Perspectives in Diabetes

The Peroxisome Proliferator-Activated Receptor-γ2 Pro12Ala Polymorphism

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Peroxisome proliferator-activated receptor (PPAR)-γ is a transcription factor that belongs to the same family of nuclear receptors as steroid and thyroid hormone receptors (1). It is activated by certain fatty acids, prostanoids, and thiazolidinediones, a novel class of insulin-sensitizing antidiabetic agents (2-4). Upon activation, it heterodimerizes with the retinoid X receptor and binds to specific PPAR-responsive elements of DNA to promote transcription of numerous target genes (5). Although the isoform PPAR-γ1 is expressed in most tissues, PPAR-γ2 is specific for adipose tissue, where it plays a key role in regulating adipogenic differentiation (6). The PPAR-γ gene is located on chromosome 3 (7), and the specific isoforms are a result of alternative mRNA splicing.

A number of genetic variants in the PPAR-γ gene have been identified. These include a very rare gain-of-function mutation (Pro115Gln) associated with obesity but not insulin resistance (8), two loss-of-function mutations (Val290Met and Pro467Leu) reported in three individuals with severe insulin resistance but normal body weight (9), the silent CAC478CAT mutation (10-12), and the highly prevalent Pro12Ala polymorphism in PPAR-γ2 (Fig. 1). The latter is the result of a CCA-GCA missense mutation in codon 12 of exon B of the PPAR-γ gene. This exon encodes the NH₂-terminal residue that defines the adipocyte-specific PPAR-γ2 isoform. The Pro12Ala polymorphism in PPAR-γ2, which is the focus of this review, was first identified in 1997 (13), and the rare allele frequencies are ~12% in Caucasians, 10% in Native Americans, 8% in Samoans, 4% in Japanese, 3% in African-Americans, 2% in Nauruans, and 1% in Chinese (14,15). In Caucasians, the ethnic group with the highest frequency, this translates into a carrier prevalence of the polymorphism of almost 25%.

ASSOCIATION WITH TYPE 2 DIABETES

The pathogenesis of type 2 diabetes is characterized by failure of β-cell function to compensate for decreased insulin sensitivity. The etiology is multifactorial, and twin studies clearly indicate a major role for involvement of genetic factors (16,17). Moreover, excess concordance rates in monozygotic versus dizygotic twins clearly suggest a contribution of genetic factors to both insulin resistance and β-cell dysfunction (18). However, due to enormous heterogeneity and probably polygenicity, very
few genetic variants have been identified to account for a substantial proportion of common type 2 diabetes.

First evidence for an association between the Pro12Ala polymorphisms in PPARγ2 and type 2 diabetes came from Japanese-Americans, in which a frequency of the rare Ala allele of 9.3% in subjects with normal glucose tolerance versus only 2.2% in patients with type 2 diabetes was observed (11). This type of association study, however, is frequently troubled by a lack of reproducibility. Nevertheless, in a study that used the powerful tool of transmission disequilibrium testing in 333 Scandinavian parent-offspring trios with abnormal glucose tolerance, only the Pro12Ala polymorphism remained significant among 16 variants with previously published evidence for association with type 2 diabetes (or related disorders) (19). This association of the Pro12Ala polymorphism with type 2 diabetes was recently replicated in two large population-based studies from Finland and Japan including well over 4,000 subjects (15,20).

Although the initial publication reported a 75% risk reduction for diabetes conferred by the Ala allele (11), of five subsequent studies (12,21–24), only one (22) was able to reproduce a significant association. However, a meta-analysis including those five plus the aforementioned data set (19) demonstrated a significant risk reduction of 21% (19). The resulting population-attributable risk was estimated as ~25% (11). In other words, if the entire population carried the Ala allele, the prevalence for type 2 diabetes would be 25% lower. In contrast, mutations responsible for monogenic mature-onset diabetes of the young, though having a major effect on the individual’s glucose homeostasis, are so rare that they hardly affect a population’s risk of type 2 diabetes (25). This underlines the importance of alleles with weak individual effect but high population prevalence, such as the (so-called) wild-type Pro allele of PPARγ2 (75% prevalence in Caucasians), and clearly indicates the prominent role of PPARγ among candidate genes for common type 2 diabetes.

To make diagnostic, preventive, or therapeutic use of a polymorphism, it is necessary to understand how and in which metabolic, genetic, or environmental context the genotype influences the phenotype. A number of recent studies on metabolic pathways, diabetic and nondiabetic subphenotypes, and gene-environment and gene-gene interaction effects have provided some useful information regarding the potential mechanism by which the Pro12Ala polymorphism reduces the risk for type 2 diabetes. Generally, a genetic variant that affects the risk for type 2 diabetes must influence insulin sensitivity, insulin secretion, or susceptibility for obesity.

**EFFECT ON INSULIN SENSITIVITY**

In the original article, significantly greater insulin sensitivity was reported in nondiabetic Ala carriers (11). However, Ala carriers also had a lower BMI, and after adjustment the difference in insulin sensitivity was no longer significant. It therefore remained unclear what the primary result of the polymorphism was. In one small study, the confounding influence of BMI (and other relevant variables) on insulin sensitivity was eliminated by pair-matching carriers of the polymorphism with wild-type control subjects (26). Glucose infusion rates during euglycemic-hyperinsulinemic clamp (the gold standard technique for measuring insulin sensitivity in humans) per se were not different; however, expressing them per unit insulin revealed a significantly greater insulin sensitivity index (26). In the largest study so far addressing this issue, a 7% greater insulin sensitivity was observed in 616 normal glucose-tolerant Swedish 70-year-old men carrying the Ala allele (27). In the same article, a 6% difference in the same direction using the minimal model estimate for insulin sensitivity was reported for 364 young Danes; however, it failed to reach statistical difference. A power calculation based on these data revealed a required sample size of ~2,000 to achieve significance. In a Chinese/Japanese sib-pair study, which by design controlled relatively well for genetic background and childhood environment, a greater estimated insulin sensitivity (homeostasis model analysis) was observed in carriers of the Ala allele (28). Interestingly, a more pronounced effect of the Pro12Ala polymorphism on insulin sensitivity was observed before the background of the common Gly972Arg polymorphism in insulin receptor substrate-1 (29). Taken together, these studies provide...
substantial evidence that the Pro12Ala polymorphism improves insulin sensitivity in humans.

In subgroups with obesity, the difference in insulin sensitivity was more pronounced (22,30,31), suggesting an interaction of the polymorphism with factors originating from adipose tissue. In healthy, lean, and normal glucose tolerant subjects, greater insulin sensitivity of glucose disposal was tightly coupled to greater insulin sensitivity of lipolysis, as measured by isotope dilution (32). This finding was in principle confirmed in a larger cohort from the same laboratory by demonstrating significantly lower free fatty acid (FFA) concentrations during insulin stimulation (33). Based on these results, it appears possible that alterations in transcriptional activity of the Ala variant in adipocytes (where PPAR-γ2 is expressed) primarily enhance insulin’s action on suppression of lipolysis, resulting in a decreased release of FFAs. Secondly, reduced availability of FFAs would then permit muscle to utilize more glucose and liver to suppress glucose production more efficiently upon insulin stimulation (34). The metabolic studies in humans were not designed to distinguish between insulin-sensitizing effects on peripheral glucose disposal and suppression of glucose production. Studies are therefore required that specifically address the question of whether this polymorphism affects glucose production and insulin clearance, two processes clearly influenced by FFA availability.

EFFECT ON INSULIN SECRETION

Currently, strong evidence for a direct effect of the Pro12Ala polymorphism on insulin secretion is lacking. However, in Japanese subjects with manifest type 2 diabetes, a lower β-cell function index (homeostasis model analysis) was reported in carriers of the Ala allele (15). Interestingly, lipid infusion designed to elevate plasma FFA concentrations fourfold resulted in a decrease in insulin secretion during hyperglycemic clamp in carriers of the Ala allele, but an increase in control subjects with two Pro alleles (35). These findings might provide a partial explanation for the above observation that β-cell function deteriorates more in Ala carriers once diabetes has developed (15). Conceivably, superimposition of secondary mechanisms—such as chronic exposure to elevated FFAs and/or hyperglycemia, which are characteristic for overt type 2 diabetes—alters (or even reverses) the effect of the genetic variant. Expression of PPAR-γ, though not specifically of the isoform PPAR-γ, in β-cells has been demonstrated (36). However, whether any of the above effects on β-cell function were direct or secondary remains open and requires further and more detailed studies in, for example, heterozygous PPAR-γ knockout animals or humans homozygous for the Ala allele.

EFFECT ON OBESITY

Because PPAR-γ plays a key role in adipocyte differentiation and body fat mass is a strong determinant of insulin sensitivity and type 2 diabetes, the influence of the Pro12Ala polymorphism on susceptibility for obesity has been of major interest. In Pima Indians, suggestive linkage (logarithm of odds = 2.0) with percentage body fat was reported for the 3p24.2-p22 locus, which is close to the region harboring the PPAR-γ gene (3p25-p24.2) (37).

Cross-sectional studies with small to moderate sample sizes yielded inconsistent results by demonstrating either no difference (21,23,28,38,39) or a modestly greater BMI in carriers of the Ala allele (10,40,41). Two sufficiently large studies (>1,000 nondiabetic subjects) found a lower BMI in Finns (11) and no difference in Japanese (15). On the other hand, longitudinal studies in selected populations with relatively small sample sizes consistently suggested greater weight gain in association with the Ala allele (20,42,43).

Thus, although cross-sectional evidence convincingly argues against susceptibility of obesity conferred by the Ala allele, the issue remains somewhat unclear. In particular, the interpretation of the longitudinal analysis is complicated by the fact that greater insulin sensitivity per se predicts future weight gain (44). Weight gain may be related to the greater insulin sensitivity particular of lipolysis (33), which would result in overproportionate retention of FFAs in stored triglycerides during physiological insulin stimulation (e.g., postprandially). In this scenario, obesity would be a consequence of increased insulin sensitivity and not directly of the Ala allele. It may be of note in this context that under a high-fat diet, heterozygous PPAR-γ-deficient mice (an animal model for reduced PPAR-γ activity) were protected from the development of insulin resistance caused by adipocyte hypertrophy, compared with wild-type littermates (45).

Clearly, any effect of this polymorphism on measures of obesity would be extremely subtle, and its demonstration would be crucially dependent on subject selection and interaction with ethnic background and other genetic or environmental factors. For example, an interaction effect of the Pro12Ala polymorphism with the Trp64Arg polymorphism in the β3-adrenergic receptor (which by itself probably does not influence body weight [46]) has been reported (47). Moreover, a greater BMI was observed in Ala carriers when the dietary polyunsaturated fat–saturated fat ratio was low, suggesting gene-nutrient interaction via the PPAR-γ locus (48).

CELLULAR MECHANISM

PPAR-γ is a master transcriptional regulator involved in the expression of probably hundreds of genes (1). Two studies have directly examined the transcriptional activity of the Ala variant of PPAR-γ in comparison to the Pro variant in experimental cell models (11,49). Reduced binding of the Ala variant to the PPAR-γ-responsive DNA elements was observed in transient transfection assays (11,49). Moreover, reduced transcription of specific genes (lipoprotein lipase and acyl-CoA oxidase) was reported for cells overexpressing the Ala variant compared with cells overexpressing the wild-type protein (11). These studies clearly indicate reduced transcriptional activity of PPAR-γ as a result of the Pro-to-Ala exchange. Because PPAR-γ2 is exclusively expressed in fat cells, any metabolic effects of the polymorphism, including those on glucose homeostasis, are likely to be secondary to alterations in adipose tissue.

There is evidence from humans that the Pro12Ala polymorphism promotes the suppression of FFA release by insulin (32,33). It is unclear, however, which genes are specifically affected by the transcriptional changes con-
ferred by the polymorphism. Conceivably, the proportion of large versus small adipocytes is involved. Activators of PPAR-γ have been shown to promote differentiation of preadipocytes to small adipocytes (50), and in small adipocytes lipolysis is more insulin sensitive than in large adipocytes (51). In addition, alterations in fat distribution (more subcutaneous and less visceral [52]) as a result of the polymorphism could mediate effects on lipolysis. In humans, PPAR-γ expression in visceral adipose tissue relative to subcutaneous adipose tissue is increased in obese subjects (53). Because visceral adipose tissue is metabolically more harmful (54), the Ala allele would be expected to have an even greater impact in obese subjects, as in fact was shown to be the case (30). Interestingly, heterozygous PPAR-γ-deficient transgenic mice had smaller adipocytes and greater insulin sensitivity than wild-type mice (45). These mice were also characterized by greater insulin sensitivity of both glucose disposal and suppression of glucose production (45,55). In these studies, insulin suppression of FFA release was not determined but would represent a prime candidate for mediating the insulin effects on glucose homeostasis.

In addition to fatty acids, adipose tissues release a number of peptide hormones that influence insulin sensitivity. These include the cytokine tumor necrosis factor-α (TNF-α) (56), resistin (57), and adiponectin (apM-1) (58). Although the insulin desensitizing evidence for TNF-α and resistin is weak for humans, adiponectin concentrations were clearly shown to be positively correlated with insulin sensitivity, even after adjusting for body fat (59), to decrease with deteriorating glucose tolerance (59), and to increase after weight reduction (60). Moreover, intravenous administration of recombinant adiponectin to rodent models of insulin resistance restored normal insulin sensitivity (61). Because all three peptides are under transcriptional control of PPAR-γ (57,62–64), any of them (and possibly other peptides yet to be identified) could mediate the Pro12Ala effect on insulin sensitivity (Fig. 2). In humans, however, it has not been studied yet whether the Pro12Ala polymorphism influences systemic or local adipocytokine concentrations.

It has been difficult to explain why, paradoxically, both reduced transcriptional activity (due to heterozygous knockout or the Pro12Ala polymorphism) and pharmacological activation (by thiazolidinediones) of PPAR-γ results in improved insulin sensitivity (65). Recent studies in heterozygous PPAR-γ-deficient mice, however, have contributed to unraveling this problem. Alterations in white adipose tissue triglycerides, hepatic triglycerides, hepatic energy consumption, and muscle energy metabolism were markedly different between the two levels of PPAR-γ activation (63), indicating that entirely different metabolic pathways must be mediating the insulin-sensitizing effect of supraphysiological stimulation (thiazolidinediones) and moderate reduction (heterozygous knockout or Pro12Ala) of PPAR-γ activity.

Comparison with other naturally occurring variants in PPAR-γ may be useful for elucidating the molecular mechanisms. The Val290Met and Pro467Leu mutations drastically reduced transcriptional activity of PPAR-γ in a dominant-negative fashion in vitro and resulted in a severely insulin resistant though lean phenotype in vivo (9). Structure-function simulations suggested that these mutations affect the orientation of helix 12 of PPAR-γ, which is important for the interaction with ligands and coactivators (9). On the other hand, these mutations are less likely to affect ligand-independent transcriptional activity, a process strongly controlled by the NH₂ terminus (66), the domain that harbors codon 12. Thus, the net result of an amino acid exchange that decreases transcriptional activity of PPAR-γ critically depends on the localization within the PPAR-γ molecule. In support of this concept, antidiabetic properties of PPAR-γ antagonists (67) or PPAR-γ modulators (68–70) have been demonstrated.

Finally, it is necessary to point out that the CCA-to-GCA missense mutation in PPAR-γ (which causes the Pro12Ala exchange) need not necessarily be the functionally relevant mutation, but rather in linkage disequilibrium with it. 
For example, the functional mutation could reside in the promoter region of the PPAR-γ gene and result in reduced expression of PPAR-γ protein. The Pro12Ala polymorphism would then merely represent a genetic marker for the relevant mutation. However, the demonstrated reduction in transcriptional activity argues against such a scenario (11,49). In addition, a systematic screening of 70 diabetic individuals failed to reveal additional missense mutations elsewhere in the PPAR-γ gene or its promoter (19). All in all, a strong case exists for Pro12Ala itself being the etiologic exchange.

SUMMARY AND CONCLUSIONS

The transcription factor PPAR-γ is a master regulator of the relationships between nutrients, susceptibility to obesity, control of peptides released from adipocytes, and insulin sensitivity. The alanine allele of the common Pro12Ala polymorphisms in the isoform PPAR-γ2 is associated with a 25% reduced risk for type 2 diabetes. It thus represents the first genetic variant with a broad impact on the risk of common type 2 diabetes. The effect of this polymorphism is probably mediated by increased insulin sensitivity, which may be secondary to more efficient suppression of FFA release from fat tissue, where the isoform PPAR-γ2 is exclusively expressed. Modulation of expression and release of adipocytokines that influence insulin sensitivity is likely also to be involved, but this remains to be demonstrated in humans. The effects on muscle, liver, and possibly other tissues ultimately influencing glucose homeostasis are secondary. The underlying molecular mechanism of this polymorphism is a moderate reduction of the ligand-independent activity of PPAR-γ. Some findings for the Pro12Ala variant differed depending on superimposition of environmental factors. This clearly suggests that so-called "gene-environment interaction" may well hinge on genetic variations, such as those occurring in the transcription factor PPAR-γ. A number of issues remain to be confirmed and further explored, including the increased vascular complications and more pronounced β-cell dysfunction associated with the Ala allele once diabetes has developed. Moreover, the likely interaction with independent modulators such as obesity, ethnicity, ratio of unsaturated to saturated fatty acids, and other common genetic polymorphisms requires further studies. The understanding of how specific modulation of PPAR-γ influences metabolism in humans may accelerate the development of novel pharmacological agents useful for preventing or treating type 2 diabetes and related disorders.

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Emerging scientific evidence has disclosed unsuspected influences between iron metabolism and type 2 diabetes. The relationship is bi-directional—iron affects glucose metabolism, and glucose metabolism impinges on several iron metabolic pathways. Oxidative stress and inflammatory cytokines influence these relationships, amplifying and potentiating the initiated events. The clinical impact of these interactions depends on both the genetic predisposition and the time frame in which this network of closely related signals acts. In recent years, increased iron stores have been found to predict the development of type 2 diabetes while iron depletion was protective. Iron-induced damage might also modulate the development of chronic diabetes complications. Iron depletion has been demonstrated to be beneficial in coronary artery responses, endothelial dysfunction, insulin secretion, insulin action, and metabolic control in type 2 diabetes. Here, we show that iron modulates insulin action in healthy individuals and in patients with type 2 diabetes. The extent of this influence should be tested in large-scale clinical trials, searching for the usefulness and cost-effectiveness of therapeutic measures that decrease iron toxicity. The study of individual susceptibility and of the mechanisms that influence tissue iron deposition and damage are proposed to be valuable in anticipating and treating diabetes complications. Diabetes 51:2348–2354, 2002

It is increasingly recognized that iron influences glucose metabolism, even in the absence of significant iron overload. In the general population, body iron stores are positively associated with the development of glucose intolerance, type 2 diabetes (1–7), and gestational diabetes (8,9). Among U.S. adults, men with newly diagnosed diabetes had an odds ratio (OR) of 4.94 (95% confidence interval [CI] 3.05–8.01) and women had an OR of 3.61 (2.01–6.48) of having elevated ferritin (9). Among newly diagnosed diabetic subjects (16) and in amelioration of endothelial dysfunction in patients with type 2 diabetes (15) a significant impact of tissue iron excess on systemic effects of diabetes is suggested by recent reports in which iron appears to influence the development of diabetic nephropathy and vascular dysfunction. In this sense, intravenous administration of deferoxamine resulted in improved coronary artery responses to cold stress testing in type 2 diabetic subjects (16) and in amelioration of endothelial dysfunction in subjects with coronary heart disease (17).

All these observations suggest that iron is more intimately linked to human pathophysiology than previously thought. In fact, iron metabolism is closely associated with the clinical presentation of numerous systemic diseases (18). Tissue iron excess contributes to produce and amplify the injury caused by free radicals as well as to modulate various steps involved in the inflammatory lesion.

In this article, we summarize the relationships between iron, insulin resistance, and type 2 diabetes and discuss the therapeutic and clinical implications of reducing body iron.

Iron stores are associated with insulin sensitivity, insulin secretion, and type 2 diabetes

Iron and insulin sensitivity. Iron stores, expressed as serum ferritin concentration, have been proposed to be a component of the insulin-resistance syndrome. Indeed, the concentration of circulating ferritin was significantly associated with centrally distributed body fatness as well as with several other measurements of obesity (19). In the apparently healthy general population, serum levels of ferritin were also positively correlated with baseline serum glucose and with the area under the curve for glucose during the glucose oral tolerance test (20,21). In gestational diabetes, both BMI and serum ferritin levels were found to be independent predictors of 2-h glucose during an oral glucose tolerance test (8,9). Ferritin levels also correlated with diastolic arterial blood pressure, even after adjustment for BMI. Of note is the beneficial effect of blood letting, a means of reducing iron stores, in the treatment of resistant hypertension (22) and in posttransplant hypertension associated with erythrocytosis (23). Serum ferritin concentration was also directly associated
with uric acid (another component of the insulin resistance syndrome) and inversely related with HDL cholesterol and the HDL\textsubscript{ld} to HDL\textsubscript{hd} ratio (21).

Insulin resistance itself, assessed by either the euglycemic clamp (24) or the minimal model (25,26), was found to be associated with total body iron stores, even in the presence of normal glucose tolerance. Dnochowski et al. (25) reported that serum concentrations of ferritin were negatively correlated with insulin sensitivity ($r = -0.58$) in subjects with hemochromatosis. Cavallo-Perin et al. (26) reported that insulin sensitivity, which correlated closely with iron overload ($r = -0.70$), was reduced by 40% in thalassemia patients. Insulin resistance also appeared to be closely linked to total body iron stores in the general population (21). Serum ferritin levels could be a useful marker of insulin resistance beyond a given threshold (20,21). In the study by Toumainen et al. (20), the increase in serum insulin concentrations was clearly apparent in the upper two quintiles of ferritin levels. In a different study, the correlation between circulating ferritin and insulin resistance was only observed in the upper two quartiles of ferritin levels (21). Below this threshold, the potential tissue effects of siderosis would be negligible.

Some comments on the specificity of serum ferritin as an indicator of iron stores seem necessary. The relationship between serum ferritin and histochemical assessment of storable tissue iron contributes to define threshold values for serum ferritin, indicating exhausted, small, normal, ample, and increased iron stores. However, the barrier between "normal" and "small" or "ample" iron stores is not well defined and remains controversial. Approximately 10% of type 2 diabetic patients with high ferritin levels had transferrin saturations greater than normal (40%). On the other hand, serum ferritin should be cautiously evaluated in patients with type 2 diabetes, because it may falsely indicate "normal iron stores." It should not be ignored that chronic inflammation could contribute, to some extent, to increased ferritin concentration (see below).

**Iron and β-cell function.** Recent in vitro studies have shown that H-ferritin mRNA is four- to eightfold higher in rat islets treated with 20 mmol/l glucose than in islets treated with 1 mmol/l glucose (27). The potential reason for the increased ferritin in the β-cell is that ferritin exhibits antioxidant properties and the β-cell is particularly sensitive to oxygen radicals. This high amount of ferritin can explain why iron is preferentially retained in the β-cell. In fact, iron deposition in islets, albeit variable, is restricted to β-cells (28).

An increase in β-cell mass was demonstrated in a small number of nondiabetic or mildly diabetic patients with iron overload (28). In agreement with this increase in β-cell mass, raised basal and stimulated C-peptide secretion were observed in type 2 diabetic patients with increased serum ferritin concentration. Furthermore, significantly lowered C-peptide secretion was found after phlebotomy-induced iron depletion, suggesting increased β-cell insulin sensitivity (15).

**Iron overload and type 2 diabetes.** Five additional pieces of scientific evidence favor the hypothesis that iron plays a role in type 2 diabetes. First, increased prevalence of hemochromatosis was found among unselected patients with type 2 diabetes. Phelps et al. (29) and Conte et al. (30) reported that diabetes confers increased risk for hereditary hemochromatosis, which was 2.4% and 1.34% higher in Australian and Italian populations, respectively.

This evidence, however, is not always consistent. The recent characterization of HFE has allowed a more direct study of the prevalence of its mutations in type 2 diabetes. Homozygosity for the C282Y change is generally associated with clinically evident hereditary hemochromatosis (83% of hemochromatosis patients are YY homozygotes). Compound heterozygotes for H63D mutation (C282Y/H63D) succumb to the disease, although with reduced penetrance. An increased frequency of C282Y mutations in subjects with type 2 diabetes has been described in some studies (31-32). Notwithstanding, at least four additional studies reported no significant differences in the prevalence of C282Y mutations between patients with type 2 diabetes and control subjects of Caucasian origin (33-36). In the Spanish population, the frequency of the H63D mutation was significantly higher in type 2 diabetic subjects (36). The H63D mutation is also associated with other nonclassical conditions of iron overload. On the other hand, it is interesting to mention that genetic hemochromatosis contributed to 1% of late-onset type 1 diabetes (37).

Second, frequent blood donations, leading to decreasing iron stores, have been demonstrated to constitute a protective factor for the development of diabetes (13). This finding is especially important given the high prevalence of increased iron stores in the general population of western countries (10) and the observation that increased iron stores appear to predict an increased incidence of type 2 diabetes (5). In experimental models, the incidence of diabetes was reduced from 78 to 22% at 120 days of age after serial blood withdrawals in the BB rat (38).

Third, a recent randomized study also suggests that iron stores may influence insulin action in type 2 diabetes (15). In this report, 28 type 2 diabetic patients with increased serum ferritin concentration and negative for C282Y mutation of hereditary hemochromatosis were randomized to blood letting (three 500 ml phlebotomies at 2-week intervals) or to observation. Insulin secretion and sensitivity were tested at baseline and at 4 and 12 months thereafter. The two groups were matched for age, BMI, pharmacological treatment, and chronic diabetes complications (15). Baseline glycated hemoglobin and insulin sensitivity were not significantly different between the two groups. A statistically significant increase in insulin sensitivity was observed in the blood-letting group (from 3.30 ± 1.81 to 3.08 ± 2.55 mg·dl\textsuperscript{-1}·min\textsuperscript{-1} at 4 months to 3.16 ± 1.85 mg·dl\textsuperscript{-1}·min\textsuperscript{-1} at 12 months, $P = 0.045$) in contrast to patients subjected to observation in whom insulin sensitivity did not significantly change (from 3.24 ± 1.9 to 3.26 ± 2.05 mg·dl\textsuperscript{-1}·min\textsuperscript{-1} at 4 months to 2.31 ± 1.35 mg·dl\textsuperscript{-1}·min\textsuperscript{-1} at 12 months). Accordingly, blood HbA\textsubscript{1c} decreased significantly only in the blood-letting group at 4 months (mean differences -0.61, 95% CI -0.17 to -1.04, $P = 0.01$).

Fourth, a novel syndrome of hepatic iron overload has been described that associates hyperferritinemia with normal transferrin saturation and is not linked to the HLA-A3 antigen, a common marker for hereditary hemochromatosis (39). This condition is known as insulin
resistance-associated hepatic iron overload (IR-HI0) and combines abnormalities in iron metabolism (isolated hyperferritinemia with normal transferrin saturation), steatohepatitis, and the insulin resistance syndrome (obesity, hyperlipidemia, abnormal glucose metabolism, and hypertension) (38–41). In IR-HIO, iron overload occurs in both hepatocytes and sinusoid cells, being higher in the latter cells in 45% of cases, a finding seen in only 3% of subjects with hemochromatosis (42). Approximately two-thirds of these patients develop steatosis, whereas the remaining third show isolated signs of inflammation (42). Thus, these patients are at high risk for developing liver fibrosis, a complication observed in 60% of all cases, even in the presence of moderate iron overload. In contrast, liver fibrosis affects only 33% of patients with hemochromatosis. Because patients with IR-HIO are prone to experience significant tissue damage and because this can be prevented with simple and inexpensive therapies (i.e., phlebotomy), higher awareness in order to diagnose the disease has been suggested.

It cannot be ruled out, however, that IR-HIO is the same process of iron overload–related insulin resistance that associates liver steatosis and fibrosis in susceptible patients. It is important to recognize that in one study, liver iron stores were found within the normal range in patients with type 2 diabetes (43) in contrast to other studies (29,30). Under this assumption, IR-HIO would be at one end of the spectrum of iron overload–related insulin resistance.

Fifth, insulin resistance features are frequently seen in patients chronically infected with the hepatitis C virus. In these subjects, BMI, elderliness, iron stores, and family history of diabetes and advanced liver fibrosis were found to predict the development of diabetes (44). Interacting pathways linking glucose and iron metabolism

**A. Insulin influences iron metabolism.** Insulin is an anabolic hormone that stimulates the cellular uptake of many nutrients, including hexoses, amino acids, cations and anions. Intestinal absorption of nonheme iron is tightly regulated in keeping with the body requirements, and absorption of iron is minimal when body iron stores are normal. Absorption of heme iron (largely provided by red meat in western countries) does not appear to be dependent on body iron content. In the steady state, circulating iron is bound to transferrin and is taken up from the blood by a high-affinity specific transferrin receptor. The transferrin-receptor complex is internalized by endocytosis and released into a nonacidic cellular compartment, where it can be used in the synthesis of essential cellular components. Insulin is known to cause a rapid and marked stimulation of iron uptake by fat cells, redistributing transferrin receptors from an intracellular membrane compartment to the cell surface (45). Insulin is also responsible for the increased ferritin synthesis in cultured rat glioma cells (46). Importantly, transferrin receptors have been shown to colocalize with insulin-responsive glucose transporters and insulin-like growth factor II receptors in the microsomal membranes of cultured adipocytes, suggesting that regulation of iron uptake by insulin occurs in parallel with its effects on glucose transport (47).

**B. Iron influences glucose metabolism.** Reciprocally, iron influences insulin action. Iron interferes with insulin inhibition of glucose production by the liver. Hepatic extraction and metabolism of insulin is reduced with increasing iron stores, leading to peripheral hyperinsulinemia (48). In fact, the initial and most common abnormality seen in iron overload conditions is liver insulin resistance (49). There is some evidence that iron overload also affects skeletal muscle (50), the main effector of insulin action.

**C. Oxidative stress influences both glucose and iron metabolism.** Oxidative stress induces both insulin resistance [by decreasing internalization of insulin (51)] and increased ferritin synthesis.

Iron is intimately linked to oxidative stress. Iron participates, through the Fenton reaction, in the formation of highly toxic free radicals, such as hydroxide and the superoxide anion, which are capable of inducing lipid peroxidation. For iron to act as a prooxidant agent, it must be in its free form. Iron can be released from ferritin by the action of reducing agents that convert Fe$^{3+}$ into Fe$^{2+}$ (52). Glycation of transferrin decreases its ability to bind ferrous iron (53) and, by increasing the pool of free iron, stimulates ferritin synthesis. Glycated holotransferrin is additionally known to facilitate the production of free oxygen radicals, such as hydroxide, that further amplify the oxidative effects of iron (53).

The fraction of unused and highly toxic iron is stored as ferritin molecules in order to be neutralized. Apoferritin, the protein fraction of ferritin, is spatially folded to create a central groove that holds oxidized iron molecules [Fe$^{3+}$]. Apoferritin is a high–molecular weight (450 kDa) multimeric protein (24 subunits of heavy and light chains) that exhibits exquisite high capacity for iron storage (4,500 mol iron per mole of ferritin). Synthesis of apoferritin is induced at both the transcriptional and posttranscriptional levels by the presence of free iron. The increase in Fe$^{2+}$ downregulates the affinity of iron-regulatory element (IRE) binding protein (BP) for its IRE binding site in the 5' region of ferritin mRNA, leading to increased ferritin translation.

The heavy chain in the apoferritin molecule exerts ferroxidase activity, catalyzing the oxidation of Fe$^{2+}$ into Fe$^{3+}$, which prevents iron-induced cyclic red-ox reactions that would spread and amplify the oxidative damage. This activity occurs under aerobic conditions, allowing the storage of intracellular iron. When concentrations of antioxidants are low, the reducing potential and anaerobiosis progressively increase, facilitating a rapid release of iron from ferritin. Additionally, the ferroxidase activity in the heavy chain is downregulated in this setting, decreasing the incorporation of iron into ferritin. The overall result of oxidative reactions is an increase in the availability of free iron from the ferritin molecule as well as from other molecules undergoing degradation, such as the heme group. These events, in turn, can enhance and amplify the process of generation of free radicals, causing cellular and tissue damage. The oxidative stress also downregulates the affinity of IRE for IRE-BP. Thus, ferritin can act both as a source or iron, which induces oxidative stress, and as a mechanism that protects against iron toxicity (54).

Hyperferritinemia is present in 6.6% of unsellected
patients with type 2 diabetes (55). Serum concentrations of ferritin are usually increased in poorly controlled type 1 and type 2 diabetic subjects, and ferritin has been shown to predict HbA1c, independently of glucose (21), probably reflecting increased oxidative stress. Short-term improvement in glycemic control is followed by variable decreases in serum ferritin concentration.

D. Cytokines influence iron and glucose metabolism. Cytokines simultaneously cause an increase in transferrin receptors on the cell surface, favoring tissue deposition of iron (56) and insulin resistance (57).

In summary, a scenario can be envisioned in which the physiological action of insulin leads to increased uptake of different nutrients and iron. Any factor causing hyperinsulinemia (weight gain, aging, repeated usual-life infections, or periodontitis) amplifies this process, determining increased deposition of iron, which in the long-term worsens insulin resistance.

Clinical and therapeutical implications of iron depletion

A. Iron depletion and diabetic metabolic control. There are historical notes regarding iron overload conditions that can be helpful in delineating the effects of iron depletion (58-60). Phlebotomy was first used in the treatment of hemochromatosis in the 1950s. Interestingly, diabetic metabolic control improved in 35–45% of patients with hemochromatosis after iron depletion (58). In 1969, Williams et al. (59) showed that diabetic patients treated with phlebotomy required less insulin than similar patients during the prephlebotomy period. In 1972, Dymock et al. (60) reported a significant reduction in total daily insulin dosage following phlebotomy.

Facchini (11) found significant reductions in insulin concentrations 1 month after performing a 550-ml phlebotomy in healthy volunteers. It has also been suggested that the increased insulin sensitivity observed in vegetarian subjects might be related to their low-iron diet. Recently, as stated above, blood letting of 1,500 ml has been demonstrated to improve insulin sensitivity and to decrease C-peptide secretion in type 2 diabetic subjects who were negative for common hemochromatosis mutations but had increased serum ferritin concentration (15).

Iron chelators also seem beneficial in optimizing diabetic metabolic control. In 1989, Cutler (61) administered deferoxamine to nine type 2 diabetic patients with hyperferritinemia who were negative for the most common hemochromatosis haplotypes. Major improvement in the diabetic metabolic control was observed in seven patients, and parallel reductions in baseline concentrations of glucose, triglycerides, and glycated hemoglobin were observed. Treatment with either insulin or oral hypoglycemic agents could be discontinued in some patients (61). Subsequently, it was reported that subcutaneous deferoxamine caused an improvement in glycated hemoglobin in another nine patients (62), although serum concentrations of C-peptide after glucose or arginine infusion did not improve significantly (62).

B. Iron depletion and chronic diabetes complications. Macrovascular disease. The general effect of catalytic iron is to convert poorly reactive free radicals, such as \( \text{H}_2\text{O}_2 \), into highly reactive ones, such as OH\(^-\) and O\(_2^-\). Free radicals and other oxidation by-products are well known factors that impair the mechanisms of vasodilatation (63) and cause endothelial depletion of endogenous antioxidants, such as ascorbic acid (64). Iron chelation blocks oxidation of LDL, and iron released from heme and ferritin favors oxidation of this lipoprotein (65). Increased iron availability is, theoretically, expected to contribute to macrovascular disease because iron has an adverse effect on endothelium (66) and accelerates the development of atherosclerosis (67). In fact, ferritin gene expression increases in the course of atherosclerotic lesion formation (68). Notwithstanding, studies performed in experimental models offer conflicting conclusions. For instance, an iron-deficient diet has been found to reduce atherosclerotic lesions (69), whereas iron overload led to a decrease in atherosclerosis (70) in the same animal model. It should be noted, however, that the possible influence of obesity, insulin resistance, or diabetes has not been explored in experimental iron-modified atherosclerosis. Moreover, humans are the only species in which genetic iron overload has been described to induce significant parenchymal damage (71).

In subjects with hemochromatosis, medium-sized arteries are characterized by an eccentric hypertrophy and decreased distensibility that are partially reversible after iron depletion (72). These findings seem to be linked to iron-induced fibrogenesis, determining an increased total collagen content in arteries from these patients. There is also some evidence for iron-dependent growth of arterial wall tissue: iron chelation by deferoxamine inhibits vascular smooth muscle cell proliferation (73).

Long-term use of the modified iron chelator hydroxyethyl starch conjugated-deferoxamine prevented endothelial dysfunction associated with experimental diabetes (74). In type 2 diabetic patients, coronary artery responses to cold stress testing improved substantially after deferoxamine administration (16). Similarly, iron chelation was shown to ameliorate the endothelial dysfunction of patients with coronary heart disease (17).

Improvement of nitroglycerine-induced vasodilatation was also observed following phlebotomy in type 2 diabetic patients in a preliminary study. The improvement in vascular reactivity paralleled the decrease in serum transferrin saturation, total hemoglobin (markers of circulating iron), and blood glycated hemoglobin (75). These observations suggest that diabetic vascular dysfunction seems partially reversible and that the circulating compartment acts as a reservoir of transition metals that directly affects vascular function (76). Increased hemoglobin, an iron-enriched protein, is deleterious for endothelial function, as normal blood vessels exposed to total and glycated hemoglobin are known to experience impaired vascular relaxation (77).

In the general population, the relationship between iron and atherosclerosis is, however, controversial (78). Thus, some animal experimental data regarding the effect of manipulating iron stores on atherosclerosis and human data showing improvement of vascular structure and function following iron depletion (16,17,72,75) are both consistent with the theory that iron contributes to the development of vascular disease. However, the current epidemiological data associating iron stores with either
Ferritin mRNA

Insulin internalisation and biological actions

Insulin

Free iron

Oxidative stress

Decreased NO synthesis
Decreased NO action
Decreased antioxidants
Increased lipid and protein peroxidation

Cell/tissue damage

Abnormal vascular reactivity

FIG. 1. Schematic representation of iron interactions with insulin resistance and oxidative stress. Insulin influences iron metabolism. Insulin stimulates ferritin synthesis and facilitates iron uptake by the cell through the translocation of transferrin receptors from the intracellular compartment to the cell surface. Conversely, iron influences glucose metabolism. Iron in a potent prooxidant that increases the cell oxidative stress, causing inhibition of insulin internalization and actions, results in hyperinsulinemia and insulin resistance. Free iron also exerts a positive feedback on ferritin synthesis, while oxidative stress increases the release of iron from ferritin. The increased oxidative stress and insulin resistance cause endothelial and tissue damage. Protein glycation, as seen in diabetes, further amplifies these abnormalities stimulating iron release from transferrin, increasing the cell oxidative stress and directly causing endothelial and tissue damage. NO, nitric oxide; TR, transferrin receptor; (+), stimulation; (-), inhibition; dotted lines, possible trafficking or iron through the cell membrane.

CONCLUSIONS

The central role of iron in biology is illustrated by the fact that this is the fourth most abundant element in Earth’s crust as well as the transition element most abundant in living organisms. Iron has additionally proven to be fundamental in the selection imposed by evolution, given its close relationship with oxygen. Although losses of this metal are only a tenth of those found in any other given mammal, iron regulation is maintained within very narrow limits in humans.

Our biochemistry and physiology are tuned to life conditions that existed before the advent of agriculture some 10,000 years ago. Hunter-gatherer societies obtained more than 56–65% of their subsistence from animal foods (81). Meat eaters, with a typical high protein and low-carbohydrate diet, have a significant higher plasma concentration of iron (82) and concomitant insulin resistance in the liver and peripheral tissues (83). It is plausible that the survival advantage of both iron and high protein-induced insulin resistance in our ancestral line was that the little available glucose from carbohydrate consumption was preserved for brain function and reproductive fetal/placental/mammary tissues (84). Nowadays, with increased life expectancy, this protective mechanism has become detrimental, with iron promoting both insulin resistance and increased oxidative stress.

The clarification of the mechanisms that regulate this interaction are proposed to contribute to improve the management of diabetes and to anticipate its possible complications. Here, we show that iron modulates insulin action in healthy individuals and in patients with type 2 diabetes. The extent of this influence should be tested in large-scale clinical trials, searching for the usefulness and cost-effectiveness of therapeutic measures that decrease iron toxicity. Of paramount importance will be the definition of “normal body iron stores” and the establishment of
early therapeutical interventions. Simple and inexpensive therapies, such as blood letting and iron chelators, are emerging as alternative and effective treatments for insulin resistance.

It will also be necessary to explore whether important elements of iron metabolism are altered in diabetes, namely the transporters DMT1, ferroportin, and MTPI, which are critical in intestinal absorption and entry of iron into the circulation, and haematin, which oxidizes Fe$^{2+}$ to Fe$^{3+}$ during this process (85). Interestingly, certain genes appear to be simultaneously involved in iron balance, inflammation, and glucose responsiveness, suggesting a link between these pathways and type 2 diabetes (85).

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**Perspectives in Diabetes**

**A Genetic Switch in Pancreatic \(\beta\)-Cells**

Implications for Differentiation and Haploinsufficiency

Jorge Ferrer

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Heterozygous mutations in the genes encoding transcriptional regulators hepatocyte nuclear factor (HNF)-1\(\alpha\) and HNF-4\(\alpha\) cause a form of diabetes known as maturity-onset diabetes of the young (MODY). Haploinsufficiency of HNF-1\(\alpha\) or HNF-4\(\alpha\) results in MODY because of defective function of pancreatic islet cells. In contrast, homozygous null mutations in mouse models lead to widespread and profound gene expression defects in multiple cell types. Thus, it is not surprising that HNF-1\(\alpha\) function is now known to have distinct properties in pancreatic \(\beta\)-cells. It controls a complex tissue-selective genetic network that is activated when pancreatic cells differentiate, and allows these cells to maintain critical specialized functions. The network contains an indispensable core component formed by a positive cross-regulatory feedback circuit between HNF-1\(\alpha\) and HNF-4\(\alpha\). This type of circuit configuration can exhibit a switch-like behavior with two stable states. In the default active state, it can serve to perpetuate network activity in differentiated \(\beta\)-cells. However, the loss of one HNF-1\(\alpha\) or HNF-4\(\alpha\) allele can increase the probability that the feedback circuit is permanently switched off, resulting in decreased expression of all four alleles selectively in \(\beta\)-cells. Such a model can serve to rationalize key aspects of the pathogenic mechanism in MODY. *Diabetes* 51:2355-2362, 2002

The genetic analysis of mendelian forms of diabetes has produced a major breakthrough in our understanding of the transcriptional programs needed for differentiated \(\beta\)-cells to function properly. During a period of \(<3\) years, mutations in five genes encoding transcriptional regulators were found to cause early-onset autosomal dominant diabetes (also known as maturity-onset diabetes of the young [MODY]) (Table 1) (1-5). The MODY3 gene, encoding an atypical homeodomain protein named hepatocyte nuclear factor (HNF)-1\(\alpha\), was incriminated in its etiology through a positional cloning strategy (2,6). HNF-1\(\alpha\) had been identified several years before, but despite a wealth of information on its function in regulating liver enriched genes, it had not been regarded as a candidate gene for diabetes (7). The identification of HNF-1\(\alpha\) led immediately to the recognition that the MODY1 gene encoded HNF-4\(\alpha\), a regulator of HNF-1\(\alpha\) (1). Because diabetes in both MODY1 and MODY3 results from a severe insulin secretory defect (6,8-10), these genetic findings indicated that HNF-1\(\alpha\) and HNF-4\(\alpha\) have an unanticipated role in controlling \(\beta\)-cell function (1,2). Shortly after these findings, a candidate gene approach led to the recognition that mutations in further \(\beta\)-cell transcriptional regulator genes caused MODY: IPF-1(MODY 4), TCF-2 (encoding HNF-1\(\beta\), MODY 5), and NeuroD-1 (3-5) (Table 1).

The identification of HNF-1\(\alpha\) and HNF-4\(\alpha\) as MODY genes has raised several fundamental questions. What are the in vivo genetic targets and the basic biological processes that are controlled by these transcriptional regulators in \(\beta\)-cells? How can mutations in genes known to regulate transcription in diverse cell types result in a phenotype that is largely restricted to pancreatic cells? Why are heterozygous mutations sufficient to cause the disease, and why is a genetically determined phenotype delayed until the second to fourth decade of life? On the other hand, it was assumed that these and possibly other transcriptional regulators involved in MODY participated in a common \(\beta\)-cell regulatory pathway, but there was no evidence to define the nature of the pathway in this particular cell type. Clearly the answers to these questions will reveal molecular mechanisms that are central in the control of differentiated \(\beta\)-cell function.

The molecular genetics of MODY has been the subject of several recent reviews (11-14). The purpose of this article is to discuss recent findings reported by several laboratories that have uncovered key genetic interactions of HNF-1\(\alpha\) and HNF-4\(\alpha\) in pancreatic \(\beta\)-cells. This information is analyzed to propose a model for the haploinsufficient mechanism in MODY.

**HNF-1\(\alpha\) and HNF-4\(\alpha\) deficiency causes dysfunction of pancreatic \(\beta\)-cells.** Humans with heterozygous HNF-1\(\alpha\) and HNF-4\(\alpha\) mutations typically develop diabetes during the second to fourth decade of life (1,6,8,13,15). Diabetes results from abnormal glucose-induced insulin secretion, rather than defective insulin action or hepatic glucose output (6,8,9,16). The \(\beta\)-cell defect cannot be ascribed solely to reduced cell mass or insulin gene transcription, as it is selective for certain stimuli (17,18).
Studies in mice with homozygous HNF-1α null mutations or expressing a dominant-negative inhibitor of HNF-1 have been conceptually consistent with the human MODY3 findings (19–25). Hnf1α−/− mice do not exhibit a conspicuous developmental block, and although they have a small pancreas (and hence reduced ϒ-cell mass), it is proportional to their small body weight (21,23). Nonetheless, minor derangements in the organization of pancreatic cells and altered adaptive regenerative capacity of β-cells likely exist (23). Insulin mRNA abundance has been shown to be either unaltered or moderately decreased in the case of the ins1 gene (22–24). Analogous to the MODY3 findings, HNF-1α−/−islet cells display severely blunted secretory responses to glucose but retain the ability to respond to depolarization induced by KCI (19,23,25). The defective glucose response results in part from a glycolytic block proximal to enolase (19), but no single target gene is likely to be incriminated as a cause, given that numerous genes involved in stimulus secretion-coupling are underexpressed in hnf1α-deficient islet cells (22,24,26). There is also no secretory response to arginine (20,23), perhaps because of decreased expression of the basic amino acid transporter BAT (24). Dominant-negative inhibition studies have additionally demonstrated effects on mitochondrial function (25). In the aggregate, it appears that a major function of HNF-1α is to ensure that the machinery that is required for β-cells to respond to glucose and other stimuli is in place.

One apparent difference between HNF-1α−null mutant mice and humans is the lack of a clear phenotype in heterozygous mice up to age 16 weeks (23,27). This discrepancy clearly needs to be assessed in greater detail, as there are several potential reasons to account for it. One is that diabetes in heterozygous humans is not present until 10–40 years after birth (13,15), which does not necessarily equate to 6–16 weeks in the life of a mouse. Another factor is the likely existence of epistatic genetic modifiers that could modulate penetrance in diverse genetic backgrounds. In keeping with this notion, heterozygosity at the hnf1a locus in mice was recently shown to have striking effects on insulin secretion in a pdx1−/−background (27). Interestingly, very late or nonpenetrance has been described in MODY3 mutation carriers, indicating not only inter- but also intraspecies phenotypic variability (8,15).

There is much less information on the role of HNF-4α in β-cells. As occurs with HNF-1α, young hnf4a−/− mice do not exhibit glucose intolerance (27), whereas germ-line homozygous null mutants do not develop beyond gastrulation (28). Conditional Cre-loxP-based inactivation studies have shown that HNF-4α has critical roles for numerous genes in adult hepatocytes (29), but analogous experiments have yet to be reported in pancreatic cells. However, the β-cell phenotype in MODY1 is extremely similar to that in MODY3 (9,10). Furthermore, dominant-negative inhibition of either HNF-1α or HNF-4α in INS-1 β-cells leads to reduced expression of a remarkably similar set of genes (25,30). This suggests that HNF-1α and HNF-4α control a common genetic program in β-cells, and it is already quite clear that this program is required for the correct function of differentiated β-cells.

The function of HNF-1α is fully dependent on cell-type context. Although homozygous HNF-1α mutations have not been reported in humans, we know that hnf1α−/−mice exhibit a severe pleiotropic phenotype in addition to diabetes (21,23,24,31–33). This results from widespread gene expression defects in liver, kidney, and other organs, leading to multiple derangements such as abnormal bile acid and cholesterol metabolism, phenylketonuria, fatty liver, growth impairment, and tubular glycosuria (21,23,24,31–33). This picture contrasts completely with that seen in heterozygous HNF-1α and HNF-4α humans, who essentially present with β-cell dysfunction and diabetes (6,8,10,16). Isolated target gene defects in other tissues have been identified, such as decreased production of apolipoproteins AII and CII in MODY1 and an altered renal glucose reabsorption threshold in MODY3, but these are comparably subtle (32,34).

The tissue specificity of heterozygous mutations cannot be ascribed to a classical dominant-negative mechanism occurring solely in β-cells. A large number of human HNF-1α and HNF-4α mutations have been carefully analyzed, and although in some instances dominant-negative effects have been identified by overexpressing mutant proteins, there is compelling evidence in many other cases (such as in promoter mutations) that the mutation simply causes loss of function (14,35–37). Furthermore, the phenotype does not appear to be more severe in cases where a dominant-negative effect is observed (15). This suggests that HNF-1α and HNF-4α have β-cell–specific functions that are uniquely vulnerable to decreased gene dosage.

There is now more detailed information on some of the β-cell–specific functions of HNF-1α. Numerous genes, including glut2 and pklr, and several transcriptional regulators exhibit markedly decreased expression in hnf1α−/−islets, but do not require HNF-1α in other tissues such as liver (22,24,38). For many (though not all) genes, it is clear that the cell-specific requirements are not related to the hyperglycemic environment or other secondary derangements. For example, several such genes are abnormally expressed prenatally or shortly after birth, at a time when there is no significant hyperglycemia (22,24,38). Furthermore, HNF-1α directly interacts with chromatin of targets such as glut2 and pklr in mouse islets (22). Although some

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**TABLE 1**

Description of six MODY genes

<table>
<thead>
<tr>
<th>Locus</th>
<th>Human gene</th>
<th>Alias</th>
<th>Protein family</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MODY1</td>
<td>HNF4A</td>
<td>HNF4α, NR2A1</td>
<td>Nuclear receptor</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>MODY2</td>
<td>GCK</td>
<td>Glucokinase, hexokinase IV</td>
<td>Hexokinase</td>
<td>Glycose, glucose sensing</td>
</tr>
<tr>
<td>MODY3</td>
<td>TCF1</td>
<td>HNF1α, HNF1</td>
<td>Atypical homeodomain</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>MODY4</td>
<td>IPF1</td>
<td>IDX1, PDX1, STF1, GSF</td>
<td>Homeodomain</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>MODY5</td>
<td>TCF2</td>
<td>HNF1β, vHNF1</td>
<td>Atypical homeodomain</td>
<td>Transcriptional regulation</td>
</tr>
<tr>
<td>MODY6</td>
<td>NEUROD1</td>
<td>NeuroD, Beta2</td>
<td>Basic helix-loop-helix</td>
<td>Transcriptional regulation</td>
</tr>
</tbody>
</table>
of the mechanisms involved in the activation of direct HNF-1α targets are beginning to be unraveled (22,39,40), it is still unclear why for some genes HNF-1α is required for activation in certain cell types but not in others.

**HNF-1α occupies a critical position in cell-specific transcriptional regulator networks.** The existence of different HNF-1α–dependent regulatory networks in diverse cell types could be one of several nonexclusive mechanisms that account for the cell-specific roles of HNF-1α. The participation of HNF-1α in transcriptional networks is well documented. The analysis of null mutant embryoid bodies has shown that HNF-3β and HNF-3α exert positive and negative control on HNF-4α, respectively (41). HNF-4α is required for normal transcription of HNF-1α in hepatocytes and endoderm-like cells (37,41–43), whereas HNF-1α controls downstream hepatic transcriptional activators, such as FXR (44). This complex network manages a vast array of targets encoding proteins involved in highly specialized liver functions (21,31,33,44).

In pancreatic islets, HNF-1α is integrated in a network that has an entirely different architecture. Several mRNAs encoding transcriptional regulators, including HNF-4α, HNF-4γ, or HNF-3γ, are dependent on HNF-1α in pancreatic cells specifically (24,38) but are expressed normally in the liver or kidney of mice lacking HNF-1α (24,38). Interestingly, HNF-1α is also dispensable for the expression of these targets in pancreatic epithelial precursor cells, and only becomes essential shortly after differentiated pancreatic cells arise (38).

The existing data indicate that the structure of the pancreatic network is complex (Fig. 1) (24,30,38,45). It is nonlinear, with cross- and autocatalytic interactions (24,27,38). Some distal targets (such as pklr or shp) are connected with HNF-1α indirectly through subsidiary activators or by both direct and indirect mechanisms (24,38,46). Most likely, any attempts to decipher the logic of this entire network design at this time would be based on a keyhole vision of a much more vast network.

**The evidence for a cross-regulatory loop between HNF-1α and HNF-4α in pancreatic cells.** It is striking that despite the complexity of this network, it is already possible to isolate a very simple substructure that exhibits a highly predictable behavior in different model systems. Thus, HNF-1α and HNF-4α have been shown to establish interdependence through direct cross-regulatory interactions in pancreatic cells (24,30,35,38) (Fig. 1).

Proof for HNF-1α dependence on HNF-4α comes from several HNF-4α–deficient models that have shown that this gene is needed for normal HNF-1α transcription in embryonic visceral endoderm and hepatocytes (37,42,43). This control is exerted through the interaction of HNF-4α with a DR1 direct repeat cis element in the HNF-1α promoter (35,47). Strongly suggestive evidence that this interaction also takes place in β-cells comes from two sources. First, dominant-negative inhibition of HNF-4α in INS-1 β-cells results in severely decreased formation of HNF-1α DNA-binding complexes and mRNA (30). Second, a loss of function mutation in the HNF-1α promoter DR1 element is linked to MODY in a large pedigree (35). This suggests that HNF-4α control of HNF-1α occurs at some point in pancreatic development, and, importantly, that this interaction is essential for pancreatic β-cell function. Although a direct genetic analysis of the requirement for HNF-4α to express HNF-1α in both precursor and differentiated pancreatic cells is still needed, the available evidence indicates that HNF-4α controls the expression of HNF-1α in multiple cell types, including embryonic endoderm, liver, and pancreatic cells (30,35,37,41–43,47).

In sharp contrast, studies using hnf1α+/− mice have shown that HNF-1α control of HNF-4α is clearly restricted to differentiated pancreatic cells (but not precursor cells), and in part to intestinal cells (24,38). Interestingly, HNF-4α transcription in pancreatic cells is driven almost exclusively by an alternate promoter known as P2 (38,45). Chromatin immunoprecipitations have shown that HNF-1α directly interacts with the P2 promoter in islets (38). HNF-1α also transactivates P2 in transient transfection assays (45). Because another promoter (P1) is the predominant transcription initiation site in adult hepatocytes and kidney, HNF-4α mRNA in hnf1α−/− mice is not grossly deranged in these tissues (24,31,38). Thus, HNF-1α is dependent on HNF-4α in multiple tissues, but in pancreatic cells, a cross-regulatory loop between these two genes is formed (Fig. 2A).

**The cross-regulatory circuit as a molecular memory device.** Once insulin-producing cells arise, mechanisms need to be in place to maintain their phenotypic properties over time and throughout consecutive rounds of cell division. Cross-regulation between HNF-1α and HNF-4α is switched on as pancreatic cells are provided the signals to differentiate (Fig. 2B) (38). Once it is installed, this type of circuit configuration can provide a stable mechanism of gene expression, as physiological perturbations of the activity of one of the two genes can be reset back to the equilibrium state by the opposite gene (48,49). This can serve to self-perpetuate the activity of both genes and their targets in differentiated cells (Fig. 2B). The notion that a transient instructive signal is sufficient to lock a genetic program that maintains differentiated functions makes
required for transition to the OFF state. Decreased gene dosage also minimistic fluctuation of HNF-1α expression falls below a threshold level. The cellular specificity of the switch mechanism matches that of the heterozygous phenotype. If disruption of the loop is instrumental in the haploinsufficient mechanism, a severe phenotype should occur only in the cells in which the circuit exists (Fig. 2A). In the absence of a loop, the predicted outcome in nonpancreatic cells is that there could be partially decreased concentration of functional HNF-1α in MODY3, or of HNF-4α and perhaps HNF-1α in MODY1, but not a severe phenotype resulting from impaired function of all four HNF-1α and HNF-4α alleles. This prediction closely fits the genetic findings. Hnf1α−/− mouse studies have clearly shown that this gene is absolutely necessary for a vast array of critical genes in liver, gut, and kidney (21,23,24,31,32), whereas heterozygosity results in a clinically significant and severe phenotype only in pancreatic islet cells (6,8,9,16,27). Furthermore, although the heterozygous HNF-1α and HNF-4α phenotypes are very similar in β-cells, the mild liver and kidney defects reported in MODY1 and MODY3 are specific for each gene (32,34).

Delayed onset of the phenotype: When does circuit breakdown take place? Although MODY1 and -3 are caused by germ-line mutations, the diabetic phenotype does not appear until 10–40 years after birth (13,15). On the other hand, we know from the hnf1α−/− mouse studies...
that interdependence is installed soon after differentiated pancreatic cells appear during embryogenesis (Fig. 2B) (38). If the phenotype is dependent on the loop mechanism, why does it take so many years to develop?

Because HNF-4α is not dependent on HNF-1α in precursor cells (and hence no loop exists), the circuit is expected to be in the default ON state as insulin cells first appear in heterozygous mutation carriers. The question thus becomes, When does circuit inactivation take place? It could occur quickly in most cells soon after interdependence is established in embryonic insulin-producing cells (Fig. 3B). However, the fact that the phenotype does not appear until many years later makes it necessary to consider the alternate possibility that inactivation may be an inefficient process.

Most likely, the loss of a single HNF-1α or HNF-4α allele does not immediately compromise circuit activity in differentiated cells. This is clearly the case in mice, as young hnf1α−/− animals express HNF-4α mRNA at nearly normal levels in pancreatic cells (S. Boj and J.F., unpublished observations). However, transcription in a single cell is an inherently noisy process (53-56); it undergoes fluctuations that are not solely environmentally determined, but also stochastic (53,57). The existence of 50% of the normal gene product can critically increase the probability that an extreme fluctuation at any moment in the lifetime of a cell surpasses a threshold required to trigger its extinction, resulting in a stochastic (53,57). The existence of 50% of the normal gene product can critically increase the probability that an extreme fluctuation at any moment in the lifetime of a cell.

The model postulated here is consistent with other parallel or consecutive pathogenic mechanisms. For example, circuit inactivation could take place long before the phenotype appears by limiting an appropriate cell growth or secretory adaptive response to demands imposed later in life, or by allowing gradual accumulation of cellular defects. On the other hand, once metabolic derangements are initiated, these are expected to contribute to the completion of the process of β-cell failure (58).

Are other MODY genes required to keep the switch turned on? In addition to HNF-1α and HNF-4α themselves, other regulators are expected to control the activity of these two genes in differentiated pancreatic cells. The bistable circuit model predicts that any circumstance that imparts a severe inhibition of the loop below a threshold level can trigger its extinction, resulting in a similar phenotype as a mutation of either HNF-1α or HNF-4α. Other MODY genes are attractive candidate sites for this type of mechanism. Strong support for such an interaction among MODY genes has been provided by a recent study indicating that IPF-1 (MODY4) may be a critical regulator of HNF-4α (45). Thomas et al. (45) discovered a mutation in the pancreatic HNF-4α promoter that segregates with diabetes in a large kindred, with a logarithm of odds score of 3.25. The mutation disrupts an IPF-1 (PDX-1) high affinity binding site that is essential for HNF-4α promoter activity (45). This suggests that the MODY4 phenotype could be mediated in part by a defective function of the HNF-1α/HNF-4α circuit (45). HNF-1β (MODY5), a paralog of HNF-1α that shares closely related DNA binding and dimerization domains (3,59), could also
regulate this circuit. \(Hnf1\beta^{-/-}\) embryos die before pancreas organogenesis but fail to express HNF-1\(\alpha\) in endoderm cells (60). HNF-1\(\beta\) also interacts with the HNF-4\(\alpha\) P2 promoter in transient transfections and in vitro binding assays (45). Furthermore, HNF-1\(\beta\) may act indirectly through IPF-1/PDX-1, as zebrafish HNF-1\(\beta\) mutants fail to express PDX-1 in the endodermal segment destined to form the pancreatic bud (61).

An additional level of complexity lies in the observation by Shih et al. (24) of decreased expression of PDX-1 in pancreatic cells of \(hnf1\alpha^{-/-}\) mice. A meaningful reduction of PDX-1 has not been observed in our own studies with embryonic and early postnatal pancreatic tissues from \(hnf1\alpha^{-/-}\) mice (22,38) or in the dominant-negative inhibition cell line experiments (25). However, the PDX-1 5' flanking region contains an evolutionary conserved HNF-1 site (62,63), and this could conceivably be variably linked to HNF-1\(\alpha\) or HNF-1\(\beta\) dependence in diverse genetic or environmental background settings. Thus, PDX-1 could lie both upstream and downstream of the HNF-1\(\alpha\)/HNF-4\(\alpha\) circuit, forming a separate regulatory loop. This notion is consistent with the existence of epistatic interactions between \(pdx1\) and \(hnf1\alpha\) loci in mice (27).

**A framework to study the mechanism of haploinsufficiency of genetic network components.** The mechanism of haploinsufficiency in human genetic diseases is greatly overrepresented among transcriptional regulator genes as compared with other functional categories (64–68). Aside from specific settings in which loss of heterozygosity and single allele expression have been described, the mechanism is generally based on the requirement of a critically narrow range of activator concentrations (64,66–68). Decreased gene dosage could lead to altered stoichiometry of multimeric regulatory complexes (68) or failure to establish cooperative interactions with other activators (69). The mechanism postulated here, for the first time to our knowledge, is that the consequences of decreased dosage of a single regulator gene can be greatly amplified in the face of a self-sustaining feedback circuit. Furthermore, it is proposed that in this setting, transient stochastic or extrinsically determined inhibitory events can serve as triggers for permanent network inactivation. Haploinsufficiency is postulated to increase both the likelihood of circuit inactivation and the stability of the inactive state. This notion broadens the implications of a study discussed earlier, whereby simulations were used to show that in the presence of a heterozygous loss of function mutation of any given gene, stochastic deactivation of the functional allele may intermittently mimic the homozygous null state (57). As cross-regulatory circuits between transcriptional regulators are extremely common (70,71), this raises the intriguing possibility that autocatalytic circuit breakdown could be involved in other haploinsufficient defects.

The model that is presented here is testable and requires experimental analysis in a \(\beta\)-cell context. For example, it is necessary to determine that extreme parameter conditions of the circuit, such as a very slow activation/inactivation rate, do not render extinction as the result of a transient event too unlikely. It is also important to know how the interplay with other network components affects the properties of the circuit. Nonetheless, the model rests on two firm premises. First, there is genetic evidence for pancre-atic-restricted interdependence of HNF-1\(\alpha\) and HNF-4\(\alpha\) which holds true irrespective of other mechanisms regulating these two genes (24,30,35,37,38,42,43,45,47). Second, the concept that positive cross-regulatory feedback circuits are capable of exhibiting multistability is well established (48–52). The model that emerges does a reasonably good job in explaining the established clinical features of MODY.

Such a framework to explain the haploinsufficient mechanism in MODY extends previous models that did not accommodate the existence of a feedback circuit, but did postulate that loss of one HNF-1\(\alpha\) allele results in insufficient activator concentrations to elicit normal target gene responses specifically in islets (36). Another model that did not incorporate this loop structure already postulated loss of function of both HNF-1\(\alpha\) alleles because of somatic loss of the wild-type allele in a subset of cells hypothesized to possess a growth advantage (14).

**Final remarks.** The obligate task of HNF-1\(\alpha\) and HNF-4\(\alpha\) in \(\beta\)-cells appears to be largely dependent on their position within a network and not solely on the linear activation of a set of critical distal target genes (24,38,45). Because one of the recognized attributes of complex networks is robustness to inactivation of random components (72), it is remarkable that the HNF-1\(\alpha\)/HNF-4\(\alpha\) subcircuit is unusually vulnerable. This vulnerability is characteristic of highly interconnected nodes within scale-free complex networks (72). An implication of such a hub position, together with the proposed bistable properties of the subcircuit, is that inhibition (perhaps even if transient) of HNF-1\(\alpha\) or HNF-4\(\alpha\) function could result in permanent damage to the differentiated state of \(\beta\)-cells. Critical regulatory mechanisms of this subcircuit may thus represent an attractive site for identifying new pathogenic mechanisms involved in the progression to \(\beta\)-cell dysfunction. Furthermore, an in-depth understanding of the design of this network may allow its manipulation and thus provide keys to build competent artificial insulin-producing cells.

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