We studied the effects of nitric oxide (NO) on the control of excess cellular heme and release of catalytically active iron. Endothelial cells (ECs) exposed to hemin followed by a NO donor have a ferritin content that is 16% that of cells exposed to hemin alone. Hemin-treated ECs experience a 3.5-fold rise in non-heme, catalytic iron 2 h later, but a hemin rechallenge 20 h later results in only a 24% increase. The addition of a NO donor after the first heme exposure prevents this adaptive response, presumably due to effects on ferritin synthesis. NO donors were found to reduce iron release from hemo-

min, while hemin accumulated in cells. A NO donor, in a dose-dependent fashion, inhibited heme oxygenase activity, measured by bilirubin production. Using low temperature EPR spectroscopy, heme oxygenase inhibition correlated with nitrosylation of free heme in microsomes. Nitrosylation of cellular heme prevented iron release, while there was heme oxygenase-dependent release of iron in cells incubated with hemin for 24 h, the addition of a NO donor blocked iron release. This indicates that NO readily nitrosylates intracellular free heme and prevents its degradation by heme oxygenase. Nitrosylation of heme was found to reduce sensitization of cells to oxidative injury.

Intravascular hemolysis is associated with endothelial cell (EC)

injury in diseases such as thrombotic thrombocytopenic purpura and disseminated intravascular coagulation. We have hypothesized that the pathogenesis of these diseases is caused in part by heme released from oxidized free hemoglobin because of the ability of heme to enter the EC membrane, where it can catalyze oxidative injury and cell death (1, 2). Heme oxygenase (HO) and ferritin provide protection and constitute an adaptive response to excess heme (3). HO is capable of releasing heme iron, while ferritin binds and stores iron in a form less apt to contribute to oxidative injury. We have previously shown that NO suppresses EC ferritin synthesis in response to hemin (4); therefore, we were interested to study the effect of NO on adaptation to heme exposure and control of reactive iron.

Control of heme-associated iron by HO and ferritin has been found to be an important level of defense in several models of injury. While acute heme exposure exacerbates oxidative injury, stimulation of HO and ferritin synthesis in the endothelium protects from subsequent exposure to hydrogen peroxide, sulfur radicals, or oxidized low density lipoprotein (3, 5). The demonstration that overexpression of HO-1 in ECs protected against heme and hemoglobin toxicity (6) has highlighted the importance of HO in the vasculature. HO and ferritin are protective in models of injury that do not directly involve heme. Sublethal UV-A irradiation of cultured skin fibroblasts induces HO-1 and ferritin and provides protection against a subsequent lethal UV-A radiation exposure. Inhibition of HO-1 induction by antisense oligonucleotide reduces protection (7, 8).

Nitrergic nerves have been found to modulate many steps in iron and heme metabolism primarily because of its affinity for heme and iron-sulfur proteins (extensively reviewed in Refs. 9 and 10). NO inhibits cytochrome P-450 and catalase activities and induces loss of heme from these proteins (11–13). NO can complex with low molecular weight iron to form dinitrosyl-iron complexes that are less reactive than unbound iron in catalyzing oxidative injury (14, 15). NO can have a protective effect by combining with hemoglobin to reduce the injury associated with generation of oxoferryl-hemoglobin (16–18). In addition to modulating iron, NO directly affects HO and ferritin protein levels by inducing HO protein synthesis (19, 20) but repressing ferritin synthesis (4, 21, 22). These opposing effects appear paradoxical, but the ultimate effect of NO on heme catabolism is unknown. In the present work, we sought to determine the effect of NO on the control of heme-associated iron.

**Experimental Procedures**

Reagents—Medium 199, Dulbecco’s modified Eagle’s medium, fetal calf serum, and Hank’s balanced salt solution were obtained from Life Technologies, Inc.; diethylamine nitric oxide adduct (DEA/NO) and spermine nitric oxide adduct (SFT/P/O) were from the Midwest Reagent Institute Inc.; 1,1-dimethyl-2-hydroxy-2-nitrosoguanidine (DEAN0) was from Alexis Corp. (San Diego, CA); polyclonal anti-human HO-1 antibody was obtained from Affinity Bioreagents, Inc. (Golden, CO); and nitrotyrosine nitric oxide adduct (SNP) was from Porphyrin Products, Inc. (Logan, UT). All other reagents were obtained from Sigma unless otherwise specified. The NO donors have the following half-lives at pH 7.4 and 37°C: DETA/NO, 20 h; SPER/NO, 39 min; and DETA/NO, 2 min (23, 24).

Cell Culture—Human umbilical vein endothelial cells (HUVECs) and porcine arterial endothelial cells (PAECs) were isolated and cultured as described previously (4, 5). A human T lymphoblast cell line (CCRF-CEM cells) (ATCC no. CCL-119) and human kidney epithelial
**Ferritin content in EC after 1 h of hemin exposure**

| Time | Medium | DETANO* | Average | Range | Average | Range |
|------|--------|---------|---------|-------|---------|-------|
| h    | ng/mg protein | ng/mg protein |         |       |         |       |
| 0    | 16.7 | 15.9−17.6 | 19.2 | 17.2−21.1 |         |       |
| 24   | 1345.8 | 1319.8−1371.8 | 201.3 | 186.4−216.2 |         |       |
| 48   | 304.4 | 228.2−379.9 | 46.8 | 42.6−51.0 |         |       |
| 72   | 136.2 | 120.5−152.0 | 9.2 | 8.1−10.4 |         |       |

* The difference between the medium and DETANO groups was significant, \( p = 0.003 \) by a weighted ANOVA.

**RESULTS**

ECs exposed to hemin will adapt by synthesizing HO and ferritin, which will enable the cell to degrade heme and sequester the released iron in a less reactive form. As we have shown previously (4), ECs exposed to hemin for 1 h have elevated ferritin content 24 h later, but the NO donor DETANO 500 \( \mu \text{M} \) (half-life of 20 h, \( \text{pH} 7.4, 37^\circ \text{C} \)) prevents the heme-induced ferritin increase (Table I). The effect of DETANO on ferritin content was statistically significant (\( p = 0.003 \) by weighted ANOVA). Thus, despite a similar exposure to hemin for 1 h, the ferritin content in the group treated with DETANO is 16% of that seen at 24 h in the group not exposed to a NO donor. If intracellular ferritin is important in the control of excess catalytically active iron, then cells treated with DETANO may be less able to sequester iron resulting from a second exposure to hemin. We wondered if the inhibitory effect of NO on ferritin synthesis would impair adaptation to excess heme with respect to the control of non-heme, catalytically active iron.

**Several studies by Gutteridge et al. (27, 28, 30) on human body fluids have shown that iron available to stimulate free radical reactions is composed of loosely bound, low molecular weight chelates. This pool of iron can be measured by the bleomycin assay with a high degree of sensitivity (0.1–0.5 \( \mu \text{M} \)) and is distinct from iron present in heme-, ferritin-, and iron-sulfur clusters except bleomycin as a control for other thiobarbituric reactive substances. The samples were read spectrophotometrically at 540 nm. Iron concentrations were calculated according to a standard curve. The values were corrected for protein content in each group.**

**Heme Oxidase Enzyme Activity—HO activity in EC microsomes was measured by bilirubin generation as described previously (4) with modification. The cells were disrupted by three cycles of freezing and thawing and sonication on ice prior to centrifugation at 18,000 \( \times g \) for 10 min at 4 \( ^\circ \text{C} \). The supernatant was added to reaction mixtures (final volume, 500 \( \mu \text{L} \)) containing 3 mg of rat liver cytosol, 20 \( \mu \text{M} \) hemin, 2 mM glucose, 12 \( \mu \text{g} \) of glucose-6-phosphate dehydrogenase, 0.8 mM NADPH, and 0–100 \( \mu \text{M} \) SPER/NO for 1 h at 37 \( ^\circ \text{C} \) in the dark. Liver cytosol was prepared by homogenizing a rat liver in 1 volume of buffer (20 mM Tris-HCl, \( \text{pH} 7.4 \), containing 1.15% KCl). The homogenate was centrifuged at 10,000 \( \times g \) for 20 min at 4 \( ^\circ \text{C} \), and the supernatant further centrifuged at 100,000 \( \times g \) for 1 h at 4 \( ^\circ \text{C} \). The supernatant was collected, and protein was measured. SPER/NO (1 \( \text{mM} \) stock) was dissolved in 10 mM NaOH and kept on ice in the dark until used. The formed bilirubin was extracted with chloroform, and the absorption was measured as the difference between 464 and 530 nm (extinction coefficient of 1.5 \( \times 10^3 \) \( \text{M}^{-1} \text{cm}^{-1} \)).**
Confluent PAECs were exposed sequentially to hemin and DETA/NO for an additional 20 h. Adaptation to heme was assessed by removal of medium containing DETA/NO and washing in buffered saline prior to recollection to 10 μM heme in serum-free medium for 1 h. After heme exposure, medium was added for an additional 1 h prior to measurement of catalytic iron. Measurements were taken in untreated cells, 1 h after an initial hemin exposure, 20 h after an initial hemin exposure, and 1 h after a second hemin exposure. Following treatment, catalytic iron was measured using the bleomycin assay as described under “Experimental Procedures.” *, p < 0.001 compared with iron content after initial exposure. #, p = 0.001 compared with cells not treated with DETA/NO. All values represent the mean of four experiments ± S.E.

Iron was measured using the bleomycin assay as described under “Experimental Procedures.” *, p = 0.02 compared with hemin-treated PAECs. All values represent the mean of two experiments performed in duplicate ± S.E.

We considered that NO might prevent the increase in catalytic iron resulting from heme exposure by preventing cellular uptake of exogenous heme or causing the displacement of heme from the cell. For example, it has previously been shown that treatment of hepatocytes with the NO donor S-nitroso-N-acetylpenicillamine results in loss of microsomal heme when measured 12 h after treatment (12). We studied the effect of NO on EC heme uptake and retention by incubating confluent monolayers of cells with 25 μM heme in serum-containing medium for 24 h in the presence of increasing concentrations of hemin, which is not different from naive ECs (Fig. 1, open circles, 22% drop in catalytic iron after 1 h incubation, respectively). Cells that were experienced 22.9 ± 8.3% iron release. These data are consistent with findings in Fig. 1. The addition of the NO donor SPER/NO (half-life 39 min, pH 7.4, 37 °C) during heme exposure (Fig. 2, third column) significantly reduces the rise in catalytic iron compared with ECs exposed to hemin alone (32 ± 3 versus 61 ± 13 nmol iron/mg protein, respectively, p = 0.02). Thus NO donors prevent both the rise in catalytic iron and the induction of ferritin synthesis resulting from excess heme.

Hibbs et al. (25) have shown that NO can induce the loss of cellular iron, raising the question as to whether the spermine/NO used in our experiments could be inducing the rapid release of cellular iron. We incubated cells that had been pre-labeled with 55Fe with 100 μM spermine/NO and measured the release of iron into the media. The spermine/NO treatment was associated with the loss of 0.9 ± 1.2 and 2.9 ± 2.1% of cellular iron after 1 and 2 h of incubation, respectively. Cells that were incubated with 100 μM DETA/NO (half-life 20 h) for 24 h experienced 22.9 ± 8.3% iron release. These data are consistent with those of Hibbs et al. (25), who found little NO-mediated iron release until after 4 h of exposure to NO. These results suggest that NO may inhibit heme degradation and prevent the rise in iron that is available to catalyze oxidative injury and drive ferritin synthesis.

In the preceding experiment, ECs were exposed to hemin and NO donor sequentially to avoid a direct interaction between hemin and NO that might alter iron release from heme. To assess direct effects of NO on hemin degradation and release of iron, we incubated cells with a NO donor and hemin simultaneously and assessed the changes in catalytic iron. As shown in Fig. 2 (second column), ECs exposed to hemin alone for 1 h experience approximately a 3-fold rise in catalytic iron (similar to findings in Fig. 1). The addition of the NO donor SPER/NO (half-life 39 min, pH 7.4, 37 °C) during heme exposure (Fig. 2, third column) significantly reduces the rise in catalytic iron compared with ECs exposed to hemin alone (32 ± 3 versus 61 ± 13 nmol iron/mg protein, respectively, p = 0.02). Thus NO donors prevent both the rise in catalytic iron and the induction of ferritin synthesis resulting from excess heme.

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NO Inhibits Heme Degradation by Heme Oxygenase

DETA/NO. Following incubation, total cellular heme was measured. We chose to expose cells to hemin over 24 h in serum-containing medium because hemin uptake is gradual in the presence of serum, and an effect of constant NO exposure (in the form of DETA/NO) on heme content over this time period should be detectable. As shown in Fig. 3, exposure of ECs to hemin results in approximately a 4-fold increase in total cellular heme over 24 h (second column). The addition of DETA/NO results in a dose-dependent increase in cellular heme (p < 0.001 by ANOVA), indicating that exposure to the NO donor resulted in accumulation rather than displacement of cellular heme.

We have found that NO donors decrease ferritin synthesis and inhibit release of catalytic iron from ferritin. Also, treatment with NO donors led to the accumulation of heme in cells exposed to exogenous heme. Taken together, these results suggest that NO might inhibit the degradation of cellular heme and release of iron. HO-1 and -2 are the enzymes thought to be primarily responsible for degrading intracellular heme, although heme can be degraded nonenzymatically by its reaction with peroxides and other oxidants (33). In experiments with a fibroblast cell line, we found that the addition of the inhibitor of HO, SnPP during heme exposure inhibited the rise in bilirubin (an iron-binding protein) to 61.3 ± 2.5% (p < 0.001 ANOVA) of that seen in cells without the inhibitor. This indicates that HO releases a significant proportion of the bilirubin detectable iron from heme degradation; therefore, we narrowed our focus to study the effect of NO on heme metabolism by HO.

We first studied whether NO could inhibit heme degradation by preventing HO-1 induction by hemin. To address this question, we measured HO-1 protein by immunoblot in ECs treated with hemin and NO donors (Fig. 4). Exposure to hemin for 1 h followed by a 3-h incubation in medium results in approximately a 2-fold increase in HO-1 protein (Fig. 4, second column). A similar hemin exposure in the presence of SPER/NO (250 μM) inhibits the rise in HO-1 to the level seen in untreated ECs (third column). Treatment of ECs with SPER/NO alone results in a slight increase in HO protein consistent with other reports demonstrating the induction of HO synthesis by NO (fourth column). This data indicates that NO donors inhibit the induction of HO-1 by hemin suggesting that NO may directly interact with heme and block downstream signaling events.

Although NO donors were found to inhibit HO-1 induction by hemin, untreated ECs possess considerable HO-1 protein, so we were interested in the effects of NO donors on HO activity in EC microsomes. Heme degradation to bilirubin and free iron by HO consists of the following steps: heme binding to HO, reduction of ferric (III) to ferrous (II) iron, binding of molecular oxygen, and oxidation of the protoporphyrin IX ring to biliverdin, which is in turn reduced by biliverdin reductase to bilirubin. We studied the effects of NO on HO activity by collecting microsomes prepared from endothelial cells incubated with hemin. We then incubated these HO-1-rich microsomes with a source of biliverdin reductase (rat liver cytosol) in the presence of 20 μM heme and SPER/NO. As shown in Fig. 5, SPER/NO from 20 to 100 μM has a dose-dependent inhibitory effect on HO activity as measured by bilirubin production (p = 0.02, ANOVA). To exclude the possibility that SPER/NO inhibits biliverdin reductase, depletes NADPH (a necessary cofactor for HO-1), or interferes with bilirubin detection, we examined its effect on biliverdin reductase activity by measuring the conversion of biliverdin to bilirubin. As shown in Fig. 6, SPER/NO had no effect on biliverdin reductase activity. This indicates that NO donors inhibit the breakdown of heme by HO but do not impair bilirubin reductase.

NO binds to reduced heme proteins (in the Fe²⁺ oxidation state) such as deoxyhemoglobin or deoxymyoglobin with high affinity; however, the affinity for oxidized heme proteins (Fe³⁺) such as methemoglobin is approximately 10⁶ lower. NO also binds to free heme (Fe²⁺) to form NO-heme adducts that have a low dissociation constant and a long half-life (approximately 9 h) (34). In aerobic solutions, protoporphyrin-bound iron will be Fe³⁺, so stable NO-heme formation is unfavorable. However, there is a strong intracellular reducing environment due to glutathione and lower oxygen tension that might favor reduction of heme (Fe³⁺) to heme (Fe²⁺), facilitating formation of NO-heme adducts. Furthermore, both NO and heme would be expected to partition in the hydrophobic compartment of cell.
membranes, thus raising the effective concentrations to drive nitrosylation reactions (35). It has been shown that hemin dissolved in liposomes can react with NO to form NO-heme (II) (16), and we wondered if a similar process would occur in cellular membranes. The relatively stable nature of NO-heme might prevent degradation by HO and thus the release of free iron.

We studied whether NO-heme forms in our HO assays by using EPR. NO may combine with heme and non-heme, low molecular weight iron to form complexes that have distinctive low temperature EPR spectra. Work by Bastian et al. (36) has shown that physiologically relevant concentrations of NO result in formation of at least three separate EPR-detectable iron species in target cells. These include the dinitrosyl-iron complex, and five- or six-coordinate nitrosyl-heme. NO-heme can be detected as a five- or six-coordinate species depending upon the absence or presence of an axial ligand opposing the plane of the NO binding site on heme iron. Five-coordinate nitrosyl-heme produces an EPR spectrum with a broad peak at $g = 2.067$ and a hyperfine triplet centered at $g = 2.009$ (16, 36–38). The spectrum resulting from six-coordinate nitrosyl-heme has a broad peak at $g = 2.067$ and a trough at $g = 1.988$. NO binds non-heme, low molecular weight iron to form dinitrosyl-iron complexes of the general formula, Fe(RS)$_2$(NO)$_2$ that have a characteristic EPR spectrum consisting of a peak at $g = 2.039$ and 2.013 (14). In non-hemin-treated cells, dinitrosyl-iron complexes form from iron displaced by NO from iron-sulfur enzymes, particularly those involved in the mitochondrial electron transport chain as shown by Drapier et al. (39). In the presence of NO and thiols such as metallothionein, available ferrous iron will form dinitrosyl-iron complexes (37). We assumed that iron released by HO due to heme degradation would be available to form dinitrosyl-iron complexes. The amount of the five- and six-coordinate nitrosyl-heme can be measured from the height of the peak at $g = 2.067$ relative to the base line. Note that the peak is referred to as the $g = 2.067$ peak, although 2.067 is not a true $g$ value. A similar nomenclature for Fe(RS)$_2$(NO)$_2$ is used in which the peak at $g = 2.039$ is used to identify this spectrum.

EPR signals from cells treated with NO are a composite of signals from varied NO-iron complexes. We exploited the differences between these spectra to qualitatively determine the nature of heme and non-heme iron in cells exposed to NO, and we used SnPP to block heme binding by HO to determine the effect of HO on the observed spectra. We first used EPR to determine the nature of the NO-iron complexes formed in cytosol and microsomes after incubation with hemin and NO donors. To address the involvement of HO in any observed NO-heme signal, we included SnPP during incubation and considered any change in the signal compared with incubation without SnPP to be due to the action of HO. As shown in Fig. 7, spectrum A, membrane-free cytosol incubated with 20 $\mu$M biliverdin, 1 mM NADPH, 1.6 mM d-glucose 6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, and SPER/NO from 20 to 100 $\mu$M. Activity was measured by bilirubin production. All values are the mean of two experiments ± the range.

FIG. 6. Biliverdin reductase activity liver cytosol in the presence of SPER/NO. Rat liver cytosol was incubated with 20 $\mu$M biliverdin, 1 mM NADPH, 1.6 mM d-glucose 6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase, and SPER/NO from 20 to 100 $\mu$M. Activity was measured by bilirubin production. All values are the mean of three experiments ± S.E.
with and without SnPP. Following this incubation, we briefly exposed the cells to 1 mM DEA/NO (half-life 2 min, pH 7.4, 37 °C) immediately prior to EPR. We were careful to use identical numbers of cells in each group (40 × 10^6 cells/group), so the EPR signals could be compared. In this experiment, NO can be considered a spin probe to detect heme versus non-heme iron. As shown in Fig. 8, spectra D and E (without and with SnPP, respectively), the predominant signal has three equally spaced lines centered at g = 2.009, which is characteristic of five-coordinate nitrosyl-heme. There is also a dinitrosyl-iron signal at g = 2.039. These signals were subtracted to obtain the signal shown in Fig. 9, spectrum F, that could be attributed to HO activity. As shown in Fig. 8, spectrum F, the subtracted signal, with principle g values at 2.039 and 2.013, is characteristic of dinitrosyl-iron. This signal is indicative of free iron released from HO-mediated degradation of heme.

To determine the effect of NO on heme degradation within cells, CCRF-CEM cells were again incubated with 25 μM hemin, but this time in the presence of the long acting NO donor DETA/NO (100 μM) for 24 h. Again, the experiment was performed with and without SnPP. As shown in Fig. 8, spectrum G (no inhibitor) and spectrum H (with SnPP), there are EPR features consistent with nitrosyl-heme and dinitrosyl-iron. The subtraction of the spectra (with and without SnPP) results in a nitrosyl-heme signal (five-coordinate), indicating that inhibition of HO resulted in less nitrosyl-heme formation. Quantitation of the spectra by double integration reveals that inhibition of HO reduces the nitrosyl-heme signal by approximately 25%. This suggests that inhibition of HO by SnPP reduces formation of nitrosyl-heme perhaps by preventing HO catalysis of NO binding to heme. More importantly, the inhibition of HO did not change the amount of dinitrosyl-iron formation (as it did in Fig. 8, spectrum F), suggesting that NO is preventing the degradation of heme by HO. To exclude errors in cell numbers that might change the relative size of the signals as an explanation of our results, we varied the scaling of the two signals before subtraction. These maneuvers did not uncover a dinitrosyl-iron signal but only changed the magnitude of the five-coordinate nitrosyl-heme signal. Based on these experiments, we can conclude that unbound cellular heme readily combines with NO intracellularly, predominantly in lipid membranes, to form nitrosyl-heme that prevents catabolism of the heme ring and release of iron.

Free heme from sources such as methemoglobin readily enters endothelial membranes, where it can exacerbate oxidative injury (2). NO has been shown to be capable of reducing the injury associated with hemoglobin by forming nitrosyl-hemoglobin and preventing oxoferryl-hemoglobin formation (16). We wondered whether exposure to NO would protect heme-treated ECs from hydrogen peroxide-mediated injury by formation of nitrosyl-heme complexes. We exposed PAECs to heme for 1 h with or without SPEr/NO followed by washing and treatment with hydrogen peroxide for 2 h. The SPEr/NO and heme were removed prior to the addition of hydrogen peroxide to prevent direct interaction between NO donor and hydrogen peroxide. As shown in Fig. 10, heme greatly exacerbates the toxicity of hydrogen peroxide, consistent with earlier reports (1, 33). The addition of SPEr/NO ameliorates the toxicity at all dose levels of hydrogen peroxide (p < 0.001), consistent with the concept that NO prevents the degradation of heme to free iron and the reactivity of the heme moiety.

**DISCUSSION**

There are several pathological states, such as disseminated intravascular coagulation, rhabdomyolysis, sickle cell disease, and thrombotic thrombocytopenic purpura, that are characterized by the release of free hemoglobin or myoglobin from he-
molysis or muscle destruction, respectively. Although plasma haptoglobin and hemopexin are important in preventing heme-mediated vascular injury by their binding of hemoglobin and heme respectively, these defenses are overwhelmed by hemolysis of over 1% of circulating red blood cells. This explains the common clinical finding of unmeasurable haptoglobin in hemolytic states. Free hemoglobin will rapidly oxidize to methemoglobin in which the affinity between heme and globin is reduced allowing the donation of heme to albumen and ECs (2, 40, 41).

Heme is composed of protoporphyrin IX and iron to form a rigid planar structure that is hydrophobic and rapidly intercalates into lipid membranes and other hydrophobic compartments when not associated with protein (1). The iron is capable of cycling between ferrous (II) and ferric (III) oxidation states under physiological conditions, and therein lies both the utility and danger of the molecule. The ability of heme to intercalate into lipid membranes and participate in the Fenton reaction to produce the hydroxyl radical makes it a potent catalyst of injury from hydrogen peroxide, oxidized LDL, and activated

**Fig. 8.** Low temperature EPR spectra of CCRF-CEM cells treated with hemin and NO donors. CCRF-CEM cells (4 x 10⁶ cells/group) were incubated for 24 h in medium (10% FBS) alone or containing 25 μM hemin. Some groups were either treated with 100 μM DETA/NO during the 24-h hemin exposure or 1 mM DEA/NO for 5 min following the hemin exposure. The effect of HO in forming the EPR signals was assessed by including the HO inhibitor 10 μM SnPP. Following incubation, the cells were pelleted, washed twice in PBS, resuspended, and frozen at 77 K for EPR. There are two separate EPR species in these samples. These include a dinitrosyl-iron Fe(RS)₂(NO)₂ signal with principle g values at 2.039 and 2.013 and a five-coordinate nitrosyl-heme signal dominated by a triplet centered at g = 2.009. A, untreated CCRF-CEM cells. B, CCRF medium (cell-free) incubated with 25 μM hemin and 100 μM DETA/NO for 24 h. C, CCRF-CEM cells incubated in medium with 100 μM DETA/NO (no hemin). D, CCRF-CEM cells incubated with 25 μM hemin and 100 μM DETA/NO. E, CCRF-CEM cells incubated with hemin and 10 μM SnPP followed by DEA/NO. F, difference spectrum obtained from subtraction of spectrum E from D. G, CCRF-CEM cells incubated with 25 μM hemin and 100 μM DETA/NO. H, CCRF-CEM cells incubated with hemin, 10 μM SnPP, and DETA/NO. I, difference spectrum obtained from subtraction of spectrum H from G. EPR parameters were as follows: field set, 3200 G; scan range, 200; receiver gain, 2 x 10⁴; temperature, 77 K; microwave power, 5 milliwatts; microwave frequency, 9.052 GHz. Curve C represents computerized averages of four acquisitions; the other curves are from a single acquisition. The curves are representative of two experiments.

**Fig. 9.** PAEC survival after exposure to hydrogen peroxide. Confluent PAECs were exposed to 5 μM hemin in serum-free medium with or without 200 μM SPER/NO for 1 h. PAECs were then washed twice with buffered saline and exposed to hydrogen peroxide in buffered saline solution for 2 h. Following hydrogen peroxide, fresh medium was replaced, and the cells were incubated for 24 h. Cell survival was assessed by neutral red assay. The protective effect of SPER/NO is significant p < 0.001 by ANOVA.
neutrophils (1, 3, 33). Heme from intracellular sites may also contribute to cellular injury as has been shown for reperfusion injury (29) in which the heme originates from P-450 cytochromes. Ferritin provides a crucial defense against heme by its ability to sequester iron in a less catalytically active form. HO plays an important role by degrading heme to release iron that can be bound by ferritin (8), although there are other pathways for heme breakdown including NADPH-cytochrome P-450 reductases and oxidation by peroxides (33, 42–44). Heme degradation is important in releasing iron for ferritin synthesis and other iron-dependent functions (45). Heme degradation drives ferritin synthesis by releasing iron that stimulates iron-regulatory protein-1 (IRP-1) activation (46, 47). Our data show that increased ferritin content in ECs is associated with less catalytic iron increase from heme. Together, HO and ferritin allow rapid shifting of iron from heme into the ferritin core, where it is less available to catalyze deleterious reactions.

The purpose of our study was to determine the effect of NO on the disposal of excess cellular heme and the resulting effects on catalytic iron. We first studied NO effects on adaptation to heme with respect to control of catalytic iron. NO represses ferritin synthesis in response to excess heme (4) as well as other forms of iron (4, 21, 22, 48, 49), resulting in a decreased capacity to control catalytic iron. Indeed, we found that inhibition of ferritin synthesis by NO donors results in cellular inability to control the catalytic iron from heme exposure (Fig. 1, 22-h time point). However, when ECs were exposed to heme in the presence of NO donors, the rise of catalytic iron after heme was inhibited (Fig. 2). One explanation may be that NO prevents the entry or enhances the release of heme from the cell; however, we found that the presence of NO donors was associated with accumulation of cellular heme (Fig. 3), implying either increased heme uptake or reduced catabolism. Another possibility may be that NO donors induce the loss of iron as it is being released by heme degradation, resulting in less bleomycin-detectable iron. In fact, it has been shown that activated macrophages cause iron depletion in tumor cells in a NO-dependent fashion (25, 50, 51). Hibbs et al. (25) showed that NO from activated macrophages induced a time-dependent loss of cellular iron, resulting in approximately 70% of cellular iron release after 24 h of exposure to NO. These studies, however, found little iron loss until 4 h after exposure to NO; this is considerably longer than the 2-h time course of our studies depicted in Fig. 2. Also, in our studies with a fibroblast cell line labeled with $^{55}$Fe, we found only 2.9% iron release after a 2-h exposure to 100 $\mu$M Sp/NO (the concentration of NO donor used in our experiments). In contrast, 100 $\mu$M Sp/NO reduced bleomycin-detectable iron 75% after heme exposure. This reduction is much higher than would be expected from direct NO effects on cellular iron. We cannot exclude, however, a specific effect of NO on the pool of iron measured by the bleomycin assay. This pool of loosely chelated iron may be particularly amenable to displacement from the cell by NO.

To further elucidate the mechanism by which NO donors prevented the increase in catalytic iron after heme, we considered the effects of NO on HO activity by measuring bilirubin production in microsomes and by using EPR to measure HO-specific formation of dinitrosyl-iron complexes. In our EPR experiments, we considered HO activity to be reflected in dinitrosyl-iron complexes that formed from heme- liberated iron, NO, and thiols. HO-specific signals were obtained by subtracting spectra taken from cells with and without HO inhibition. We found that NO donors, in a dose-dependent fashion, dramatically inhibited microsomal HO activity measured by bilirubin production, but NO donors had no effect on biliverdin reductase activity. However, we repeated the HO assays, performed EPR on the reaction mixtures, and found that NO-heme complexes formed in the aerobic solution. This finding suggested that NO bound heme in microsomal membranes, thus sequestering it from HO-mediated catabolism.

To assess whether NO-heme formation reduced iron release from HO, we incubated cells with NO donors and heme with or without a HO inhibitor to determine HO-specific iron release. Our EPR data showed that the HO-specific dinitrosyl-iron complex signal was lost, but a five-coordinate nitrosyl-heme signal formed in the presence of continuous NO (Fig. 8, spectrum 1). If NO had no effect on HO activity but simply caused loss of free iron from cells, then our EPR experiments would show a HO-dependent dinitrosyl-iron signal in NO donor-treated cells (albeit reduced in amplitude compared with non-NO donor-treated cells), due to the HO-dependent release of iron from heme to form the signal. This finding is apparent in the cells exposed to heme for 24 h and pulsed with NO donor prior to EPR. Although NO may cause loss of iron from the cell, our data more strongly suggest that NO prevents heme degradation as the reason for NO donor inhibition of heme-induced catalytic iron increase.

The nitrosylation of heme appears to diminish both the induction of HO synthesis and its enzymatic activity. Previous work has shown that NO alone can induce HO synthesis (19, 20, 52–54) and reduce heme content of cells (12, 19), which on the surface seems at odds with our results. For example, Yee et al. (19) have shown that the short acting NO donor S-nitroso-N-acetylenicilamin results in elevation of HO activity (10 h after exposure), loss of heme iron (14 h after exposure), and an expansion of non-heme iron. While their results appear to show that NO fosters HO-mediated heme degradation, an important difference is that their measurements of heme and non-heme iron were taken after exhaustion of the NO donor (as demonstrated by their EPR experiments showing loss of the g = 2.039 dinitrosyl-iron signal). In our experiments, measurements of catalytic iron were taken at the time corresponding to approximately one half-life of the NO donor when NO release would be ongoing. In the setting of continued NO release, we found less catalytic iron resulting from HO activity than in control cells. We hypothesize that during NO production, NO binds the heme moieties of heme proteins causing displacement, perhaps by disrupting the axial ligand. As the concentration of NO de-
clines, previously formed nitrosyl-heme will dissociate, freeing heme to drive HO induction. In this model, stimulation of HO synthesis occurs, not due to NO directly but as a result of heme released from heme proteins. In support of this hypothesis, we found that NO donors inhibit the amount of HO synthesized in response to heme. This suggests that nitrosylation of heme inhibits the downstream control elements that activate HO gene transcription and translation.

Nitrosylation of heme generally does not occur in aerobic solutions due to the oxidation state of the iron, so we were initially surprised to find nitrosyl-heme signals in endothelial microsomes incubated with heme and NO under aerobic conditions. We did not find nitrosyl-heme signals in cell-free medium after prolonged incubation with heme and DETA/NO (Fig. 8, spectrum B) unless the incubation was carried out anaerobically and after the addition of dithionite (data not shown). The addition of HO-rich microsomes facilitated the formation of nitrosyl-heme, despite the aerobic environment, suggesting that the hydrophobic milieu and/or a reducing environment facilitated the reduction of heme to allow nitrosylation. The partitioning coefficients of heme and NO favor localization in hydrophobic compartments (see Ref. 35). The expected increase in relative concentrations of NO and heme may favor nitrosylation reactions.

Due to the lack of purified HO, we cannot comment on specific interactions between NO and HO or the HO-heme complex, but the EPR data showing loss of nitrosyl-heme after HO inhibition suggests that HO may catalyze the reaction between NO and heme. This is plausible because HO is known to reduce heme (III) to heme (II) to allow binding of molecular oxygen that is necessary to oxidize the protoporphyrin IX ring. NO has a high affinity for reduced heme proteins, and NO is known to bind the HO-heme (II) complex (55). NO may also inhibit HO activity by binding the HO-oxymel complex, resulting in oxidation of heme (II) to heme (III) with nitrite production. Although the exact mechanism is not clear, our data show that NO prevents the degradation of heme to bilirubin and prevents the release of the heme-associated iron.

Previous work has shown that heme exacerbates oxidative injury due to its ability to intercalate into membranes and participate in the breakdown of lipid components (1). NO has been shown to reduce the oxidative injury associated with t-butyl hydroperoxide treatment of hemoglobin-containing cells by binding heme and preventing oxoferryl-hemoglobin formation and subsequent oxidation of membrane phospholipids (16, 17). We found that NO donors reduce the sensitization to oxidative injury resulting from exposure to heme. In these experiments, NO and heme were removed prior to hydrogen peroxide exposure to eliminate a direct NO reaction with hydrogen peroxide. We could not detect nitrosyl-heme formation in medium, and we could not detect loss of heme from ECs over the course of the short term exposure (data not shown), so the observed protection by NO is not explained by differences in heme uptake. The mechanism of protection is likely to be similar to that proposed by Gorbunov et al. (16) in their studies using hemoglobin containing cell lines exposed to t-butyl hydroperoxide. They found that nitrosylation of hemoglobin prevented oxoferryl-hemoglobin formation by the reaction between t-butyl hydroperoxide and heme, thus preventing participation of the heme iron in lipid oxidation.

An unresolved issue in our experiments pertains to the nature and fate of the nitrosyl-heme complexes. Hemin is hydrophobic, due to the protoporphyrin IX ring and will intercalate into hydrophobic compartments. A brief exposure to heme results in its uptake by the cellular membrane (1). The EPR spectra from the cells incubated with NO donors and heme show predominantly a signal from the five-coordinate heme (that is lacking an axial ligand) and resembles those found from liposomes incubated with hemin and NO (16). The half-life of nitrosylated heme is estimated to be 9 h, suggesting that this species may be long lived in cells (34). If the complexes remain in the lipid membranes, there may be deleterious effects due to the retention of heme in the lipid milieu. While the nitrosylation of heme may be viewed as protective, the ability of reactive oxygen species to oxidize NO leaving reactive heme would reduce any sustained protective benefit. Prevention of nitrosyl-heme formation would allow HO-mediated degradation and storage of iron in ferritin that may ultimately be more effective in reducing the potential for injury. Based on our data, we propose a model for heme metabolism in the presence of NO that is depicted in Fig. 10. In the presence of NO, excess heme may become nitrosylated in the microsomal compartment, where it would remain until NO dissociates to allow its degradation. This would occur when NO levels decline, favoring the dissociation of NO-heme. The effect of NO would be to act as a buffer against excess heme by reducing its redox activity and slowing its release of free iron, thus protecting cells from both heme and iron-catalyzed injury. As NO levels decline, dissociation of NO from heme will allow degradation and storage in ferritin. Although there is little doubt that NO has a pronounced effect on IRP-1 activation and ferritin mRNA translation, our work supports a concept that NO also changes the availability of heme-associated iron (10). We found that NO readily binds heme in microsomal membranes and also inhibits its degradation and release of iron. Although it is unknown what effect nitrosyl-heme may have on IRP-1 activation, heme-associated iron alone does not activate IRP-1 until it is released by oxidation of the protoporphyrin IX ring (56, 57). Thus, nitrosylation of heme is likely to contribute to the inhibitory effect of NO on ferritin synthesis induced by heme.

Acknowledgment—We thank Owen W. Griffith, Ph.D. for helpful suggestions and comments.

REFERENCES
1. Balla, G., Vellorelloti, G. M., Muller-Eberhard, U., Eaton, J., and Jacob, H. S. (1991)Lab. Invest. 64, 649–655
2. Balla, J., Jacob, H. S., Balla, G., Nath, K., Eaton, J. W., and Vercellotti, G. M. (1993)Proc. Natl. Acad. Sci. U. S. A. 90, 9285–9289
3. Balla, G., Jacob, H. S., Balla, J., Rosenberg, M., Nath, K., Apple, F., Eaton, J. W., and Vercellotti, G. M. (1992)J. Biol. Chem. 267, 18148–18153
4. Juckett, M. B., Weber, M., Balla, J., Jacob, H. S., and Vercellotti, G. M. (1996)Free. Radical Biol. Med. 20, 63–73
5. Juckett, M. B., Balla, J., Balla, G., Jessurun, J., Jacob, H. S., and Vercellotti, G. M. (1996)Proc. Natl. Acad. Sci. U. S. A. 93, 6798–6802
6. Abraham, N. G., Lavrovsky, Y., Schwartzman, M. L., Stoltz, R. A., Levere, R. D., Gerritsen, M. E., Shubara, S., and Kappas, A. (1995)Proc. Natl. Acad. Sci. U. S. A. 92, 6798–6802
7. Vile, G. F., and Tyrrell, R. M. (1993)J. Biol. Chem. 268, 14678–14681
8. Vile, G. F., Basu-Mudak, S., Walton, C., and Tyrrell, R. M. (1994)Proc. Natl. Acad. Sci. U. S. A. 91, 2607–2610
9. Ponka, P. (1997)Blood 89, 1–25
10. Hentze, M. W., and Kuhn, L. C. (1996)Proc. Natl. Acad. Sci. U. S. A. 93, 8175–8182
11. Nakano, R., Sato, H., Watanabe, A., Ito, O., and Shimizu, T. (1996)J. Biol. Chem. 271, 8570–8574
12. Kim, Y. M., Bergonia, H. A., Muller, C., Pitt, B. R., Watkins, W. D., and Lancefield, R. J., Jr. (1992)J. Biol. Chem. 270, 5710–5713
13. Khatsonos, O. G., Gross, S. S., Rikkind, A. B., and Vane, J. R. (1993)Proc. Natl. Acad. Sci. U. S. A. 90, 11147–11151
14. Mulesch, A., Mordvinov, P. I., and Vane, J. R. (1995)J. Lipid Res. 36, 1303–1308
15. Sergent, O., Griffon, B., Morel, I., Chevanne, M., Dubos, M. P., Cillard, P., and Cillard, J. (1997)Hepatology 25, 122–127
16. Gorbunov, N. V., Yaslich, J. C., Gammad, A., Thamplant, R., Ritter, B. V., Kisin, E. R., Elsayed, N. M., and Kagan, V. E. (1997)J. Biol. Chem. 272, 12328–12341
17. Gorbunov, N. V., Oxiorn, A. N., Day, B. W., Zaivesa-Rivera, B., Kagan, V. E., and Elsayed, N. M. (1995)Biochemistry 34, 6689–6699
18. Kanner, J., Harel, S., and Granit, R. (1991)Arch. Biochem. Biophys. 289, 150–156
19. Yee, E. L., Pitt, B. R., Billiar, T. R., and Kim, Y. M. (1996)Am. J. Physiol. 270, L15–L21
20. Motterlini, R., Foresti, R., Intaglietta, M., and Winslow, R. M. (1996)Am. J. Physiol. 270, H107–H114
21. Drapier, J. C., Hirling, H., Wietzerbin, J., Kaldy, P., and Kuhn, L. C. (1993) EMBO J. 12, 3643–3649
22. Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G., Wachter, H., and Hentze, M. W. (1993) EMBO J. 12, 3651–3657
23. Mosradian, D. L., Hutsell, T. C., and Keefer, L. K. (1995) J. Cardiovasc. Pharmacol. 25, 674–678
24. Keefer, L. K., Nims, R. W., Davies, K. M., and Wink, D. A. (1996) Methods Enzymol. 268, 281–293
25. Hibbs, J. B., Taintor, R. R., and Vavrin, Z. (1984) Biochem. Biophys. Res. Commun. 123, 716–723
26. Kuross, S. A., Rank, B. H., and Hebbel, R. P. (1988) Blood 71, 876–882
27. Gutteridge, J. M., Rowley, D. A., and Halliwell, B. (1982) Biochem. J. 206, 605–609
28. Gutteridge, J. M., Rowley, D. A., and Halliwell, B. (1981) Biochem. J. 199, 263–265
29. Paller, M. S., and Jacob, H. S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7002–7006
30. Gutteridge, J. M., Mumby, S., Koizumi, M., and Taniguchi, N. (1996) Biochem. Biophys. Res. Commun. 229, 806–809
31. Mumby, S., Margarson, M., Quinlan, G. J., Evans, T. W., and Gutteridge, J. M. (1997) Intensive Care Med. 23, 635–639
32. Berger, T. M., Polidori, M. C., Dabbagh, A., Evans, P. J., Halliwell, B., Morrow, J. D., Roberts, I. J., II, and Frei, B. (1997) J. Biol. Chem. 272, 15656–15660
33. Balla, G., Jacob, H. S., Eaton, J. W., Belcher, J. D., and Vercellotti, G. M. (1991) Arterioscler. Thromb. 11, 1700–1711
34. Kharitonov, V. G., Sharma, V. S., Magde, D., and Koesling, D. (1997) Biochemistry 36, 6814–6818
35. Liu, X., Miller, M. J. S., Joshi, M. S., Thomas, D. D., and Lancaster, J. R., Jr. (1996) Proc. Natl. Acad. Sci. U. S. A. 95, 2175–2179
36. Bastian, N. R., Yim, C. Y., Hibbs, J. B., Jr., and Samlowski, W. E. (1994) J. Biol. Chem. 269, 5127–5131
37. Kennedy, M. C., Gan, T., Antholine, W. E., and Petering, D. H. (1993) Biochem. Biophys. Res. Commun. 196, 632–635
38. Henry, Y., Durcroç, C., Drapier, J. C., Servent, D., Pellat, C., and Guissani, A. (1991) Eur. J. Biochem. 20, 1–15
39. Drapier, J. C., and Hibbs, J. B. (1986) J. Clin. Invest. 78, 790–797
40. Bunn, H. P., and Jandl, J. H. (1968) J. Biol. Chem. 243, 465–475
41. Balla, J., Nath, K. A., Balla, G., Juckett, M. B., Jacob, H. S., and Vercellotti, G. M. (1995) Am. J. Physiol. 268, L321-L327
42. Doherty, J. C., FIRNEISZ, G. D., and Schacter, B. A. (1984) Arch. Biochem. Biophys. 235, 657–664
43. Schaefer, W. H., Harris, T. M., and Guengerich, F. P. (1985) Biochemistry 24, 3254–3263
44. Cantoni, L., Gibbs, A. H., and De, M. F. (1981) Int. J. Biochem. 13, 823–830
45. Poss, K. D., and Tonegawa, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10919–10924
46. Philpott, C. C., Klausner, R. D., and Rouault, T. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7321–7325
47. Rouault, T. A., Hentze, M. W., Wright-Caughman, S., Harford, J. B., and Klausner, R. D. (1988) Science 241, 1207–1210
48. Gray, N. K., Quick, S., Goossen, B., Constable, A., Hirling, H., Kuhn, L. C., and Hentze, M. W. (1993) Eur. J. Biochem. 218, 657–667
49. Pantopoulos, K., and Hentze, M. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1267–1271
50. Wharton, M., Granger, D. L., and Durack, D. T. (1988) J. Immunol. 141, 1311–1317
51. Drapier, J. C., and Hibbs, J. B., Jr. (1986) J. Immunol. 140, 2829–2838
52. Willis, D., Tomlinson, A., Frederick, R., Paul-Clark, M. J., and Willoughby, D. A. (1995) Biochem. Biophys. Res. Commun. 214, 1152–1156
53. Kurata, S., Matsumoto, M., and Yamashita, U. (1996) J. Biochem. (Tokyo) 120, 49–52
54. Takahashi, K., Hara, E., Suzuki, H., Sasano, H., and Shibahara, S. (1996) J. Neurochem. 67, 482–489
55. Sun, J., Wilks, A., Ortiz de Montellano, P. R., and Loehr, T. M. (1993) Biochemistry 32, 14151–14157
56. Eisenstein, R. S., Garcia-Mayol, D., Pettingell, W., and Munro, H. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 688–692
57. Haile, D. J., Rouault, T. A., Harford, J. B., and Klausner, R. D. (1990) J. Biol. Chem. 265, 12786–12789