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Dietary Iron Overload Induces Visceral Adipose Tissue Insulin Resistance

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Increased iron stores associated with elevated levels of the iron hormone hepcidin are a frequent feature of the metabolic syndrome. The aim of this study was to assess the effect of dietary iron supplementation on insulin resistance and the role of hepcidin in C57Bl/6 male mice fed a standard or iron-enriched diet for 16 weeks. Iron supplementation increased hepatic iron and serum hepcidin fivefold and led to a 40% increase in fasting glucose due to insulin resistance, as confirmed by the insulin tolerance test, and to threefold higher levels of triglycerides. Iron supplemented mice had lower visceral adipose tissue mass estimated by epididymal fat pad, associated with iron accumulation in adipocytes. Decreased insulin signaling, evaluated by the phospho-Akt/Akt ratio, was detected in the visceral adipose tissue of iron overloaded mice, and gene expression analysis of visceral adipose tissue showed that an iron-enriched diet up-regulated iron-responsive genes and adipokines, favoring insulin resistance, whereas lipoprotein lipase was down-regulated. This resulted in hyperresistinemia and increased visceral adipose tissue expression of suppressor of cytokine signaling-3 (Socs3), a target of resistin and hepcidin implicated in insulin resistance. Acute hepcidin administration down-regulated lipoprotein lipase and up-regulated Socs3 in visceral adipose tissue. In conclusion, we characterized a model of dysmetabolic iron overload syndrome in which an iron-enriched diet induces insulin resistance and hypertriglyceridemia and affects visceral adipose tissue metabolism by a mechanism involving hepcidin up-regulation. *(Am J Pathol 2013, 182: 2254–2263; http://dx.doi.org/10.1016/j.ajpath.2013.02.019)*

The dysmetabolic iron overload syndrome (DIOS), characterized by fatty liver, increased ferritin levels, and increased body iron stores in the presence of insulin resistance (IR), is observed in 15% to 30% of patients with the metabolic syndrome (MetS)[1] and is associated with increased risk of type 2 diabetes (T2D) and organ damage.[2] Dietary iron intake has been associated with T2D,[3] and iron depletion decreased IR in controlled studies.[4] Differently from genetic iron overload disorders,[5] DIOS is characterized by preserved up-regulation of the iron hormone hepcidin,[1] which inhibits iron absorption and recycling by binding and inactivating the cellular iron exporter ferroportin-1.[6] Therefore, the pathogenesis of iron accumulation in DIOS has been related to altered iron trafficking associated with steatosis, hepatic inflammation, and IR.[1,7]

DIOS is also associated with altered release of adipokines,[8] iron treatment decreases insulin-stimulated glucose transport and increased lipolysis in adipocytes *in vitro*,[9] and increased ferritin levels and iron indices have been associated with adipose tissue insulin resistance in a population study.[10]

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In obesity models, iron accumulation induced IR by still unclear mechanisms, whereas dietary restriction or chelation protected against diabetes and decreased IR.[11–15] Mice with iron overload caused by down-regulation of hepcidin due to deletion of the hemochromatosis gene (*Hfe*) are characterized by decreased glucose oxidation in skeletal muscle despite increased uptake,[11] leading to higher hepatic glucose output but not to hyperglycemia or dyslipidemia.[12] Recently, increased hepcidin levels have been associated with MetS in the general population,[16] but the role played by hepcidin in the pathogenesis of metabolic abnormalities could not be investigated in previous experimental models, which, unlike DIOS, were not characterized by increased hepcidin.

Therefore, the aims of this study were to assess whether dietary iron overload leading to increased hepcidin release influenced glucose and lipid metabolism and insulin resistance in C57Bl/6 mice and to investigate the mechanisms by analyzing insulin signaling alterations in target tissues and the role of hepcidin.

Materials and Methods

Animal Model

Six-week-old, male C57Bl/6 mice were purchased from Charles River (Charles River, Calco, Italy), housed at constant room temperature (23°C) under 12-hour light/dark cycles with *ad libitum* access to water in compliance with the Principles of Laboratory Animal Care (NIH publication 86-23), and fed either standard iron concentration diet (8 mg/kg; control, measured by atomic absorption spectrometry in regular commercial chow diet) or an iron-enriched diet (IED; 3% carbonyl-iron, a highly pure form of iron, which does not contain carbohydrates)[17] for 16 weeks. Experiments were conducted in 15 mice per group, unless otherwise specified. Body weight and food intake were measured weekly. In a second set of experiments, supplemental fructose was administered as 30% fructose in drinking water [high-fructose diet (HFRD)], as a model of fatty liver.[18]

A glucose tolerance test (GTT; 2 g/kg of glucose)[19] and an insulin tolerance test (ITT; 0.75 U/kg)[20] were performed after prolonged overnight fasting at 16 weeks; afterward animals were anesthetized and sacrificed. For hepcidin treatment, 8-week-old male mice ($n = 5$) fed control diet were injected i.p. with 20 μg of hepcidin-25 in 0.3 mL of PBS, with a second dose injected 20 hours later,[21] and sacrificed at 24 hours. A comparable control group ($n = 5$) was injected with PBS only.

Blood was collected, and liver and epididymal visceral adipose tissue (VAT) were dissected and flash frozen in liquid nitrogen. Samples of liver and VAT were also collected for quantification of iron by atomic absorption spectrometry and histologic analysis. The experimental protocol was approved by the University of Milan and Italian Ministry of Health review boards (protocol 13/10).

Biochemical Measurements

Blood glucose was measured by an Accu-Check glucometer (Roche Diagnostics AVIVA, Mannheim, Germany). Serum iron, plasma triglycerides, and alanine aminotransferases levels were measured with an automated analyzer. Plasma insulin, resistin, tumor necrosis factor-α, and IL-6 levels were determined using a multiplex assay (kit MADPK-71K; Millipore, Billerica, MA). Measurement of lipid peroxidation (thiobarbituric acid reactive substances) was performed using the thiobarbituric acid reaction as previously described.[22] Total iron regulatory protein (IRP) activity, reflecting cytosolic iron availability (the higher IRP activity, the lower iron availability), was measured by RNA band shift assay, as described.[23]

Serum hepcidin was measured by mass spectrometry using a method based on surface-enhanced laser desorption/ionization time of flight. A synthetic hepcidin analog (Hepcidin 24; Peptides International, Louisville, KY) was used as an internal standard.[24]

Isolation of Tissue RNA and qPCR

RNA was isolated from tissues by the Trizol reagent (Life Technologies, Carlsbad, CA). First-strand cDNA was synthesized starting from 1 μg of total RNA using the VILO random examers synthesis system (Life Technologies). Real-time quantitative PCR (qPCR) was performed by using the SYBR green chemistry (Fast SYBR Green Master Mix; Life Technologies). All of the reactions were delivered in triplicate with the 7500Fast Real-Time PCR system (Life Technologies) in a 25-μL final volume. Primers are listed in Table 1. Custom Taqman array plates were purchased from Life Technologies, and the reactions were delivered in a 10-μL final volume. Evaluated target and control genes are presented in Supplemental Table S1.

Western Blot Analysis

Cell extracts were prepared from 20 mg of tissue, and equal amounts of proteins (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane (BioRad, Hercules, CA). Membranes were incubated with polyclonal rabbit Akt antibody (C#9272; Cell Signaling Technology, Danvers, MA), polyclonal rabbit phospho(p)-Akt antibody (#9271; Cell Signaling), polyclonal rabbit Srebp1c antibody (sc-366; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit C/EBPα antibody (sc-61; Santa Cruz Biotechnology), and polyclonal goat β-actin antibody (sc-1615; Santa Cruz Biotechnology).

Histological Analysis

Liver and VAT tissue samples were fixed in 10% PBS-buffered formalin. All tissues were embedded in paraffin within 48 hours of formalin fixation. Histologic staining was performed using H&E and Perls Prussian blue method to