Blood Letting in High-Ferritin Type 2 Diabetes

Effects on Insulin Sensitivity and β-Cell Function

José Manuel Fernández-Real,[1] Georgina Peñarroja,[2] Antoni Castro,[2] Fernando García-Bragado,[2] Ildefonso Hernández-Aguado,[3] and Wifredo Ricart[1]

Iron-related insulin-resistance is improved by iron depletion or treatment with iron chelators. The aim of this study was to evaluate insulin sensitivity and insulin secretion after blood letting in patients who had high-ferritin type 2 diabetes and were randomized to blood letting (three phlebotomies [500 ml of blood] at 2-week intervals, group 1) or to observation (group 2). Insulin secretion and sensitivity were tested at baseline and 4 and 12 months thereafter. The two groups were matched for age, BMI, pharmacologic treatment, and chronic diabetic complications. All patients were negative for C282Y mutation of hereditary hemochromatosis. Baseline glycated hemoglobin (6.27 ± 0.9% vs. 6.39 ± 1.2%), insulin sensitivity (2.75 ± 1.8 vs. 3.2 ± 2.1 mg · dl^{-1} · min^{-1}), and area under the curve for C-peptide ($AUC_{C\text{-}peptide}$; 38.7 ± 11.6 vs. 37.6 ± 14.1 ng · ml^{-1} · min^{-1}) were not significantly different between the two groups of patients. Body weight, blood pressure, blood hematocrit levels, and drug treatment remained essentially unchanged during the study period. As expected, serum ferritin, transferrin saturation index, and blood hemoglobin decreased significantly at 4 months only in patients who received blood letting. In parallel to this changes, blood HbA_{1c} decreased significantly only in group 1 subjects (mean differences, −0.61; 95% CI, −0.17 to −1.048; $P = 0.01$). $AUC_{C\text{-}peptide}$ decreased by −10.2 ± 6.3% after blood letting. In contrast, a 10.4 ± 6.4% increase in $AUC_{C\text{-}peptide}$ was noted in group 2 subjects at 4 months ($P = 0.032$). At 12 months, $AUC_{C\text{-}peptide}$ returned to values not significantly different from baseline in the two groups of subjects. At 4 months, the change in insulin sensitivity from baseline was significantly different between the two groups (80.6 ± 43.2% vs. −8.6 ± 9.9% in groups 1 and 2, respectively, $P = 0.049$). At 12 months, the differences between the two groups were even more marked (55.5 ± 24.8% vs. −26.8 ± 9.9%; $P = 0.005$). When the analysis was restricted to those subjects who completed the follow-up until 12 months, results did not show differences compared with the changes observed at 4 months, except for insulin sensitivity. A statistically significant increase in insulin sensitivity was observed in the blood-letting group (from 2.30 ± 1.81 to 3.08 ± 2.55 mg · dl^{-1} · min^{-1} at 4 months, to 3.16 ± 1.85 mg · dl^{-1} · min^{-1} at 12 months; $P = 0{,}045$) in contrast with group 2 subjects (from 3.24 ± 1.9 to 3.26 ± 2.05 mg · dl^{-1} · min^{-1} at 4 months, to 2.31 ± 1.35 mg · dl^{-1} · min^{-1} at 12 months). In summary, blood letting led simultaneously to decreased blood HbA_{1c} levels and to changes in insulin secretion and insulin resistance that were significantly different from those observed in a matched observational group of subjects with high-ferritin type 2 diabetes. The mechanisms for improvement in peripheral insulin sensitivity after blood letting should be investigated further. *Diabetes* 51:1000–1004, 2002

Iron is a transition metal that can easily become oxidized and thus act as an oxidant. 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. Increased accumulation of iron affects insulin synthesis and secretion in the pancreas (1,2) and interferes with the insulin-extracting capacity of the liver (3). Iron deposition in muscle decreases glucose uptake because of muscle damage (4). Conversely, insulin stimulates cellular iron uptake through increased transferrin receptor externalization (5). Thus, insulin and iron can mutually potentiate their effects, leading, after a vicious cycle, to insulin resistance and diabetes. We (6) and others (7) have described a relationship between serum ferritin and several components of the insulin resistance syndrome in seemingly healthy subjects. Serum ferritin was proportional to serum glucose concentration, diastolic blood pressure, HDL cholesterol, and insulin resistance (6). In fact, the higher the ferritin levels, the higher the incidence of type 2 diabetes in recent epidemiological studies (8,9).

Iron chelating agents and blood donation can prevent the development of diabetes in transfusional iron overload (10,11). Blood letting is commonly used in the treatment of hemochromatosis (12) and has been consistently demonstrated to lower iron stores and to be safe in individuals with diabetes (13). We found no previous controlled clinical trials of the effect of blood letting on metabolic control, insulin secretion, or insulin action in high-ferritin type 2 diabetes. For that reason, we carried out a clinical trial to test the effect of decreased stored iron on these parameters.

RESEARCH DESIGN AND METHODS

Inclusion and exclusion criteria. Patients with type 2 diabetes, according to American Diabetes Association criteria, were prospectively recruited from

From the [1]Unit of Diabetes, Endocrinology and Nutrition, University Hospital of Girona "Dr. Josep Trueta," Girona, Spain; the [2]Department of Internal Medicine, University Hospital of Girona "Dr. Josep Trueta," Girona, Spain; and the [3]Division of Preventive Medicine and Public Health, University "Miguel Hernández," Alicante, Spain.

Address correspondence and reprint requests to José M. Fernández-Real, Unit of Diabetes, Endocrinology and Nutrition, Hospital de Girona "Dr. Josep Trueta," Ctra. França s/n, 17007 Girona, Spain. E-mail: endocrino@htrueta.scs.es.

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AUC, area under the curve.

diabetes outpatient clinics on the basis of the following: *1*) serum ferritin >200 ng/ml on two separate determinations, with at least a 1-month interval, and *2*) stable metabolic control in the previous 6 months, as defined by stable HbA_{1c} values. The latter criterion is important because serum ferritin concentrations are significantly increased in patients with poorly controlled diabetes (14,15), and short-term improvement in glucose control is associated with variable decreases in serum ferritin concentration (15).

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 >80 g/day in men and >40 g/day in women, or serum transaminase activity more than twice the upper limit of normal; *4*) an elevated serum creatinine concentration; *5*) 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 subjects 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 that included 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 dilation 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 who were considered to be eligible to participate in the study met with the doctor 4 weeks before the study, 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. Patients who had diabetes with elevated serum ferritin concentrations were randomized according to a randomization table that included age, BMI, and blood glycated hemoglobin, to blood letting (group 1) or to observation (group 2). To exclude sex bias, we initially studied only men. The blood letting intervention consisted of three phlebotomies at 2-week intervals at study weeks 0, 2, and 4. Each time, 450 g (500 ml) of blood was drawn. After phlebotomy, blood volume was restored to normal within 24–48 h by hemodilution. This hemodilutional effect is usually manifested at 1 week postphlebotomy, with significant reductions in the hematocrit levels, which return to baseline levels after 4 weeks (16). Thus, the patients were studied at baseline, at 4 months, and at 12 months after blood letting. All subjects were instructed to keep their usual treatment with insulin or hypoglycemic agents, diet, and exercise during the study period. One of the researchers monitored usual medication every 2 months.

Measurements. Each subject was studied in the research laboratory in the postabsorptive state. The room was quiet, lights were dimmed, and 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 are 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.

Insulin sensitivity. The experimental protocol started between 8:00 and 9:30 A.M. after an overnight fast. All of the subjects were on a weight-maintaining diet before the test. A bolus of human Actrapid insulin (0.1 unit/kg; Novo-Nordisk, Copenhagen, Denmark) was administered into an antecubital vein, and blood was sampled from a vein on the dorsum of the same hand. To arterialize the venous blood, the hand was placed on a hot box at a constant temperature of 40°C for 20 min before the start of the study and kept there until the end of the test. Sampling was carried out every minute until 15 min after the injection of insulin. Insulin sensitivity was indicated by the first-order rate constant for disappearance rate of glucose K_{ITT} estimated from the slope of the regression line of the logarithm of blood glucose against time during the first 3–15 min.

β-Cell function. Plasma C-peptide was determined basally and 6 and 10 min after the injection of 1 mg i.v. glucagon (Novo-Nordisk). Area under the curve of glucose ($AUC_{glucose}$) and C-peptide ($AUC_{C\text{-}peptide}$) were then calculated using the trapezoidal method.

TABLE 1
Clinical variables of subjects

Variable	Group 1 (n = 13)	Group 2 (n = 15)	P
Age (years)	54.4 ± 8.2	55.7 ± 8	NS
Smokers (n)	3	2	NS
Pharmacologic treatment			
Insulin	1	2	NS
Biguanides	6	5	NS
Sulphonylureas	6	3	NS
Acarbose	1	1	NS
Statins	2	3	NS
Fibrates	1	3	NS
ACE1	2	2	NS
β-blockers	1	1	NS
Aspirin	2	1	NS
Allopurinol	1	1	NS
Diabetic complications			
Macroangiopathy	1	2	NS
Diabetic retinopathy	2	4	NS
Microalbuminuria	3	4	NS

ACEI, angiotensin-converting enzyme inhibitors; NS, not significant.

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-performance liquid chromatography with the use of a fully automated glycated hemoglobin analyzer system (Hitachi L-9100, Hitachi-Merck, Rahway, NJ). Serum ferritin was determined by Microparticle Enzyme ImmunoAssay (AXSYMTM; Abbot Laboratories, Abbott Park, IL), with a coefficient of variation intra- and interassay <6%. Serum transferrin, transferrin saturation index, and C-reactive protein (the lowest limit of detection was 0.1 mg/dl; Beckman, Fullerton, CA), iron (Hitachi 917), and whole-blood hemoglobin level and hematocrit levels (EDTA sample; Coulter Electronics, Hialeah, FL) were determined by routine laboratory tests.

Serum C-peptide concentrations were measured using a fluorometric immunoassay (EG & G Wallac, Wallac Oy, Turku, Finland) with intra- and interassay coefficients of variation <6%. Total serum cholesterol was measured through the reaction of cholesterol esterase/cholesterol oxidase/peroxidase, using a BM/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.

Statistical methods. Descriptive results of continuous variables are expressed as mean ± 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 χ^2 test for comparisons of proportions and unpaired or paired t tests for comparisons of quantitative variables. To determine whether therapeutic strategy had any effect on insulin sensitivity, insulin secretion, or glycated hemoglobin, we performed a repeated measures ANOVA. We did a separate analysis for each variable. Also, we assessed the effect of treatment for those subjects who completed the follow-up at 4 months (n = 28) and for those who were evaluated at 12 months of follow-up. The assumption of equality of covariance matrices was checked by the Box's M test.

RESULTS

The clinical and biochemical characteristics of the study patients are provided in Tables 1 and 2. The two groups of patients were comparable in age, BMI, waist/hip ratio, systolic and diastolic blood pressure, proportion of smokers, serum ferritin, pharmacologic treatment, and chronic diabetic complications. Of note is the near identical baseline blood HbA_{1c} levels because the patients were stratified according to this parameter. Baseline insulin sensitivity (2.75 ± 1.8 vs. 3.2 ± 2.1 $mg \cdot dl^{-1} \cdot min^{-1}$) or

TABLE 2
Baseline and follow-up of clinical and biochemical variables in the study subjects

Variable	Group 1				Group 2			
	Baseline	4 months	12 months (n = 8)	P	Baseline	4 months	12 months (n = 10)	P
BMI (kg/m^2)	28.7 ± 2.3	29.1 ± 2.7	29.6 ± 2	NS	30.5 ± 3.2	30.2 ± 3.7	30.8 ± 3.2	NS
Systolic blood pressure (mmHg)	135.1 ± 23.9	133.3 ± 22.6	132 ± 20	NS	137.3 ± 17.5	140 ± 15.3	135.6 ± 11.2	NS
Diastolic blood pressure (mmHg)	82 ± 15.9	83.6 ± 12	71.2 ± 12	NS	82.1 ± 7.5	84.6 ± 7	85 ± 10.6	NS
Hb (g/dl)	14.72 ± 1.1	14.12 ± 1.2*	14.3 ± 0.8	0.038	14.8 ± 1.1	15.1 ± 0.8	14.9 ± 1.1	NS
Hematocrit (%)	43 ± 3.3	42.6 ± 1.7	43 ± 2.8	NS	43.4 ± 2.7	44.3 ± 2.7	43.8 ± 3.6	NS
Ferritin (ng/ml)	460 ± 109	232 ± 110*	222 ± 104*	<0.0001	566 ± 369	507 ± 438	346 ± 200†	0.048
Transferrin saturation index (%)	33.9 ± 11.3	19.89 ± 6.1*	31 ± 7.4†	<0.001	33.4 ± 9	31 ± 12.5	33.9 ± 14.6	NS
C-reactive protein (mg/dl)	0.50 ± 0.6	0.57 ± 0.5	0.31 ± 0.25	NS	0.3 ± 0.2	0.7 ± 1.3	0.31 ± 0.25	NS
Fasting glucose (mg/dl)	168 ± 44	161 ± 31	158 ± 20	NS	154 ± 38	165 ± 45	151 ± 49	NS
Fasting C-peptide (ng/ml)	2.86 ± 1.1	2.76 ± 1.4	3.05 ± 1.1	NS	2.38 ± 1	2.65 ± 1.4	2.8 ± 1.4	NS
Cholesterol (mg/dl)	185 ± 33	182 ± 30	192 ± 32	NS	194 ± 36	195 ± 40	184 ± 31	NS
LDL cholesterol (mg/dl)	99 ± 44	109 ± 24	107 ± 43	NS	106 ± 43	117 ± 32	91.3 ± 34	NS
Triglycerides (mg/dl)	181 ± 67	194 ± 70	184 ± 72	NS	159 ± 84	156 ± 87	141 ± 33	NS

*Significantly different from baseline; †significantly different from the same parameter at 4 months.

$AUC_{C\text{-peptide}}$ (38.7 ± 11.6 vs. 37.6 ± 14.1 ng · ml^{-1} · min^{-1}) were not significantly different between the two groups of patients. Body weight, blood pressure, blood hematocrit levels, as the majority of the biochemical parameters that were measured, and drug treatment did not significantly change in either group (Tables 1 and 2). C-reactive protein excluded significant acute inflammation in all subjects during the study period and remained essentially unchanged.

As expected, serum ferritin, transferrin saturation index, and blood hemoglobin decreased significantly at 4 months only in patients who received blood letting (Table 2). In parallel to these changes, blood HbA_{1c} decreased significantly only in these patients (mean differences, −0.61; 95% CI, −0.17 to −1.048; P = 0.01). They maintained the similar agents for the treatment of diabetes and showed a nonsignificant tendency toward increased number of hypoglycemic events. None of them required hospitalization.

In parallel to decreasing HbA_{1c}, $AUC_{C\text{-peptide}}$ decreased by −10.2 ± 6.3% in group 1 patients after blood letting. In contrast, a 10.4 ± 6.4% increase in $AUC_{C\text{-peptide}}$ was noted in group 2 subjects at 4 months (P = 0.032 for the difference; Fig. 1). At 12 months, $AUC_{C\text{-peptide}}$ returned to values not significantly different from baseline in the two groups of subjects. When the analysis was restricted to those 18 subjects who completed the follow-up until 12 months, similar results were obtained (group 1: $AUC_{C\text{-peptide}}$ from 45.5 ± 14.5 to 41.2 ± 15.5 ng · ml^{-1} · min^{-1} at 4 months to 43.3 ± 16.4 ng · ml^{-1} · min^{-1} at 12 months; group 2: $AUC_{C\text{-peptide}}$ from 42.2 ± 15 to 45.9 ± 13.4 ng · ml^{-1} · min^{-1} at 4 months to 47.2 ± 19.1 ng · ml^{-1} · min^{-1} at 12 months).

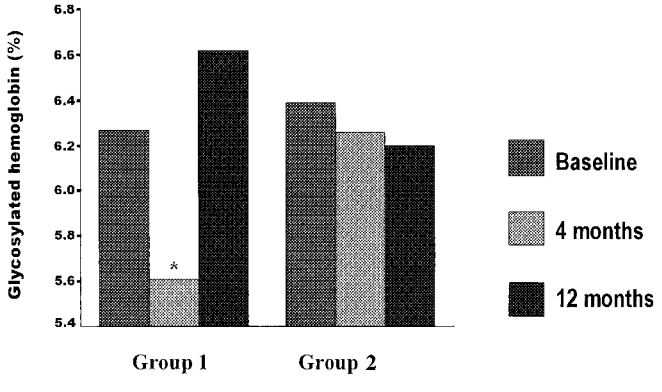

FIG. 1. Changes of glycated hemoglobin, after blood letting (group 1 subjects) or observation (group 2). *Significantly different from baseline (P = 0.01).

Within groups, insulin sensitivity was highly reproducible, as indicated by the correlations observed in the insulin sensitivity index at 4 months versus baseline and at 12 months versus baseline (all $r > 0.75$, $P < 0.01$). At 4 months, the change in insulin sensitivity from baseline was significantly different between the two groups of patients (80.6 ± 43.2% vs. −8.6 ± 9.9% in groups 1 and 2, respectively; P = 0.049; Fig. 2). At 12 months, the differences remained significant and were even more marked (55.5 ± 24.8% vs. −26.8 ± 9.9%; P = 0.005). When the analysis was restricted to those 18 subjects who completed the follow-up until 12 months, results did not show differences compared with the changes observed at 4 months, except for insulin sensitivity. A statistically significant increase in insulin sensitivity was observed in the blood-letting group (from 2.30 ± 1.81 to 3.08 ± 2.55 mg · dl^{-1} · min^{-1} at 4 months, to 3.16 ± 1.85 mg · dl^{-1} · min^{-1} at 12 months; P = 0.045) in contrast with group 2 subjects (from 3.24 ± 1.9 to 3.26 ± 2.05 mg · dl^{-1} · min^{-1} at 4 months, to 2.31 ± 1.35 mg · dl^{-1} · min^{-1} at 12 months).

Mean ferritin decrease of group 2 patients at 12 months was exclusively due to one subject with serum ferritin >1,000 ng/ml. After exclusion of this subject, mean serum ferritin at 12 months was not statistically different from baseline or from 4 months in this group of subjects.

DISCUSSION

The findings of the present study suggest that blood letting might contribute as an adjuvant treatment in patients who have type 2 diabetes with increased serum ferritin concentrations. This is a preliminary observation that needs to be confirmed in a larger sample of subjects. In our patients, blood letting led simultaneously to decreased blood HbA_{1c} levels and to changes in insulin secretion and insulin resistance that were significantly different from those

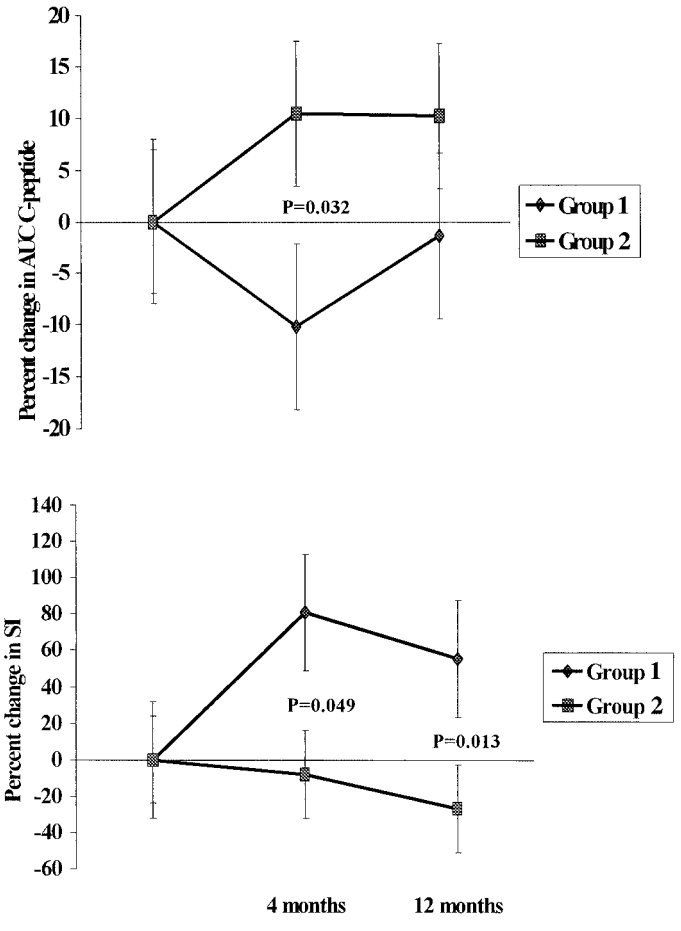

FIG. 2. Changes in insulin secretion ($AUC_{C\text{-}peptide}$ glucagon) and insulin sensitivity after blood letting (group 1 subjects) in comparison with group 2 subjects.

observed in a matched observational group of subjects with high-ferritin type 2 diabetes. $AUC_{C\text{-}peptide}$ was significantly lower after blood letting, suggesting lower endogenous insulin requirements for improved metabolic control. In fact, significant reductions in insulin secretion accounted for the majority of the overall increase in S_I after improved metabolic control in recent studies (17). Decreased stimulated C-peptide after blood letting evoked a predominance of β-cell downregulation in response to improved β-cell insulin sensitivity. In healthy volunteers with normal glucose tolerance and normoferritinemia, Facchini (18) found decreased postload plasma glucose and insulin concentrations 1 month after a 500-ml phlebotomy. Bleeding was also found to decrease serum glucose and triglycerides in patients with diabetes (13).

Increased iron stores predicted the development of diabetes in epidemiological studies (8,9). It is interesting that a lower prevalence of diabetes was recorded among frequent blood donors in a recent report (19). Perhaps the most striking aspect was the longevity of effects of blood letting in our study. The changes in insulin sensitivity were maintained even 1 year after the procedure. A possible explanation is that blood letting removes free transition metals from the body and a substantial time is needed for levels to build up again to those before therapy. The complex process of advanced glycation end product formation produces reactive oxygen species by metal-catalyzed reactions. Advanced glycation end products themselves bind transition metals (20), potentiating their toxic effects, including insulin resistance. Reactive oxygen species interfere with insulin signaling at various levels, impairing insulin uptake through a direct effect on insulin receptor function (21) and inhibiting the translocation of GLUT4 in the plasma membrane (22). Decreasing iron stores would ameliorate insulin resistance by reducing this cascade of events.

The initial and the most common defect in patients with an earlier stage of damage induced by iron overload is liver-mediated insulin resistance (23). Hepatic iron overload syndrome, unrelated to hereditary hemochromatosis, has been recently described and is characterized by hyperferritinemia, normal transferrin saturation, and increased prevalence of glucose tolerance and diabetes (24). Our patients with diabetes share different characteristics of this syndrome except that all subjects had normal liver enzymes. Liver enzymes did not substantially change during the study (data not shown). However, improved hepatic insulin sensitivity after blood letting cannot be excluded.

The decrease in glycated hemoglobin after blood letting was similar to that obtained after deferoxamine therapy in patients with type 2 diabetes (−0.5% [25] and −0.6% [26]), in parallel to decreased transferrin saturation index and serum ferritin concentration. Blood hematocrit did not significantly change, excluding hemodilution as a confounding factor (16). Long-term treatment of diabetic rats with hydroxyethyl starch–conjugated deferoxamine did not modify serum insulin or blood glucose but caused a reduction in glycated hemoglobin (27). The iron chelator DETAPAC also reduced glycation of albumin in vitro by elevated glucose concentration (28). Transition metals play an important role in protein glycation induced by hyperglycemia. In fact, both glycated hemoglobin and serum glucose are strongly associated with serum ferritin levels even in healthy subjects (6,7,29).

The impact of iron depletion on metabolic control and insulin sensitivity in patients with diabetes needs to be confirmed in a large-scale study for their important public policy implications. Excess iron storage in patients with type 2 diabetes unrelated to primary hemochromatosis is increasingly recognized. Moreover, patients with diabetes showed increased prevalence for mutations of hereditary hemochromatosis in some studies (30,31). An adequate and safe therapy will be needed for these patients, and blood letting might be one of them.

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