Iron overload in Hepc1−/− mice is not impairing glucose homeostasis

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Abstract Diabetes Mellitus is found with increasing frequency in iron overload patients with hemochromatosis. In these conditions, the pancreas shows predominant iron overload in acini but also islet β-cells. We assess glucose homeostasis status in iron-overloaded hepcidin-deficient mice. These mice presented with heavy pancreatic iron deposits but only in the acini. The β-cell function was found unaffected with a normal production and secretion of insulin. The mutant mice were not diabetic, responded as the control group to glucose and insulin challenges, with no alteration of insulin signalling in the muscle and the liver. These results indicate that, β-cells iron deposits-induced decreased insulin secretory capacity might be of primary importance to trigger diabetes in hemochromatotic patients.

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1. Introduction

In mammals, iron homeostasis is complex and depends on the regulated absorption of dietary iron by mature enterocytes of the duodenum and iron recycling by macrophages [1]. These two fundamental processes are deregulated in hereditary hemochromatosis (HH), leading, over time, to transferrin saturation and iron deposition in parenchymal cells [2]. HH is a highly prevalent iron overload disorder that is, unless recognized and treated, fatal. As the disease progresses, patients develop iron-induced tissue damage resulting in serious illnesses including cirrhosis, hepatomas, cardiomyopathy, arthritis, endocrinopathies and diabetes. HH is genetically heterogeneous depending on distinct clinical and genetic entities. Classical hemochromatosis is associated with the historical and most prevalent form of HH, almost always caused by mutations in HFE and rarely in transferrin receptor 2 (TIR2) (reviewed in [3]). Juvenile hemochromatosis (JH) is a rare form of the disease characterized by the early and severe onset of symptoms, in particular cardiac and endocrine defects. Most JH families have mutations in the recently cloned hemoujuvelin (HJV) gene (reviewed in [3]). For a very small subset of patients, mutations have been identified in hepcidin (HAMP), which encodes a small circulating 25-amino-acid cysteine-rich peptide that constitutes the master regulator of iron homeostasis (reviewed in [3]). The circulating peptide acts to limit gastrointestinal iron absorption and serum iron by inhibiting dietary intestinal iron absorption and iron recycling by the macrophages [4]. To limit iron egress, hepcidin binds to ferroportin, the transmembrane iron transporter necessary for iron transfer out of intestinal epithelial cells and macrophages [5], thereby inducing its internalization and subsequent degradation [6–8]. Most of the iron overload syndromes known to date (primary hemochromatosis and secondary iron overloads) imply a reduction of hepcidin secretion. In contrast, hypersecretion of hepcidin seems to play a determining role in anemia of chronic disease [9].

Diabetes is part of the triad originally used to define fully penetrant HH. However, so far, the pathophysiology of the diabetes associated with HH is poorly understood and the actual prevalence of diabetes in HH is still subjected to debate [10]. It is generally admitted that two mechanisms contribute to the development of hyperglycemia and diabetes in hemochromatotic patients; liver iron overload, leading to insulin resistance, and pancreatic β-cell iron accumulation, resulting in cell damage and diminished insulin secretion [11–13]. Further support for a toxic role of iron in the β-cell has been reported in patients with thalassemia [10] and in rats subjected to experimental iron overload [14,15]. Since studies of the mechanisms of hemochromatosis-associated diabetes have been limited in mouse models, we sought to analyze glucose metabolism in our recently generated Hepc1 knockout mice. These mice presented an iron overload phenotype similar to that observed in HH, with increased iron deposition in the liver and the pancreas, representing therefore a good model to assess to what extend iron can induce impairment in glucose homeostasis in mice.

2. Materials and methods

2.1. Animals

Hepcidin knockout mice were previously described [16]. All the studies were carried on F1 hybrids on a mixed C57BL/6 X 129 background. Animals were cared for in accordance with the “European convention for the protection of laboratory animals”. Animals were maintained in a temperature- and light-controlled environment and were given free access to tap water and food (standard laboratory mouse chow, AO3, iron content 280 mg/kg, UAR, France).
2.2. Iron measurements and immunohistochemistry
For histology, tissues were fixed in 4% formaldehyde, and embedded in paraffin. Sections were immersed in Perls’ solution (1:1, 2% HCl and 2% potassium ferrocyanide) to visualize ferric (non-heme) iron and counterstained with nuclear fast red using standard procedures. Insulin immunochemistry was performed as previously described [17].

2.3. Blood parameters
Blood was withdrawn from the tail using EDTA-aprotinin as the anticoagulant. Blood glucose levels were assessed using a glucometer (Glucotrend II, Roche Laboratories, France). Plasma insulin concentrations were assessed using a rat insulin ELISA kit with a mouse insulin standard (Crystal Chem. Inc., Chicago, IL, USA).

2.4. Glucose and Insulin Tolerance Tests
OGTT was performed as previously described on mice fasted overnight for 12 h [18]. Blood glucose levels were determined at 0, 20, 40, 60, and 80 min after an oral bolus of glucose (3 g/kg of body weight). Blood was also collected at t = 0 and after 20 min for determination of plasma insulin concentration. For insulin tolerance test (ITT), animals fasted for 4 h (from 10:00 AM to 14:00 PM) were injected intraperitoneally with 0.5 U/kg of insulin and glucose levels were measured at 0, 15, 30, 60 and 90 min post-injection [18].

2.5. Insulin signalling
For insulin signalling experiments, following an overnight fast, mice were anesthetized with a ketamine/xylazine mix and injected with 5 U/kg of regular human insulin (Actrapid Penfill; NovoNordisk Inc.) via the portal vein. Three and five minutes after the injection of insulin bolus, livers and muscles were removed, respectively, and frozen in liquid nitrogen. Immunoblot analysis of the insulin signalling was performed as described on AKT phosphorylation [18].

3. Results
3.1. Hepc1<sup>−/−</sup> animals accumulated iron in the exocrine pancreas and presented normal plasma and pancreatic levels of insulin
We previously reported accumulation of iron in the pancreas of Hepc1<sup>−/−</sup> mice [16]. Further examination of pancreatic iron accumulation by staining histological sections for iron showed that iron accumulated predominantly in the exocrine pancreas, with sparing of the islets (Fig. 1A). This massive iron accumulation was not associated with fibrosis nor with pancreatitis as judged by the absence of collagen deposition (trichrome staining, not shown) and normal levels of pancreatic enzymes (lipase and amylase activities measured in the plasma of Hepc1<sup>−/−</sup> mice, not shown). The function of the β-cells in the Hepc1<sup>−/−</sup> mice was next analyzed. The insulin content in the pancreas of Hepc1<sup>−/−</sup> mice was determined by immunochemistry and showed no difference with the controls (Fig. 1B). Furthermore, plasma insulin levels in fed animals was not affected in the mutant mice (0.73 ± 0.44 ng/ml in Hepc1<sup>−/−</sup> mice, n = 7, vs 0.71 ± 0.33 in control mice, n = 9).

These results suggest that the iron overload of the exocrine pancreas is not affecting the production and secretion of insulin in Hepc1<sup>−/−</sup> mice.

3.2. Unaltered glucose homeostasis in Hepc1<sup>−/−</sup> animals
To further explore the effects of iron overload on insulin sensitivity and glucose effectiveness, oral glucose and insulin tolerance tests were performed. As shown in Fig. 2A, Hepc1<sup>−/−</sup>
mice were not intolerant to glucose and even displayed a tendency, although not statistically significant, to an increased glucose tolerance. Importantly, there was no significant difference in blood glucose levels at the fasted state between the mutant and control mice indicating the absence of hyperglycemia in mutant mice. Insulin levels were comparable in Hepc1<sup>−/−</sup> and control mice in the fasting state as well as 20 min after the glucose challenge (Fig. 2B). Finally, insulin tolerance test revealed no significant differences in insulin sensitivity between the Hepc1<sup>−/−</sup> and the control mice (Fig. 2C). However, although not significant, the tendency of impaired reduction in blood glucose concentration after intraperitoneal administration of insulin in Hepc1<sup>−/−</sup> mice prompted us to further analyze the insulin resistance state of the mutant mice by studying the activation of the insulin signalling pathway. To that purpose, AKT phosphorylation was evaluated after insulin injection in liver and muscle of overnight-fasted control and mutant mice. As shown in Fig. 3, the stimulation by insulin of Akt phosphorylation was similar in livers and muscles of control and Hepc1<sup>−/−</sup> mice. Collectively, these results suggest

![Fig. 2. Glucose and insulin tolerance analyses in hepc1 knockout mice.](image-url)

(A) A bolus of glucose (3 g/kg body weight) was administered in control (Hepc1<sup>+/+</sup> and Hepc1<sup>+/−</sup> mice) and Hepc1<sup>−/−</sup> mice aged of 12 months (control, n = 8 and Hepc1<sup>−/−</sup> mice, n = 8). Tail vein glucose levels were determined at the indicated times. (B) Plasma insulin levels were determined before and 20 min after glucose injection in the same cohort. (C) Insulin (0.5 U/kg body weight) was administered ip, and blood glucose levels were determined at the indicated times (control, n = 8 and Hepc1<sup>−/−</sup> mice, n = 8). Data are the mean ± S.E.M. Statistical analysis was performed using Student’s t-test (unpaired, two tailed).
that whole body insulin sensitivity is preserved in Hepc1/−/− mice and that the liver iron overload of mutant mice is not inducing an hepatic insulin resistance.

4. Discussion

In humans, different studies support a β-cell defect in HH and a pathogenic role of iron in the β-cell [12,13,19]. Importantly, it was reported that impaired insulin secretory responses may be reversed to some extend by therapeutic phlebotomies [12]. In the recent report of McClain et al. [13], the authors demonstrated that the high prevalence of abnormal glucose homeostasis primarily involved insulin secretory defects in individual with HH. The authors reported that hemochromatotic patients with impaired glucose tolerance were not insulin-resistant but presented with a decrease in insulin response to glucose, while hemochromatotic patients with diabetes exhibited further declines in insulin response to glucose, insulin-resistance or both [13].

The resistance of mice to iron-induced diabetes was recently exemplified in the Hfe−/− knockout mice which, despite an heavy iron overload, were not described as being diabetic [20]. A complete study of glucose metabolism was also reported in Hfe−/− mice i.e. the mouse model of classical hemochromatosis [21]. The authors found that the Hfe−/− mice were not glucose intolerant nor insulin-resistant. However, a 1.7 fold increase of iron in isolated pancreatic islets was described resulting in decreased insulin secretion secondary to β-cell apoptosis. Loss of β-cell mass and desensitization of glucose-induced insulin secretion in the mutant Hfe−/− mice. In Hepc1/−/− mice, we were unable to detect any increase of β-cells apoptosis with activated caspase 3 (not shown). The reason of the iron loading resistance of the Hepc1/−/− β-cell is not clear and deserves further examination. However, even with the β-cell dysfunction in the Hfe−/− mice, these mice are able to compensate and do not develop diabetes [21].

The resistance of iron loaded mice to develop diabetes is unclear. Of consideration for the Hepc1/−/− model is the age and the genetic background of the mice. The study was performed on 12-month-old mice on a mixed 129 and C57BL6 genetic background. It will be interesting to determine whether altered glucose homeostasis could be seen with aging and when mice are backcrossed on the C57BL6 strain, which is known to be a more diabetes-prone strain.

In conclusion, abnormal glucose tolerance and diabetes are seen at significant frequencies in humans with HH and the loss of insulin secretory capacity is the primary event leading to hemochromatosis related diabetes [13]. Our study, together with the data of the literature, suggest that mice, for some unknown reasons are resistant to the toxic effects of iron-induced organ damage and that diabetes is not a characteristic of mouse models of HH. Consequently, these models are not suitable to study the pathogenesis of diabetes associated with hemochromatosis.

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References


