

# Erythrocyte Hemolysis and Hemoglobin Oxidation Promote Ferric Chloride-induced Vascular Injury

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## Abstract

The release of redox-active iron and heme into the blood-stream is toxic to the vasculature, contributing to the development of vascular diseases. How iron induces endothelial injury remains ill defined. To investigate this, we developed a novel ex vivo perfusion chamber that enables direct analysis of the effects of FeCl<sub>3</sub> on the vasculature. We demonstrate that FeCl<sub>3</sub> treatment of isolated mouse aorta, perfused with whole blood, was associated with endothelial denudation, collagen exposure, and occlusive thrombus formation. Strikingly exposing vessels to FeCl<sub>3</sub> alone, in the absence of perfused blood, was associated with only minor vascular injury. Whole blood fractionation studies revealed that FeCl<sub>3</sub>-induced vascular injury was red blood cell (erythrocyte)-dependent, requiring erythrocyte hemolysis and hemoglobin oxidation for endothelial denudation. Overall these studies define a unique mechanism of Fe<sup>3+</sup>-induced vascular injury that has implications for the understanding of FeCl<sub>3</sub>-dependent models of thrombosis and vascular dysfunction associated with severe intravascular hemolysis.

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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 (FeCl<sub>3</sub>) widely used to induce vascular injury and thrombosis in experimental animal models (22). High concentrations of FeCl<sub>3</sub> 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 FeCl<sub>3</sub>-induced thrombi are rich in platelets, fibrin, and red blood cells (23-26). However, the mechanism(s) by which FeCl<sub>3</sub> induces vascular injury has not been clearly defined. FeCl<sub>3</sub> 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 LDL3 (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 FeCl<sub>3</sub> 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 FeCl<sub>3</sub> on the vasculature. Our studies demonstrated that FeCl<sub>3</sub> 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 FeCl<sub>3</sub>-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, FeCl<sub>2</sub> and FeCl<sub>3</sub> solutions (60% (w/v) stock), hemin (protoporphyrin IX), Fe<sup>3+</sup>-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% FeCl<sub>3</sub> 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 CO<sub>2</sub>, 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 from healthy volunteers who had given informed consent with the approval of the Monash University Human Ethics Committee. Murine blood was collected via the inferior vena cava. Platelets, leukocytes, and red blood cells were isolated and prepared according to published protocols (28). Plasma was prepared by centrifugation (1500 × g) for 15 min at 37 °C. Each isolated blood cell component was reconstituted back to its original whole blood concentration, measured using an automated blood cell counter (Sysmex, Roche Applied Science) prior to perfusion through isolated vessel segments. In all studies whole blood/isolated cell preparations were maintained at 37 °C.

**Ex Vivo Vascular Injury and Thrombosis Model**—Isolated aortas were mounted on a vessel chamber primed with Krebs buffer and maintained at physiological pH by infusing carbogen gas through the buffer at 37 °C. Anticoagulated human whole blood or reconstituted blood cell preparations were labeled with the membrane dye DiI<sub>C18</sub> (1:1000). Blood cell preparations, metHb (0.38 mg/ml), or hemin (1 mM) were perfused through aortas at 0.12 ml/min using a Harvard syringe pump (Harvard Apparatus). In background studies we confirmed that this perfusion rate was optimal for efficient leukocyte-platelet and leukocyte-endothelial cell interactions. Moreover we noted no major differences in cell adhesion to the vasculature when using whole blood from different human donors or between human and mouse blood preparations as described previously (29). Images and videos of vessel wall-blood cell interactions were observed using a Olympus BX51 fluorescence microscope fitted with a 10× long working distance lens (Olympus, Tokyo, Japan) and analyzed using a Hamamatsu camera coupled to Image ProPlus software (MediaCybernetics). Results were calculated as the mean ± S.D. and expressed as fluorescence intensity. Vascular injury was induced by removing the Krebs buffer surrounding the aorta and topically bathing the vessel in 6% FeCl<sub>3</sub> or 6% FeCl<sub>2</sub> for 10 min either before perfusion (with washout two times with Krebs buffer) or during perfusion.

FeCl<sub>2</sub> was made up to 6% (w/v) in saline. Saline was bubbled with nitrogen for 15 min prior to addition of FeCl<sub>2</sub>. The FeCl<sub>2</sub> solution was then subsequently bubbled with nitrogen for 30 min prior to all treatments to prevent the solution from autooxidizing. In some experiments the carbon monoxide donor tricarbonyldichlororuthenium(II) dimer (10 μm) was added to whole blood, or carbon monoxide gas was bubbled directly into whole blood prior to perfusion in the presence of FeCl<sub>3</sub>. The antioxidants tempol (1 mM) or catalase (5000 units/ml) were incubated with whole blood for 10 min prior to perfusion through isolated aortas. Blood components were further isolated before ex vivo perfusion, including red blood cells (RBCs) (see "Plasma Hemoglobin and Lipid Peroxidation Measurements"), plasma, leukocytes, and platelets via density centrifugation. Resuspended RBC ghosts (membranes) were prepared via RBC hemolysis with hypotonic cold water solution, and membranes were isolated via ultracentrifugation (14,000 rpm for 10 min) and perfused through aorta as describe above. At the conclusion of the experiments, aortas were fixed in 4% paraformaldehyde for subsequent analysis by SEM or immunofluorescence as described below. Endothelial cells were intact and viable in isolated aortic preparations as assessed by PECAM-1 staining by immunohistochemistry and nitric oxide (NO)-dependent endothelial relaxation as described previously (29).

**Histology**—Mouse aortas were fixed with 4% paraformaldehyde for at least 48 h prior to alcohol and xylene 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% OSO<sub>4</sub> in 100 mM Na<sub>2</sub>HPO<sub>4</sub>, NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4 for 30 min. The fixed vessels were dehydrated by successive immersions in increasing concentrations of ethanol followed by drying in increasing concentrations of hexamethyldisilazine in ethanol. The coverslips on which the dehydrated vessels were attached were mounted on SEM stubs and coated with gold prior to using a Hitachi 5570 scanning electron microscope. Images were acquired at ×300 and ×2500 magnification.

**Immunofluorescence Studies**—Collagen exposure on the luminal surface of aorta was examined by incubating open vessel segments with a Texas Red-conjugated anti-collagen polyclonal antibody (20 min at 37 °C) (10 μg/ml), then washed two times in Krebs buffer, fixed in 4% paraformaldehyde, and mounted onto coverslips for confocal analysis. Each vessel was visualized using a Leica SP5 confocal microscope using a 40× long working distance lens and laser/lens set on an excitation of 535 nm and emission of 585 nm. A conjugated isotype-matched antibody (10 μg/ml) was used as a negative control.

**Plasma Hemoglobin and Lipid Peroxidation Measurements**—Hirudin-treated whole blood was collected as described above, and RBCs were isolated by centrifugation at 200 × g for 30 min. Platelet-rich plasma was removed and layered for density centrifugation for removal of leukocytes. Isolated RBCs were washed three times with washing buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM glucose). Treatment of whole blood or washed RBCs with FeCl<sub>3</sub>, FeCl<sub>2</sub>, and FeCl<sub>3</sub>-EDTA used in concentrations ranging from 0 to 0.25% was performed for the indicated time points (0-15 min). Incubations with tempol (1 mM), catalase (5000 units/ml), or haptoglobin (25 mg/dl) were performed for 10 min before induction of hemolysis. Whole blood or washed RBC preparations were

centrifuged at 1500 × g for 15 min at 4 °C, and the plasma or supernatant was collected. Total hemoglobin levels in plasma or supernatant samples were measured using a commercially available enzyme-linked immunosorbent assay (Bethyl Laboratories, Inc.) according to the manufacturer's instructions. Lipid peroxidation levels in each sample were assessed by measuring thiobarbituric acid-reactive substances according to the manufacturer's instructions (Cayman).

Statistics—Values are presented as the mean ± S.D. Grouped data were compared using a one-way analysis of variance followed by Tukey's post hoc test. Significance was accepted at  $p < 0.05$ . All data are a minimum of  $n = 6$ , including representative images shown.

## RESULTS

**Effect of Ferric Chloride on Vascular Injury and Arterial Thrombosis**—To gain insight into the mechanism(s) by which FeCl<sub>3</sub> 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 FeCl<sub>3</sub> on the vasculature. In initial studies, we compared the vascular injury and thrombosis response in isolated mouse aortic preparations treated with FeCl<sub>3</sub> (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% FeCl<sub>3</sub> 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/FcR  $\gamma$ -chain (FcR  $\gamma$ -/- 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 FeCl<sub>3</sub> on aortic endothelium, FeCl<sub>3</sub> was perfused through isolated aorta independently of flowing blood. Strikingly no endothelial denudation or collagen exposure was evident following prolonged exposure to FeCl<sub>3</sub> (6%) (Fig. 1C). Subsequent perfusion of anticoagulated whole blood through FeCl<sub>3</sub>-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 FeCl<sub>3</sub> 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 FeCl<sub>3</sub> (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 FeCl<sub>3</sub> alone (data not shown). To investigate whether FeCl<sub>3</sub> induced RBC hemolysis, total extracellular hemoglobin (Hb) levels were examined. Treatment of whole blood with FeCl<sub>3</sub> resulted in a dose-dependent increase in Hb levels with up to 200 µg/ml Hb released at 0.25% FeCl<sub>3</sub> ( $p < 0.05$ ;  $n = 6$ ) (Fig. 2A). Analysis of higher concentrations of FeCl<sub>3</sub> was not possible because of 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 FeCl<sub>3</sub> treatment of isolated aorta in the presence of flowing blood ( $157 \pm 45$  µg/ml), whereas FeCl<sub>3</sub> 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 FeCl<sub>3</sub> 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 FeCl<sub>3</sub>-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 FeCl<sub>3</sub> were examined on isolated washed RBCs. As demonstrated in Fig. 2D, washed RBCs were highly sensitive to the hemolytic effects of FeCl<sub>3</sub> with 3-fold higher levels of Hb released by 0.25% FeCl<sub>3</sub> 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 FeCl<sub>3</sub>-induced RBC hemolysis by ~50%. The hemolytic effects of iron on RBCs appeared to be dependent on its catalytic activity as catalytically active FeCl<sub>2</sub> also induced hemolysis, albeit slightly less potently than FeCl<sub>3</sub> (Fig. 2E), whereas Fe<sup>3+</sup> (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 FeCl<sub>3</sub>- and FeCl<sub>2</sub>-induced RBC lipid peroxidation and hemolysis; dose-response studies were performed. FeCl<sub>3</sub> 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 FeCl<sub>2</sub> 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,  $\geq 99\%$  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% FeCl<sub>3</sub> with hemin (1 mM) perfusions caused no greater endothelial injury than FeCl<sub>3</sub> or hemin (1 mM) alone (data not shown), whereas FeCl<sub>3</sub> in the presence of low concentrations of metHb (0.38 mg/ml) induced extensive vascular injury similar to that observed with FeCl<sub>3</sub> in the presence of whole blood (Figs. 4A and 1D, respectively). Notably the concentrations of hemin (heme) and metHb used in these studies were comparable to the levels generated by FeCl<sub>3</sub> 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 FeCl<sub>3</sub>, did not cause a greater level of vascular injury than did FeCl<sub>3</sub> alone (data not shown), confirming that Hb release and subsequent oxidation are likely to be the predominant mechanism underlying FeCl<sub>3</sub>-induced vascular injury.

**Vascular Protective Effects of Antioxidants**—By-products of heme and iron metabolism, such as heme oxygenase-dependent 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 FeCl<sub>3</sub>. 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 FeCl<sub>3</sub>-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 FeCl<sub>3</sub> 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 FeCl<sub>3</sub>. Third, reintroduction of washed RBCs or purified metHb in the presence of FeCl<sub>3</sub> led to a similar level of vascular injury as observed with whole blood, whereas isolated RBC membranes and heme, even in the presence of FeCl<sub>3</sub>, produced relatively mild injury. Finally the time course for FeCl<sub>3</sub>-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 FeCl<sub>3</sub>-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 redoxactive 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 these processes in isolation are unlikely to be sufficient to induce severe vascular injury and occlusive thrombus formation independent of RBC hemolysis and Hb oxidation. Our studies suggest a model in which iron-dependent free radical generation (the Fenton reaction) is sufficient to induce lipid peroxidation and mild hemolysis and endothelial perturbation; however, progressive red cell damage, vascular injury, and thrombus formation appears to be critically dependent on Hb oxidation, setting up a potentially hazardous hemolysis/oxidative cycle (see schematic in Fig. 6). As outlined below, iron-dependent Hb oxidation generates a number of redox-active molecules that have known adverse effects on the vasculature, perturbing endothelial, leukocyte, and platelet function and contributing to a proinflammatory and prothrombotic state (1, 2, 5).

Our demonstration that severe hemolysis, vascular injury, and thrombosis are only induced when  $\text{Fe}^{3+}$  was combined with isolated RBCs or metHb and are not induced by  $\text{Fe}^{3+}$ , 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  $\text{Fe}^{3+}$  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 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  $\text{Fe}^{3+}$  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 ( $\text{H}_2\text{O}_2$ ), 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  $\text{FeCl}_3$  is consistent with an important role for ROS and  $\text{H}_2\text{O}_2$  in this process.  $\text{H}_2\text{O}_2$  can further react with Hb to generate  $\text{Fe}^{4+}$  and protein radicals (38).  $\text{Fe}^{4+}$  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  $\text{FeCl}_3$ .

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<sub>3</sub>-induced thrombus formation. The topical application of FeCl<sub>3</sub> 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<sub>3</sub> are primarily localized to the endothelium with uptake of iron through endothelial pinocytotic processes (25, 27). Consistent with this are studies demonstrating Fe<sup>3+</sup>-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<sub>3</sub>-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<sub>2</sub>O<sub>2</sub>), 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<sub>3</sub> 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<sub>3</sub> 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.

<sup>3</sup>The abbreviations used are: LDL, low density lipoprotein; DiIC18, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; metHb, methemoglobin; SEM, scanning electron microscopy; RBC, red blood cell; Hb, hemoglobin; HO-1, heme oxygenase-1; ROS, reactive oxygen species.

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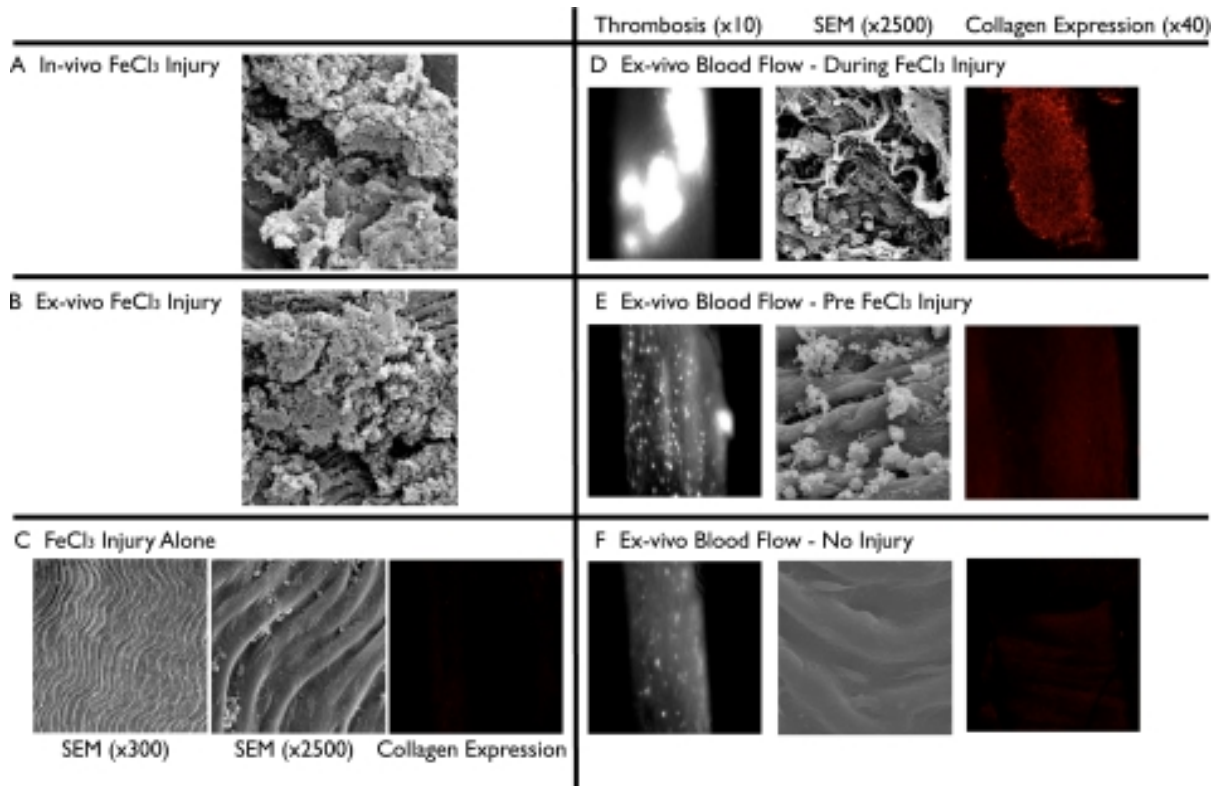
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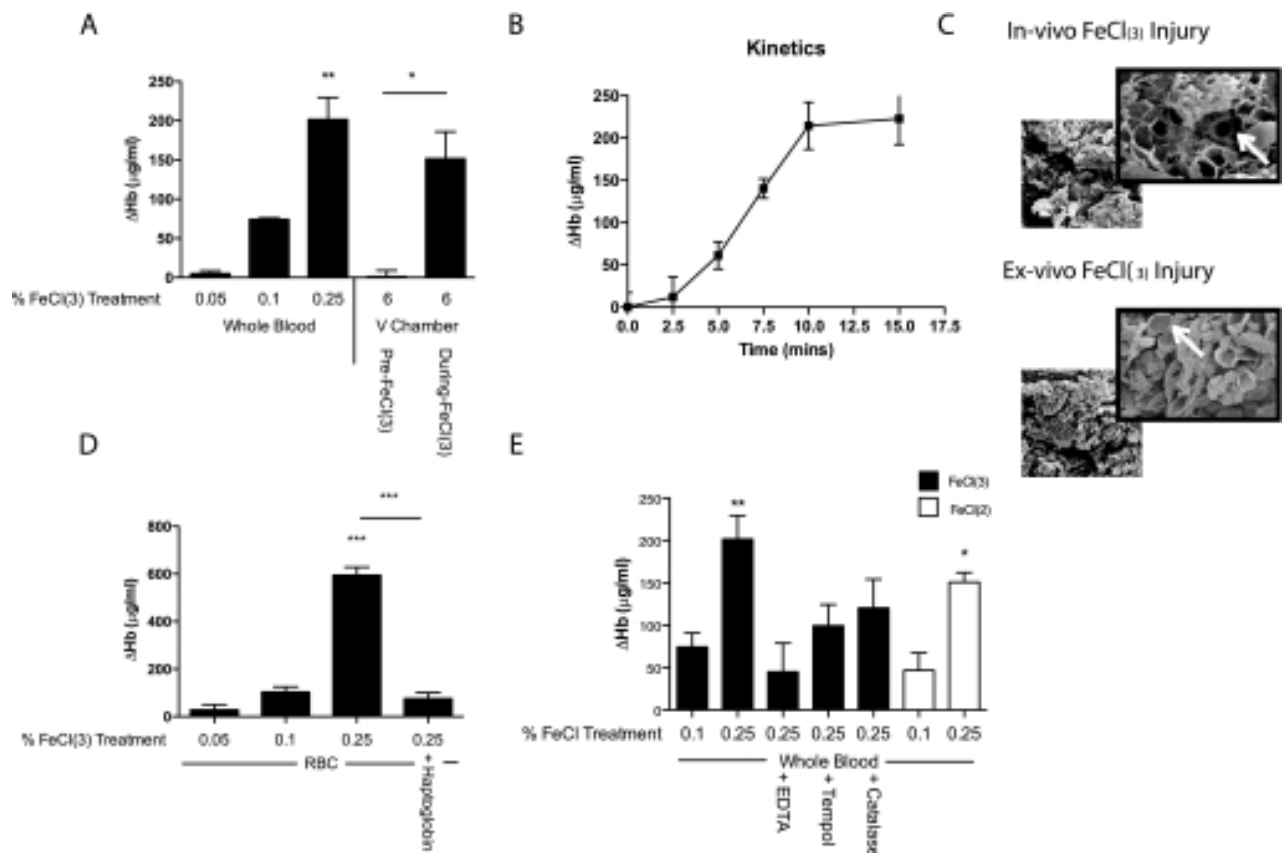
## Figures

FIGURE 1.



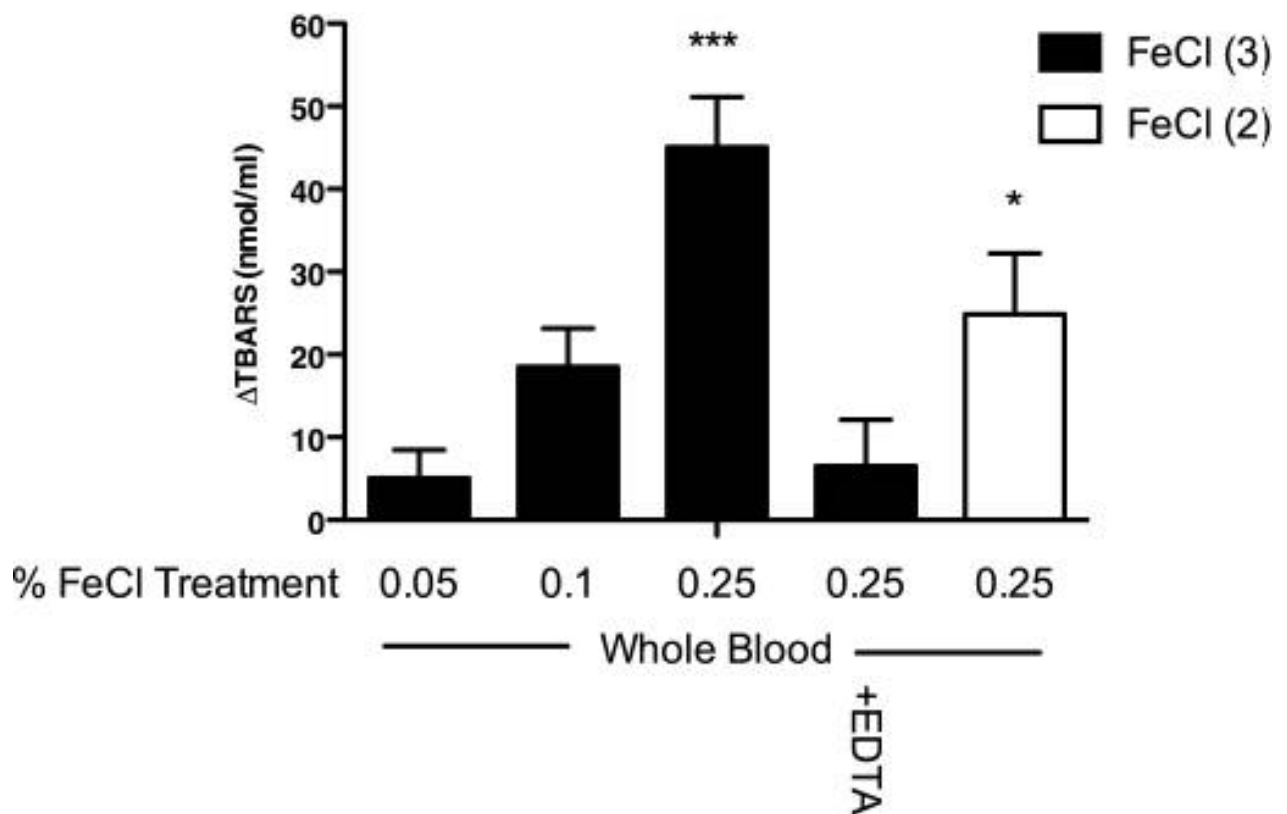
**FeCl<sub>3</sub>-induced vascular injury and thrombus formation *in vivo* (A) and *ex vivo* (B-E).** A, the abdominal aorta was exposed and separated from the inferior vena cava for a distance of 2-3 mm immediately inferior to the renal arteries. FeCl<sub>3</sub> (6% (w/v) solution) was applied topically 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 FeCl<sub>3</sub>. C, SEM (×300, *left image*; ×2500, *middle image*) and confocal microscopy (collagen, ×40; *right image*) of the luminal surface of aorta after exposure to FeCl<sub>3</sub> 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 FeCl<sub>3</sub> during blood flow. E, fluorescence microscopy (*left*), SEM, and confocal images (collagen) of the luminal surface of aorta after exposure to FeCl<sub>3</sub> 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  $n = \geq 6$  experiments.

FIGURE 2.



**Effect of FeCl<sub>3</sub> on erythrocyte hemolysis.** A, anticoagulated whole blood was treated with increasing amounts of FeCl<sub>3</sub> (0, 0.05, 0.1, and 0.25%) for 10 min at 37 °C. Intact RBCs were pelleted by centrifugation at 1500 × g for 10 min, and the cell-free plasma supernatant was removed for Hb quantitation. Total Hb levels were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions and expressed as μg/ml increases (Δ) in Hb above those present in the plasma supernatant of untreated whole blood samples. *Pre-FeCl* (*V Chamber*) refers to Hb levels in plasma samples from whole blood collected from the vessel chamber. Note that the vessel had been pretreated with 6% FeCl<sub>3</sub> (topically) for 10 min prior to blood perfusion. *During-FeCl* (*V Chamber*) refers to Hb levels in plasma from whole blood perfused through isolated vessels in the presence of topically applied FeCl<sub>3</sub>. B, kinetics of RBC hemolysis (0-15 min) following FeCl<sub>3</sub> (0.25%) addition to whole blood. C, SEM images of *in vivo* and *ex vivo* thrombosis models (×300 and ×3500, respectively) showing RBC hemolysis at the sites of vascular injury. *Arrows* highlight examples of hemolyzed RBCs. D, washed RBCs were treated with the indicated concentrations of FeCl<sub>3</sub> (0-0.25%) with or without haptoglobin (25 mg/dl). RBCs were pelleted, and Hb levels were measured in the supernatant and expressed as μg/ml increases (Δ) in Hb above those present in the supernatants of untreated washed RBCs. E, the indicated concentration of FeCl<sub>3</sub> or FeCl<sub>2</sub> (0-0.25%) was added to whole blood in the presence of control buffer, EDTA, tempol (1 mM), or catalase (5000units/ml). \*, \*\*, and \*\*\* represent  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ , respectively, relative to untreated controls or relative to indicated samples (*black line*) (analyzed by analysis of variance followed by Tukey's post hoc test). *Error bars* represent S.D.

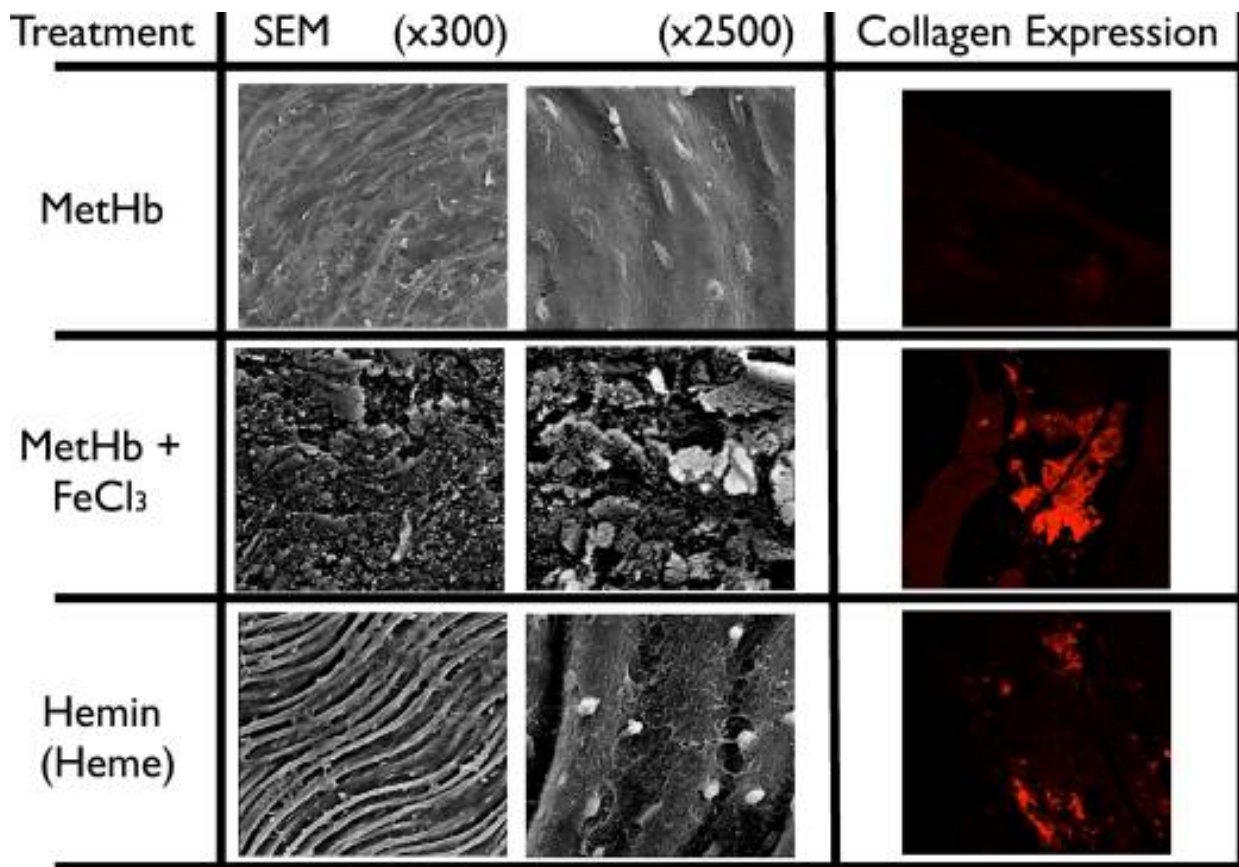
FIGURE 3.



**Lipid peroxidation of erythrocytes.** Anticoagulated (hirudin) whole blood was collected, and RBCs were washed as described under “Experimental Procedures.” RBCs were treated with the indicated concentrations of  $\text{FeCl}_3$  (0-0.25%),  $\text{FeCl}_2$  (0 and 0.25%), or 0.25%  $\text{FeCl}_3$  + EDTA for 10 min, and then lipid peroxidation levels in each sample were quantitated by measuring thiobarbituric acid-reactive substances (TBARS) according to the manufacturer's instructions (Cayman). Data are presented as the change ( $\Delta$ ) from control saline-treated cells. \* and \*\*\* represent  $p < 0.05$  and  $p < 0.001$ , respectively, from control treated samples (analyzed by analysis of variance followed by Tukey's post hoc test).

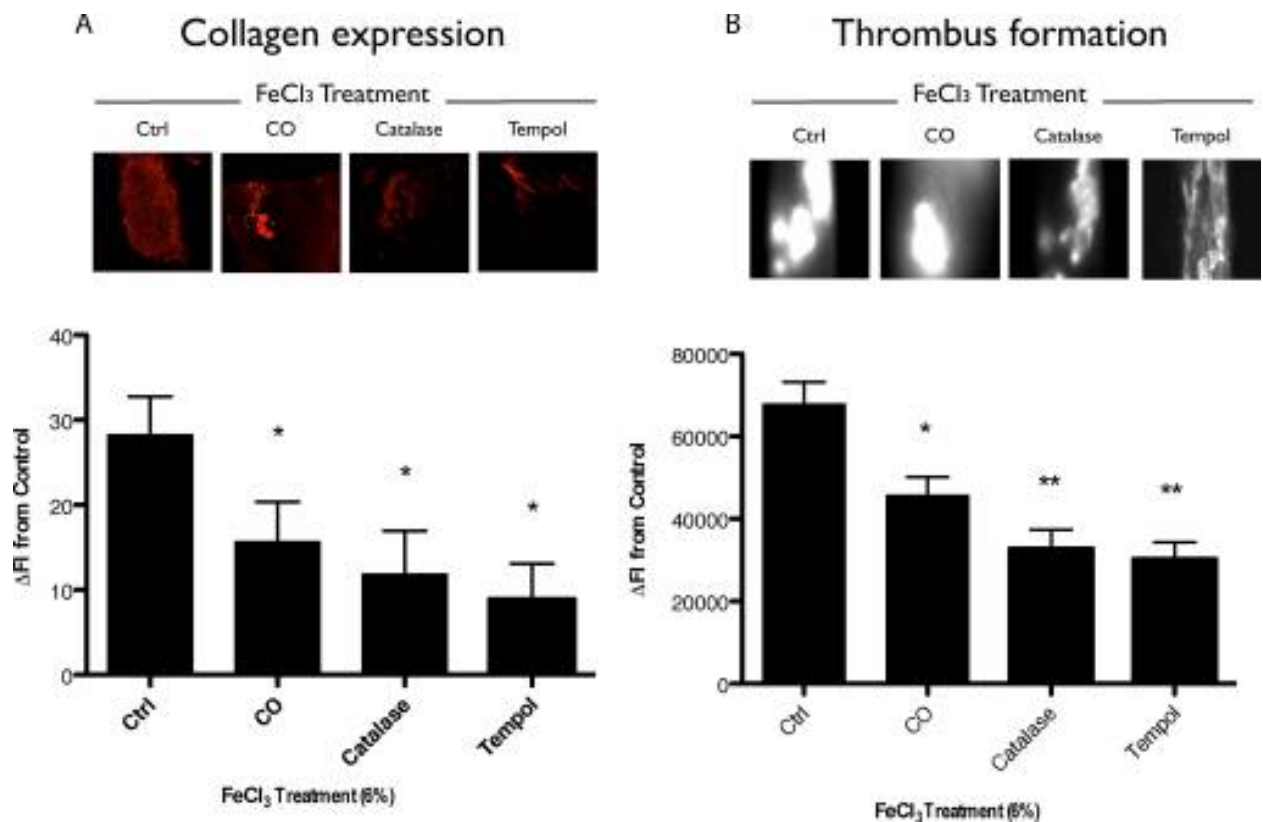


FIGURE 4.



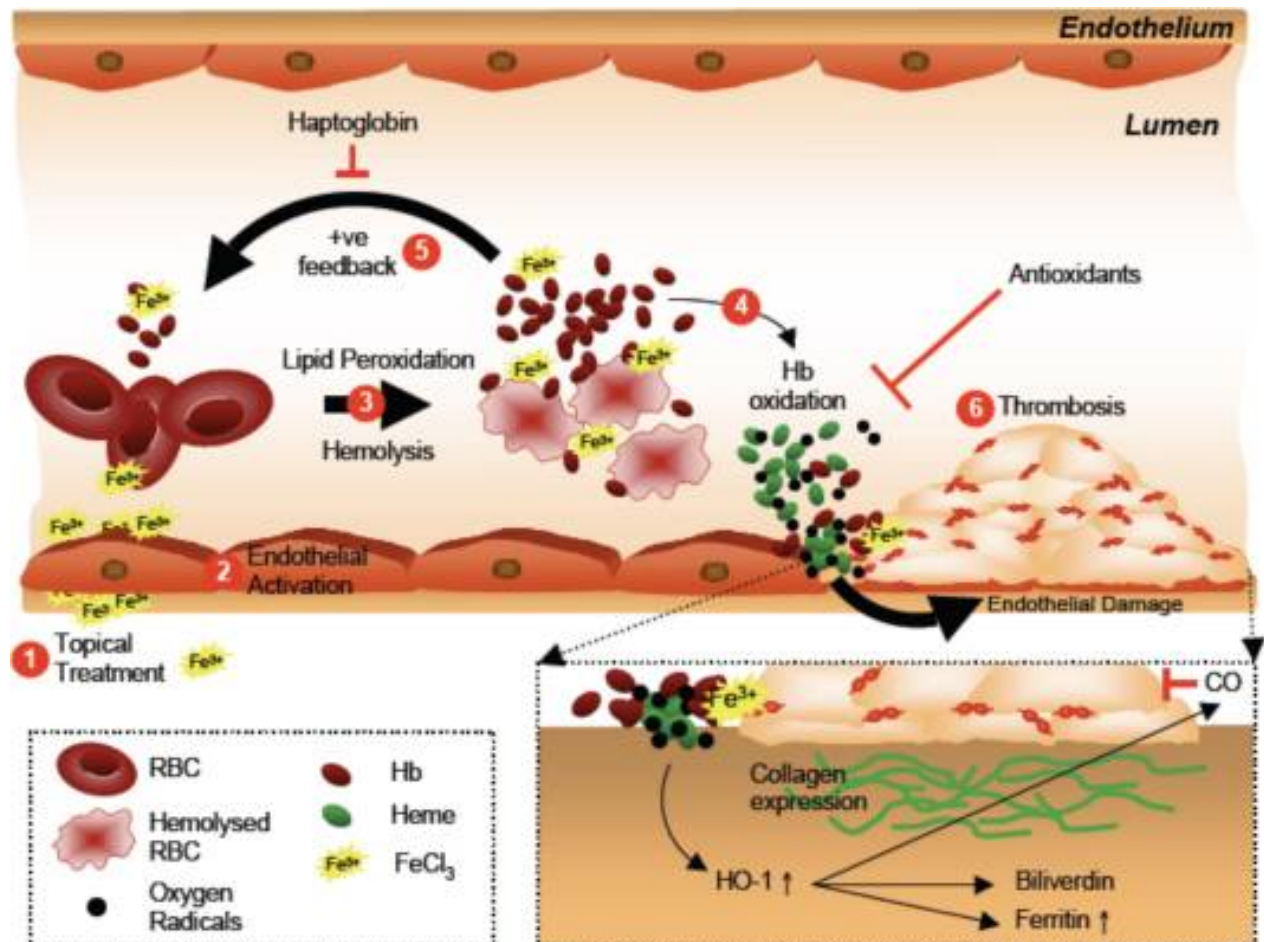
**The effects of metHb and hemin on vascular injury.** Aorta was dissected from euthanized mice and mounted *ex vivo* prior to perfusion with metHb (0.38 mg/ml), hemin (1 mm), or metHb (0.38 mg/ml) in the presence of 6% FeCl<sub>3</sub> for 10 min. All vessels were examined for vascular injury via SEM and for collagen expression by confocal microscopy as described in [Fig. 1](#). Each of the images presented is representative of those from  $n = \geq 6$  experiments.

FIGURE 5.



**Effect of CO donors, catalase, or tempol on FeCl<sub>3</sub>-induced vascular injury and thrombosis.** Aorta was dissected from euthanized mice and mounted *ex vivo* prior to perfusion with anticoagulated fluorescently labeled whole blood in the presence of topical FeCl<sub>3</sub> (6% for 10 min). In the indicated experiments, the carbon monoxide donor tricarbonyldichlororuthenium(II) dimer (10 μm)(CO), catalase (5000 units/ml), or tempol (1 mM) was added to whole blood prior to the application of FeCl<sub>3</sub>. Collagen expression (A) and thrombus formation (B) was analyzed by confocal microscopy and fluorescence microscope, respectively, as described under “Experimental Procedures.” Representative images are shown, and the fluorescence intensities (FI) ± S.D. of collagen expression (types I and III) and platelet accumulation after FeCl<sub>3</sub> treatment are shown. Data are presented as the change (Δ) in fluorescence intensity relative to saline-treated controls (Ctrl). \* and \*\* represent *p* < 0.05 and *p* < 0.01, respectively, relative to FeCl<sub>3</sub> treatment alone (analyzed by analysis of variance followed by Tukey's post hoc test; *n* = 6 experiments).

FIGURE 6.



**Schematic illustration of the  $FeCl_3$ -induced hemolysis/Hb oxidation cycle that induces further red cell damage, vascular injury, and thrombosis.** Our studies demonstrate that topically applied  $FeCl_3$  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  $Fe^{3+}$  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  $H_2O_2$ , 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  $H_2O_2$  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.