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Iron Regulation of Pancreatic Beta-Cell Functions and Oxidative Stress

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Abstract

Dietary advice is the cornerstone in first-line treatment of metabolic diseases. Nutritional interventions directed at these clinical conditions mainly aim to (*a*) improve insulin resistance by reducing energy-dense macronutrient intake to obtain weight loss and (*b*) reduce fluctuations in insulin secretion through avoidance of rapidly absorbable carbohydrates. However, even in the majority of motivated patients selected for clinical trials, massive efforts using this approach have failed to achieve lasting efficacy. Less attention has been given to the role of micronutrients in metabolic diseases. Here, we review the evidence that highlights (*a*) the importance of iron in pancreatic beta-cell function and dysfunction in diabetes and (*b*) the integrative pathophysiological effects of tissue iron levels in the interactions among the beta cell, gut microbiome, hypothalamus, innate and adaptive immune systems, and insulin-sensitive tissues. We propose that clinical trials are warranted to clarify the impact of dietary or pharmacological iron reduction on the development of metabolic disorders.

Contents

INTRODUCTION

Pancreatic beta-cell failure is a precondition in the pathogenesis of diabetes, except in rare cases involving severe mutations in the insulin receptor signaling pathway causing insulin resistance insurmountable by compensatory increases in secreted insulin. This *sine qua non* relationship has long been appreciated in type 1 diabetes (T1D), which is caused by an immune-mediated selective destruction of beta-cell mass that leads to absolute insulin deficiency (10). Only recently has it

become generally accepted that type 2 diabetes (T2D), conventionally believed to be caused by insufficient insulin action, is a beta-cell disease with consequently relative insulin deficiency (103).

This paradigm shift is supported by strong epidemiological, genetic, and pathophysiological evidence. Even people with morbid obesity and grave insulin resistance are not doomed to develop diabetes provided they are not genetically destined for a failing functional beta-cell mass (104). Indeed, the majority of genes predisposing to unusual monogenic or common polygenetic T2D regulate beta-cell development, regeneration, nutrient sensing, or secretory function (8, 56). An important basis for the clinical pathophysiology of T2D is the hyperbolic relationship between insulin resistance and insulin secretion (103), often termed the Starling curve of the beta cell: With increasing insulin requirements—physiologically observed during growth, puberty, and pregnancy and pathologically observed in obesity, in certain endocrine disorders, or during treatment courses with drugs provoking insulin resistance—the inherent plasticity of the functional beta-cell mass will strive to compensate by proportional increments in secreted insulin, titrated to achieve a metabolic homeostasis within amazingly narrow boundaries. Nutrient excess is a major signal for beta-cell compensation (167) that encompasses increased insulin biosynthesis and exocytosis in the active beta-cell mass; recruitment of quiescent beta-cell mass; beta-cell hypertrophy and hyperplasia; neogeneration from beta-cell precursors; and transdifferentiation from other endocrine cells, ductal cells, or even exocrine acinar tissue. It is only when these compensatory mechanisms fail that glucose intolerance and later T2D develop.

The molecular mechanisms that determine the switch between physiological compensation and pathological beta-cell decompensation in T2D are incompletely understood, and three hypotheses prevail. The first purports that elevated circulating nonesterified free fatty acids (FFAs) during evolving obesity and insulin resistance, and later elevated blood glucose as glucose dyshomeostasis becomes manifest, lead to a synergistic beta-cell toxic action (termed glucolipotoxicity) (167). This theory has been challenged by the lack of detectable elevated FFAs in insulin-resistant individuals (105). The second hypothesis contends that nutrient excess leads to a shift from beta oxidation to lipid storage with accumulation of toxic lipid intermediates (48), a concept termed lipid partitioning. In the third theory, excess nutrients and islet amyloid polypeptide cosecreted with insulin are perceived as danger-associated molecular patterns that activate islet macrophage and/or beta-cell inflammasomes to process proinflammatory procytokines, such as pro-interleukin-1 (proIL-1) and proIL-18, in turn causing inflammatory beta-cell damage (206).

Most of these proposed mechanisms involve the generation of oxidative stress in which iron plays a key role as a catalyst of the Fenton reaction, highlighting the importance of iron in the regulation of beta-cell function and stress, which is the focus of this review. A recent surge of original papers on this topic, which has not yet been covered by many reviews (77), provides further evidence of the timeliness of the present article.

A more important and translational perspective for this review is the appalling nature of the global health threat of T2D, which bluntly speaking is a problem that is out of control. Bleak figures recently released by the International Diabetes Federation reveal that worldwide, 387 million people (8.3% of the population) are affected with diabetes, a number projected to reach 592 million in 2035; in 2014, approximately 5 million people died from the disease; and costs for multifactorial prevention and treatment of late diabetic complications were $612 billion ($1 out every $9 spent on health care) in 2014 and are increasing at a dramatic pace (93).

No causative therapy or curative treatment for diabetes exists. Although T2D is in principle preventable by lifestyle measures, effectiveness of such measures has been shown only in selected populations. Weight reduction is generally transient even in highly motivated research groups (202), and the impact of public health campaigns on rates of obesity or diabetes morbidity and mortality is uncertain, with motivation and adherence being major stumbling blocks. It is therefore

imminent to assess the possibilities for more selective, focused interventions that are amenable for large-scale implementation and that do not have serious adverse effects. Nutritional guidelines directed at changes in the intake of micronutrients might be more successful than guidelines directed at changes in macronutrients.

This review assesses the evidence for a role of iron in metabolic and inflammatory beta-cell failure and death. Reminded by John Donne's famous 1624 quote, "No man is an island," also no pancreatic islet is an island. Therefore, we do not focus strictly on the pancreatic beta cell, but rather take a broader view on the integrative physiological and pathophysiological interactions between the effects of iron on the beta cell and the actions of iron on metabolism and immunity relevant to diabetes pathogenesis. Through this approach, we demonstrate that appropriate adjustments of iron levels and handling have clinical benefits beyond protecting insulin secretion. The provoking and controversial message of the review is that nutritional therapy that is proven safe and effective in clinical trials and is aimed at reducing iron intake to obtain an iron saturation in the low-normal range (without causing frank anemia) may be necessary to dam up the diabetes tsunami. In high-risk individuals, pharmacological iron chelation may be warranted.

NUTRITION, IRON, AND THE GUT

Iron is required for normal beta-cell function, but in excess it becomes toxic (77). Because the body cannot synthesize iron itself and lacks a physiological mechanism for excreting iron, iron levels are carefully balanced via iron absorption from nutritional sources. How iron is absorbed by the enterocytes of the gut, and how iron levels affect the composition of the gut microbiota, are topics addressed in the following sections.

The human body contains approximately 3.5 g iron, of which most is found in erythrocytes in hemoglobin (approximately 2.3 g). The remaining iron is localized in skeletal muscle fibers in myoglobin (0.35 g), liver (0.2 g), macrophages (0.5 g), and bone marrow (0.15 g) (192). The body loses approximately 1–2 mg iron/day via sloughing of epithelial cells and menstruation (92). To offset this loss and maintain iron stores, the body absorbs 1–2 mg iron/day from external sources. A typical Western diet provides 15 mg iron/day, from which only approximately 10% is absorbed. Iron absorption can, however, be increased up to 20-fold to recover from blood loss (67).

Dietary iron exists as heme and nonheme iron. Heme iron is found in hemoglobin and myoglobin and is derived from red meat, fish, and poultry; nonheme iron stems mainly from fruits and vegetables. The iron source in a typical Western diet is mainly heme iron (approximately 90%). Whereas heme iron is readily absorbable via incorporation into porphyrins, nonheme iron exists primarily as ferric iron (Fe^{3+}), which must be reduced to ferrous iron (Fe^{2+}) to be absorbed. Nonheme iron thus has a lower bioavailability than heme iron, but absorption of nonheme iron can be increased by additional intake of substances such as ascorbic acid (e.g., from citrus fruits). If ascorbic acid is not tolerated, iron uptake is often ensured by supplementation with heme iron. The diet contains natural inhibitors of nonheme iron absorption, including phytate (which is found in vegetables) and polyphenols (which are present in tea and coffee). The bioavailability of heme iron is thought to be less affected by the aforementioned substances, although calcium is believed to inhibit absorption of both heme and nonheme iron (83).

Intestinal Handling of Iron

No physiological mechanism exists for the excretion of iron, unlike other minerals. Iron balance is thus regulated through iron absorption, iron recycling, and mobilization of iron from the liver and other tissue stores (186). Although the absolute absorption of dietary iron is small, it is a tightly regulated process because both iron deficiency and overload have detrimental effects. Absorption

of heme and nonheme iron takes place mainly at the duodenal enterocytes, albeit by different mechanisms. Following reduction of ferric iron to ferrous iron by the ferrireductase duodenal cytochrome b reductase (Dcytb) located in the apical membrane, nonheme iron is transported into the enterocyte via the divalent metal transporter 1 (DMT1). Heme iron is easily absorbed, likely via heme carrier protein 1, although the exact mechanism is not understood. Once inside the enterocyte, it is believed that heme iron is released from its porphyrin structure by hemeoxygenase, thereby enabling it to enter the same pathways as nonheme iron in the form of ferrous iron.

Inside the enterocyte, a fraction of ferrous iron binds to ferritin and is thereby stored. Iron can be released from this ferritin store if additional iron is needed for oxygen transport or energy metabolism. However, the majority of iron is released from the enterocyte via ferroportin, localized at the basolateral membrane, to enter the bloodstream. Total body iron is therefore found either in iron-containing proteins such as hemoglobin and myoglobin, circulating bound to transferrin, or in storage bound to ferritin, primarily in the liver and bone marrow.

In situations of iron deficiency or overload, the body alters the level of absorption by regulating Dcytb, DMT1, and ferroportin. A key regulator of systemic iron homeostasis is the hormone hepcidin. Hepcidin is mainly synthesized in the liver, but secretion of hepcidin may also occur from macrophages, pancreatic islets, and adipose tissue (41). Hepcidin is secreted in response to iron overload and is believed to decrease iron levels by binding to and degrading ferroportin, thus preventing cellular release of iron into the blood stream (152).

Iron and the Microbiome

The iron-absorbing enterocytes of the duodenum normally coexist peacefully with the gut microbiota, and ingested iron sustains the gut microbiome and its physiological functions (**Figure 1**). The gut microbiome is now recognized to be an important regulator of metabolic homeostasis. Animal studies suggest that a skewing of the normal compositional balance causes obesity and metabolic diseases (174), although the cause-effect relationship in humans is still debated. However, bacterial pathogens also depend on iron that they acquire from their host to colonize and potentially cause disease (155). Nutritional immunity refers to a first-line defense system in which nutritional iron is sequestered to prevent colonization of pathogenic bacteria.

Environmental factors, such as socioeconomic status, cultural traditions, and agriculture, influence diet and thereby the worldwide variation of nutritional levels of iron. Increasing evidence demonstrates how the nutritional value of ingested foods is affected by the composition of the gut microbiome as well as how the composition of food influences gut microbiota (106).

In terms of micronutrients such as iron, the focus has been mainly on the effect of inadequate levels, that is, iron deficiency. Iron deficiency is the most common nutritional deficiency, causing varying degrees of symptoms from fatigue and poor performance to mental retardation and perinatal mortality in the infants of iron-deficient mothers. To ensure adequate levels of iron and prevent the harmful effects of iron deficiency, foods are fortified and supplements are used. However, if not properly monitored, iron fortification and supplementation may result in excess iron intake, which can increase pathogen abundance and intestinal inflammation of the gut (96, 192). Interestingly, the composition of gut microbes can also affect the iron-related protein signature of intestinal enterocytes (44). Accordingly, enterocytes of germ-free mice exhibit high levels of proteins involved in apical iron uptake (Dcytb and DMT1), whereas levels of proteins involved in iron storage or basolateral export (ferritin or ferroportin, respectively) are decreased. Following microbial colonization, the protein signature is reversed, favoring proteins involved in iron storage and export rather than iron uptake. Hence, complex cross talk takes place between the gut microbiota and the iron-absorbing enterocytes of the duodenum.

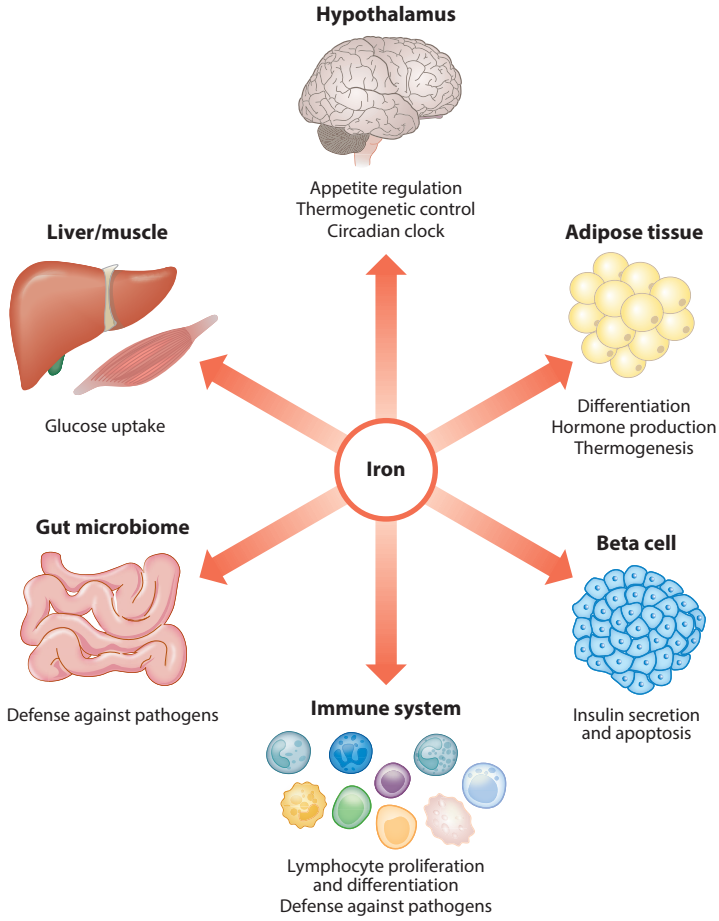

Figure 1

Tissue iron levels control body metabolism. In the brain, iron levels of the hypothalamus are altered by obesity and are believed to control neuronal function, possibly affecting neurons controlling hunger and satiety. Iron is also essential for regulation of the circadian clock and for perception of cold. Adipose tissue iron levels are important for adipocyte differentiation, production of leptin and adiponectin, and function of brown adipose tissue. Iron controls glucose homeostasis by regulating beta-cell insulin secretion, muscle glucose uptake, and liver gluconeogenesis. In addition, iron levels are important for defense against pathogens in the gut microbiome and for lymphocyte differentiation in the immune system.

IRON AND METABOLIC HOMEOSTASIS

Over the past two decades, it has become increasingly clear that appropriate iron levels are critical for maintaining body metabolism, and elevated iron stores (expressed as serum ferritin concentrations) have been proposed to be a component of the metabolic syndrome (59, 63). Although the exact role of iron in metabolic homeostasis is not fully understood, a number of mechanisms have been proposed, which we discuss in the following sections.

Iron and Hypothalamic Control

Iron is important in maintaining neuronal function and survival in the hypothalamus. Interestingly, hypothalamic neurons express low levels of the iron exporter ferroportin (16). Whether the low

expression of ferroportin in hypothalamic neurons reflects a physiological need for constantly elevated iron levels is not settled.

Increased iron accumulation is seen in hypothalami from obese individuals compared to controls and correlates with increased hepatic iron accumulation (9). Obesity is known to induce an inflammatory response in the hypothalamus with the transcription factor nuclear factor (NF)-κB as central mediator. Hypothalamic inflammation causes neuronal dysfunction via increased reactive oxygen species (ROS) production and endoplasmic reticulum (ER) stress, resulting in perturbed appetite regulation and energy homeostasis, further increasing weight gain and insulin resistance (22, 221). In addition, intracerebroventricular injections of low-dose tumor necrosis factor-alpha (TNF-α) induce hypothalamic inflammation and also result in decreased expression of brown adipocyte thermogenetic genes, hyperinsulinemia, and hepatic and skeletal muscle insulin resistance (3).

Iron and Temperature Regulation

Iron status regulates body temperature and metabolic rate. Iron-depleted or anemic rats and humans have lower body core temperature, correlating with decreased metabolic rate and heat production (176). Anemic patients have a reduced threshold for cold-induced shivering and elevated levels of norepinephrine (176), which activate brown adipocytes and lead to increased heat production in an attempt to restore normal body temperature.

Normal iron status is also important for normal brown adipocyte function (133). Interestingly, insulin induces iron uptake in brown adipocytes. Although the precise mechanism is unknown, insulin promotes iron uptake and accumulation in other adipocytes by increasing the number and distribution of transferrin receptors on the cell surface, as transferrin receptors colocalize with glucose transporters. The insulin-induced iron uptake in brown adipocyte is associated with increased lipofuscin granules, a marker of cellular degeneration/senescence (110), suggesting a harmful effect of insulin-induced iron uptake in brown adipocytes.

Iron and Appetite Regulation

Iron treatment of anemic rat pups results in restoration of hypothalamic iron levels (169), suggesting a functional iron uptake system in the hypothalamus. Additionally, dietary iron is positively correlated with nitric oxide synthase (NOS) levels in the hypothalamus (109). NOS produces nitric oxide, which is an important messenger of appetite regulation, and NOS inhibition causes decreased food intake (193). Decreased iron levels in the hypothalamus in rats fed an iron-depleted diet correlate with decreased levels of gamma-aminobutyric acid (GABA)-synthesizing enzyme glutamic acid decarboxylase and GABA-degrading enzyme GABA-T (128), suggesting that iron is also important for regulation of intraneuronal signaling. Dietary iron supplementation in excess of the daily requirement increases appetite, suggesting that inappropriate iron intake can induce obesity. Iron and copper (Cu) supplementation of a high-fat diet accelerates weight gain and increases adipose tissue in rats (198). These observations suggest direct effects of iron on hypothalamic appetite regulation.

Iron and Adipocyte Biology

Adipocytes require iron for normal function and differentiation. Adipocyte iron is primarily regulated by its high expression of ferroportin (65). Ferritin light (L) and heavy (H) chains form aggregates to generate iron-storage ferritin protein in the cytoplasm. The ferritin L:H chain ratio

is lowered during adipocyte differentiation due to increased expression of ferritin H chain, which is important for protection against lipid peroxidation resulting from the ferroxidase activity of ferritin H that oxidizes ferrous iron to ferric iron (25, 64).

Alterations in adipocyte mitochondrial iron content and mitochondrial Fe-S proteins affect adipocyte differentiation and insulin sensitivity (29, 146). Although the precise mechanism is unknown, it is proposed that iron enhances mitochondrial biogenesis in adipocytes, as iron chelation leads to reduced expression of genes involved in both mitochondrial biogenesis and adipogenesis (146). Knockdown of mitoferrin (Mfrn) 1 and 2, two mitochondrial iron-storage proteins, reduces mitochondrial iron content and consequently oxygen consumption and adenosine triphosphate (ATP), which results in decreased expression of adipocyte-specific genes (29). Epididymal white adipose tissue lipolysis depends on serum transferrin and iron-mediated oxidation via an unknown mechanism (177).

Excess iron increases adipocyte ROS formation and induces adipocyte dysfunction. Thus, treatment of adipocytes with iron decreases transcription of leptin, a hormone produced by adipocytes that negatively regulates appetite via interference of the cAMP response element-binding protein binding sites of the leptin promoter (68). In T2D patients, increased iron levels are associated with decreased levels of adiponectin and thus lower adipocytokine-mediated insulin sensitivity due to iron-mediated transcriptional repression by forkhead box protein O1 (FOXO1) in adipocytes (65). In mice fed a high-fat diet, adipocyte-specific overexpression of the mitochondrial 2Fe-2S iron uptake inhibitor protein mitoNEET led to decreased mitochondrial iron content and decreased mitochondrial function. Despite promoting further obesity owing to the combination of compensatory increased lipid influx and reduced oxidative capacity, the decreased mitochondrial iron content was associated with improved insulin sensitivity via enhanced adiponectin release (113).

Iron in Muscle and Liver Glucose Metabolism

In muscle and liver, iron levels are important for insulin signaling and glucose uptake. Mice fed an iron-restricted diet for 78 days had increased expression of the insulin receptor and glucose transporter 4 (Glut4) in skeletal muscle, compared to mice fed with iron-supplemented diet (141). Accordingly, iron supplementation in pregnant rats impaired glucose tolerance by decreasing expression of Glut4 in skeletal muscle (82). In Hfe knockout mice [a model of hereditary hemochromatosis (HH)′, the increased muscle iron contents resulted in activation and phosphorylation of muscle 5'AMP-activated protein kinase (AMPK), probably secondary to reduced Glut4-mediated glucose uptake, thereby aiming at restoring glucose disposal (88).

In C57Bl/6 mice, dietary iron supplementation leading to increased hepatic iron levels enhanced the production of ROS and oxidative stress while impairing insulin signaling in hepatocytes (7, 89). The harmful effects of iron overload in hepatocytes were associated with NF-κB activation, phosphorylation and activation of the MAPK p38 and ERK, and disruption of the balance between pro- and antiapoptotic proteins of the B-cell lymphoma-2 (Bcl-2) family (7).

Excess iron is believed to cause dysfunctional insulin action in muscle and liver via several mechanisms that are not yet fully understood. One mechanism is believed to be activation of stress pathways with the formation of ROS, which via hydroxylation of phenylalanine residues of insulin results in reduced affinity of the insulin receptor for insulin. ROS can also activate FOXO1, promoting insulin resistance, and AMPK, promoting glucose uptake and fatty acid oxidation (188).

Taken together, these findings indicate that iron is important for key processes in the maintenance of metabolic homeostasis, such as neuronal function and survival, body temperature and metabolic rate regulation, adipocyte function and differentiation, and insulin signaling in liver and muscle. Evidence suggests that iron levels need to be maintained within a narrow range, as excess

iron is associated with obesity and ROS formation, which contributes to adipocyte dysfunction and impaired insulin signaling in muscle and liver (**Figure 1**).

IRON AND THE INNATE AND ADAPTIVE IMMUNE SYSTEMS

Beta-cell failure in both T1D and T2D is associated with islet infiltration, in the case of T1D with both adaptive and innate immune cells, and in the case of T2D with only innate immune cells. In T1D, islet inflammation is triggered by an autoimmune process, whereas T2D is associated with low-grade systemic and local inflammation in many tissues, including the hypothalamus, adipose tissue, liver, and pancreatic islets. The systemic low-grade inflammatory condition is indicated by slightly but significantly elevated C-reactive protein, proinflammatory cytokines and chemokines, and number and activation levels of leukocyte populations (47).

Iron is closely interconnected with the immune system. Both inflammatory and infectious stimuli result in the production of hepcidin (12). Bacteria require iron to achieve full virulence, and the production of hepcidin thereby serves as a first-line defense system by reducing cellular ferroportin-mediated iron release as detailed in the following section. Deprivation of iron decreases the pathogenicity of several bacteria, such as *Legionella pneumophila* and *Mycobacterium tuberculosis* (157). In contrast, excess iron has been shown to interact with and strengthen the severity of infections, including malaria and human immunodeficiency virus (HIV) (155). Individuals with gross iron overload due to thalassemia and HH have increased susceptibility to infections, further demonstrating the bimodal effects of iron on infectious defense (67). The more direct effects of iron on innate and adaptive immunity are elucidated in the following sections.

Iron and Innate Immunity

Tight regulation of iron homeostasis serves as a defense mechanism used by the innate immune system against invading pathogens. The sequestration of circulating iron, which restricts bacterial iron utilization, is referred to as nutritional immunity. This iron-withholding strategy is achieved by different means. Hepcidin, which is secreted from the liver in response to iron overload, is also released in response to infectious or inflammatory stimuli (144). Accordingly, activation of pattern-recognition receptors leads to production of the cytokine interleukin (IL)-6, which induces expression of hepcidin via the signal transducer and activator of transcription-3 pathway and bone morphogenic protein signaling. Hepcidin promotes ferritin synthesis while degrading ferroportin in macrophages, neutrophils, hepatocytes, and enterocytes, thereby lowering iron release to the circulation.

In addition, hepcidin-independent mechanisms act to strengthen the iron-limiting defenses against intracellular pathogens. These include downregulation of DMT1-mediated iron absorption, increase in ferritin synthesis, and production of glycoproteins, such as lactoferrin, that sequester free iron (26). During long-term inflammation, hepcidin-induced iron sequestration in macrophages limits the availability of iron for erythropoiesis and thereby contributes to the syndrome anemia of inflammation, also known as anemia of chronic disease (166).

Most pathogens have developed high-affinity iron uptake mechanisms in order to compete with host-mediated iron sequestration by three main mechanisms: induction of siderophores, heme acquisition, and transferrin/lactoferrin receptors (190).

Iron and Adaptive Immunity

Adaptive immune function depends on the activation, proliferation, and differentiation of antigen-specific B and T lymphocytes, which acquire iron via transferrin receptor (TfR)-1. Although B

lymphocytes do not seem to be affected by TfR-1deficiency, reduced TfR-1attenuates differentiation of T lymphocytes. Why T lymphocytes are particularly sensitive to iron deprivation is unknown. Several animal and human studies show that iron deficiency is associated with impaired lymphocyte proliferation and alters the cytokine profile of activated lymphocytes favoring T-helper type 1 and 2 cells (32). The mechanism is believed to involve reduced activity and translocation of protein kinase C, which in turn results in inhibition of renewal of the lymphocyte pool (114).

Thus, although the effects of iron deficiency on lymphocyte function and the consequences for adaptive immunity have yet to be fully elucidated, the evidence reviewed above underlines that changes in iron homeostasis may well have an impact on the pathogenesis of diabetes through microbial activators of autoimmunity, target beta-cell destruction, and systemic low-grade inflammation.

IRON AND REACTIVE OXYGEN SPECIES

Iron catalyzes the generation of ROS via the Fenton reaction, in which ferrous iron is oxidized via reaction with hydrogen peroxide to generate ferric iron, hydroxide, and the hydroxyl radical. The hydroxyl radical has a very short half-life of approximately 10^{-9} seconds (187). However, during that short reaction time, the hydroxyl radical will oxidize lipids, proteins, carbohydrates, RNA, and DNA if not dealt with by the cell (187). Therefore, cellular ROS damage occurs when ROS formation exceeds the capacity of cells to neutralize ROS. The extremely rapid kinetics of hydroxyl radical formation makes it evident that strategies that target formation of ROS to prevent disease caused by oxidative stress may be superior to antioxidant approaches limited by inadequate reaction constants and stoichiometry.

The cellular antioxidant system can be divided into two defense types: enzymatic and nonenzymatic. The enzymatic defense system relies on enzymes that actively convert ROS to less harmful molecules, whereas the nonenzymatic defense system either quenches or scavenges ROS or chelates oxidizing metals such as iron (187). The beta cell expresses the classic antioxidant enzymes, for example, the hydrogen peroxide–forming cytosolic copper/zinc (Cu/Zn) superoxide dismutase (SOD), mitochondrial manganese (Mn) SOD, and glutathione peroxidase, but only at levels corresponding to 15–40% of those of the liver; catalase is not expressed by the beta cell (126). Additionally, islets express approximately 30% of the liver level of the enzyme glutamyl-cysteine ligase, which facilitates the synthesis of the nonenzymatic antioxidant glutathione, binds intracellular ferrous iron and makes it less reactive, and serves as a transport molecule of iron to organelles or storage proteins (84, 200). This low expression of antioxidant enzymes makes the beta cell vulnerable to increases in ROS formation. It has been suggested that beta-cell defense against ROS-induced damage is exerted mainly through expression of peroxiredoxins, which are the only ROS-inducible antioxidant enzymes (15, 212). The single most important metabolic stress–induced ROS mediating beta-cell apoptosis is the hydroxyl radical, which is the product of the Fenton reaction (69). Furthermore, the primary ROS produced in beta cells upon palmitic acid exposure is hydrogen peroxide (69), confirming the potentially deadly combination of metabolic stress and iron.

Mitochondrial and Cytosolic ROS

There is a general distinction between mitochondrial and cytosolic ROS. Mitochondrial ROS are formed in the electron transport chain (ETC) by Fe-S proteins such as complex I and III, and their production is enhanced upon increased mitochondrial function or mitochondrial

dysfunction. Cytosolic ROS are generated by enzymes, for example, nicotinamide adenine dinucleotide phosphate oxidase, or nonenzymatically by catalysis in the Fenton reaction.

The labile iron pool (LIP) is the chelatable pool of iron in the cytoplasm, nucleus, and mitochondria (19). The cytosolic Fenton reaction is catalyzed by the cytosolic LIP, which in comparison with the mitochondrial and nuclear LIPs is believed to be the largest cellular LIP, and it serves as a readily accessible source of iron for immediate metabolic processes (19). The size of the LIP is proportional to the ROS formation via Fenton chemistry (72).

The primary cellular iron consumer is the mitochondrion, which needs iron in the ETC to provide cellular energy. Antioxidant enzymes, such as Mn SOD and Cu/Zn SOD, convert superoxide anions generated in the ETC to hydrogen peroxide, thereby providing substrate for the Fenton reaction (17). This apparent paradox is likely related to the need of ROS as signaling molecules. Interestingly, mitochondria from *Hfe* knockout mice, a hemochromatosis model with intracellular iron overload, have decreased respiratory capacity and decreased Cu, Mn, and Zn contents, correlating with decreased Mn SOD activity due to unmetallated apoprotein (101). This suggests that iron not only generates ROS through the LIP and Fenton reaction, but also modulates mitochondrial antioxidant defense through altered metal ion uptake.

IRON AND BETA-CELL FUNCTION

Even though it has been known for a quarter of a century that transferrin is the only nondispensable factor when culturing beta-cell lines (153), the beta-cell iron metabolism is poorly understood. However, it is believed to be similar to most other cell types, apart from the enterocyte (**Figure 2**).

Circulating transferrin-bound iron is taken up after binding to TrfR. The transferrin-TrfR complex is thereafter endocytosed with DMT1 and the metalloreductase six transmembrane epithelial antigen of prostate family member 3 (STEAP 3). Inside the endosome, iron is released from transferrin and reduced via decrease of pH by the proton pump v-ATPase and the metalloreductase STEAP 3. Iron is thereafter released to the cytoplasmic LIP through DMT1 over the proton gradient provided by v-ATPase. Iron is delivered to the cytosolic LIP as ferrous iron, from where it is distributed to ferritin for storage, or to the nucleus, ER, mitochondria, and Fe-S proteins for functional utilization by iron chaperones, such as lipocalin 2 and poly(rC) binding protein (PCBP) 1 and 2 (77). Electron microscopy has established that ferritin-bound iron is stored in the beta cell close to the plasma membrane or in insulin granules (139). Beta-cell iron is believed to be exported through ferroportin, the activity of which is inhibited by endocrine, paracrine, or autocrine actions of hepcidin (2, 170).

Islets and beta cells express TrfR (78, 132); the mitochondrial iron-storage protein frataxin (43); the cytosolic iron-storage proteins ferritin H and L chains (135); the iron-export regulatory hormone hepcidin, which is located in the insulin granules (2, 111); the iron chaperone lipocalin 2 (27, 78); and v-ATPase (154); these findings confirm that beta cells possess a classic iron metabolism. However, clarification of the functional importance of these iron-handling proteins in the beta cell is limited to DMT1.

The understanding of beta-cell mitochondrial and ER iron metabolism is of particular interest owing to the importance of the mitochondria and ER in beta-cell function and dysfunction and the ensuing therapeutic implications. Several proteins control iron transport to the mitochondria and ER. DMT1 is expressed on the mitochondrial outer membrane (213, 214), and seems to facilitate beta-cell mitochondrial iron uptake in parallel with the classic mitochondrial iron transporters Mfrn 1 and/or 2 (158). Beta-cell mitochondrial iron uptake seems to be regulated by the outer mitochondrial membrane 2Fe-2S protein [C-X-C-X2-(S/T)-X3-P-X-C-D-G-(S/A/T)-H] (CDGSH)

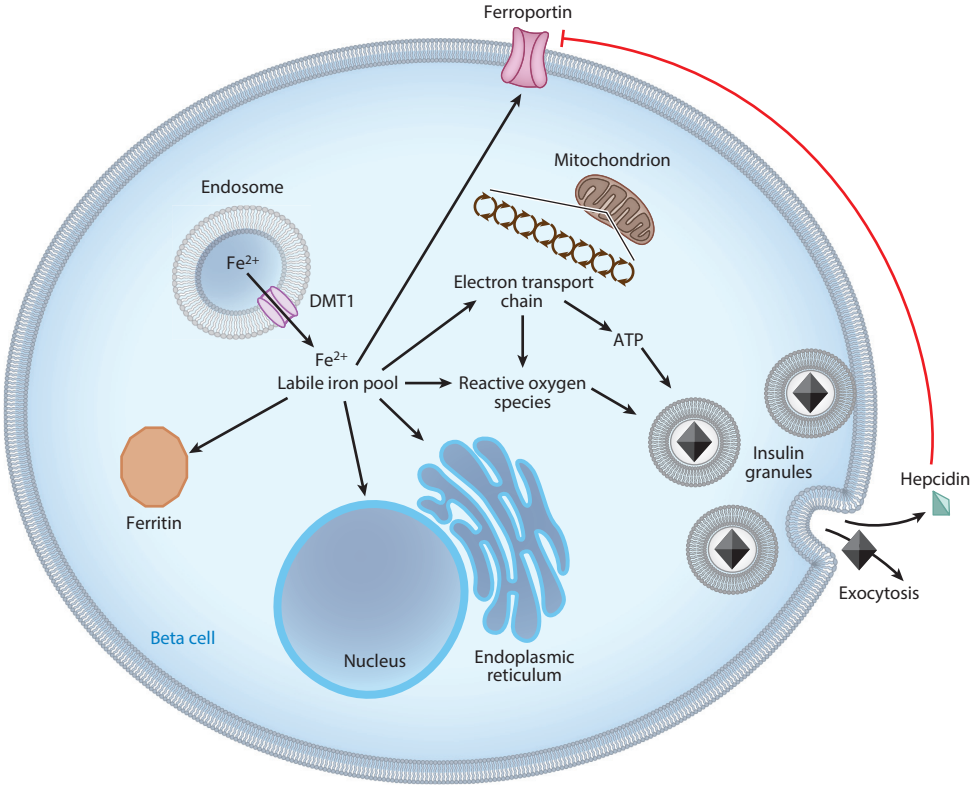

Figure 2

Beta-cell iron is imported to the cytosol after endocytosis of transferrin-transferrin receptor complexes and transported from the endosome by divalent metal transporter 1 (DMT1). Inside the cytosol, free ferrous iron enters the labile iron pool, where it is distributed for storage in ferritin; functionally in the nucleus, endoplasmic reticulum, or mitochondria; or exported by ferroportin. Beta cells produce hepcidin that is secreted with insulin, and hepcidin inhibits iron secretion via ferroportin. Beta-cell iron is important for insulin secretion, either by generation of reactive oxygen species through the Fenton reaction or by maintaining the electron transport chain in the mitochondria, resulting in adenosine triphosphate (ATP) production and subsequent insulin exocytosis.

iron sulfur domain 1 also known as mitoNEET (210), an inhibitor of mitochondrial iron uptake (113). Interestingly, the insulin-sensitizing drug pioglitazone, which activates nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), binds to and stabilizes mitoNEET (36, 156), thereby inhibiting iron delivery to the mitochondria, which results in decreased mitochondrial LIP (196). These findings suggest that insulin sensitivity is regulated via iron-mediated mitochondrial ROS production.

Iron delivery to the ER has not been reported in the beta cell, and to our knowledge no beta-cell ER iron transport protein has been identified. The 2Fe-2S protein iron sulfur domain 2 (Miner 1), which belongs to the same family as mitoNEET, does localize to the ER in other cells, is important for ER integrity (37, 209), and could possibly regulate ER iron transport.

Iron is important for normal insulin secretion. In beta-cell-specific *DMT1* knockout islets, glucose-stimulated insulin secretion is reduced (78). The proton pump v-ATPase a3 is highly expressed in islets, and a *null* mutation of this pump reduces insulin secretion (194). Since

v-ATPases are involved in acidification of insulin granules and endosomes for iron release to the LIP, it is difficult to discern if the effect of the knockout is due to altered iron metabolism, insulin granule acidification, or both. Additionally, hepcidin is secreted from beta cells upon glucose stimulation, which suggests that shutting down iron export via binding to ferroportin is a positive feedback mechanism in iron regulation during glucose-stimulated insulin secretion (2).

Two mechanisms of direct iron-mediated insulin secretion are possible: via maintenance of the electron flow through the ETC or via ROS production. Because (*a*) four out of five complexes in the ETC contain Fe-S proteins or heme, (*b*) ETC complexes I and II contain Fe-S proteins, and (*c*) ETC complex IV contains both heme iron and Fe-S protein, maintenance of beta-cell mitochondrial iron is of utmost importance for the production of ATP and thereby insulin secretion by triggering closure of the ATP-dependent potassium channels and membrane depolarization.

Mitochondrial-derived ROS is important for beta-cell function because it serves as an amplifying signal that increases insulin secretion (125, 175). ROS-mediated amplification of insulin secretion is mediated by ROS-dependent activation of ryanodine receptor 2, which promotes ER calcium release to the cytosol (130); the ER calcium release will potentiate the exocytotic effect of increases in cytosolic calcium derived from opening of the voltage-dependent calcium channels upon membrane depolarization. Whether cytosolic LIP-catalyzed ROS contributes to insulin secretion remains unknown.

Iron may contribute indirectly to beta-cell insulin secretion by maintaining nutrient sensing and responses to hypoxia. This hypothesis, however, is underinvestigated. The nutrient master regulator AMP-to-ATP ratio, which activates the cellular energy sensor AMPK, is regulated by iron. Thus, mice fed iron-rich diet have increased AMP-to-ATP ratio and AMPK activity in liver and muscle, and iron-depleted diet reduces AMPK subunits $\alpha 2$, $\beta 2$, and $\gamma 3$ in rat gastrocnemius muscle (90). The mechanism of iron regulation of AMPK is currently unknown. However, at least two possible pathways of iron-mediated regulation of AMPK activity exist: Either (*a*) AMPK acts as a redox sensor and is therefore regulated by ROS formed by the LIP, or (*b*) AMPK is regulated directly by the activity of the ETC that is in turn dependent on cellular iron content (34, 185).

Apart from its direct role in the ETC, iron may affect beta-cell metabolism through the transcription factor hypoxia-inducible factor (HIF) 1α regulating aerobic and anaerobic glycolysis and transition from cell proliferation to a cell-survival state (134). Iron delivery to prolyl hydroxylases and asparaginyl hydroxylase by PCBP1 and PCBP2 promote HIF1α degradation (151). In this way, iron controls the stress response to hypoxia and subsequent glycolysis and cell survival. Thus, decreased iron levels might promote HIF1α-mediated cell-survival gene expression.

In addition to controlling nutrient and oxygen sensing, excess iron is also suspected to perturb the circadian clock in beta cells. The circadian clock is controlled by a complex cascade of activating and repressing transcription factors that respond to changes in light (reviewed in 70). Heme biosynthesis is tightly regulated by the circadian clock via regulation of the rate-limiting enzyme in heme synthesis 5′-aminolevulinate synthase 1 (Alas1) (102). Conversely, heme also controls the circadian clock by binding the nuclear receptor subfamily 1, group D, member 2 (Reverb-α), and via the nuclear receptor corepressor and histone deacetylase 3 complex, heme promotes transcriptional repression of the circadian clock component aryl hydrocarbon receptor nuclear translocator-like (Bmal1) (217). In addition, dietary iron has been shown to affect the hepatic heme synthesis via control of Reverb-α and PGC-1α-mediated Alas1 transcription (189) (**Figure 3**).

In human beta cells, the circadian clock controls insulin secretion (11). Beta-cell-specific genetic deletion of the circadian clock component Bmal1 impairs insulin secretion in vitro and in vivo (136). Interestingly, the binding partner of HIF1α, HIF1β (or aryl hydrocarbon receptor

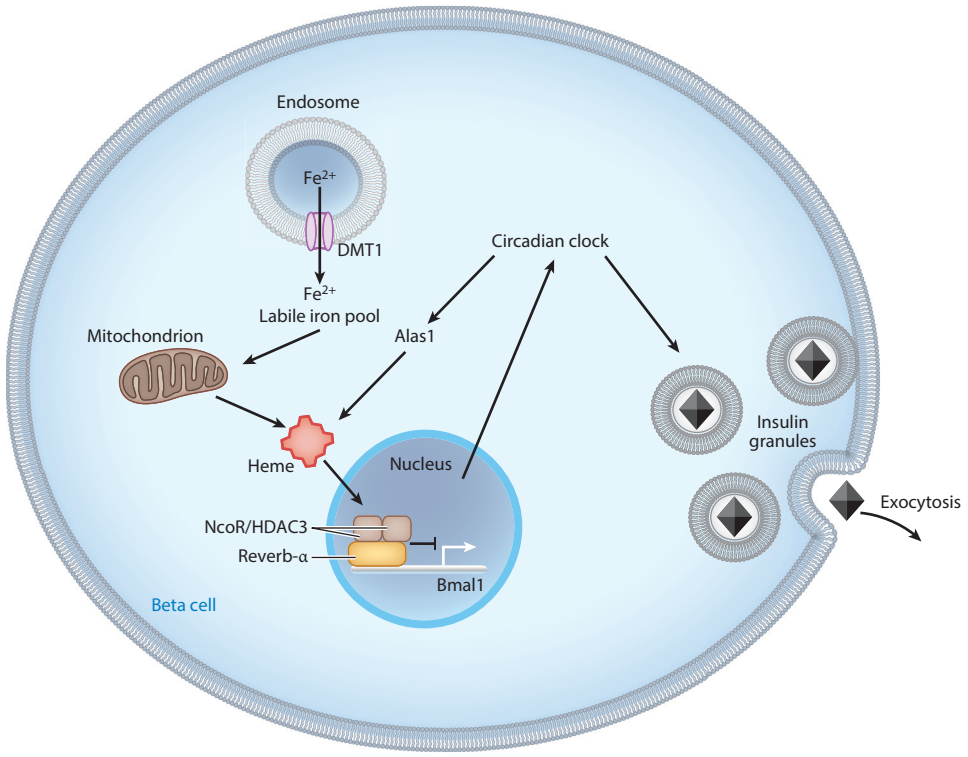

Figure 3

Iron is hypothesized to regulate the beta-cell circadian clock via heme. Heme is synthesized in the mitochondria, and its synthesis is controlled by the rate-limiting enzyme 5′-aminolevulinate synthase 1 (Alas1); in addition, expression of Alas1 is controlled by dietary iron. Heme can bind nuclear receptor subfamily 1, group D, member 2 (Nr1d2 or Reverb-α) and promote transcriptional repression of aryl hydrocarbon receptor nuclear translocator-like (Bmal1) together with and via the nuclear receptor corepressor (NcoR) and histone deacetylase (HDAC) 3 complex. This process is a central step in circadian clock regulation, which is of key importance in insulin secretion regulation.

nuclear translocator) is decreased with HIF1α and BMAL1 in islets from T2D patients (74) and regulated by the circadian-clock-controlled gene albumin D element-binding protein (*Dbp*) in mouse islets (150). This mechanism is impaired in islets of the Wolfram syndrome model *Wfs1*$^{-/-}$ *Ay/a* mouse that spontaneously develops obesity, beta-cell ER stress, and insulin resistance, resulting in decreased *Dbp* and subsequent *HIF1β* expression (150). Taken together, these results suggest a tightly controlled mechanism for regulation of hypoxic response in the beta cell by the circadian clock, with iron as a central actor. Studies of the regulation of beta-cell iron metabolism by the circadian clock would be highly relevant to confirm this theory.

In addition to controlling beta-cell function and metabolism, iron seems to have a role in beta-cell differentiation. Thus, the tumor suppressor gene *CDC14A* mRNA contains binding sites for iron-responsive element in its 3′ UTR, suggesting a direct role of iron in regulating the cell cycle (181). Furthermore, iron chelation induces cell cycle arrest in G1 phase in a human neuroblastoma cell line and in porcine liver cells (21, 31). Cell cycle control might be dependent on TrfR, since TrfR overexpression in the Chinese hamster ovary TRVb cell line abrogates iron-chelator-mediated cell cycle arrest (107), confirming the importance of iron and

iron-metabolism proteins in cell cycle regulation. This remains to be directly demonstrated in beta cells.

IRON AND BETA-CELL DYSFUNCTION

Animal models of the genetic iron-overload disorder HH have confirmed that iron overload is particularly harmful for the beta cell and have shed light upon the molecular mechanism of iron overload. In the *Hfe* knockout mouse (hemochromatosis model), iron accumulates in the islets, causing increased protein oxidation, decreased insulin secretion, and increased apoptosis (39). In addition, rat islets cultured with pharmacologically relevant concentrations of iron have increased DNA oxidation and decreased viability (137). Furthermore, patients with transfusional iron overload have increased iron deposition in beta cells (131). Ultra-structurally, beta cells of rats treated with ferric nitrilotriacetate have clumped nuclear chromatin, dilated nuclear membrane, dilated ER, and light secretory granules (184). Of note, the observed apoptotic and necrotic features were restricted to beta cells; alpha and delta cells accumulated iron but did not undergo apoptosis or necrosis (184).

Obesity, metabolic stress, and T2D alter body iron homeostasis. Iron absorption and retention are increased in the leptin-deficient *ob/ob* mouse, which becomes obese and develops T2D (55). The mitochondrial iron-storage protein frataxin is decreased in islets from T2D donors compared to controls (43), suggesting that islets from T2D patients have impaired iron homeostasis. The antioxidant protein hemeoxygenase 1 (HO1), which cleaves the heme ring in heme b, causing release of ferrous iron, is upregulated in islets by high glucose and hydrogen peroxide (51, 100); whether HO1-mediated heme degradation causes an increase in the beta-cell LIP has not been investigated. The iron-storage protein ferritin is highly expressed in rat islets compared to other tissues, such as liver, skeletal muscle, and heart, and exposure for 24 hours to 20 mM glucose increases the synthesis of ferritin in rat islets (135).

Reducing beta-cell iron improves function and protects from harmful ROS production in T2D animal models. Dietary iron restriction or pharmacological iron chelation improves beta-cell function in *ob/ob* mice (38). Furthermore, beta-cell-specific *DMT1* knockout mice are protected from high-fat-diet-induced diabetes by improved insulin secretion (78). Additionally, changing diabetic *ob/ob* mouse diet from normal to low iron content improves glucose tolerance (38), which suggests that iron reduction not only prevents but also reverses the development of T2D.

Apart from regulating ROS formation through the Fenton reaction, iron induces beta-cell dysfunction via other mechanisms, for example, through iron-mediated degradation of HIF-1α (30, 151). HIF-1α is decreased in islets of T2D patients, and beta-cell-specific *HIF-1α* knockout reduces insulin secretion and ATP synthesis, suggesting impaired mitochondrial function (30). In addition, iron overload decreases the beta-cell antioxidant defense by inhibiting Mn uptake and Mn SOD activity (101). Accordingly, dietary Mn supplementation prevents high-fat-diet-induced diabetes by restoring Mn SOD activity and thereby improving beta-cell insulin secretion and mitochondrial function and decreasing protein peroxidation (124).

In T2D patients, deposits of human islet amyloid polypeptide (hIAPP), a protein cosecreted with insulin, accumulate in the extracellular matrix surrounding the islets, causing beta-cell failure (76). The plaques of hIAPP can potentially increase the availability of iron to the islet, since heme can bind hIAPP, thereby serving as a reservoir providing iron directly to the beta cell (148). In addition, amyloid beta itself can reduce ferric iron to ferrous iron (91). Interestingly, the amyloid precursor protein is important for iron export via ferroportin in neurons (215), and iron itself induces production of amyloid precursor protein, particularly the highly aggregative amyloid species Aβ42 in retinal pigment epithelial cells (75). It is unknown if hIAPP facilitates iron export

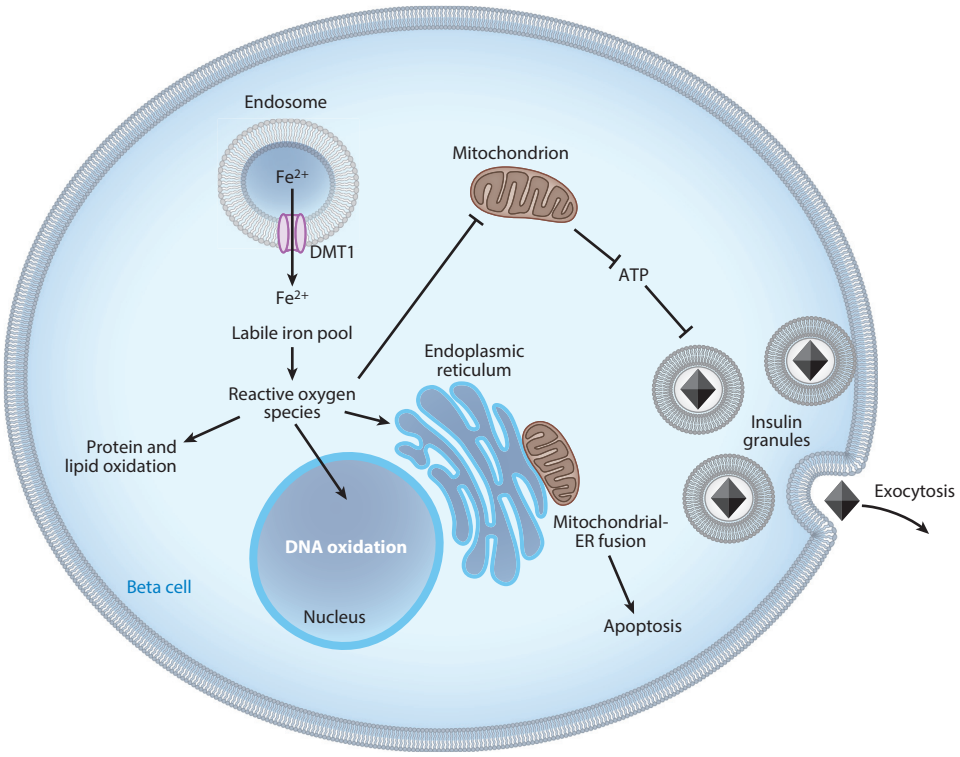

Figure 4

The labile iron pool is central in iron-mediated beta-cell toxicity due to reactive oxygen species (ROS) formation via the Fenton reaction. Increased beta-cell ROS formation causes protein, lipid, and nucleic acid oxidation. In addition, ROS can inhibit mitochondrial function, resulting in decreased adenosine triphosphate (ATP) production and impairment of insulin secretion. Increased iron-mediated ROS production induces beta-cell apoptosis, which possibly is mediated through mitochondrial–endoplasmic reticulum interaction. Abbreviation: DMT1, divalent metal transporter-1.

via ferroportin in beta cells or if hIAPP aggregates inhibit that export. However, it seems that iron induces formation of hIAPP, resulting in increased aggregate formation and extracellular iron accumulation in the islet.

Increasing evidence suggests that iron partakes in beta-cell destruction in T2D (38, 78). Whether iron takes part in the apoptotic mechanism in metabolic stress–induced beta-cell apoptosis is unknown. The 2Fe-2S proteins Miner1 and mitoNEET are proposed to facilitate ER-mitochondrial cross talk in diabetes (37), a mechanism that recently has been discovered to be induced by metabolic stress in hepatocytes, resulting in insulin resistance and development of T2D (4). This mechanism might be an attractive pharmaceutical target, since pioglitazone, which binds mitoNEET, improves islet function in a rodent T2D model (94).

In summary, evidence indicates that iron-induced beta-cell dysfunction in T2D is caused by increased iron uptake through DMT1, which (*a*) increases ROS production, causing lipid, protein, and nucleic acid oxidation; (*b*) decreases mitochondrial function; and (*c*) possibly induces mitochondrial-ER interactions; all of these mechanisms contribute to impaired insulin secretion and apoptosis (**Figure 4**).

Apoptosis and Ferroptosis

Iron induces beta-cell death via increased ROS formation. However, the mechanistic detail is not understood. An intriguing hypothesis is that iron-induced beta-cell death occurs via induction of ferroptosis.

Ferroptosis is a recently described form of cell death, triggered by the antitumor small molecule erastin in cancer cells, which involves increased cellular iron accumulation and iron-dependent cytosolic ROS formation via reduced cysteine uptake, and that can be prevented by iron chelation (45). Unlike cells in classic apoptosis, ferroptotic cells do not exhibit organelle swelling, condensed chromatin, or autophagy. Interestingly, HO1, which is upregulated by metabolic stress in beta cells, accelerates erastin-induced lipid peroxidation and ferroptosis (115), a mechanism that could be due to the iron-releasing properties of HO1. Glutathione peroxidase 4 (Gpx4) is an antioxidant and inhibitor of ferroptosis (216), and Gpx4 knockout causes rapid onset of ferroptosis in neurons (28). In high-calorie-diet-fed KK mice (a mouse model that spontaneously develops hyperglycemia due to beta-cell dysfunction), Gpx activity is decreased in islets (129). Pharmacological inhibition of Gpx synthesis in beta cells induces glucose-mediated beta-cell dysfunction in vitro (197), further pointing to a possible induction of ferroptosis in beta cells by metabolic stress.

IRON AND INSULIN SENSITIVITY

Insulin resistance challenges the pancreatic beta cell not only by increasing insulin secretory demands to compensate for reduced insulin action in muscle, fat, and liver, but also by the adverse effects of inhibiting beta-cell autocrine insulin signaling (112, 211). Excess iron may affect insulin sensitivity in all of these tissues and thereby contribute to the development of T2D. The role of iron and insulin sensitivity is addressed in this section.

Metabolic Syndrome, Iron, and Type 2 Diabetes

Although it is well established that an excess of macronutrients, such as fat and carbohydrates, is involved in the pathogenesis of T2D, an increasing number of studies demonstrate that micronutrients, such as iron, are also important factors in the development of T2D (59). Accordingly, increased ferritin levels (i.e., increased levels of body iron stores) are associated with increased risk of T2D. In patients with HH, circulating levels of ferritin are inversely correlated with insulin sensitivity (87). In contrast, lowering the levels of ferritin (e.g., using iron chelators such as desferoxamine) protects against T2D (9), and phlebotomy improves insulin sensitivity and glycemic control. Animal studies show that iron deficiency is associated with increased insulin sensitivity, whereas several human cross-sectional studies support the correlation between elevated ferritin levels and decreased insulin sensitivity (172). A large cross-sectional study reported an association between elevated ferritin levels and the metabolic syndrome (98). Furthermore, high levels of ferritin and transferrin were associated with increased prevalence of the metabolic syndrome in a substudy of the Data from an Epidemiological Study on the Insulin Resistance Syndrome (DESIR) cohort (204). The mechanisms underlying iron-induced diabetes are not yet fully elucidated but are believed to involve impaired insulin secretion from pancreatic beta cells, insulin resistance, and hepatic dysfunction (195), as reviewed above.

GENETICS AND EPIDEMIOLOGY OF IRON OVERLOAD AND DIABETES

The association between diabetes and elevated iron levels was first discovered in patients with HH by Trousseau in 1865 (201). More than a century later, a prospective study found that moderate

elevations of body iron stores, much lower than those seen in patients with hemochromatosis, are associated with increased risk of T2D in a healthy population (180). Subsequently, several epidemiological studies have confirmed the association between iron overload and risk of T2D (49, 99, 145, 172) as well as other insulin-resistant states, such as metabolic syndrome (98), polycystic ovary syndrome (52), and gestational diabetes (18). Eventually, the organ damage caused by oxidative stress due to elevated iron levels shortens survival (50).

Genetic Disorders (Hereditary Hemochromatosis)

HH is an autosomal recessive disorder and is the most common genetic iron-overload disorder associated with diabetes. Individuals with HH are treated with phlebotomy, which reduces ferritin and transferrin saturation concentrations; this treatment improves glycemic control via increased insulin sensitivity and secretory capacity if initiated before irreversible iron-overload-mediated tissue dysfunction has developed (1).

Most cases of HH occur as the result of two missense mutations (C282Y and H63D) in the *HFE* gene (58). Mutations in genes encoding TfR-2, ferroportin, hemojuvelin, and hepcidin are more rare causes of HH (164). The molecular functions of the *HFE* gene are not fully understood; however, it is evident that *HFE* plays an important role in regulating hepcidin expression, and that *HFE* mutations lead to an inappropriately low hepcidin concentration and consequently dysregulated and increased intestinal iron absorption (5). HH is usually associated with ferritin levels higher than 1,000 μg/L (172).

In a genome-wide association study (191), one of the common missense mutations (C282Y) responsible for HH was found to be significantly associated with hemoglobin A1c (HbA1c) concentrations; however, no association existed with C282Y and risk of T2D. The prevalence of diabetes among patients with HH ranges from 22% to 63% in studies (205), and numerous explanations likely exist for the reported variation in prevalence. First, diabetes mellitus is a common disease, and the penetrance of HH is highly variable (203). Likewise, the development of diabetes in patients with HH is age dependent, with the disease typically manifesting in late adulthood; therefore, inclusion of younger adults without diabetes in studies will automatically yield a lower prevalence even though these individuals may be at risk of developing diabetes later in life.

Nongenetic Causes of Iron Overload

Aging is associated with cellular iron accumulation. In men, HH onset is typically at middle age (163), whereas in women, who regularly lose iron during menstruation, onset is typically after menopause. However, increased iron stores may also be related to nutrition, sedentary lifestyle, smoking, alcohol, oral iron supplementations, or repeated blood transfusions.

Nutrition

A recent meta-analysis evaluated the association between dietary iron and T2D in five prospective studies (6). In all of these studies, individuals with high intake of heme iron were consistently found to have a 30% increased risk of T2D compared to people with low intake. Dietary nonheme iron was not associated with risk of T2D in these studies. In another study, consumption of red meat and alcohol enhanced the uptake of heme iron, whereas consumption of milk was found to decrease the bioavailability of heme (161). These results are confirmed by a study reporting that individuals who consume a vegetarian diet have increased insulin sensitivity compared to those who eat meat (127). Furthermore, the Mediterranean diet, which is considered a low-iron-available diet in which

iron-absorption inhibitors (e.g., polyphenols, phytates, and dairy products) prevail over enhancers (e.g., ascorbic acid and red meat), is also associated with a low risk of diabetes (127).

It is possible that the reported results above are confounded by macronutrient intake and total daily calorie intake, since higher intake of heme iron may be a marker for an unhealthy lifestyle, including higher consumption of saturated fat and carbohydrates, which may be associated with increased risk of T2D. Although all of the above studies adjusted for several lifestyle factors in multivariable analyses, confounding remains a concern.

Sedentary Lifestyle and Obesity

Sedentary lifestyle and obesity are strong risk factors for T2D (183). Obesity is associated with a chronic low-grade inflammation due to slightly increased production of cytokines, such as TNF-α, IL-1β, and IL-6, that contribute to the development of insulin resistance and beta-cell failure (47, 85). Wenzel et al. (207) were the first to describe a significantly lower mean serum iron concentration in an obese compared to a lean control population. Additionally, ferritin levels are positively associated with body mass index and visceral fat mass (95, 219). This apparent paradox can be explained by viewing obesity as a chronic low-level inflammatory state with increased sequestration of iron that leads to low circulating iron concentration and increased intracellular ferritin levels (see Iron and Innate Immunity section above). In turn, excess intracellular iron instigates a vicious circle and fosters oxidative stress, thereby reinforcing the state of chronic inflammation.

In contrast, physical activity reduces diabetes risk by helping to maintain a normal body weight and by improving insulin sensitivity. This reduction in risk could be linked to iron metabolism, as a study of Finnish males found that higher levels of leisure-time physical activity were associated with reduced levels of stored iron (117).

Smoking

Smoking affects iron homeostasis. A person who smokes one packet of cigarettes per day inhales 1.12 μg of iron (138). In fact, inhalation of iron in tobacco smoke increases iron content in alveolar macrophages as much as four- to fivefold. Inhaled iron can be disseminated via macrophages, resulting in systemic iron overloading (71, 159). This alteration in iron homeostasis induces oxidative stress and inflammation, which could contribute to development of T2D and other diseases.

In a prospective cohort study involving 144 pregnant women, smoking was correlated with increased levels of ferritin and higher body iron in the mothers (160). Interestingly, a negative correlation was found between maternal smoking and the total body iron levels of their infants (160). Nicotine causes vasoconstriction, which in turn restricts uteroplacental blood flow (118), and a possible explanation for decreased iron stores in infants of smoking mothers is a decreased placental transport of nutrients and oxygen to the fetus. Hypoxia increases erythropoiesis, which also could explain the decreased iron stores in infants.

Alcohol

Alcohol is known to cause disturbances in iron homeostasis through several mechanisms, including downregulation of the secretion of hepcidin (79). Thus, patients with alcoholic liver disease frequently present with increased body iron stores and hepatic iron concentration. Even mild to moderate alcohol consumption has been shown to increase the prevalence of iron overload (79, 208), and the effect of alcohol on iron stores is dependent on both the individual's past alcohol

intake and the type of alcohol consumed, as beer has a greater effect than wine (208). Interestingly, one study reported that high intake of nonheme iron was associated with a reduced risk of diabetes mellitus only among those who did not consume alcohol, suggesting a potential interaction between nonheme iron and alcohol (123).

Iron Supplementation

Supplemental oral iron intake is beneficial in iron-deficient but not in iron-replete individuals. A recent meta-analysis showed that dietary vitamin and mineral supplements were associated with increased mortality, possibly due to supplemental iron (149).

Iron supplementation is particularly recommended during pregnancy to prevent iron-deficiency anemia caused by the increased need for iron of both the mother and the fetus. Iron-deficiency anemia detected prior to midpregnancy is associated with increased risk of preterm birth. During the third trimester, maternal anemia is usually not associated with increased risk of adverse pregnancy outcomes and may be an indicator of an expanded maternal plasma volume (182). Iron supplementation may improve pregnancy outcome when the mother is iron deficient; however, given the toxic properties of excess iron, it is also possible that prophylactic supplementation will increase the risk of adverse pregnancy outcome when the mother is not iron deficient.

In a study examining 197 pregnant women, 99 women received 100 mg of iron supplementation from week 28 of pregnancy through the remainder of pregnancy, whereas the rest of the group received placebo (168). At delivery, there was no difference between the two groups in transferrin saturation and ferritin measured in the cord blood; however, mean length at birth and Apgar score were significantly higher in newborns whose mothers had received iron supplements. Because iron measurements did not differ, it is likely that these results were due to other factors. Some investigators have suggested that supplemental iron during pregnancy may cause harm because high hemoglobin concentrations are associated with impaired placental perfusion due to increased viscosity of the blood, which can result in preeclampsia, preterm birth, and low birth weight (discussed in 179). In contrast, others suggest that an increased concentration of hemoglobin caused by iron supplementation is not associated with risk of adverse outcomes (discussed in 179). So far, no evidence indicates that routine iron supplementation during pregnancy has been of any benefit other than preventing maternal iron deficiency (23). This issue warrants comprehensive further investigation.

Transfusion

Excess iron from nondietary sources may occur in patients with diseases related to ineffective erythropoiesis, such as beta-thalassemia major, who receive chronic erythrocyte transfusion to maintain adequate hemoglobin levels (162, 178). Because diseases with ineffective erythropoiesis cause anemia, hepcidin levels are low, which leads to increased iron absorption from the gut, thereby exacerbating the transfusion iron overload. In beta-thalassemia major, iron accumulates in the pancreatic beta cells beginning in early childhood, leading to decreased insulin secretion (199). However, diabetes may also be caused by hepatic iron overload, leading to insulin resistance (24, 142, 195). The prevalence of diabetes in patients with beta-thalassemia major is estimated to be 6% to 14% (42). Iron chelation may improve glucose metabolism by reducing insulin resistance and improving beta-cell function (20, 35, 57, 165). Primary determinants of risk of diabetes in beta-thalassemia major are male sex, poor compliance with iron chelator treatment, advanced age at the start of intensive chelation therapy, liver cirrhosis or severe fibrosis, liver iron concentration, and duration of transfusion therapy (42, 57, 66).

Gestational Diabetes Mellitus

In light of the focus on iron supplementation during pregnancy, it has been speculated that high iron stores in the beginning of the pregnancy might contribute to a greater risk of gestational diabetes mellitus (GDM), since it has been found that iron overload contributes to the pathophysiology of T2D (220). For example, it has been reported that serum ferritin levels were higher among Chinese women with increased glucose tolerance diagnosed during 28 to 30 weeks of pregnancy compared to those with normal glucose tolerance (121). In another study, the same investigators reported that mean ferritin levels were higher among women with GDM compared to those without GDM (47.4 versus 22.5 pmol/l) (119). Likewise, a study investigating possible risk factors of GDM in pregnant women with a diagnosis of GDM compared to normoglycemic pregnant women found that women diagnosed with GDM had higher plasma ferritin levels than the control group (97). The women with GDM had an increased risk of giving birth to neonates with higher birth weights compared to the control group, even though the case and control groups were matched with regard to body mass index, maternal age, and gestational age (97). These results conflict with research that was reviewed above (see the Iron Supplementation section) (179), which suggests that iron supplementation results in low birth weight. However, it may indicate that high iron stores in the beginning of pregnancy and prophylactic iron supplementation taken during the pregnancy have different effects on the infant.

Taken together, the reviewed evidence indicates that routine iron supplementation should be individualized on the basis of ferritin levels to minimize the risk of GDM. This approach is supported by a study of iron-deficiency anemia in pregnant women who were reported to have a reduced risk of GDM (120). However, information is lacking about the effect of low-dose iron supplementation and development of GDM, and randomized trials of iron supplementation for pregnant women are warranted to investigate the association between iron-induced oxidative stress and risk of GDM. It is of high importance to clarify the role of iron in the risk of GDM because women with a history of GDM are at an elevated risk of developing T2D later in life (122).

Insulin Resistance

Functionally, iron-overload-mediated diabetes is characterized by both insulin resistance (24, 46, 80, 142, 143) and insulin deficiency (171, 188) and may therefore mimic both T2D and idiopathic T1D. Patients with HH are more prone to develop diabetes if insulin resistant because their insulin secretory capacity is decreased, although it is usually not absent (140, 188). In the general population, elevated fasting serum ferritin levels are associated with surrogate measures of both impaired beta-cell function and decreased insulin sensitivity (13). In patients with diabetes, it has been shown that iron depletion ameliorates HbA1c levels, insulin secretion, insulin resistance, and vascular dysfunction (61). Also, as mentioned above, phlebotomy may improve insulin secretory capacity (1, 81) and insulin sensitivity (62) if instituted early, and it reverses impaired glucose tolerance in patients with HH (81, 87). The pathogenic explanation for insulin resistance may be hepatic dysfunction due to hepatic iron overload (33, 73, 195) and decreased skeletal muscle glucose oxidation (89).

CLINICAL INTERVENTION STUDIES

Clinical intervention studies that show improvement in diabetic states after reduction in iron stores are necessary to prove causality between iron overload and the development of T2D. Unfortunately, few clinical intervention studies have examined the effect of iron depletion in individuals with T2D, and those that have were generally inadequately powered and used open-label designs.

Phlebotomy

Blood donations result in a significant reduction in circulating ferritin levels and improved insulin sensitivity (60). However, it is important to note that blood donors are highly selected (a bias termed the healthy donor effect): Donors are likely to have a healthier lifestyle than nondonors, and donors must pass rigorous and regular donation testing.

We are aware of only one clinical trial examining the glycemic status after phlebotomy treatment among individuals diagnosed with T2D (61). Twenty-eight patients with T2D underwent bloodletting of 500 ml three times at two weekly intervals and achieved significant improvement in insulin sensitivity and decreased levels of HbA1c compared with a matched nonbloodletting control group (61). The improvement in insulin sensitivity was sustained, whereas the HbA1c level was reversed to baseline after 12 months.

In an uncontrolled study, a group of glucose-intolerant individuals with ferritin levels within normal limits underwent phlebotomy aimed at near iron deficiency. These persons achieved decreased levels of fasting glucose after treatment. However, the results were reversed after a six-month period of iron repletion, with normalization of iron levels. Furthermore, two clinical trials have examined the glycemic status following phlebotomy treatment among healthy individuals with normal ferritin levels (14, 54). In one of the studies (54), fasting glucose was improved by 19% after four weeks of phlebotomy; in the other study (14), HbA1c was improved, with a difference in percentage points of −2.9 three weeks after phlebotomy.

A few intervention studies with phlebotomy have been conducted in patients with T2D or metabolic syndrome to examine the effect on surrogate cardiovascular endpoints (i.e., blood pressure, vascular function, and glucose) and on hard endpoints such as cardiovascular disease, total and cause-specific mortality, and cancer. These studies have shown improvements in vascular function (62) and insulin sensitivity (61) and decreases in cholesterol (86, 116), HbA1c (62), and blood pressure (86). However, larger randomized, sham-controlled, multicenter studies are needed in order for phlebotomy therapies to be instituted as clinical practice in at-risk individuals.

Iron Chelation Therapy

So far, only three studies (40, 108, 173) have examined whether reducing serum iron by chelation therapy prevents or delays the development of T2D. The studies show contradictory outcomes and are described in detail in a recent review (77).

It has been speculated that chelation therapy is a potential therapeutic approach to reduce cardiovascular event rates because increased ferritin levels have been found in atherosclerotic coronary arteries (218). A randomized, double-blind, placebo-controlled trial studied the effect of ethylene diaminetetraacetic acid chelation on cardiovascular endpoints in patients who had suffered from a myocardial infarction at least six weeks before enrollment (53). Interestingly, chelation therapy reduced the numbers of cardiovascular endpoints in patients with diabetes; in contrast, the treatment had no effect in patients without diabetes. However, ethylene diaminetetraacetic acid chelates not only iron but also minerals and other metals such as calcium, Mn, lead, cadmium, aluminum, Cu, and Zn. Information about iron stores before and after the chelation therapy is missing, and it is therefore not possible to conclude if these results are due to decreased iron levels. Larger and longer-term randomized, controlled trials are required to address both the beneficial and potential adverse effects of iron chelation therapy.

CONCLUSIONS, KNOWLEDGE GAPS, AND PERSPECTIVES

In contrast to the frustrating clinical experiences in preventing beta-cell destruction in individuals at risk for autoimmune T1D or in patients with autoimmune T1D who receive islet transplants as a

curative approach, prevention of T2D seems so simple and intuitive: Nutritional advice to achieve weight loss by correcting the imbalance between energy intake and expenditure will reduce insulin resistance, alleviate the burden on the pancreatic beta cell of compensatory insulin secretion, and restore metabolic homeostasis. But then there is always a simple answer to a complicated problem, and it is always wrong.

Although the causes of obesity respect the laws of mass constancy, the complex etiology and pathogenesis of obesity, the metabolic syndrome, and T2D include genetic, epigenetic, and intrauterine elements; psychological and social factors; disturbances of appetite, diurnal rhythm, and thermogenesis; interactions with the microbiome; deregulation of gene expression; dysregulation of translational and posttranslational circuits; signaling pathway derangements; and alterations in substrate metabolism and several cellular effector pathways, eventually resulting in insulin resistance and beta-cell damage characteristic of the fulminant T2D phenotype.

Adjustment of caloric and macronutrient consumption and physical activity levels may correct some of these intertwined causal factors, but the introduction of, and continued compliance to, dietary and exercise interventions are challenged by long-established lifestyle habits, and such interventions are successful long term in merely 10% to 15% of subjects despite highly intensive and costly health-care efforts. Although idealistic, this approach to the growing diabetes problem is naive, and a desperate need exists for more targeted nutritional and/or pharmacological strategies.

Growing evidence supports mounting of redundant responses in the pancreatic beta cell, the culprit in diabetes development, to inflammatory and metabolic stress in T1D and T2D, since these beta-cell stressors are generally believed to provoke an exaggerated and unopposed generation of oxidative and nitroxidative reactive intermediates, which normally function at low levels as physiological signaling molecules regulating beta-cell function. Excess ROS also impair insulin signaling in fat, liver, and muscle. Iron is a micronutrient of key importance in catalyzing the generation of ROS in the Fenton reaction. Iron also affects microbiome composition, hypothalamic appetite regulation and thermogenesis, adipocyte differentiation, and innate and adaptive immunity. The emerging preclinical and clinical evidence described in this review links iron handling in insulin-sensitive tissues and the beta cell with risk of diabetes development. Taken together, the available evidence points to iron and iron handling as promising targets of intervention.

However, important gaps remain to be filled in our knowledge about the physiological roles of iron to be spared by interventions, the pathophysiological molecular mechanisms of iron-mediated cellular damage, and the safety and efficacy of iron-reducing strategies in animal models and patients. It is amazing that fundamental questions of importance in understanding general iron metabolism remain to be solved: What are the precise molecular mechanisms of action of hepcidin and the functions of the hemochromatosis wild-type gene product? How does iron overload affect microbiome composition? Why do hypothalamic neurons tend to preserve high normal iron levels? How is iron transport regulated in insulin-sensitive tissues and brown fat, and how precisely does iron regulate insulin sensitivity? How does iron overload affect innate and adaptive immune function?

Also unanswered are an equal number of questions more specifically related to the physiological and pathophysiological roles of iron in the beta cell: Does the beta cell possess cell-specific iron-handling processes? How does iron regulate insulin secretion? What are the mechanisms behind the insulin secretory defects in iron deprivation or knockout of iron import in animals, and what are the compensatory mechanisms that alleviate the phenotypic consequences? Is beta-cell selective destruction in iron overload models and diseases in humans due to such processes or merely to low ROS defense? Or do inadequate antioxidative defense mechanisms inadvertently increase the LIP in beta cells, as seems to be case with glucose-induced hemeoxygenase 1? What is the role for hypoxia and hypoxia-induced pathways for beta-cell nutrient sensing and stress coping? What is

the role of iron in the beta-cell cell cycle and in circadian secretory rhythmicity? Does islet amyloid contribute to beta-cell iron accumulation? Is beta-cell iron handling affected by metabolic stress? What is the role of ferroptosis in beta-cell demise in diabetes?

Translational exploitation of current and future basic insights will require progress in human studies: Although common genetic variants and dietary factors for risk of T2D and obesity have been extensively studied (127, 147), how these genetic variants and dietary iron intake interact remains unknown. Limits of normal plasma hemoglobin and serum ferritin in women differ from those in men due to women's regular loss of blood (and iron) through menstruation before menopause. As a result, men are more exposed to adverse health outcomes associated with elevated iron stores than women. Could men benefit from having the same upper limits of hemoglobin and ferritin as women? Should lower limits of normal serum ferritin be proposed for people with metabolic diseases? Should women with diabetes (T1 or T2), obesity, or the metabolic syndrome receive oral iron supplementation? Does bloodletting provide protection against diabetes when the confounding impact of the healthy donor effect is eliminated? To what degree is the circulating iron level genetically determined, and can it be stably modified by approaches such as reducing iron in diets, eliminating routine iron supplementation in iron-replete subjects, inhibiting iron absorption through nutrients, changing ascorbic acid intake, or changing gut luminal pH? Should all women take iron supplementation during pregnancy, and if so, in which periods? Should women with GDM discontinue iron supplementation? Does a reduction in dietary iron intake reduce the risk of developing T2D?

Carefully designed and high-powered randomized, controlled clinical trials are required to answer these questions. The outcomes of such trials are needed to generate an evidence base for clinical guidelines and public health campaigns aimed at reducing iron intake and to provide the rationale for pharmacological intervention trials using third-generation iron chelators to lower iron saturation in individuals at risk for developing metabolic diseases.

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