Chronic Iron Administration Increases Vascular Oxidative Stress and Accelerates Arterial Thrombosis

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Background—Iron overload has been implicated in the pathogenesis of ischemic cardiovascular events. However, the effects of iron excess on vascular function and the thrombotic response to vascular injury are not well understood.

Methods and Results—We examined the effects of chronic iron dextran administration (15 mg over 6 weeks) on thrombosis, systemic and vascular oxidative stress, and endothelium-dependent vascular reactivity in mice. Thrombus generation after photochemical carotid artery injury was accelerated in iron-loaded mice (mean time to occlusive thrombosis, 20.4±8.5 minutes; n=10) compared with control mice (54.5±35.5 minutes, n=10, P=0.009). Iron loading had no effect on plasma clotting, vessel wall tissue factor activity, or ADP-induced platelet aggregation. Acute administration of DL-cysteine, a reactive oxygen species scavenger, completely abrogated the effects of iron loading on thrombus formation, suggesting that iron accelerated thrombosis through a pro-oxidant mechanism. Iron loading enhanced both systemic and vascular reactive oxygen species production. Endothelium-dependent vasorelaxation was impaired in iron-loaded mice, indicating reduced NO bioavailability.

Conclusions—Moderate iron loading markedly accelerates thrombus formation after arterial injury, increases vascular oxidative stress, and impairs vasoreactivity. Iron-induced vascular dysfunction may contribute to the increased incidence of ischemic cardiovascular events that have been associated with chronic iron overload. (Circulation. 2003;107:2601-2606.)

Key Words: thrombosis • free radicals • arteries

Although iron is essential for many physiological processes, excess iron can lead to tissue damage by promoting the generation of reactive oxygen species (ROS) through the Fenton reaction.1 Primary iron overload occurs in patients with hereditary hemochromatosis, which is one of the most common recessive genetic disorders.2 Carriers of the hemochromatosis gene can have moderate iron overload compared with age-matched normal subjects3 and have higher serum free iron levels.4 Secondary tissue iron overload can result from frequent blood transfusions or parenteral iron infusions.5 Although controversial, several lines of evidence suggest that iron excess may predispose to vascular disease. Elevated body iron stores were associated with an increased risk of myocardial infarction in a large cohort of Finnish men.6 Carriers of the hemochromatosis gene appear to be at increased risk of myocardial infarction and cardiovascular death, an effect synergistic with other risk factors associated with increased oxidative stress, such as hypertension and smoking.7,8 Because iron is capable of supporting lipid peroxidation,9,10 acceleration of atherosclerosis development has been postulated as a potential mechanism by which iron overload may increase the risk of ischemic cardiovascular events. However, animal and human studies have not found a consistent effect of iron overload on atherogenesis.11–15

By promoting the generation of ROS, excess iron may predispose to ischemic cardiovascular events by mechanisms independent of atherosclerotic burden.16 Thrombotic arterial occlusion after plaque rupture, leading to unstable angina and myocardial infarction, constitutes the major cause of morbidity and mortality associated with coronary artery disease. Increased levels of oxidative stress have been demonstrated in patients with unstable angina,17 suggesting that ROS may contribute to plaque disruption and thrombosis.18 In vitro studies have shown that iron acutely promotes platelet reactivity19 and that iron chelation inhibits activation of tissue factor (TF).20 However, the effects of chronic iron overload on arterial thrombosis are unknown.

Iron may also have deleterious effects on vascular function. Locally enhanced vascular production of ROS decreases the bioavailability of nitric oxide (NO), impairing vasorelaxation and promoting platelet adhesion and aggregation.21 Therefore, a dynamic interaction exists between endothelial function and thrombosis, both of which may be influenced by oxidative stress. Yet, limited data are available on the effects
of chronic iron overload on ROS production within the arterial wall or on vascular function.

We conducted a series of animal experiments to test the hypotheses that iron overload contributes to the development of vascular disease by promoting thrombosis after arterial injury, by catalyzing ROS generation in the blood vessel wall, and by adversely affecting endothelium-dependent vasoreactivity. Our results support an important role of iron excess in thrombosis and vascular dysfunction that may help to explain the postulated association between elevated body iron stores and ischemic cardiovascular events.

Methods

Animals

Eight- to 10-week-old C57BL/6 male mice (Jackson Labs, Bar Harbor, Maine) were given 1.5 mg of iron dextran (Sigma Chemical Co) diluted in 0.9% NaCl by intraperitoneal injection every 3 to 5 days for a total of 10 doses over 6 weeks. Control animals received saline. Mice were fed a laboratory rodent diet (PMI Feeds Inc, Formula 5001) containing 198 ppm iron and distilled water. The University of Michigan Committee on Use and Care of Animals approved all experimental procedures.

Carotid Artery Thrombosis Model

Photochemical vascular injury was performed in an unblinded fashion as previously described. Where indicated, 100 mg/kg DL-cysteine hydrochloride (Sigma) in PBS or an equal volume of PBS alone was injected into the tail vein 5 minutes after initiation of injury. Occlusion time was defined as the interval from initiation of injury until a reduction in carotid blood flow to 0 mL/min persisting for ≥1 minute. The arterial vasculature was perfusion fixed with 10% formalin at the completion of the protocol. The carotid arteries and livers were paraffin-embedded, sectioned, and stained with hematoxylin and eosin or Prussian blue.

Coagulation and Platelet Function Assays

Blood hemoglobin concentration was measured with Drabkin’s reagent in a chromogenic assay (Sigma). Platelet counts were determined in whole blood with the use of an automated cell counter (Hemavet, CDC Technologies). An Amelung clotting apparatus (Hemavet, CDC Technologies) was used to measure the prothrombin time (PT) and the activated partial thromboplastin time (aPTT). TF activity in carotid artery homogenates was measured by a previously described clotting-based assay. Platelet aggregation was performed as described previously.

Vascular Studies

Aortic rings (4 mm) were suspended in individual organ chambers filled with PSS buffer (in mmol/L: NaCl, 99.0; KCl, 4.7; CaCl₂, 1.9; MgSO₄, 1.2; K₂HPO₄, 1.0; NaHCO₃, 25.0; and glucose, 11.1; pH 7.4) containing indomethacin (10 μmol/L), aerated continuously with 5% CO₂ in O₂ at 37°C. The vessels were preconstricted with gradual doses of l-phenylephrine (0.15 μmol/L) to reach a stable contraction plateau that approached 40% to 50% of peak tension generated with maximal dose KCl. The rings were then exposed to graded doses of acetylcholine (1 nmol/L to 1 μmol/L), followed by nitroglycerin (1 mmol/L to 10 μmol/L). Results were reported as percent relaxation for each concentration of agonist and as ED₅₀, that is, the agonist dose at which 50% of maximal relaxation was achieved. In situ detection of ROS formation in the vessel wall was assessed with the use of the oxidatively active fluorescent dye 2’,7’-dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes). Unfixed aortic ring segments were frozen in OCT and cut into 30-μm cross sections. DCFDA (5 μmol/L) was applied to each section for 5 minutes at room temperature. Before application of DCFDA, some cross sections were pretreated for 20 minutes at 37°C with N-2-mercaptoethanol (MPG, 100 μmol/L) or catalase (350 U/mL), scavengers of hydroxyl radical and H₂O₂ respectively. Arteries were scanned with a laser confocal microscope (Biorad MRC-600) as described.

Biochemical Analyses

Serum albumin, total cholesterol, iron, and total iron-binding capacity were measured by means of chromogenic assays (Sigma). Twenty-four-hour urine samples were collected in metabolic cages. Urinary 6-keto prostaglandin F₁α and 11-dehydro thromboxane B₂, the stable metabolites of prostacyclin and thromboxane A₂ (TXA₂), respectively, were measured by ELISA (Cayman Chemical). NO production was assessed by use of a colorimetric assay for urinary nitrate/nitrite concentration (Cayman Chemical). Nonheme iron content (expressed in μg/g dry wt tissue) was measured in livers, spleens, and aortas as previously described. Thiobarbituric acid reactive substances (TBARS) were measured with the use of a commercially available assay according to the manufacturer’s instructions (Oxi-tet, Zepptomixter Corporation). Malondialdehyde (MDA)-LDL levels were measured by ELISA, and plasma free F₂-isoprostanes were measured by stable isotope dilution mass spectrometry.

Statistical Analyses

Thrombosis times of iron-loaded and control animals were compared by proportional hazards modeling with SAS 8.2 (SAS Institute). All other results were expressed as mean±SD and analyzed by 2-tailed Student’s t tests for unpaired data.

Results

After 6 weeks of iron loading, mice appeared healthy and weighed the same as saline-treated animals (29.9±2.0 g for each group). There was no difference in serum albumin (3.2±0.2 g/dL versus 3.0±0.3; P=0.14, n=8 per group) or total cholesterol (81±3 mg/dL versus 77±12; P=0.28, n=8 per group) between iron-loaded and control groups, suggesting adequate nutritional status and normal hepatic synthetic function. The mean heart/body weight ratio (4.18±0.54; n=8 per group) and lung/body weight ratio (4.79±0.54 mg/g versus 4.65±0.54; P=0.51, n=10 per group) did not differ between iron-loaded and control groups, respectively.

Iron Contents and Histology

Serum and tissue iron parameters are summarized in Table 1. Livers of iron-loaded animals had normal architecture with no evidence of inflammation, necrosis, or scarring. Prussian blue staining revealed iron deposits predominantly localizing to tissue macrophages (Figure 1, A and B). Although nonheme iron content was elevated 4-fold in the aortas of iron-loaded mice, Prussian blue staining did not detect any
carotid arterial iron (Figure 1, C and D). Noninjured carotid arteries appeared histologically normal by hematoxylin and eosin staining in both groups (data not shown).

**Thrombosis Studies**

Thrombus formation after photochemical carotid artery injury was accelerated in iron-loaded animals compared with control animals (Figure 2). Mean occlusion times were $20.4 \pm 8.5$ minutes in the iron-loaded animals versus $54.5 \pm 35.5$ minutes in the saline-treated animals ($P<0.01$; $n=10$ for each group). Occlusion occurred in $<30$ minutes in 9 of 10 iron-loaded mice but only in 2 of 10 control animals. We performed a series of experiments to examine potential mechanisms by which iron overload could accelerate thrombus formation. To determine whether the effects of iron were attributable to generation of ROS, the aminothiol DL-cysteine, a nonspecific ROS scavenger, was administered 5 minutes after initiation of vascular injury. DL-Cysteine caused a statistically insignificant prolongation of the mean occlusion time of control animals (to $88.0 \pm 29.9$ minutes, $n=5$, $P=0.23$, Figure 2). In contrast, DL-cysteine significantly inhibited thrombus formation in the iron-loaded animals (mean occlusion time increased to $75.5 \pm 42.6$ minutes, $n=5$, $P<0.01$). Administration of PBS alone had no effect on occlusion times in either group (data not shown).

The effects of iron loading on coagulation parameters and platelet function were also examined. Whole blood hemoglobin concentration, platelet counts, PT, aPTT, vessel wall TF activity, and ADP-induced platelet aggregation did not differ significantly between groups (Table 2). Production of two important mediators of platelet activation, prostacyclin and TXA$_2$, was assessed by measuring their respective urinary metabolites, 6-keto prostaglandin F$_1$-$\alpha$ and 11-dehydro thromboxane B$_2$. Urinary 6-keto prostaglandin F$_1$-$\alpha$ levels were significantly elevated in iron-loaded mice versus control mice ($22.7 \pm 4.2$ ng/mg creatinine [$n=4$] versus $8.8 \pm 2.0$ [n=5], respectively; $P=0.0003$), and there was a trend toward higher 11-dehydro thromboxane B$_2$ levels ($6.2 \pm 2.7$ ng/mg creatinine [$n=8$] versus $4.1 \pm 2.6$ [n=10]; $P=0.12$).

**Vascular ROS Production and Systemic Oxidative Stress**

The production of ROS within the arterial wall was assessed in situ by incubating aortic cross sections from iron-loaded and control mice with the oxidatively active fluorescent dye.

### Table 2. Hemostatic and Coagulation Parameters

<table>
<thead>
<tr>
<th></th>
<th>Control Animals</th>
<th>Iron-Loaded Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin concentration, g/dL</td>
<td>$15.9 \pm 1.1$ (8)</td>
<td>$16.3 \pm 0.8$ (8)</td>
</tr>
<tr>
<td>Platelet count, 10$^9$/L</td>
<td>$1280 \pm 95$ (5)</td>
<td>$1216 \pm 179$ (5)</td>
</tr>
<tr>
<td>PT, s</td>
<td>$10.4 \pm 0.5$ (7)</td>
<td>$10.3 \pm 0.4$ (7)</td>
</tr>
<tr>
<td>aPTT, s</td>
<td>$32.1 \pm 2.9$ (7)</td>
<td>$34.3 \pm 3.3$ (7)</td>
</tr>
<tr>
<td>Carotid tissue factor activity, s</td>
<td>$36.9 \pm 2.6$ (10)</td>
<td>$35.4 \pm 3.0$ (10)</td>
</tr>
<tr>
<td>ADP-induced platelet aggregation, %</td>
<td>$51.2 \pm 8$ (5)</td>
<td>$53.6 \pm 8$ (5)</td>
</tr>
</tbody>
</table>

Number of animals per group is indicated in parentheses. $P>0.05$ for all comparisons.
DCFDA. No ROS were detectable in cross sections (n=3 per artery) from control animals (n=3, Figure 3A). In contrast, cross sections from iron-loaded animals (n=3) demonstrated markedly increased DCFDA staining, particularly within the adventitia (Figure 3B). In some sections, ROS were also detected in the intima (Figure 3C). Catalase and MPG, scavengers of H₂O₂ and OH⁻, respectively, almost completely abolished this signal (Figure 3, D and E). Systemic markers of oxidative stress, including TBARS, MDA, and F₂-isoprostanes, were also significantly elevated in iron-loaded animals versus control animals (Table 3).

Vascular Reactivity and NO Production
To determine if the enhanced vascular production of ROS after iron loading was associated with endothelial dysfunction, vascular reactivity studies were performed. In response to the endothelium-independent agonist nitroglycerin, there was no significant difference in peak relaxation (∼99.1±0.9% versus ∼99.3±1.2, P=NS) or ED₅₀ (∼7.52±0.13 log [M] versus ∼7.81±0.06, P=NS) of aortic rings from iron-loaded mice versus control mice (Figure 4A), suggesting that iron did not adversely affect smooth muscle function. In contrast, in response to the endothelium-dependent agonist acetylcholine, aortic rings from iron-loaded mice had significantly impaired peak relaxation (∼54.8±13.4% versus ∼93.2±0.1, P=0.05) and a shift in the ED₅₀ (∼6.24±0.49 log [M] versus ∼7.33±0.03, P<0.01) compared with control vessels (Figure 4B), suggesting decreased NO bioavailability. Consistent with these results, urinary nitrate/nitrite levels were lower in iron-loaded mice (760±640 nmol NO/mg creatinine, n=6) than in control mice (1700±980, n=9, P=0.06).

Discussion
We have examined the impact of chronic iron administration on the thrombotic response to arterial injury, vascular production of ROS, and endothelium-dependent vasoreactivity, three components of vascular function that play major roles in the pathogenesis of ischemic vascular disease. The principal finding of our study is that chronic iron administration markedly accelerates the thrombotic response to arterial injury. This effect was observed at a level of tissue iron overload comparable to that observed in human iron storage diseases (500 to 14 000 µg/g wet liver). This prothrombotic effect of iron may represent a mechanism by which iron overload could increase the risk of cardiovascular death and myocardial infarction, as suggested by several epidemiological studies, independent of a potential effect on atherosclerotic burden.

We hypothesized that iron overload could promote thrombosis through direct effects on the coagulation system. TF is a transmembrane protein that is essential for initiation of coagulation. Although previous studies have shown that TF expression is acutely enhanced under conditions of oxidative stress in cultured cells, we did not detect any difference in vascular TF activity between the iron-loaded and saline-treated animals. Taken together with the absence of effects on

TABLE 3. Measures of Systemic Oxidative Stress

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th></th>
<th>Liver</th>
<th></th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=5)</td>
<td>Iron-Loaded (n=4)</td>
<td>Control (n=5)</td>
<td>Iron-Loaded (n=4)</td>
<td>Control (n=5)</td>
</tr>
<tr>
<td>TBARS, nmol/mL MDA</td>
<td>4.4±0.7</td>
<td>6.3±0.7</td>
<td>4.9±0.9</td>
<td>11.3±2.3</td>
<td>7.8±1.3</td>
</tr>
<tr>
<td>MDA*</td>
<td>91±8.2</td>
<td>137±10.2</td>
<td>3.3±0.4</td>
<td>4.0±0.6</td>
<td>...</td>
</tr>
<tr>
<td>F₂-isoprostanes, pg/mL</td>
<td>70.2±10.1</td>
<td>99.3±10.5</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

P<0.05 for each control vs iron-loaded comparison, for each parameter, at every site.

*Values expressed as U MDA-LDL/L for serum and U MDA-protein/mg total protein for liver.
abolished by MPG and catalase, suggesting that iron in osteoblasts in the aortas of iron-loaded mice was nearly completely abrogated the effects of iron loading on thrombus formation, significantly prolonging the time to vessel occlusion. This suggests that iron may accelerate thrombosis in this model by a pro-oxidant mechanism. Future studies will be helpful in clarifying whether iron enhances thrombosis after redox-independent vascular injury.

In conclusion, chronic parenteral administration of iron dextran markedly accelerates the thrombotic response to carotid artery injury, increases systemic and vascular ROS production, and impairs endothelium-dependent vasorelaxation in mice. Although the relevance of these mouse studies to vascular disease and thrombosis in humans is unknown, these data suggest that iron overload may increase the propensity for ischemic vascular events by impairing vascular function and increasing thrombotic risk.

Acknowledgments
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