Iron and heme-containing moieties are indispensable for the normal transport of oxygen in the blood; however, once released into the bloodstream these molecules are highly toxic to the vasculature because of their pro-oxidative effects on the endothelium (1–3). Humans have therefore evolved sophisticated iron transport and sequestration systems as well as heme-metabolizing enzymes to rapidly clear iron and heme from the circulation (4, 5). There is growing evidence that defects in these natural protective mechanisms lead to endothelial dysfunction and vascular disease, and as a consequence, methods that reduce the pro-oxidative effects of iron and heme may have therapeutic benefit (2).

Clinical syndromes associated with marked intravascular hemolysis and circulating free hemoglobin, such as sickle cell disease, paroxysmal nocturnal hemoglobinuria, thalassemias, and hereditary spherocytosis, lead to endothelial dysfunction, thrombosis, and vascular disease (5–10). Similarly administration of purified recombinant hemoglobin to humans promotes vascular injury and arterial thrombosis, precipitating acute myocardial infarction (11–13). Some of these vascular effects are related to nitric oxide scavenging by excess plasma hemoglobin, whereas others are linked to cytotoxic, proinflammatory, and pro-oxidant effects of iron-containing hemoglobin and heme (14–19). Interestingly elevated levels of body iron stores are associated with an increased risk of myocardial infarction, and carriers of the hemochromatosis gene have an increased risk of myocardial infarction and cardiovascular death (20, 21). Whether the pro-oxidative effects of iron per se are proatherogenic remains controversial; however, in the context of erythrocyte-dependent release of hemoglobin and heme, redox-active iron is likely to play an important role in promoting vascular dysfunction.

The well defined pro-oxidative properties of redox-active iron have been exploited experimentally with topical application of ferric chloride (FeCl₃) widely used to induce vascular injury and thrombosis in experimental animal models (22).

High concentrations of FeCl₃ induce profound injury to the vasculature, leading to endothelial denudation, and collagen and tissue factor exposure, leading to the rapid formation of vaso-occlusive thrombi. Histologically FeCl₃-induced thrombi are rich in platelets, fibrin, and red blood cells (23–26). However, the mechanism(s) by which FeCl₃ induces vascular injury has not been clearly defined. FeCl₃ can have direct pro-oxidative effects on endothelial cells as a result of the Fenton reaction, leading to hydroxyl radical generation and lipid peroxidation (1, 3). It can also mediate vascular injury indirectly through oxidative modification of LDL (3, 14). A recent study has demonstrated transfer of ferric ions through the vasculature, penetrating the internal elastic membrane and emerging through the endothelium via an endocytic/exocytic pathway, leading to the development of ferric oxide aggregates in the vascular lumen (27). Although the direct cytotoxic effects of redox-active iron on endothelial cells have been well established in vitro, the importance of this mechanism to the severe vascular injury and thrombus formation induced by topical FeCl₃ in vivo remains unclear.

To gain insight into this, we developed a novel ex vivo perfusion chamber that enables direct analysis of the effects of FeCl₃ on the vasculature. Our studies demonstrated that FeCl₃ alone
induces relatively mild injury to endothelial cells with severe vascular injury only observed in the presence of flowing blood. Whole blood fractionation studies revealed that FeCl₃-mediated vascular injury is dependent on erythrocyte hemolysis and hemoglobin oxidation, defining a unique mechanism of iron-induced vascular injury.

EXPERIMENTAL PROCEDURES

Materials—DiIC₁₈ fluorescent cell dye was purchased from Invitrogen. Sucrose gradient media, FeCl₂ and FeCl₃ solutions (60% (w/v) stock), hemin (protoporphyrin IX), Fe²⁺-EDTA, oxidized hemoglobin (methemoglobin (metHb)), purified haptoglobin, and the carbon monoxide donor tricarbonyldichlororuthenium(II) dimer were from Sigma. Goat anti-collagen (types I and III) polyclonal antibody and conjugated secondary antibody were purchased from EMD Chemicals. The thiobarbituric acid-reactive substance assay was from Cayman Chemicals. All other chemicals were purchased from Sigma-Aldrich.

In Vivo Thrombosis Model—All procedures involving mice were approved by the Alfred Medical Research and Education Precinct animal ethics committee. Male and female C57Bl/6 mice (20–30 g) were anesthetized by an intraperitoneal injection of sodium pentobarbitone (30 mg/kg), and body temperature was maintained at 37 °C by a thermoblanket (Harvard Apparatus, Hugstetten, Germany). A tracheotomy was performed, and mice were ventilated with room air via a respiratory pump (MiniVent Type 845; Harvard Apparatus). The aorta was exposed via a midline incision through the abdomen followed by retraction of intestines. The aorta was carefully dissected away from the inferior vena cava for a distance of 2–3 mm immediately inferior to the renal arteries. The exposed aorta was topically bathed in a 6% FeCl₃ solution for 10 min after which the mice were euthanized with sodium pentobarbitone (100 mg/kg intravenously). The aorta was removed and fixed in 4% paraformaldehyde for subsequent histological and scanning electron microscopy (SEM).

Isolation of Mouse Aortas—Male and female C57Bl/6 mice (20–30 g) were euthanized via inhalation of CO₂, and aortas were isolated and removed through a midline abdominal incision. Using a stereomicroscope, excess tissue and fat were removed from the outer surface of the aortas, which were placed in Krebs buffer on ice until experimentation (~1 h).

Preparation of Whole Blood and Isolated Blood Cell Fractions—Anticoagulated (hirudin-treated) whole blood was collected via the inferior vena cava. Murine blood was isolated and removed through a midline abdominal incision. Using a stereomicroscope, excess tissue and fat were removed from the outer surface of the aortas, which were placed in Krebs buffer on ice until experimentation (~1 h).

Histology—Mouse aortas were fixed with 4% paraformaldehyde for at least 48 h prior to alcohol and xyleno processing followed by paraffin embedding. Serial sections (5-mm thick) were cut and stained using Cartairs stain. Images were visualized using an Olympus BH2-RFCA microscope (Olympus) using ×20 and ×40 objectives.

Scanning Electron Microscopy—Aortas were cut open and glued onto coverslips with the lumen uppermost. Vessels were then incubated with 1% OSO₄ in 100 mM Na₂HPO₄, NaH₂PO₄, pH 7.4 for 30 min. The fixed vessels were dehydrated by successive immersions in increasing concentrations of ethanol fol-
**FeCl₃, Hemoglobin Oxidation, and Vascular Injury**

**FIGURE 1. FeCl₃-induced vascular injury and thrombus formation in vivo (A) and ex vivo (B–E).** A, the abdominal aorta was exposed and separated from the inferior vena cava for a distance of 2–3 mm immediately inferior to the renal arteries. FeCl₃ (6% w/v solution) was applied topically to the exposed aorta for 10 min. Mice were then culled, and the aorta was dissected and immediately fixed in 4% paraformaldehyde. The aortic lumen was exposed and prepared for SEM analysis (×300). B–F, for ex vivo analysis dissected aortas were mounted in a perfusion chamber and perfused with buffer or anticoagulated fluorescently labeled blood preparations (0.12 ml/min). B, represents SEM (×300) of aorta perfused with whole blood while exposed to topical FeCl₃. C, SEM (×300, left image; ×2500, middle image) and confocal microscopy (collagen, ×40; right image) of the luminal surface of aorta after exposure to FeCl₃ in the absence of blood. D, fluorescence microscopy (left), SEM (×2500), and confocal images (collagen, ×40) of the luminal surface of aorta after exposure to FeCl₃ during blood flow. E, fluorescence microscopy (left), SEM, and confocal images (collagen) of the luminal surface of aorta after exposure to FeCl₃ and washout prior to blood flow. F, fluorescence microscopy (left), SEM, and confocal images (collagen) of the luminal surface of aorta with resting blood flow (no treatment). Note that all fluorescence microscopy images are after 5 min of perfusion and are included as supplemental videos. Each of the images presented is representative of n = 6 experiments.

Thrombosis—To gain insight into the mechanism(s) by which FeCl₃ induces vascular injury and in particular whether the effects are principally due to direct or indirect effects on the endothelium, we designed a novel ex vivo vessel chamber that enabled direct analysis of the effects of FeCl₃ on the vasculature. In initial studies, we compared the vascular injury and thrombus response in isolated mouse aortic preparations treated with FeCl₃ (ex vivo) with those occurring in intact aorta in vivo. As demonstrated in Fig. 1, topically exposing aorta in vivo (Fig. 1A) or ex vivo (Fig. 1B) to 6% FeCl₃ resulted in major vascular injury as evidenced by endothelial denudation, collagen exposure, and the subsequent formation of arterial thrombi (Fig. 1D and supplemental Video 1). Platelet thrombus formation in the ex vivo model occurred in the presence of hirudin (Fig. 1B); however, it was critically dependent on collagen activation of platelets as thrombus formation failed to develop using platelets deficient in the major collagen receptor GPVI/FcRγ-chain (FcRγ⁻/⁻ mice) (data not shown). Histological examination revealed that thrombi formed in vivo were rich in platelets, fibrin, and RBCs (supplemental Fig. 1) consistent with previous reports (25). Although thrombi formed in the ex vivo model were principally composed of platelets, a high proportion of RBCs were also present in these thrombi (supplemental Fig. 1), a
surprising finding given that RBC accumulation is typically fibrin-dependent (30). Oxidized iron can induce endothelial dysfunction directly through free radical generation and lipid peroxidation or indirectly through oxidative modification of LDL (3). To investigate the direct effects of FeCl₃ on vascular injury, FeCl₃ was perfused through isolated aorta independently of flowing blood. Strikingly no endothelial denudation or collagen exposure was evident following prolonged exposure to FeCl₃ (6%) (Fig. 1C). Subsequent perfusion of anticoagulated whole blood through FeCl₃-pretreated vessels was associated with an increase in leukocyte and platelet adhesion to the vessel wall (Fig. 1E and supplemental Video 2); however, relative to untreated aorta (Fig. 1F and supplemental Video 3) and treatment in the presence of flowing blood (Fig. 1D and supplemental Video 1), thrombus formation was not observed. These studies indicate that although FeCl₃ can perturb endothelial function, leading to an increase in platelet and leukocyte adhesion, in isolation it does not induce severe vascular injury or significant thrombus formation.

Ferric Chloride-mediated Vascular Injury Is Erythrocyte-dependent—To investigate whether severe vascular injury was dependent on oxidative modification of plasma (e.g. LDL) or cellular components, blood fractionation experiments were performed. Removal of either platelet, leukocyte, or plasma components from whole blood did not prevent severe vascular injury in response to FeCl₃ (data not shown). In contrast, selectively removing RBCs prevented endothelial denudation and collagen exposure, resulting in a relatively mild perturbation of endothelial function similar to that observed with FeCl₃ alone (data not shown). To investigate whether FeCl₃ induced RBC hemolysis, total extracellular hemoglobin (Hb) levels were examined. Treatment of whole blood with FeCl₃ resulted in a dose-dependent increase in Hb levels with up to 200 μg/ml Hb released at 0.25% FeCl₃ (p < 0.05; n = 6) (Fig. 2A). Analysis of higher concentrations of FeCl₃ was not possible because
FeCl₃, Hemoglobin Oxidation, and Vascular Injury

FeCl₃, Hemoglobin Oxidation, and Vascular Injury

marked precipitating effects on plasma proteins. Analysis of Hb release from ex vivo vessel chamber experiments revealed a significant (p < 0.05; n = 4) increase in Hb levels following FeCl₃ treatment of isolated aorta in the presence of flowing blood (157 ± 45 μg/ml), whereas FeCl₃ pretreatment of vessels prior to blood perfusion caused no hemolysis (Fig. 2A). Analysis of the time course of hemolysis in whole blood revealed a rapid linear increase in Hb levels, peaking 10 min after FeCl₃ addition (Fig. 2B), a time course consistent with the rapid hemolysis and vascular injury observed in the ex vivo aortic thrombosis model. Further evidence that hemolysis was likely to be directly relevant to FeCl₃-induced vascular injury was obtained from SEM analysis of both in vivo and ex vivo aortic models, which demonstrated marked RBC fragmentation at sites of major injury (Fig. 2C, SEM insets).

To investigate whether iron-induced RBC hemolysis occurred directly or required the presence of plasma proteins, the effects of FeCl₃ were examined on isolated washed RBCs. As demonstrated in Fig. 2D, washed RBCs were highly sensitive to the hemolytic effects of FeCl₃ with 3-fold higher levels of Hb released by 0.25% FeCl₃ relative to whole blood (Fig. 2A). Haptoglobin, the major Hb-binding protein in whole blood, modulated the sensitivity of RBCs to iron-induced hemolysis as addition of purified haptoglobin to washed RBCs markedly reduced RBC hemolysis (Fig. 2D). To investigate whether oxidative injury was important for hemolysis, whole blood was treated with the superoxide dismutase mimetic tempol or the hydrogen peroxide-metabolizing enzyme catalase. As demonstrated in Fig. 2E, both tempol and catalase reduced FeCl₃-induced RBC hemolysis by ~50%. The hemolytic effects of iron on RBCs appeared to be dependent on its catalytic activity as catalytically active FeCl₃ also induced hemolysis, albeit slightly less potently than FeCl₃ (Fig. 2E), whereas Fe³⁺ (ferric ions) complexed to EDTA was less effective.

Lipid peroxidation is a reported mechanism of iron-induced membrane damage (31, 32). To investigate whether there was a correlation between FeCl₃- and FeCl₂-induced RBC lipid peroxidation and hemolysis; dose-response studies were performed. FeCl₃ treatment resulted in a dose- (Fig. 3) and time-dependent (data not shown) increase in RBC lipid peroxidation, which correlated closely with hemolysis (Fig. 2B). This increase in lipid peroxidation was dependent on catalytically active iron as it was induced by FeCl₃ with a potency similar to hemolysis and was markedly reduced by EDTA (Fig. 3).

Ferric Chloride Oxidation of Hemoglobin Is Associated with Severe Vascular Injury—Oxidative modification of Hb produces metHb and a variety of other oxidizing molecules, including heme (2). In all experiments, ≥99% of Hb used was in an oxidized form (metHb) as measured by a blood gas analyzer. Therefore we examined the direct effects of metHb on the vasculature in our ex vivo arterial perfusion model. Perfusion of metHb alone did not induce significant vascular injury, whereas the heme substitute hemin induced mild endothelium denudation associated with a low level of collagen exposure and platelet aggregate formation on the perturbed endothelium (Fig. 4). Topical treatment with 6% FeCl₃ with hemin (1 mM) perfusions caused no greater endothelial injury than FeCl₃ or hemin (1 mM) alone (data not shown), whereas FeCl₃ in the presence of low concentrations of metHb (0.38 mg/ml) induced extensive vascular injury similar to that observed with FeCl₃ in the presence of whole blood (Figs. 4 and 1D, respectively). Notably the concentrations of heme (heme) and metHb used in these studies were comparable to the levels generated by FeCl₃ treatment of vessel preparations perfused with whole blood (data not shown). In control studies we confirmed that isolated red cell membranes, either alone or in combination with FeCl₃, did not cause a greater level of vascular injury than did FeCl₃ alone (data not shown), confirming that Hb release and subsequent oxidation are likely to be the predominant mechanism underlying FeCl₃-induced vascular injury.

Vascular Protective Effects of Antioxidants—By-products of heme and iron metabolism, such as heme oxygenase-dependant generation of carbon monoxide (CO), have antioxidant and
anti-inflammatory protective functions against iron-induced vascular damage (33); therefore we examined the impact of CO donors on collagen exposure and thrombus formation in the ex vivo vascular injury model. Pretreating anticoagulated whole blood with tricarbonyldichlororuthenium(II) dimer, a CO donor, significantly reduced collagen exposure (Fig. 5A) and thrombus formation (Fig. 5B) induced by FeCl₃. A similar protective effect was observed when carbon monoxide gas was bubbled directly into whole blood prior to vessel perfusion (data not shown). Furthermore preincubating whole blood with either catalase or tempol prior to perfusion led to a significant reduction in collagen exposure and thrombus formation (Fig. 5, A and B, respectively), confirming a major role for oxidative stress in FeCl₃-induced vascular injury and thrombus formation.

**DISCUSSION**

The studies presented here have defined a key role for RBC hemolysis and Hb oxidation in promoting iron-induced vascular injury and thrombosis. Moreover they have demonstrated that released Hb plays an important role in exacerbating RBC hemolysis, establishing a damaging hemolysis/oxidative cycle that drives further red cell damage, vascular injury, and thrombosis. Several lines of evidence support a major role for iron-induced RBC hemolysis and hemoglobin oxidation in promoting severe vascular injury and thrombosis. First, topical application of FeCl₃ to isolated arteries prior to the exposure of flowing blood promoted only minor perturbation of the endothelial surface and minimal thrombus growth. Second, removal of the RBC component of blood but not all other elements prevented severe vascular injury in response to FeCl₃. Third, reintroduction of washed RBCs or purified metHb in the presence of FeCl₃ led to a similar level of vascular injury as observed with whole blood, whereas isolated RBC membranes and heme, even in the presence of FeCl₃, produced relatively mild injury. Finally the time course for FeCl₃-induced lipid peroxidation and RBC hemolysis was consistent with the rapid development of vascular lesions and thrombi in vivo and ex vivo. This combined with the scanning electron microscopy studies demonstrating prominent RBC fragmentation at the site of injury provides strong evidence that hemolysis is a key feature of FeCl₃-induced vascular damage. Overall these studies demonstrate a major role for Hb-derived oxidative products in iron-dependent vascular injury and thrombosis and raise the possibility that similar oxidative processes may contribute to
the vasculopathy and thrombotic complications associated with severe hemolytic diseases.

There is growing evidence for an important role for redox-active iron and heme-containing moieties in promoting endothelial dysfunction and vascular diseases with current evidence supporting a major role for free radical generation and LDL oxidation in this process (3). The studies presented here demonstrate that topically applied FeCl₃ alone induces relative mild endothelial injury (1), leading to leukocyte and platelet adhesion to the vessel wall (2) independent of subendothelial collagen exposure and thrombus formation. Subsequent Fe³⁺ transport to the vascular lumen causes lipid peroxidation of red blood cell membranes, inducing limited hemolysis (3). Oxidative modification of released Hb by iron generates H₂O₂, ROS, and protein radicals and leads to the release of lipophilic heme (4). These redox-active molecules are likely to markedly increase RBC hemolysis, establishing a potential hazardous hemolysis/Hb oxidation cycle (5). Iron-dependent hemolysis can be limited by sequestering oxidized Hb with haptoglobin and by neutralizing ROS and H₂O₂ with tempol and catalase, respectively. Excessive production of Hb-derived oxidation products plays a major role in inducing severe vascular injury, collagen exposure, and thrombus development (6). Natural antioxidants, such as HO-1-derived CO and superoxide dismutase, are likely to play an important role in limiting the toxic effect of iron/Hb oxidation products on the vasculature. +ve, positive.

Our demonstration that severe hemolysis, vascular injury, and thrombosis are only induced when Fe³⁺ was combined with isolated RBCs or metHb and are not induced by Fe³⁺, heme, and metHb in isolation suggests an important role for oxidized by-products of Hb in inducing cell damage. Under normal physiological conditions, free Hb in the circulation is spontaneously oxidized to metHb or heme (5, 34). Although metHb is capable of activating endothelial cells by stimulating IL-6, IL-8, and E-selectin (35), we demonstrated that in the absence of free Fe³⁺, it does not cause severe vascular injury or thrombosis. Similarly heme generation per se does not appear to be sufficient to induce severe vascular injury independently of RBC hemolysis and Hb oxidation.
of oxidized Hb. Free heme is lipophilic and intercalates into the membrane of endothelial cells increasing vascular permeability and ICAM-1, VCAM-1, and E-selectin expression (19). As a consequence, infused heme (hemin) has been demonstrated to enhance leukocyte adhesion to the vessel wall (17, 18), a finding confirmed in the current study. In addition to its direct effect on cells, heme also induces cell damage indirectly through oxidative modification of LDL (36). It is possible that the toxic effects of Fe^{3+} and free Hb are partially due to the formation of reactive oxygen species as a direct effect of Hb oxidation. For example, ROS generation from Hb auto-oxidation is known to produce cellular damage (37). Furthermore superoxide can react with Hb to produce hydrogen peroxide (H_2O_2), a well-defined pro-oxidative molecule that causes vascular injury (1). The demonstration that tempol and catalase reduce hemolysis, vascular injury, and thrombosis in response to FeCl_3 is consistent with an important role for ROS and H_2O_2 in this process. H_2O_2 can further react with Hb to generate Fe^{4+} and protein radicals (38). Fe^{4+} is a potent oxidant capable of damaging a broad range of lipid, amino acid, and nucleic acid substrates, and globin radicals can produce inter- and intramolecular cross-linkages between heme and amino acids (39). Thus a multitude of redox-active processes linked to Hb oxidation are likely to contribute to the vascular injury and thrombotic response induced by FeCl_3.

There is growing recognition of the clinical importance of intravascular hemolysis and cell-free Hb in the pathogenesis of a broad range of diseases (5). First, in general, the damaging effects of iron, Hb, and heme on the vasculature occur under situations in which the in vivo protective mechanisms against these molecules are overwhelmed or inadequate. This would include situations of severe vascular hemolysis, such as sickle cell disease, paroxysmal nocturnal hemoglobinuria, severe thalassemias, and hereditary spherocytosis, whereby elevated levels of cell-free Hb overwhelm the normal clearance processes, leading to Hb oxidation, vascular injury, and thrombosis, hallmark features of severe hemolytic syndromes (7–10). Second, the infusion of hemoglobin blood substitutes, whereby chemically modified or genetically engineered Hb molecules are infused at levels that greatly exceed the body’s capacity to neutralize free Hb, results in oxidative injury, vascular dysfunction, and thrombotic events in ischemic patients (11–13). Third, clinical situations associated with the accumulation of RBCs outside the vessel lumen, including intracerebral hemorrhage, RBC extravasation at sites of venous insufficiency, and RBC accumulation in atherosclerotic plaques, lead to increased oxidative stress and tissue injury (14, 40). For example, in hemorrhagic stroke iron-dependent oxidative processes exacerbate neural injury (41), whereas in atherosclerotic plaques, Hb oxidative products accelerate lesion progression (3, 42). Finally, redox-active damage by released Hb is likely to be magnified by deficiency of one or more components of the Hb scavenging system, such as haptoglobin, hemopexin, or heme oxygenase-1 (HO-1). Clinical studies have confirmed endothelial dysfunction and vasculopathy in a patient with HO-1 deficiency, and similarly, mice lacking HO-1 have increased vascular injury and thrombotic complications (43, 44). Furthermore mice lacking both haptoglobin and hemopexin have increased cell-free Hb and heme, leading to tissue inflammation (45).

The findings presented here shed new insight into the underlying mechanisms of FeCl_3-induced thrombus formation. The topical application of FeCl_3, to vessels in the microcirculation or larger arteries is one of the most common experimental models used to examine thrombus development (22). It is widely assumed that the effects of FeCl_3 are primarily localized to the endothelium with uptake of iron through endothelial pinocytic processes (25, 27). Consistent with this are studies demonstrating Fe^{4+}-rich membrane-enclosed particles transmigrating into the endothelium and exocytosed into the lumen. It is assumed that this accumulation of iron produces endothelial toxicity and denudation, leading to the exposure of subendothelial elements that promote thrombus formation (27). We demonstrated that iron chelation by EDTA prevents oxidative injury to RBC membranes presumably because of the increased hydrophilic nature of the iron-EDTA complex, reducing solubility of the iron chelate in the cell membrane. Although direct toxic effects of iron on the vasculature undoubtedly contribute to the thrombotic response, RBC hemolysis and Hb oxidation also appear to play a major role. One of the major deleterious effects of plasma Hb on the vasculature is the sequestration of endothelially derived NO (2). Cell-free Hb binds rapidly and irreversibly to nitric oxide, abolishing its ability to induce vasodilation and inhibit platelet activation (9). RBCs are also a rich source of nucleotides, including ADP and ATP, which play a major role in promoting platelet activation and thrombus growth (46). Our demonstration that hemolyzed RBCs are prominent in the platelet-rich thrombi of FeCl_3-treated vessels raises the possibility that RBC-derived nucleotides play a significant role in enhancing thrombus development. Finally redox-active molecules, including those derived from Hb oxidation (ROS and H_2O_2), can potentiate platelet and leukocyte activation and enhance thrombus formation (47). Although it should be emphasized that the findings presented here have only addressed the effects of FeCl_3 on vessel injury in macrovessels, they nonetheless indicate that the effects of redox-active iron on thrombus formation are likely to be considerably more complex than previously thought.

Finally the demonstration that antioxidants have a substantial protective effect against iron-induced vascular damage raises the possibility that antioxidant strategies preventing localized iron/Hb-dependent oxidative injury may ultimately have significant therapeutic benefit. The demonstration that CO donors significantly reduce vascular injury and thrombosis in response to FeCl_3 is consistent with its known antioxidant, antiapoptotic, and anti-inflammatory properties (33). Physiologically CO generation from heme metabolism by HO-1 plays an important role in the vascular protective effects of HO-1 (33). It is tempting to speculate that antioxidant therapy in combination with strategies that enhance the vascular protective effects of HO-1 may be highly effective at reducing vascular complications associated with localized iron-dependent Hb oxidation.

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FeCl₃, Hemoglobin Oxidation, and Vascular Injury