Oxidative Stress, β-Cell Apoptosis, and Decreased Insulin Secretory Capacity in Mouse Models of Hemochromatosis

ROBERT C. COOKSEY, HANI A. JOUIHAN, RICHARD S. AJIOKA, MARK W. HAZEL, DEBORAH L. JONES, JAMES P. KUSHNER, AND DONALD A. MCCAIN

Research Service of the Veterans Affairs Medical Center (R.C.C., D.A.M.), and Departments of Medicine and Biochemistry, University of Utah School of Medicine (R.C.C., H.A.J., R.S.A., M.W.H., D.L.J., J.P.K., D.A.M.), Salt Lake City, Utah 84132

The pathogenesis of diabetes associated with hemochromatosis is not known. We therefore examined glucose homeostasis and β-cell function in mouse models of hemochromatosis. Mice with targeted deletion of the hemochromatosis gene (Hfe−/−) on the 129/Sv genetic background exhibited a 72% increase in iron content in the islets of Langerhans compared with wild-type controls. Insulin content was decreased in Hfe−/− mice by 35%/pancreas and 25%/islet. Comparable decreases were seen in the mRNA levels of β-cell-specific markers, ins1, ins2, and glucose transporter 2. By 6–8 months, islets from Hfe−/− mice were 45% smaller, associated with increased staining for activated caspase 3 and terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling. Islets from Hfe−/− mice were also desensitized to glucose, with half-maximal stimulation of insulin secretion seen at 16.7 ± 0.9 mM glucose in perfused islets compared with 13.1 ± 0.6 mM glucose in wild-type animals. Carboxyl protein modification, a marker for oxidative stress, was increased by 58% in Hfe−/− islets. Despite decreased islet size, Hfe−/− mice exhibited enhanced glucose tolerance. Fasting serum insulin levels were comparable between Hfe−/− and Hfe+/+ mice, but were 48% lower in the Hfe−/− mice 30 min after challenge. Similar results were seen in mice carrying an Hfe mutation analogous to the common human mutation (C282Y) and in mice fed excess dietary iron. Hfe−/− mice on the C57BL6 background exhibited decreased glucose tolerance at 10–12 months due to an inability to increase insulin levels as they aged. We conclude that iron excess results in β-cell oxidant stress and decreased insulin secretory capacity secondary to β-cell apoptosis and desensitization of glucose-induced insulin secretion. This abnormality alone, however, is insufficient to cause diabetes. (Endocrinology 145: 5305–5312, 2004)

Heredity hemochromatosis type 1 is transmitted as an autosomal recessive trait and occurs at a frequency of approximately five per 1000 in populations of northern European extraction (1, 2). Most patients with hemochromatosis are homozygous for a single nucleotide substitution in the hemochromatosis gene (Hfe), resulting in a change from cysteine to tyrosine at amino acid 282 in the Hfe protein (C282Y) (3). The Hfe gene is located near the human leukocyte antigen A locus, and encodes a 343-amino acid protein (Hfe) that has a wide tissue expression and is involved in the regulation of gastrointestinal iron absorption, although the precise mechanism of its action remains unknown. The Hfe protein exists as a heterodimer with β2-microglobulin that binds to and reduces the affinity of the transferrin receptor for iron-saturated transferrin (4). Phe-notypic expression of hemochromatosis may vary from a fully penetrant clinical syndrome (skin pigmentation, cirrhosis, arthritis, endocrinopathy, and cardiomyopathy) to a simple laboratory abnormality, namely an elevated transferrin saturation without organ injury (1, 5). There is an increasing appreciation, however, that significant morbidity may be present in the affected population even before clinical presentation (6).

Although diabetes is part of the classic presentation of hemochromatosis, little is known about its pathogenesis. It is assumed, but unproven, that excess iron stores are directly responsible for the diabetes. The relative contributions of insulin deficiency, hepatic dysfunction, and insulin resistance to hemochromatosis-associated diabetes, however, are not known. We have therefore examined insulin secretory capacity in mouse models of hemochromatosis and mice with dietary iron overload. Our results demonstrate that iron overload in the β-cell results in decreased insulin secretion secondary to β-cell apoptosis, loss of β-cell mass, and desensitization of glucose-induced insulin secretion. These defects alone, however, are well compensated and not sufficient to cause diabetes.

Materials and Methods

Experimental animals

Mice were of the 129/SvEvTac and C57BL6 genetic background. Targeted mutagenesis was used to produce a knockout (Hfe−/−) and the equivalent C282Y mutation (HfeC282Y) in the mouse (7, 8). The mutation was bred onto the appropriate background for at least eight generations. Dietary iron overload was produced by supplementing the diet of wild-type mice with 20 g/kg carboxyl iron (Teklad T91013, Harlan, Indianapolis, IN). The control diet contained 0.33 g/kg iron (TD8640). Age- and sex-matched wild-type 129/SvEvTac (Hfe−/−) and C57 (Hfe−/−) mice were used as controls. Procedures were approved by the institutional animal care and use committee of University of Utah.

Abbreviations: AIRg, Acute insulin response to glucose; GLUT, glucose transporter; HBSS, Hanks’ balanced salt solution; IPGTT, ip glucose tolerance testing; PPARα, peroxisomal proliferator-activated receptor α; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling.

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Islet isolation

Islets were isolated using the intraductal Liberase RI digestion technique as described previously (9). Briefly, mice were killed by cervical dislocation, and 3 ml Hanks’ balanced salt solution (HBSS) containing 0.3 mg/ml Liberase RI Purified Enzyme Blend (Roche, Indianapolis, IN) were injected into the pancreas through the bile duct. Pancreata were excised and incubated at 37 C for 12 min, briefly shaken, and filtered through a nylon mesh. The islets were then further purified by hand-picking under a dissecting microscope to eliminate any exocrine tissue.

Perfusion studies

Islets were perfused as previously described (10, 11). Briefly, size-matched islets (13–16/study) were isolated and placed on a 62-µm monofilament nylon mesh (Small Parts, Inc., Miami Lakes, FL) inside a 13-mm filter holder (Swinnex, Millipore Corp., Bedford, MA). For basal insulin secretion, islets were perfused in Krebs-Ringer bicarbonate buffer containing 3 mm glucose, 1 mg/ml BSA, 10 mm HEPES (pH 7.3), 1× MEM amino acid solution, 1× MEM nonessential amino acid solution (Invitrogen Life Technologies, Inc., Grand Island, NY), and 5 mM NaHCO3 at a flow rate of 1 ml/min for 30 min before collection of fractions. The Krebs-Ringer bicarbonate buffer was maintained at 37 C in a water bath and was gassed with 95% O2/5% CO2. After the first 30-min period, glucose concentrations perfusing the islets were increased to a final concentration of 35 mM over a period of 100 min using a linear gradient. Fractions (1 ml) of perfusion buffer containing glucose and the released insulin were collected at 1-min intervals. Glucose levels were measured using a Glucose Analyzer 2 (Beckman Instruments, Inc., Fullerton, CA). Insulin levels were assessed by RIA (Sensitive Rat Insulin RIA Kit, Linco Research, Inc., St. Charles, MO).

Determination of tissue iron levels in liver and islets

Iron content was measured on acid hydrolysates of tissue by either atomic absorption spectroscopy or a colorimetric assay (12). Approximately 100 mg liver or 150 islets were used for iron measurements.

Histology and determination of β-cell mass

For terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL) and caspase 3 immunohistochemistry, pancreata (six per group) were fixed in 10% formalin and paraffin-imbbedded, and 10-µm serial sections were collected. DNA fragmentation analysis was performed using TUNEL according to manufacturer's protocol (In Situ Cell Death Detection Kit, Roche, Nutley, NJ) (13) with slight modification. Briefly, sections were deparaffinized and blocked in 3% H2O2/PBS solution for 5 min at room temperature. Samples were then placed in 10 ml 0.1 M citrate buffer, pH 3.0, and subjected to microwave irradiation at 770 watts for 5 min, followed by a 15-min cooling period. Samples were then washed twice with PBS, incubated in permeabilization solution at 37 C, washed in PBS, and blocked again in 20% normal sheep serum, 3% BSA, and 1% (wt/vol) Roche Blocking reagent for 30 min at room temperature. Signal conversion was accomplished by incubating slides in 50 µl Converter-POD solution (1:3 anti-fluorescein antibody conjugated with horseradish peroxidase), followed by washing with PBS. Finally, 3,3'-diaminobenzidine substrate solution was applied for 30 sec, reactions were stopped with dH2O, and tissue sections were counterstained with hematoxylin and visualized under the light microscope. Sections for analysis of activated caspase 3 were deparaffinized, rehydrated, treated with 3% H2O2, and blocked with 5% horse serum in HBSS, followed by treatment with cleaved caspase 3 primary antibody (Cell Signaling Technology, Beverly, MA) and donkey antirabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Four slides per pancreas with an average of 10 islets/slide were used in a blinded fashion.

For determination of islet size, deparaffinized sections (10 µm/sec-
agrose gel electrophoresis confirmed the absence of nonspecific products. Quantitation of cDNA products was accomplished by the Light-Cycler software, using the second derivative maximum or threshold cycle at which the fluorescence clearly increases above background fluorescence. Standard curves of log cDNA amount vs. crossing point cycle number were constructed for each run of the four transcripts of interest. Results for each sample were normalized by dividing the relative amount of each transcript by the relative amount of cyclophilin-A transcript (averaged from triplicate determinations) generated from that sample.

**Determination of intracellular protein oxidation in pancreatic islets**

Cell extracts were prepared from freshly isolated islets by incubation in 100 μl lysis buffer [50 mm Tris-HCl (pH 8.0), 150 mm NaCl, 10% (vol/vol) glycerol, 1% (vol/vol) Nonidet P-40, 1 mm NaF, 2 mm Na3VO4, 1 mm phenylmethylsulfonylfluoride, 1 mm EDTA, and protease inhibitor cocktail tablets; Roche]. Protein carbonyl content was measured according to manufacturer protocol (OxiBlot Protein Oxidation Detection Kit, Chemicon International, Temecula, CA). Briefly, protein samples (5 μg/lane) were derivatized with dinitrophenyl hydrazine, fractionated by 10% SDS-PAGE, and electroblotted to Immobilon-P transfer membranes (Millipore Corp.). The derivatized proteins were sequentially reacted with rabbit antidinitrophenyl and horseradish peroxidase-conjugated goat antirabbit IgG antibodies and visualized by chemiluminescence (Amersham Bioscience, Little Chalfont, UK). Equal protein loading on gels was verified by Coomassie Blue staining. Densitometry measurements were obtained using a UMAX Astra 3450 scanner (UMAX Technologies, Fremont, CA) and NIH Image 1.63 (Bethesda, MD).

**Statistical procedures**

Descriptive statistics are represented as the average ± se. A t test (two-tailed) was used to compare differences between experimental and control groups.

**Results**

**Increased tissue iron concentrations in Hfe<sup>−/−</sup> mice**

Iron concentrations were measured in liver and in pancreatic islets (100 islets/determination; three to five determinations per group). Hfe<sup>−/−</sup> mice had a 72% increase in the level of iron in the islets of Langerhans compared with controls (Fig. 1A; 0.055 ± 0.013 vs. 0.032 ± 0.009 pg/islet; P < 0.05). Hepatic iron was similarly elevated (Fig. 1B; 738 ± 22 vs. 171 ± 45 μg/wet weight; P < 0.01). The iron was elevated by 5 wk of age and remained elevated thereafter (not shown).

**Decreased pancreatic and islet insulin content resulting from β-cell apoptosis and decreased β-cell mass**

We next assessed the effect of iron accumulation on insulin content and β-cell mass. The total pancreatic insulin content (Fig. 2A; P < 0.03) and insulin content per islet (Fig. 2B; P = 0.01) were both lower in Hfe<sup>−/−</sup> mice at 6–8 months of age. The loss of insulin was paralleled by decreased mRNA levels in the total pancreas of the two rodent insulin genes, ins1 and ins2; the liver and β-cell-specific glucose transporter, GLUT2; and the nuclear receptor, PPARα. Ins1 and ins2 mRNA levels, normalized to total pancreatic cyclophilin-A, were decreased in Hfe<sup>−/−</sup> mice to 73% and 68%, respectively, of the levels in wild-type controls (Fig. 2C; P = 0.0002 for both ins1 and ins2). The mRNA for GLUT2 was decreased to 55% of the level in Hfe<sup>+/+</sup> mice (P = 0.001). PPARα mRNA was decreased by 24%, although this result was not statistically significant.

**Decreased glucose sensitivity in Hfe<sup>−/−</sup> mice**

To assess whether there were additional functional defects in glucose-induced insulin secretion, the glucose sensitivity of islets from 8-wk-old mice was determined. Islets were perfused with a linearly increasing gradient of glucose concentrations, and insulin was measured in the perfusate. Islets from the Hfe<sup>−/−</sup> mice were significantly less sensitive to glucose stimulation of insulin secretion compared with control islets, as revealed by a rightward shift in the glucose dose-response curve. Half-maximal insulin secretion was seen at 16.7 ± 0.9 mm glucose in Hfe<sup>−/−</sup> islets compared with 13.1 ± 0.6 mm in wild-type islets (Fig. 5; P < 0.006). These results were confirmed by perfusion with static concentrations of glucose. Namely, Hfe<sup>−/−</sup> islets did not increase insulin secretion when the glucose concentration was increased from 3 to 5 mm, whereas wild-type islets exhibited an increase of 15% (not shown).
Hfe mRNAs for ins1 and ins2 (*, H11006/H11005). Insulin content. The glucose tolerance associated with the decrease in islet insulin content. There were no differences in body weight (Hfe+/+, 21.1 ± 0.8; Hfe−/−, 21.9 ± 0.7 g) or pancreatic weight (Hfe−/−, 0.21 ± 0.01; Hfe+/+, 0.24 ± 0.01 g) in these animals. B, Isolated islets from Hfe−/− mice contained significantly less insulin than controls (0.12 ± 0.01 vs. 0.16 ± 0.01 μg insulin/islet; 10–12 mice/group; *, P < 0.01). C, Lower expression of mRNAs for ins1 and ins2 (*, P < 0.002) and GLUT2 (⁎, P < 0.001) in Hfe−/− mice compared with wild-type controls. Values represent the percent change in Hfe−/− samples (n = 4 separate pancreata) compared with the Hfe+/+ control samples (n = 4).

Increased levels of oxidized protein in islets of Hfe−/− mice

We next determined whether islets isolated from Hfe−/− mice exhibited markers of increased cellular oxidative stress. Protein modification by carbonyl groups was assessed in islets from wild-type and Hfe−/− mice. Densitometric quantification of protein immunoblots revealed a significant increase of 30% in the density of total carbonyl protein modifications in islets from Hfe−/− mice (Fig. 6; P < 0.001). Normal glucose tolerance despite decreased insulin secretory capacity in Hfe−/− mice

Mice of 10 wk of age were next examined for any loss of glucose tolerance associated with the decrease in islet insulin content. The Hfe−/− and Hfe+/+ mice did not differ in weight (21.3 ± 0.5 g for Hfe−/− and 20.6 ± 0.7 g for Hfe+/+; not significantly different). Fasting glucose levels were slightly elevated in the Hfe−/− (100 ± 3 mg/dl for Hfe−/− and 86 ± 2 for Hfe+/+; P < 0.001; Fig. 7A). Surprisingly, however, the Hfe−/− mice had no impairment in glucose tolerance compared with wild-type controls, with significantly lower blood glucose values 60 and 120 min after an ip glucose challenge (1 mg/g body weight; Fig. 7B). The overall area under the glucose curve was 14% lower in Hfe−/− mice compared with controls (P < 0.05; Fig. 7B). The incremental area under the glucose curve was decreased by 52% in the Hfe−/− mice compared with controls (7927.8 ± 476 mg/min·dl for Hfe+/+ and 3772 ± 226 mg/min·dl for Hfe−/−; P < 0.001; n = 21 mice/group; not shown). Fasting serum insulin levels were comparable in Hfe−/− and Hfe+/+ mice (Fig. 7C; 17% lower in Hfe−/−; nonsignificantly different, P = 0.18). Insulin levels at 30 min were 48% lower in Hfe−/− mice (P = 0.001). Abnormal glucose tolerance did not develop in male or female mice as old as 12–14 months, even with additional iron loading by dietary iron or ip iron-dextran (not shown). Insulin tolerance testing revealed no significant differences in insulin sensitivity for blood glucose levels between the Hfe−/− mice and the wild-type controls (Fig. 7D).

Decreased insulin secretion and insulin content, but normal glucose tolerance, in two other models of iron overload

We next examined two other models of iron overload in mice. In the first model, mice harbor a mutation in the Hfe gene that results in the equivalent C282Y mutation found in the majority of hereditary hemochromatosis in humans (Hfe80%). In the second, iron overload was induced by main-
Age-dependent decrease in glucose tolerance in C57BL6
Hfe<sup>−/−</sup> mice

To determine whether diabetes would develop with hemochromatosis in a strain more susceptible to diabetes, we cross-bred the Hfe<sup>−/−</sup> mice onto the C57BL6 background for at least eight generations to generate C57BL6 Hfe<sup>−/−</sup> mice. We examined these mice for changes in glucose tolerance associated with aging. C57BL6 Hfe<sup>−/−</sup> mice did not normally compensate for aging with hyperinsulinemia, as demonstrated by the reduced fasting insulin levels in mice 10–12 months of age (Fig. 9A). This inability to increase insulin levels with age was associated with a modest decrement in glucose tolerance in the C57BL6 Hfe<sup>−/−</sup> mice compared with their age-matched, wild-type counterparts (Fig. 9B).

Discussion

We have demonstrated that iron overload induced by targeted inactivation of the Hfe gene in mice results in decreased insulin secretory capacity. This decrease is due to apoptosis and loss of pancreatic islet size and is associated with increased markers of oxidative stress in the affected islets, as might be expected if the initiating factor is excess iron. Indeed, the similarity of the phenotypes in Hfe<sup>−/−</sup>, mice bearing the mutation found in the majority of human hemochromatosis (Hfe<sup>Wt</sup>), and mice fed excess dietary iron suggests that β-cell failure and β-cell apoptosis are a direct result of iron toxicity. The basis for the sensitivity of the β-cell, in particular, to iron is not completely understood. One possibility is...
that the β-cell simply accumulates more iron than other cells, based on relatively high levels of expression of transmembrane divalent metal transporters (16) needed to facilitate zinc uptake for packaging insulin in secretory granules. The high saturation of transferrin, which is the phenotypic hallmark of hemochromatosis, is associated with the presence of nontransferrin-bound iron in plasma, and nontransferrin-bound iron is readily taken up by cells via divalent metal transporter-1 (17, 18). Potentially high iron fluxes through this pathway might explain the high levels of apo-ferritin in normal β-cells (19). The β-cell may also be particularly susceptible to oxidative damage, perhaps based on the nearly exclusive reliance upon mitochondrial metabolism of glucose for glucose-induced insulin secretion. Oxidative damage to mitochondria may explain both the impairment of glucose sensing for insulin secretion as well as subsequent apoptosis.

Part of the classic clinical description of human hemochromatosis is diabetes, but the true prevalence of diabetes and the relative contributions to that diabetes of insulin secretory defects, insulin resistance, and altered glucose production by the liver have not been systematically examined. One study of human subjects with hemochromatosis demonstrated a significant decrease in the acute insulin response to glucose (AIRg) during an iv glucose tolerance test (20), but this was true in subjects both with and without diabetes. Reduction of iron stores by phlebotomy increased the AIRg by 35%. Similar impairment of the β-cell response in the face of iron overload has been seen in patients with thalassemia major (21) and in rats subjected to experimental iron overload (8). Other studies have demonstrated insulin resistance in subjects with diabetes and hemochromatosis (22), particularly in the presence of hepatic disease (23), and phlebotomy can result in improved insulin sensitivity (24).

The mouse models of hemochromatosis, like many humans with the disease, do not develop diabetes despite de-
creased insulin secretory capacity. This is true in both the 129/Sv and the more diabetes-prone C57BL6 strains, although in the latter, a modest degree of glucose intolerance did develop with aging. There are several possible explanations for this. First, there may simply be a quantitative requirement for more time or more iron overload to lead to loss of a critical mass of β-cells. We have followed mice for up to 14 months, and we have fed Hfe−/− mice excess carbonyl iron or injected iron dextran ip to accentuate the iron overload, but in none of these cases has diabetes developed (not shown). These maneuvers do not rule out the possibility that a critical threshold of iron-induced damage still has not been reached. In surgical pancreatectomy models, resection of 85–95% of the pancreas is generally required to reproducibly induce hyperglycemia in the short term (25). Resection of 70–85% of the pancreas results in hyperglycemia in a period of weeks (26), and up to 50% resection is generally well tolerated in an otherwise metabolically normal individual (27). Of note, however, older Hfe−/− mice did exhibit normal glucose homeostasis despite chronic decreases in insulin levels after glucose challenge of up to 68% (not shown), close to the threshold of the 70% loss of insulin that can result in diabetes.

Another possible explanation for the failure to develop diabetes is that a second insult to the organs involved in fuel homeostasis is needed. That is, with the levels of iron overload found in typical hemochromatosis, it may be unusual to develop diabetes purely on the basis of decreased insulin secretion. If, however, insulin resistance were to develop concomitantly with the insulin secretory defect, diabetes might result. Consistent with this, the Hfe−/− mice were not insulin resistant, as evidenced by normal insulin testing. There is evidence to support the hypothesis that insulin resistance may be needed in hemochromatosis before diabetes will develop. For example, insulin resistance can be seen in subjects with diabetes resulting from hemochromatosis (22, 23) or iron overload (24). The origin of this insulin resistance may be multifactorial. Insulin resistance is a hallmark of typical type 2 diabetes (28–30), and it is possible that those individuals with hemochromatosis who develop diabetes are those who are also genetically at risk for type 2 diabetes. In fact, if type 2 diabetes is a disease that results when insulin resistance is no longer compensated because of β-cell failure (28), one would predict that in kindreds with both hemochromatosis and typical type 2 diabetes, the “built-in” β-cell insufficiency resulting from hemochromatosis might result in earlier and/or more severe presentations of type 2 diabetes.

Another potential mechanism for insulin resistance and progression to diabetes in individuals with hemochromatosis is iron-induced liver damage. This possibility is supported by the known relationship between diabetes and cirrhosis of any etiology. For example, there is a 24% prevalence of diabetes in cirrhotic subjects with hepatitis C, whereas hepatitis without cirrhosis is not associated with diabetes (31). Fasting insulin levels in the cirrhotic subjects with diabetes are significantly elevated, consistent with a major role for insulin resistance in the diabetic phenotype (31). Treatment of hepatitis C with interferon improves insulin sensitivity by nearly 3-fold, but does not affect insulin secretion (32). The relationship between diabetes and hepatic damage in hemochromatosis is also supported by our review of records of individuals with hemochromatosis. Of 104 clinically affected male probands, 32 (31%) had diabetes, and of these, 23 had biopsy-proven cirrhosis, five had moderate fibrosis, and only four had normal liver architecture (Kushner, J. P., unpublished observations). Thus, the failure of Hfe−/− mice to progress to diabetes may be related to the fact that these mice do not develop cirrhosis or significant liver disease (Ajioka, R., and J. P. Kushner, unpublished observations).

An interesting observation made in these studies is that insulin deficiency is compensated and does not result in significant glucose intolerance. We have observed the same phenomenon in humans with hemochromatosis. Although other studies have demonstrated insulin resistance with hemochromatosis after the onset of diabetes (21, 22), when these individuals are studied before the onset of diabetes, there is a decrease in AIRg that is nearly fully compensated by increased insulin sensitivity (McClain, D., J. Kushner, and D. Abraham, manuscript in preparation). It is known that organisms respond to hyperinsulinemia by compensatory decreases in insulin sensitivity (33–36), and the converse (increased insulin sensitivity with hypoinsulinemia) has been observed in animal models (37). The basis for this in-
crease in insulin sensitivity is currently under study and may involve decreased adiposity resulting from hypoinsuline-
ma. Hepatic glucose production may also be dysregulated
because of hepatic iron overload, although fasting glycemia
and responses to prolonged fasting (not shown) are not
markedly different in Hfe–/– mice.

In summary, we report that iron overload in mouse mod-
els of hemochromatosis results in a loss of insulin secretory
function, which involves decreased adiposity resulting from hypoinsuline-
ma, decreased insulin sensitivity, and increases in insulin-resistant
muscle. These findings suggest that iron overload in humans may
also decrease insulin sensitivity and increase insulin resistance.

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Address all correspondence and requests for reprints to: Dr. Donald A. McClain, Division of Endocrinology, University of Utah School of Medicine, 30 North 2030 East, Salt Lake City, Utah 84132. E-mail: donald.mcclain@hsc.utah.edu.

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