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Iron overload in *Hepc1*$^{-/-}$ mice is not impairing glucose homeostasis

G. Ramey[1], A. Faye[1], B. Durel, B. Viollet, S. Vaulont*

Institut Cochin, Département Endocrinologie Métabolisme et Cancer, Paris, F-75014, France
Inserm, U567, Paris, F-75014, France
CNRS, UMR 8104, Paris, F-75014, France
Université Paris 5, Faculté de Médecine René Descartes, UM 3, Paris, F-75014, France

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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 hemochromatosic patients.**

Keywords: Diabetes; Hepcidin; Iron metabolism; Glucose homeostasis; Insulin sensitivity

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, endocrinopaties 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 (TfR2) (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 hemojuvelin (*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 hemochromatosic patients; liver iron overload, leading to insulinoresistance, 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).

*Corresponding author. Fax: +33 1 44412421.
E-mail address: vaulont@cochin.inserm.fr (S. Vaulont).

[1]The first two authors contributed equally to this work.

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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. $Hepc1^{-/-}$ 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 $Hepc1^{-/-}$ 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 $Hepc1^{-/-}$ mice, not shown). The function of the β-cells in the $Hepc1^{-/-}$ mice was next analyzed. The insulin content in the pancreas of $Hepc1^{-/-}$ 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 $Hepc1^{-/-}$ 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 $Hepc1^{-/-}$ mice.

3.2. Unaltered glucose homeostasis in $Hepc1^{-/-}$ 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, $Hepc1^{-/-}$

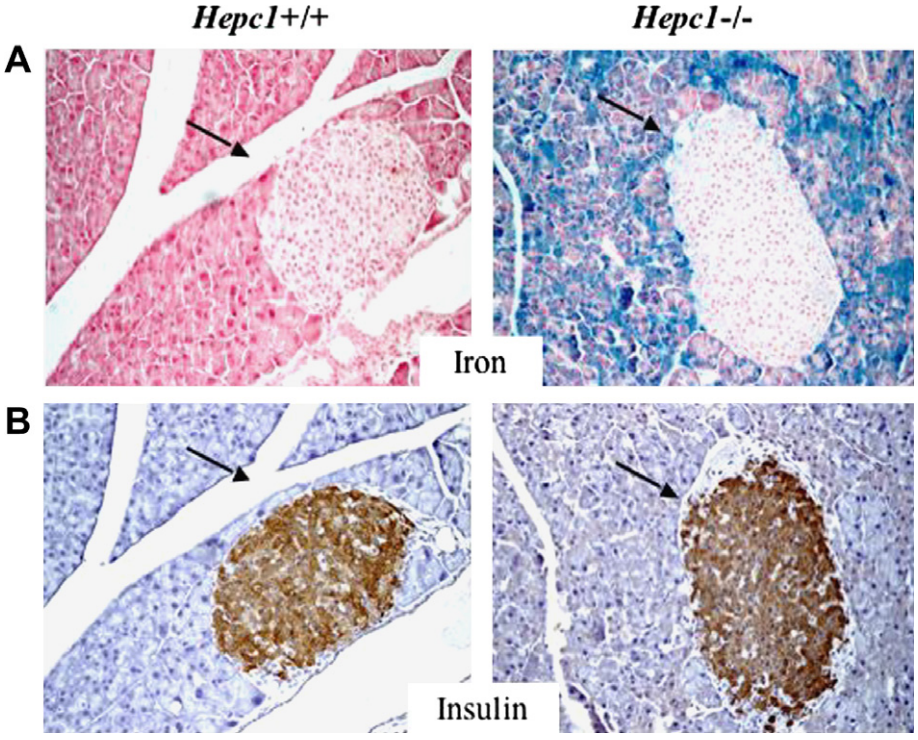

Fig. 1. Pancreas iron and insulin content in *hepc1* knockout mice. Typical pancreas sections (original magnification 20×) from $Hepc1^{+/+}$ and $Hepc1^{-/-}$ mice aged of 8 months. For Perls' staining (A), non-heme iron stains blue. For insulin immunohistochemistry (B), insulin stains brown. The arrows show the islets.