyer, R. F. (1986) *J. Biol. Chem.* **261,** 13182–13185
11. Levi, S., Luzzago, A., Cesareni, G., Cozzi, A., Franceschinelli, F., Albertini, A., and Arosio, P. (1988) *J. Biol. Chem.* **263,** 18086–18092
12. Balla, G., Balla, J., Rosenberg, M., Nath, K., Jacob, H. S., and Vercellotti, G. M. (1991) *Clin. Res.* **39,** 724 (abstr.)
13. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) *J. Clin. Invest.* **52,** 2745–2756
14. Antonov, A. S., Nikolaeva, M. A., Klueva, T. S., Romanov, Yu A., Babaev, V. R., Bystrevskaya, V. B., Perov, N. A., Repin, V. S., and Smirnov, V. N. (1986) *Atherosclerosis* **59,** 1–19
15. Vercellotti, G. M., Yin, H. Q., Gustafson, K. S., Nelson, R. D., and Jacob, H. S. (1988) *Blood* **71,** 1100–1107
16. Ponka, P., Borova, J., Neuwirt, J., and Fuchs, O. (1979) *FEBS Lett.* **97,** 317–321
17. Ponka, P., Schulman, H. M., and Wilczynska, A. (1982) *Biochim. Biophys. Acta* **718,** 151–156
18. Broxmeyer, H. E., Cooper, S., Levi, S., and Arosio, P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 770–774
19. Tenhunen, R., Marver, H. S., and Schmid, R. (1970) *J. Lab. Clin. Med.* **75,** 410–421
20. Maines, M. D., and Kappas, A. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 4293–4297
21. Leonard, T. B., Graichen, M. E., Dahm, L. J., and Dent, J. G. (1986) *Biochem. Pharmacol.* **35,** 3057–3063
22. Brown, A., Lutton, J. D., Nelson, J., Abraham, N. G., and Levere, R. D. (1987) *Blood Cells* **13,** 123–136
23. Ibrahim, N. G., Lutton, J. D., and Levere, R. D. (1987) *Br. J. Haematol.* **50,** 17–28
24. Rogers, J., and Munro, H. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2277–2281
25. Giegel, J. L., Brotherton, M. M., Cronin, P., D'Aquino, M., Evans, S., Heller, Z. H., Knight, W. S., Krishnan, K., and Sheiman, M. (1982) *Clin. Chem.* **28,** 1894–1898
26. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Keyse, S. M., Applegate, L. A., Tromvoukis, Y., and Tyrrell, R. M. (1990) *Mol. Cell. Biol.* **10,** 4967–4969
28. Eisenstein, R. S., Garcia-Mayol, D., Pettingell, W., and Munro, H. N. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 688–692
29. White, K., and Munro, H. N. (1988) *J. Biol. Chem.* **263,** 8938–8942
30. Mariash, C. N., Seelig, S., and Oppenheimer, J. H. (1982) *Anal. Biochem.* **121,** 388–394
31. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356
32. Abraham, N. G., Lin, J. H.-C., Schwartzman, M. L., Levere, R. D., and Shibahara, S. (1988) *Int. J. Biochem.* **20,** 543–558
33. Lin, J.-J., Daniels-McQueen, S., Patino, M. M., Gaffield, L., Walden, W. E., and Thach, R. E. (1990) *Science* **247,** 74–77
34. Simionescu, M., and Simionescu, N. (1991) *Cell Biol. Rev.* **25,** 1–80
35. Galbraith, R. A., Sassa, S., and Kappas, A. (1985) *J. Biol. Chem.* **260,** 12198–12202
36. Vincent, S. H., Grady, R. W., Shaklai, N., Snider, J. M., and Muller-Eberhard, U. (1988) *Arch. Biochem. Biophys.* **265,** 539–550
37. Gutteridge, J. M. C., and Smith, A. (1988) *Biochem. J.* **256,** 861–865
38. Alam, J., Shibahara, S., and Smith, A. (1989) *J. Biol. Chem.* **264,** 6371–6375
39. Lin, J. J., Patino, M. M., Garfield, L., Walden, W. E., Smith, A., and Thach, R. E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6068–6071
40. Levi, S., Luzzago, A., Franceschinelli, F., Santambrogio, P., Cesoreni, G., and Arosio, P. (1989) *Biochem. J.* **264,** 381–388
41. Varani, J., Fligiel, S. E. G., Till, G. O., Kunkel, R. G., Ryan, U. S., and Ward, P. A. (1985) *Lab. Invest.* **53,** 656–663
42. Vennerstrom, J. L., and Eaton, J. W. (1988) *J. Med. Chem.* **31,** 1269–1277
43. Hunter, G. C., Dubick, M. A., Keen, C. L., and Eskelson, C. D. (1991) *Proc. Soc. Exp. Biol. Med.* **196,** 273–279
44. Nath, K. A., Balla, G., Vercellotti, G. M., Balla, J., Jacob, H. S., Levitt, M. D., and Rosenberg, M. E. (1992) *J. Clin. Invest.*, in press