Blood Letting in High-Ferritin Type 2 Diabetes

Effects on vascular reactivity

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OBJECTIVE — In a recent study, iron chelation with deferoxamine led to improvement of endothelial dysfunction in patients with coronary artery disease. We tested the hypothesis that decreasing circulating iron stores might improve vascular dysfunction in patients with type 2 diabetes and increased serum ferritin concentration.

RESEARCH DESIGN AND METHODS — A total of 28 type 2 diabetic male patients with serum ferritin levels >200 ng/ml (~18% of consecutive type 2 diabetic men attending our outpatient clinic) were randomized to iron depletion (three extractions of 500 ml blood at 2-week intervals; group 1A) or to observation (group 1B). C282Y mutation was absent in all patients. Vascular reactivity (high-resolution external ultrasound) was evaluated at baseline and at 4 and 12 months thereafter. The two groups of patients were matched for age, BMI, pharmacological treatment, and chronic diabetic complications.

RESULTS — Endothelium-dependent vasodilation remained essentially unchanged in both groups of patients. In contrast, the vasodilation induced by glyceryl trinitrate (GTN) improved significantly after iron depletion ($P = 0.006$). These changes occurred in parallel to decreases in transferrin saturation index and HbA_{1c} levels (-0.6%, $P < 0.05$) only in group 1A patients. The best predictor of the modifications in endothelium-independent vasodilation was the change in HbA_{1c} levels. Changes in endothelium-independent vasodilation also correlated with the change in serum ferritin ($r = -0.45$, $P = 0.04$). At 12 months, transferrin saturation index and GTN-induced vasodilation returned to values similar to those at baseline in both groups of subjects.

CONCLUSIONS — Iron depletion improves vascular dysfunction in type 2 diabetic patients with high ferritin concentrations. The mechanisms by which these changes occur should be further investigated.

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Ferritin gene expression increases in the course of atherosclerotic plaque formation (1). Iron is a transition metal that can easily become oxidized and thus act as an oxidant. A possible link between iron and atherogenesis has been suggested by the finding that iron chelation blocks oxidation of LDL, whereas iron released from heme and ferritin favors oxidation of LDL (2). In fact, the general effect of catalytic iron is to convert poorly reactive free radicals such as H_2O_2 into highly reactive ones, such as the hydroxyl radical. In vitro addition of oxygen-derived free radical scavengers or antioxidants can reverse defective endothelium-dependent relaxation in experimental diabetes (3–6) or in diabetic patients (7). Impaired vascular responses to vasodilators, such as acetylcholine and glyceryl trinitrate (GTN), are usually found in patients with type 2 diabetes (8–10).

In experimental models, iron has an adverse effect on endothelium (11) and accelerates the development of atherosclerosis (12,13). In patients with iron overload, midsize arteries are characterized by an eccentric hypertrophy and decreased distensibility (14). These findings seem to be linked to iron-induced fibrogenesis, determining an increased total collagen content in arteries from these patients (15). There is also some evidence of iron-dependent growth of arterial wall tissue (16). Iron chelation by deferoxamine inhibits vascular smooth muscle cell proliferation (17).

Diabetes-induced endothelial dysfunction is prevented by long-term treatment with the modified iron chelator hydroxyethyl starch–conjugated deferoxamine (18). The coronary artery responses to cold pressor test and papaverine are improved by acute administration of deferoxamine in type 2 diabetic patients (19). Deferoxamine also improved nitric oxide (NO)-mediated vasodilation in patients with coronary artery disease (20). Iron depletion, commonly used to treat patients with hemochromatosis, has been demonstrated to be safe in diabetic subjects (21). Blood letting has been shown to increase the oxidation resistance of serum VLDLs/LDLs in men who regularly smoke (22). Oxidized low LDL impairs cyclic GMP–mediated dilations (23), and, thus, endothelial function is expected to improve after iron depletion. We found no previous controlled clinical trials of the effect of iron depletion on vascular function. For that reason, we carried out a clinical trial to test the effects

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Abbreviations: CV, coefficient of variation; GTN, glyceryl trinitrate; MDA, malondialdehyde; NO, nitric oxide.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

of reducing stored and circulating iron on endothelial function in type 2 diabetic subjects with increased serum ferritin.

RESEARCH DESIGN AND METHODS

Inclusion and exclusion criteria

A total of 28 type 2 diabetic men were prospectively recruited from diabetes outpatient clinics on the basis of the following criteria: *1*) serum ferritin level >200 ng/ml on two separate determinations, with at least a 1-month interval, and *2*) stable metabolic control in the previous 6 months. Approximately 18% of consecutive type 2 diabetic men attending our outpatient clinic fulfilled these criteria. Exclusion criteria included the following: *1*) clinically significant hepatic, neurological, endocrinologic, or other major systemic disease, including malignancy; *2*) history or current clinical evidence of hemochromatosis, or presence of the Cys282Tyr mutation; *3*) history of drug or alcohol abuse, defined as consuming over 80 g/day, or serum transaminase activity over twice the upper limit of normal; *4*) an elevated serum creatinine concentration; *5*) an acute major cardiovascular event in the previous 6 months; *6*) acute illnesses and current evidence of acute or chronic inflammatory or infective diseases; *7*) transfusion history, or iron or vitamin therapies in the previous year; *8*) history of disturbances in iron balance (e.g., hemosiderosis from any cause, atransferrinemia, paroxysmal nocturnal hemoglobinuria, or iron deficiency); and *9*) mental illness rendering the subject unable to understand the nature, scope, and possible consequences of the study. Informed written consent was obtained after the purpose, nature, and potential risks were explained to the subjects. The experimental protocol was approved by the Hospital Ethics Committee.

Study protocol

All patients underwent a full medical history including age, duration of diabetes, BMI, eating habits, smoking habits, blood pressure, total cholesterol, and a full examination to screen for diabetic complications. The clinical diagnosis of diabetic retinopathy was based on the examination of the ocular fundus after dilatation of the pupils by experienced ophthalmologists. Simplex retinopathy was defined as one or more microaneurysms or hemorrhages. Diabetic macroangiopathy complications were diagnosed according to clinical findings, Doppler sonography, and angiopathy. Persistent microalbuminuria was defined as albumin excretion rate of 30–300 mg/day. Patients considered eligible to participate in the study met with the doctor 4 weeks before, every 2 months during the first 4 months, and every 4 months thereafter. The patients were instructed to record any episode of symptomatic hypoglycemia daily.

Study design

Diabetic patients with elevated serum ferritin concentrations were randomized to either an iron depletion group (group 1A; $n = 13$) or an observation group (group 1B; $n = 15$) according to a randomization table that included age, BMI, and HbA_{1c}. The iron depletion intervention consisted of three extractions at 2-week intervals at weeks 0, 2, and 4. Each time, 450 g (500 ml) of blood was drawn. After blood donation, blood volume was restored to normal within 24–48 h by hemodilution. This hemodilutional effect was usually manifest 1 week after phlebotomy, with significant reductions in the hematocrit levels, which returned to baseline level after 4 weeks (24). Thus, the patients were studied at baseline, at 4 months, and at 12 months after iron depletion. All subjects were instructed to keep their usual treatment with insulin or hypoglycemic agents, diet, and exercise during the study period.

Measurements

Each subject was studied in the research laboratory in the postabsorptive state. The room was quiet, lights were dimmed, and the temperature was controlled at 23°C. BMI was calculated as weight (in kilograms) divided by height (in meters) squared. The subjects waist was measured with a soft tape midway between the lowest rib and the iliac crest. The hip circumference was measured at the widest part of the gluteal region. The waist-to-hip ratio was then calculated. Blood pressure was measured in the supine position on the right arm after a 10-min rest; a standard sphygmomanometer of appropriate cuff size was used, and the first and fifth phases were recorded. Values used in the analysis were the average of three readings taken at 5-min intervals. Alcohol, caffeine, and all medications, including sulfonylurea, metformin, and insulin, were withheld within 12 h of the different tests.

Brachial artery vascular reactivity

A high-resolution external ultrasound (128XP/10 mainframe with a 7.5-MHz linear array transducer) (Toshiba SSH-140A) was used to measure changes in brachial artery diameter in response to reactive hyperemia (leading to flow-mediated endothelium-dependent dilation) and in response to 400 μg sublingual GTN, an endothelium-independent direct smooth muscle dilator, as described by Celermajer et al. (25). The lumen diameter of the artery was defined as the distance between the leading edge of the echo of the near wall–lumen interface to the leading edge of the far wall–lumen interface echo (26). All scans were taken electrocardiogram-triggered coincident with the R wave–end diastolic. All images were recorded with an super-VHS videotape (Panasonic MD-830AG). Endothelial-dependent vasodilation was elicited with hyperemia induced by inflation of a pneumatic tourniquet placed around the forearm, distal to the scanned part of the artery, up to a pressure of 300 mmHg for 5 min, followed by sudden deflation. This maneuver is recognized to increase shear stress on the endothelial cells, which in turn releases NO-producing vasodilation, allowing endothelial function to be tested (27). Endothelial-dependent vasodilation is expressed as the percentage of change in the arterial diameter 1 min after hyperemia. Endothelial-independent vasodilation is induced after sublingual administration of a 400-μg metered dose of GTN, an exogenous NO donor (Solinitrina spray; Almirall Prodesfarma, Barcelona, Spain) and expressed as the percentage of change in the arterial diameter 3 min later. Reactive hyperemia is calculated as the percentage change between the maximum flow recorded in the first 15 s after cuff deflation and the flow during the resting scan.

A first scan was recorded after 10 min of resting in a quiet room in the supine position. Then the tourniquet was inflated for 5 min. A second scan was recorded during 90 s beginning 10 s before cuff deflation. After at least 10 more minutes of rest, a new control scan was recorded. A last scan was recorded from 2 min after GTN administration during 70 s. All images registered on a super-VHS tape were analyzed afterward by two

Table 1—*Baseline and follow-up of brachial artery vascular reactivity*

	Group 1A (n = 9)				Group 1B (n = 8)			
	Baseline	4 Months	12 Months	*P*	Baseline	4 Months	12 Months	*P*
Baseline vessel size (mm)	4.28 ± 0.41	4.14 ± 0.53*	4.16 ± 0.55	0.03	4.28 ± 0.55	4.44 ± 0.6	4.29 ± 0.50	NS
Baseline flow (ml/min)	0.50 ± 0.07	0.48 ± 0.123	0.52 ± 0.15	NS	0.59 ± 0.16	0.62 ± 0.23	0.55 ± 0.21	NS
Endothelium-dependent vasodilation (%)	2.7 ± 3.7	4.3 ± 4	2.9 ± 2.5	NS	4.3 ± 3.9	3.8 ± 4.7	4.4 ± 4.8	NS
Endothelium-independent vasodilation (%)	10.6 ± 3.1	18.4 ± 7*	12 ± 7	0.006	13.2 ± 8.7	12.4 ± 6.3	12.8 ± 5.4	NS

Data are means ± SD. *Significantly different from baseline.

independent observers blinded to the randomization of the subject and the stage of the experiment. Each observer analyzed the arterial diameter of four cardiac cycles for each condition, and these measurements were averaged. Before the initiation of the study in diabetic subjects, validation of this technique was performed through the evaluation of inter- and intraobserver repeatability in 22 healthy subjects (12 men and 10 women, mean age 30.1 years [95% CI 27.1–33.2], BMI 22.6 kg/m^2 [21.3–23.8]). Measurements were performed by two observers (A and B). Intraclass coefficient of correlation of fixed effects between observers A and B was 0.90. Coefficient of variation (CV) between means obtained by observers A and B was 9%. The CV obtained by a same observer was 3%. The repeatability (95% CI) was 0.27 mm (observer A). The CV was 4% for observer B, with a repeatability (95% CI) of 0.39 mm. Within-subject variability for 5 consecutive days (five subjects) showed a CV of 6% (observer A) and 2% (observer B). The GTN-induced vasodilation correlated with basal artery diameter ($r = -0.67$; $P = 0.025$) and flux-mediated vasodilation ($r = 0.68$; $P = 0.021$).

Analytical determinations

The serum glucose concentrations were measured in duplicate by the glucose oxidase method with the use of a Beckman Glucose Analyzer II (Beckman Instruments, Brea, CA). HbA_{1c} was measured by high-pressure liquid chromatography with the use of a fully automated glycosylated hemoglobin analyzer system (Hitachi L-9100). Serum ferritin was determined by microparticle enzyme immunoassay (AXSYMTM; Abbot Laboratories, Abbott Park, IL), with a intra- and interassay CV of <6%. Serum transferrin, transferrin saturation index, C-reactive protein (Beckman, Fullerton, CA), iron (Hitachi 917), and whole-blood hemoglobin level and hematocrit levels (EDTA sample; Coulter Electronics, Hialeah, FL) were determined by routinary laboratory tests. Serum C-peptide concentrations were measured using a fluorimetric immunoassay (EG & G Wallac, Wallac Oy, Turku, Finland) with intra- and interassay CVs of <6%. Total serum cholesterol was measured through the reaction of cholesterol esterase/cholesterol oxidase/peroxidase, using a Hitachi 747. HDL cholesterol was quantified after precipitation with polyethylene glycol at room temperature. LDL cholesterol was calculated using the Friedewald formula, when applicable. Total serum triglycerides were measured through the reaction of glycerol/phosphate/oxidase and peroxidase.

Plasma NO_2^-/NO_3^- levels were measured with the Nitric Oxide Colorimetric Assay Kit (Calbiochem-Novabiochem, San Diego, CA) based on a Griess reaction. Amounts of nitrite in the plasma were estimated by a standard curve obtained from enzymatic conversion of KNO_3 to nitrite.

Lipid peroxidation was evaluated through a modification of the thiobarbituric acid method, in which the concentration of malondialdehyde (MDA), a stable end by-product of the metabolism of lipid peroxides, is specifically measured by high-pressure liquid chromatography, according to Esterbauer and Cheeseman (28).

Statistical methods

Descriptive results of continuous variables are expressed as means ± SD. Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene's test, and then variables were given a log-transformation if necessary. We used the χ^2 test for comparisons of proportions, and we used one-way ANOVA with Bonferroni correction, unpaired, or paired *t* tests for comparisons of quantitative variables.

RESULTS — The two groups of patients were comparable in age (group 1A, 54.4 ± 8.2 vs. group 1B, 55.7 ± 8 years), BMI (28.7 ± 2.3 vs. 30.5 ± 3.2 kg/m^2), waist-to-hip ratio, systolic blood pressure (135.1 ± 23.9 vs. 137.3 ± 17.5 mmHg), diastolic blood pressure (82 ± 15.9 vs. 82.1 ± 7.5 mmHg), proportion of smokers (n = 3 vs. n = 2), serum ferritin (460 ± 109 vs. 566 ± 369 ng/ml), pharmacological treatment (insulin, biguanides, sulfonylureas, acarbose, statins, fibrates, ACE inhibitors, β-blockers, aspirin, and allopurinol), and chronic diabetic complications (macroangiopathy, diabetic retinopathy, and microalbuminuria). Of note is the near-identical initial blood HbA_{1c} levels (6.27 ± 0.95 vs. 6.39 ± 1.2%) because the patients were stratified according to this parameter. Baseline endothelium-dependent and -independent vasodilations were not significantly different in the two groups of patients (Table 1). After the study intervention, body weight, blood pressure, and blood hematocrit levels comprised the majority of the biochemical parameters that were measured, and drug treatment did not significantly change in either group (4-month follow-up: BMI: group 1A: 29.1 ± 2.7 vs. group 1B: 30.2 ± 3.7; systolic blood pressure: 133.3 ± 22.6 vs. 140 ± 15.3; diastolic blood pressure: 71.2 ± 12 vs. 85 ± 10.6; hematocrit: 42.6 ± 3.7 vs. 44.3 ± 2.7; fasting glucose: 161 ± 31 vs. 165 ± 45; fasting C-peptide: 2.76 ± 1.4 vs. 2.65 ± 1.4; cholesterol: 182 ± 30 vs. 195 ± 40; LDL cholesterol: 109 ± 24 vs. 117 ± 32; and triglycerides: 194 ± 70 vs. 156 ± 87). As expected, serum ferritin, transferrin saturation index, and blood hemoglobin decreased significantly at 4 months only in patients after iron depletion (4-

month follow-up: ferritin: group 1A: 232 ± 110 vs. group 1B: 507 ± 438; transferring saturation index: 19.89 ± 6.1 vs. 31 ± 12.5; and hemoglobin: 14.12 ± 1.2 vs. 15.1 ± 0.8). In parallel to these changes, blood HbA_{1c} decreased significantly only in these patients (mean differences −0.61, 95% CI −0.17 to −1.05; $P = 0.01$). They maintained the similar agents for the treatment of diabetes and showed a nonsignificant tendency toward an increased number of hypoglycemic events. None of them required hospitalization. C-reactive protein excluded significant acute inflammation in all subjects during the study period and remained essentially unchanged (4-month follow-up: group 1A: 0.57 ± 0.5; group 1B: 0.7 ± 1.3). At baseline, serum MDA (a surrogate marker of free radical production) was not associated with any parameter of iron metabolism ($r < 0.27$, NS). Serum MDA and NO_2^-/NO_3^- did not significantly change during the 4-month period in either group (4-month follow-up: MDA: group 1A: 15.6 ± 7.4 vs. group 1B: 19.2 ± 8.5; NO_2^-/NO_3^-: group 1A: 299 ± 157 vs. 268 ± 76).

Endothelium-dependent vasodilation did not change significantly in either group of subjects (Table 1). In contrast, GTN-induced vasodilation improved significantly after iron depletion in group 1A patients in parallel to changes in HbA_{1c} and the transferrin saturation index (Fig. 1). Significant improvements in GTN-induced vasodilation were observed in nearly 80% of patients, whereas it remained essentially unchanged in the rest of patients. No significant differences in clinical or biochemical characteristics were found between the subjects who responded to phlebotomy versus the subjects who did not respond. No significant changes were observed in group 1B patients. The best predictor of the observed changes in endothelium-independent vasodilation was the change in HbA_{1c} levels when all subjects were considered as a whole ($r = -0.48$, $P = 0.02$). Changes in endothelium-independent vasodilation also correlated with the change in serum ferritin ($r = -0.45$, $P = 0.04$), and a tendency was observed with the change in blood total hemoglobin in group 1A subjects ($r = -0.48$, $P = 0.09$, $n = 9$). Although no significant changes in endothelium-dependent vasodilation was observed in either group, post hoc analysis indicated that it was associated with changes in endothelium-independent vasodilation ($r = 0.55$, $P < 0.01$) and also with the decrease in HbA_{1c} ($r = -0.43$, $P = 0.02$) when all the subjects were considered as a whole. At 12 months of follow-up, all clinical and biochemical parameters remained unchanged in the control group. In the phlebotomy-treated group, hemoglobin, transferrin saturation, and HbA_{1c} returned to baseline levels, whereas ferritin concentrations remained significantly lower compared with baseline. Similarly, all vascular reactivity parameters remained unchanged at 12 months of follow-up in the control group, whereas vessel size and endothelium-independent dilation returned to baseline levels in the treated group.

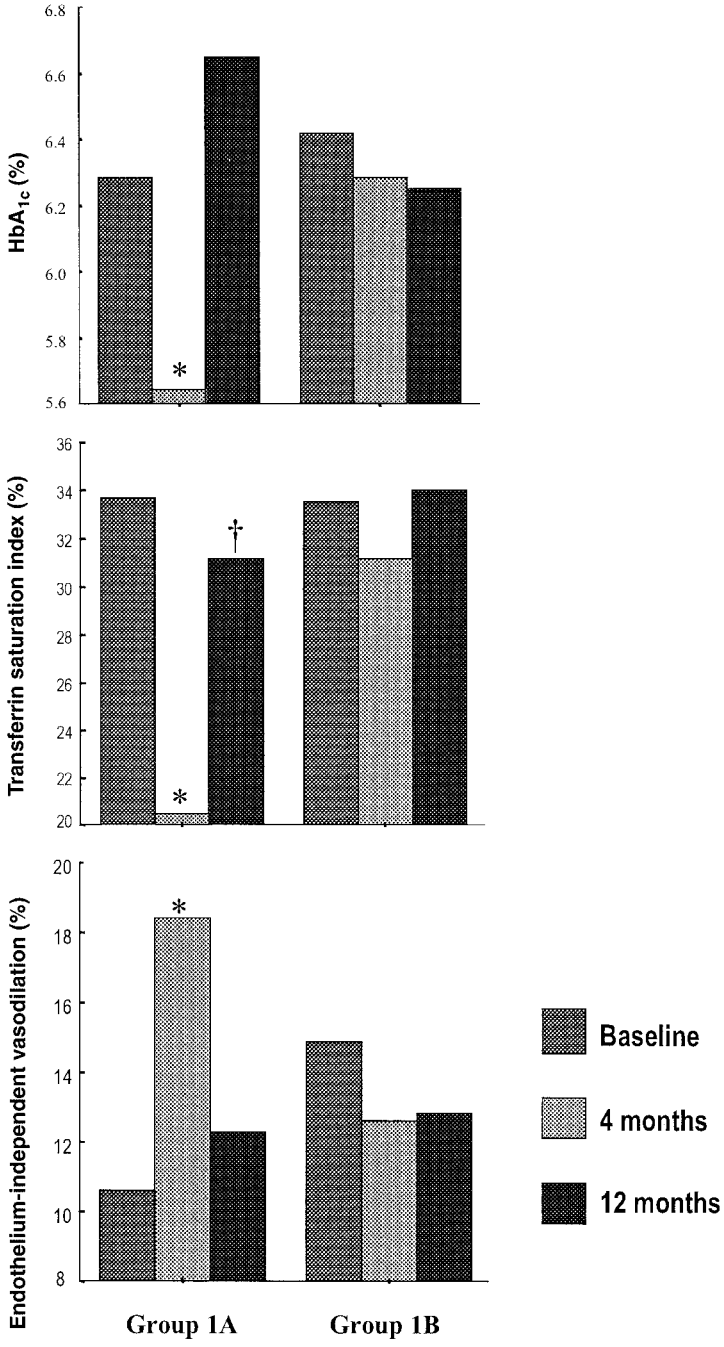

Figure 1—*Changes in HbA_{1c}, transferrin saturation index, and endothelium-independent vasodilation after blood letting (group 1A subjects) or sham blood letting (group 1B). *Significantly different from baseline; †significantly different from the same parameter at 4 months.*

CONCLUSIONS — Transition metals and active oxygen species act as vascular smooth muscle cell growth factors (16). Iron overload causes an early alteration of arterial wall structure and function in humans, characterized by eccentric hypertrophy (14). A thicker vascular wall implies that the poorly vascularized intimal layer is less easily reached by oxygen and other nutrients. This hypertrophy is reversible after iron depletion (14), whereas iron chelation by deferoxamine inhibits vascular smooth muscle cell proliferation (17). In the present study, we observed increased GTN-induced vasodilation of forearm conduit vessels in type 2 diabetic patients after iron depletion. Vasodilation in response to GTN is mediated by vascular smooth muscle cells. Thus, iron depletion results in increased arterial distensibility (14) and raised vasodilatory response as surrogates of improved smooth muscle cell function. These findings occurred in parallel to decreased HbA_{1c} concentrations (Fig. 1). The parallel modifications observed in these variables suggest that they are inter-related events. Transition metal–catalyzed reactions play a major role in the development of vascular dysfunction in experimental diabetes (29). Although our study cannot elucidate the precise mechanisms involved, iron is known to generate reactive oxygen species, especially hydroxyl radical, via Fenton chemistry. The importance of hydroxyl radicals for impaired endothelial function in diabetes has been stressed elsewhere (18,29).

Superoxide reacts with NO to produce peroxynitrite, which impairs vasodilation and can nitrosylate proteins to alter their function (30). Iron chelation with deferoxamine improves vascular dysfunction in patients with coronary artery disease (20) and experimental models (18). Our observation of improved vascular responses to GTN after decreasing iron stores evokes a scenario in which biochemical rather than structural defects are present and may be reversible. Changes in transferrin saturation index, blood hemoglobin, and HbA_{1c}, but not in serum ferritin (an indicator of tissue iron stores), mirrored the wax and wane of vascular responses. These findings suggest that blood itself is an important source of transition metals that impair vascular function. Exposure of normal blood vessels to HbA_{1c} has been shown to inhibit vascular relaxation (31). Endothelial cells are able to incorporate cell-free hemoglobin, creating a new way for circulating hemoglobin to be in close contact with NO (32).

Arterial smooth muscle responses to increasing doses of GTN are significantly lower in diabetic subjects (33–35). In the larger study sample to date, diabetes, larger vessel size, and reduced endothelium-dependent dilation were all independently associated with impaired GTN-related vasodilation on multivariate analysis (35). These findings are in line with the parallel changes observed in endothelium-dependent and -independent vasodilation ($r = 0.55$, $P < 0.01$) and the reduction in arterial diameter after the improved responses to GTN found in this study.

In addition to decreased responsiveness of vascular smooth muscle to NO, the mechanisms responsible for endothelial dysfunction and reduced endothelium-dependent vasodilation in patients with diabetes are not completely understood. Decreased synthesis or release of NO by endothelial cells is one possibility. We observed no significant changes in serum NO_2^-/NO_3^- concentration and no significant variations in endothelium-dependent vasodilation during the study. Another possible mechanism is increased inactivation of endothelium-derived NO by oxygen-derived free radicals, which damages endothelial membrane receptors for vasodilator agonists (3,4). We observed that vascular dysfunction did not run in parallel with the serum MDA concentration. However, we studied only diabetic patients with stable metabolic control, and thus the lack of significant modifications in endothelium-dependent responses could be related to good baseline risk factor control. The decrease in HbA_{1c} after iron depletion was similar to that obtained after deferoxamine therapy in type 2 diabetic patients (−0.5 and −0.6%, respectively [36,37]), in parallel to decreased transferrin saturation index and serum ferritin concentration. Blood hematocrit did not significantly change, excluding hemodilution as a confounding factor (24). Bleeding produced a significant decrease in serum glucose in diabetic patients (21) and healthy subjects (38). Long-term treatment of diabetic rats with hydroxyethyl starch–conjugated deferoxamine did not modify serum insulin or blood glucose but caused a reduction in HbA_{1c} (39). This chelator also reduced glycation of albumin in vitro through elevated glucose concentrations. Transition metals play an important role in protein glycation induced by hyperglycemia. In fact, both HbA_{1c} and serum glucose are strongly associated with serum ferritin levels, even in healthy subjects (40,41). Increased iron stores predicted the development of diabetes in epidemiological studies (42,43). Interestingly, a lower prevalence of diabetes was observed among blood donors in a recent study (44). The impact of iron depletion on vascular dysfunction and metabolic control in diabetic patients needs to be confirmed in a large-scale study because of the important public health implications.

Current research is examining strategies that might improve endothelial function. The early implementation of such strategies in the disease process would prevent or delay atherogenesis. Up until now, the therapies that have demonstrated to improve endothelial function have been lipid lowering (45,46), oral administration of L-arginine (47), ACE inhibition (48), estrogen therapy (49), administration of antioxidants (vitamin C) (7,50) and insulin (10), and allopurinol treatments (51), among others, with the latter two being the only treatments tested in diabetic patients. Our findings contribute to a greater understanding of the factors that modulate the vasorelaxant response to NO. Whether blood letting should be considered adjuvant therapy in high-ferritin type 2 diabetes requires further study.

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