Iron Chelation Improves Endothelial Function in Patients With Coronary Artery Disease

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Background—Some epidemiological studies have shown that increased iron stores are associated with increased cardiovascular events. Redox-active iron may contribute to lipid peroxidation, endothelial cell activation, and generation of reactive oxygen species (especially hydroxyl radical, via Fenton chemistry). Increased oxidative stress is associated with impaired action of endothelium-derived nitric oxide in patients with atherosclerosis.

Methods and Results—To test the hypothesis that reducing vascular iron stores would reverse endothelial dysfunction, we examined the effects of the iron chelator deferoxamine (500 mg intra-arterially over 1 hour) on vasomotor function in forearm resistance vessels of patients with coronary artery disease by venous occlusion plethysmography. Patients with coronary artery disease had impaired endothelium-dependent vasodilation in response to methacholine compared with healthy control subjects ($P < 0.001$). Deferoxamine infusion decreased serum iron levels ($P < 0.001$). Deferoxamine improved the blood flow response to methacholine in patients with coronary artery disease ($P < 0.01$ by 2-way repeated-measures ANOVA) but had no effect on the response to sodium nitroprusside. In normal volunteers, deferoxamine had no effect on the response to methacholine. The nitric oxide synthase inhibitor $N^G$-monomethyl-$L$-arginine abolished augmentation of the methacholine response associated with deferoxamine. The hydroxyl radical scavenger mannitol had no effect on the methacholine response.

Conclusions—Deferoxamine improved nitric oxide–mediated, endothelium-dependent vasodilation in patients with coronary artery disease. These results suggest that iron availability contributes to impaired nitric oxide action in atherosclerosis. (Circulation. 2001;103:2799-2804.)

Key Words: iron ■ nitric oxide ■ endothelium ■ coronary disease

Numerous epidemiological studies have found an association between markers of increased iron stores and risk of coronary heart disease.1–3 This relationship appears to be stronger for ischemic events than for the presence of atherosclerosis per se,4 and recent data suggest that iron may be an important early in the development of atherosclerosis.5,6 The adverse effect of iron is potentiated by other risk factors, such as hypercholesterolemia.7 This hypothetical link and its mechanisms, however, are not without controversy.8

Despite the somewhat inconclusive epidemiological evidence, a number of mechanisms have been proposed to explain this association. Redox-active iron can initiate lipid peroxidation,9 an important early event in the development of atherosclerosis. In animal models of atherosclerosis, vascular iron deposition is closely related to progression of atherosclerosis and LDL oxidation.6 Recent evidence also suggests that redox-active iron may contribute to endothelial cell10 and platelet activation.11 These effects may be due to generation of reactive oxygen species, especially hydroxyl radical, via Fenton chemistry. Iron is also involved in many enzyme systems, however, including nitric oxide (NO) synthase (NOS), and non–protein-bound iron may directly inactivate endothelium-derived NO (EDNO).12 Thus, a potential mechanism for iron-related cardiovascular disease risk may be endothelial dysfunction.

The endothelium is critical in regulating vasomotor tone, platelet activity, leukocyte adhesion, and vascular smooth muscle proliferation through the release of several paracrine factors, including NO.13 Although endothelial dysfunction has been associated with the presence of atherosclerosis,14 impaired EDNO activity has also been shown in patients with atherosclerosis risk factors without overt vascular disease.14,15 Indeed, endothelial dysfunction not only may be an early marker for cardiovascular risk but also may contribute to the pathogenesis of atherosclerosis.11 Increased oxidative stress is associated with impaired EDNO bioactivity and may be a key early mechanism in the development of atheroma.9

Deferoxamine, a specific iron chelator, forms a stable complex with ferric iron, decreasing its availability for the production of reactive oxygen species.16 Deferoxamine may decrease endothelial cell activation in response to TNF-$\alpha$10 and collagen-induced whole-blood platelet aggregation.11 In

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higher concentrations (>0.5 mmol/L), deferoxamine may also scavenge reactive oxygen species. A recent study demonstrated that acute intravenous administration of deferoxamine improved coronary vasomotor responses to cold pressor testing and to flow increase in diabetics. We hypothesized that redox-active iron in the vasculature contributes to endothelial dysfunction in atherosclerosis. This study aimed to test this hypothesis by examining the effect of deferoxamine on endothelium-dependent vasodilation in patients with coronary artery disease (CAD).

Methods

Patients referred to Boston University Medical Center with significant CAD were eligible for study. Healthy control subjects without known risk factors for atherosclerosis were recruited by advertisement. The presence of CAD was confirmed by a history of percutaneous or surgical revascularization or by the presence of ≥1 coronary stenosis >50% on angiography. The Boston Medical Center Institutional Review Board approved the study. Volunteers provided written, informed consent.

Vasoactive medications were withheld for ≥12 hours before study, and long-acting vasoactive drugs were withheld for ≥24 hours. Volunteers with uncontrolled hypertension, heart failure, or unstable angina were excluded, as were those with diabetes mellitus (hypoglycemic treatment or fasting glucose >140 mg/dL) or anemia and those who had taken antioxidant vitamins, estrogen replacement therapy, or iron supplements within 1 month. Patients with CAD were taking aspirin (325 mg/d) when studied.

Protocol

Forearm blood flow was measured by venous occlusion plethysmography, as previously described. Blood pressure was measured via the arterial catheter.

The following intra-arterial drug infusion protocol was performed: (1) serial 5-minute infusions of the endothelium-dependent vasodilator methacholine (0.3, 1.0, 3.0, and 10 μg/min, Roche) or the endothelium-independent vasodilator sodium nitroprusside (0.3, 1.0, 3.0, and 10 μg/min, Elkins-Sinn); (2) dextrose control for 30 minutes to reestablish control conditions; (3) the iron chelator deferoxamine (Desferal, Novartis) 500 mg over 1 hour at 8.3 mg/min or the hydroxyl radical scavenger mannitol (25%, Fujisawa) at 200 mg/min; and (4) repeat methacholine or nitroprusside infusions. After deferoxamine, dextrose infusion was continued until resting blood flow was reestablished before readministration of methacholine or nitroprusside. Mannitol infusion was continued to reestablish control conditions; (3) the iron chelator deferoxamine (Desferal, Novartis) 500 mg over 1 hour at 8.3 mg/min or the hydroxyl radical scavenger mannitol (25%, Fujisawa) at 200 mg/min for 10 minutes; and (4) repeat methacholine or nitroprusside infusions. After deferoxamine, dextrose infusion was continued until resting blood flow was reestablished before readministration of methacholine or nitroprusside. Mannitol infusion was continued during readministration of methacholine. Estimated forearm blood concentrations of deferoxamine and mannitol were 0.42 and 55 mmol/L, respectively, based on resting forearm blood flow of 2.5 mL · min⁻¹ · 100 mL tissue⁻¹ and estimated forearm volume of ~1 L. In 10 additional studies, the NOS inhibitor N⁶-monomethyl-L-arginine (L-NMMA) was commenced at 1 mg/min 5 minutes before deferoxamine and was coinfused with deferoxamine and subsequent methacholine infusions to assess the contribution of NO to these responses. In 7 further studies, this latter protocol was repeated without deferoxamine to assess the contribution of NO to the methacholine response alone. Blood flow and blood pressure were measured for the last 2 minutes of each infusion.

Biochemical Analyses

Blood samples were obtained from the intra-arterial catheter with no concomitant drug infusion and after the first sample was discarded. Serum iron (reference range 65 to 175 μg/dL) was measured colorimetrically with Ferrozine (Roche Diagnostics) as chromogen. Total iron-binding capacity (reference range 250 to 450 μg/dL) was calculated from the sum of serum iron and unsaturated iron-binding capacity. Serum ferritin (reference range 10 to 322 ng/mL) was measured by chemiluminescence sandwich immunoassay with an automated chemiluminescence system (Bayer). Serum osmolality was measured by freezing point depression with a micro-osmometer. Hemoglobin, total cholesterol, HDL cholesterol, triglycerides, and glucose were measured by automated analyzer (Hitachi-917). LDL cholesterol was calculated by use of the Friedewald formula. Total iron-binding capacity, μg/dL 42.6 ± 28.0

Statistical Analysis

Data are mean ± SD, except in the figures (mean ± SEM). Baseline characteristics for the CAD and normal groups were compared by unpaired t test, χ², or Fisher’s exact test as appropriate. The effects of treatment on forearm blood flow or iron parameters were examined by 1-way or 2-way repeated-measures ANOVA with Student-Newman-Keuls post hoc comparison as appropriate. We explored the relations between serum iron or ferritin concentration, conventional atherosclerosis risk factors, and methacholine responses by linear regression. Variables with a univariate P value <0.10 were entered into a multiple linear regression model.

Results

Baseline Characteristics

A total of 54 volunteers were studied, including 28 CAD patients and 26 healthy control subjects. They participated in 78 separate studies. Clinical characteristics are contained in the Table. CAD patients were older and had higher hemoglobin and lower HDL cholesterol than control subjects. As expected, CAD patients also had a higher prevalence of hypercholesterolemia, hypertriglyceridemia, lipid-lowering treatment, family history of CAD, hypertension, and current or recent history (≥1 year) of smoking. In CAD patients, medications included aspirin (100%), β-blockers (89%), calcium antagonists (36%), ACE inhibitors (21%), and nitrates (21%).
As shown in Figure 1A, baseline flow was similar in CAD patients and control subjects, 2.9±1.1 and 2.9±1.2 mL·min⁻¹·dL tissue⁻¹, respectively. Intra-arterial infusion of methacholine increased flow in both groups. Vasodilation was attenuated in CAD patients, however (P<0.001). The response to the highest dose of methacholine (10 μg/min) was 12.1±4.5 mL·min⁻¹·dL tissue⁻¹ in 15 CAD patients and 16.7±6.9 mL·min⁻¹·dL tissue⁻¹ in 14 control subjects. Blood pressure was unaffected by methacholine. By contrast, vasodilation to the highest dose of nitroprusside (10 μg/min) was similar in 10 CAD patients (14.0±7.0 mL·min⁻¹·dL tissue⁻¹) and 12 control subjects (15.6±6.7 mL·min⁻¹·dL tissue⁻¹), P=0.65 (Figure 1B). After colinear variables had been excluded, univariate predictors of peak methacholine response among all participants were the presence of CAD (r=−0.32, P=0.026), LDL cholesterol (r=−0.32, P=0.027), and HDL cholesterol (r=0.29, P=0.046). Among CAD patients, univariate predictors of peak methacholine response were LDL cholesterol (r=−0.58, P=0.004), total cholesterol (r=−0.44, P=0.03), and serum iron (r=−0.38, P=0.078). By multivariate analysis, the independent predictors of peak methacholine response were LDL cholesterol and presence of CAD (adjusted R²=0.21, P=0.005).

**Forearm Blood Flow in Patients With CAD and Control Subjects**

![Figure 1A](http://circ.ahajournals.org/)

**Figure 1.** A, Forearm blood flow (FBF) responses were examined in 15 patients with CAD (●) and 14 control subjects (▲). Methacholine-induced, endothelium-dependent vasodilation was lower in patients with CAD (P<0.001). *P<0.05 by Student-Newman-Keuls post hoc comparison. B, FBF responses were examined in 10 patients with CAD (●) and 12 control subjects (▲). Sodium nitroprusside-induced, endothelium-independent vasodilation was similar in both groups (P=0.65).

**Effect of Deferoxamine on Iron Parameters**

Serum iron and total iron-binding capacity were similar in both groups at baseline (Table). Serum ferritin, however, tended to be higher in CAD patients than in control subjects (127±108 versus 76±68 ng/mL, P=0.13). Deferoxamine reduced serum iron by 54%, from 85±26 to 39±24 μg/dL (n=35, P<0.001). Serum iron was still depressed after protocol completion (after restesting vascular function: 68±27 μg/dL, n=22, P=0.002), although it was higher than immediately after cessation of deferoxamine infusion (P<0.001). These changes were similar in CAD patients and control subjects. Deferoxamine did not affect serum ferritin (P=0.33).

**Effect of Deferoxamine on Resting Flow**

During infusion of deferoxamine in 15 CAD patients, resting flow approximately doubled, from 2.8±1.2 to 5.2±1.8 mL·min⁻¹·dL tissue⁻¹, and persisted at this level during the infusion (Figure 2, P<0.001). Resting flow returned to baseline a mean of 27±11 minutes after cessation of the infusion. Deferoxamine infusion also increased resting flow (with similar duration of increase) in 14 control subjects from 2.7±1.1 to 6.8±2.0 mL·min⁻¹·dL tissue⁻¹, P<0.001. This increment was greater in control subjects than CAD patients (P=0.021). Infusion of the NOS inhibitor L-NMMA reduced resting flow in 10 CAD patients by 33% (P=0.014). Coinfusion of L-NMMA with deferoxamine attenuated the increase in resting flow compared with patients given deferoxamine alone (P<0.001); however, the percent and absolute increases in flow were comparable during coinfusion of L-NMMA.

**Effect of Deferoxamine on Flow Responses**

Iron chelation with deferoxamine augmented peak methacholine-induced vasodilation from 12.1±4.5 to 14.9±5.5 mL·min⁻¹·dL tissue⁻¹ (Figure 3A, P<0.01) in 15 CAD patients. To determine whether augmentation of vascular function with deferoxamine was due to NO, we examined the response to deferoxamine with L-NMMA. As shown in Figure 3B, deferoxamine had no effect in the presence of L-NMMA. Indeed, coinfusion of L-NMMA with deferoxamine impaired methacholine-induced vasodilation (P=0.01). In 7 further studies in CAD patients, L-NMMA infusion without deferoxamine impaired methacholine-induced vasodilation to a similar extent.
P, 0.001), such that the methacholine-induced vasodilation with L-NMMA was similar with or without deferoxamine (Figure 3B, P > 0.61). Deferoxamine, however, did not affect dose-dependent vasodilation to methacholine in 14 control subjects (peak response 16.7 ± 6.9 versus 16.3 ± 8.0 mL · min⁻¹ · dL tissue⁻¹). In separate studies in 10 CAD patients, deferoxamine did not affect vasodilation to nitroprusside (Figure 4).

**Effect of Mannitol on Flow Responses**
Infusion of mannitol, a hydroxyl radical scavenger, for 10 minutes in 10 CAD patients increased resting flow by 71%, (P<0.001), such that the methacholine-induced vasodilation with L-NMMA was similar with or without deferoxamine (Figure 3B, P = 0.61). Deferoxamine, however, did not affect dose-dependent vasodilation to methacholine in 14 control subjects (peak response 16.7 ± 6.9 versus 16.3 ± 8.0 mL · min⁻¹ · dL tissue⁻¹). In separate studies in 10 CAD patients, deferoxamine did not affect vasodilation to nitroprusside (Figure 4).

**Discussion**
This study demonstrated that endothelium-dependent dilation of forearm microvessels to methacholine is impaired in patients with proven CAD compared with healthy control subjects, whereas endothelium-independent vasodilation is preserved. This endothelial dysfunction was ameliorated after iron chelation with deferoxamine. Augmentation of methacholine-induced endothelium-dependent vasodilation with deferoxamine was abrogated by NOS inhibition with L-NMMA. Deferoxamine had no effect on endothelium-independent vasodilation with sodium nitroprusside. Importantly, deferoxamine infusion did not affect endothelial function in control subjects. These findings suggest that redox-active iron contributes to impaired bioactivity of EDNO in the microvasculature of patients with CAD. Although redox-active iron may contribute to the production of hydroxyl radicals via Fenton chemistry, coinfusion of the hydroxyl radical scavenger mannitol had no effect on endothelial function, suggesting that extracellular hydroxyl radical formation does not contribute to vascular dysfunction in this setting.

These findings are consistent with 1 previous human study.¹⁷ In that study, intravenous bolus injection of deferoxamine improved flow-mediated dilation and the response to cold pressor testing in conduit coronary arteries of diabetic patients with angiographically normal coronaries but no other risk factors. Several important differences, however, exist between these 2 studies. First, Nitenberg and colleagues¹⁷ did not confirm that deferoxamine had an effect on NO bioactivity, because the effect of NOS inhibition was not examined. Second, vasomotor changes with cold pressor testing are complex and reflect activation of the sympathetic nervous system as well as endothelial function.¹⁹ Third, flow-mediated dilation in the coronary circulation is not solely NO-dependent.²⁰ Thus, our major new finding is the role of iron in impaired bioactivity of EDNO in patients with CAD.
Iron and NO interactions are critical to the bioactivity of NO. For example, activation of guanylate cyclase occurs by reversible NO binding to ferrous heme iron, and NO can bind both ferric and ferrous heme in NOS to cause feedback inhibition. Moreover, NO may regulate cellular iron storage. Several experimental studies, however, support the hypothesis that excess redox-active iron may adversely affect EDNO and shed light on potential mechanisms for our findings. Iron and copper are known to stimulate LDL and membrane lipid peroxidation, and these transition metal ions are present in human atherosclerotic lesions in sufficient quantity to catalyze this reaction. Oxidized LDL and lipid peroxyl radicals formed during LDL oxidation may inactivate NO. To the extent that it is possible that lipid peroxidation might be inhibited in the time frame of this study, one mechanism for our observations may be inhibition of lipid peroxidation. A second explanation is that iron depletion may increase NO production, because high concentrations of iron decrease endothelial and inducible NOS activity. Finally, non–protein-bound iron may directly inactivate NO, and iron may be mobilized from ferritin by superoxide, which is increased in inflammation and atherosclerosis.

A commonly proposed mechanism for findings associated with deferoxamine is decreased iron-catalyzed hydroxyl radical formation via the Haber-Weiss reaction. Indeed, some propose that hydroxyl radical production is the likely mechanism by which iron might reduce NO bioactivity in vivo. The reaction kinetics between NO and hydroxyl radical make this hypothesis plausible, because the rate constant should be similar to the diffusion-limited reaction of NO and superoxide. The hydroxyl radical scavenger mannitol did not affect EDNO-mediated vasodilation in our study, however, suggesting that the mechanism by which iron chelation augmented EDNO bioactivity was not decreased hydroxyl radical production. This is consistent with the knowledge that hydroxyl radicals have a half-life of only nanoseconds and have diffusion-controlled chemistry in vivo owing to their facile reaction with nearly any adjacent molecules. Although our conclusion that deferoxamine did not augment EDNO bioavailability by decreasing hydroxyl radical production is limited by the fact that mannitol is poorly cell-permeable, deferoxamine is also poorly cell-permeable and probably chelated extracellular iron during the time course of this study. Nevertheless, the effect of intracellular hydroxyl radical scavenging on EDNO bioactivity warrants further investigation.

The present study also demonstrated that intra-arterial deferoxamine increased resting blood flow. This effect is unlikely to explain improved methacholine-induced vasodilation, because baseline conditions were reestablished before methacholine infusion was repeated. The percent and absolute increases in flow with deferoxamine were comparable with and without confusion of L-NMMA in patients with CAD. These findings suggest that factors other than NO contribute to this direct vasodilator effect. The resting flow increment, however, was greater in control subjects than in patients with CAD. Whether NO contributed to greater vasodilation in control subjects has not been determined by this study, although healthy participants may have greater flow-mediated dilation of forearm resistance vessels secondarily to increased flow associated with intra-arterial deferoxamine than patients with CAD. Mannitol increased resting flow to a similar extent. This finding is consistent with previous studies and most likely reflects increased serum osmolality, because hypertonic glucose also increases resting flow and serum osmolality. In the latter study, mannitol infusion did not affect endothelium-dependent vasodilation, confirming the present findings.

The present study also demonstrated endothelial dysfunction in forearm microvessels in patients with CAD and demonstrated that the presence of CAD independently predicts the methacholine response. Although LDL cholesterol was also an independent predictor of methacholine-induced vasodilation, LDL levels were not different between patients with CAD and control subjects. This apparent paradox may be explained by lipid-lowering therapy in patients with CAD and confirms that despite active risk factor control, patients with CAD still have endothelial dysfunction. Although the groups were not age-matched, age was not a predictor of endothelial function in this study. Extensive previous work has shown brachial artery conduit vessel endothelial dysfunction in patients with CAD. Numerous studies have also demonstrated impaired forearm resistance vasculature function in association with atherosclerosis risk factors such as hypercholesterolemia, hypertension, and diabetes. An association between forearm microvascular dysfunction and CAD, however, has not previously been established.

A potential limitation to this study is that serum iron levels returned toward normal by protocol completion, when endothelial function was retested, owing to the short half-life of deferoxamine and possible intracellular iron-store mobilization. Thus, we may have underestimated the benefit of lowering iron levels on EDNO bioactivity, because the effect may be greater if iron was lower throughout the second methacholine infusion. Second, we demonstrated only modest impairment of endothelial function in our patients with CAD, probably related to good risk factor control. Thus, augmentation of NO-mediated vasodilation might have been greater if we had studied patients with more risk factors, who typically demonstrate more profound endothelial dysfunction. Third, because this was a human study, the effect of deferoxamine on tissue iron cannot be assessed.

Considerable epidemiological data now suggest that increased body iron stores are a risk factor for cardiovascular events. Some of the controversy regarding this association may relate to use of inaccurate or inappropriate indicators of iron stores. Although recent data suggest that increased stored iron associated with hemochromatosis heterozygosity is not associated with increased atherosclerosis per se, this genetic marker is associated with increased cardiovascular events. Endothelial dysfunction owing to increased vascular iron could potentially explain the association between iron and cardiovascular events, because endothelial dysfunction is commonly present in patients with atherosclerosis, and endothelial disruption is a key pathophysiological mechanism in acute ischemic syndromes.

This study suggests that redox-active iron may contribute to endothelial dysfunction in patients with atherosclerosis,
although this does not appear to be related to increased hydroxyl radical availability. These novel findings may partially explain the association between increased iron stores and risk of cardiovascular events observed in epidemiological studies.

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