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Iron overload induces changes of pancreatic and duodenal divalent metal transporter 1 and prohepcidin expression in mice

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ABSTRACT

It is well known that the iron content of the body is tightly regulated. Iron excess induces adaptive changes that are differentially regulated in each tissue. The pancreas is particularly susceptible to iron-related disorders. We studied the expression and regulation of key iron proteins in the pancreas, duodenum and liver, using an animal model of iron overload (female CF1 mice injected i.p. with iron saccharate, colloidal iron form). Divalent metal transporter 1, prohepcidin and ferritin (pancreas, duodenum, liver) were assessed by immunohistochemistry; divalent metal transporter 1 (pancreas, duodenum) by Western blot. In the iron overloaded mice, prohepcidin expression increased in islets of Langerhans and hepatocytes, and divalent metal transporter 1 expression decreased in cells of islets and in enterocytes. In the iron overloaded mice, ferritin expression decreased in islets of Langerhans and increased in acinar cells; hemosiderin was localized in connective tissue cells. The inverse relationship between divalent metal transporter 1 and prohepcidin may indicate a negative regulation by hepcidin, and hence reduction of iron stores in islets of Langerhans. Our data showed that in iron overloaded mice model, induced by colloidal iron form, a coordinated expression of key iron proteins in the pancreas, duodenum and liver may occur. Further research will be necessary to determine the adaptive responses induced by iron in the pancreas.

Introduction

Over the last decade remarkable advances have been made in understanding iron metabolism. These studies led to the identification and characterization of novel proteins that interact with other well documented proteins such as ferritin and the transferrin proteins (Gkouvatsos et al., 2012). It is well known that iron homeostasis is maintained by a balance between iron uptake from the diet by duodenal enterocytes and by iron recycling from senescent erythrocytes in the reticuloendothelial system (Nicolas et al., 2001).

Hepcidin, the major regulator of iron stores, regulates cellular iron efflux by inducing ferroportin (FPN) degradation, the only iron exporter identified to date (Nemeth et al., 2004; D'Anna et al., 2009). Therefore in the presence of high hepcidin levels, iron efflux from FPN-expressing cells, such as enterocytes and macrophages, is significantly decreased (Chung et al., 2009).

Another key protein of iron metabolism is divalent metal transporter 1 (DMT1), involved in both iron uptake and release into the target cell. DMT1 is expressed at the apical membrane of duodenal enterocytes and in the endocytic compartment of peripheral tissues where it releases iron internalized throughout the transferrin system (Andrews, 1999). DMT1 has been studied in the duodenum where modulation of dietary iron absorption is the main mechanism for regulating body iron balance, noting that DMT1 expression is reduced when iron-rich foods are consumed, but increases when iron intake is restricted (Canonne-Hergaux et al., 1999). However in the liver, high levels of dietary iron produce an increase in DMT1 expression in hepatocytes, promoting iron acquisition, whereas low levels of iron decrease hepatic DMT1 expression, causing a reduction in iron accumulation (Trinder et al., 2000).

As regards hepcidin, the essential role of this peptide in the maintenance of systemic iron balance has been demonstrated in mouse models (Ganz and Nemeth, 2006; D'Anna et al., 2011). Hepcidin production by the liver is modulated in response to several stimuli such as hypoxia, inflammation and iron overload (Lee and Beutler, 2009). It has been reported that mice lacking hepcidin expression develop systemic iron overload, a disorder characterized by severe iron overload in several organs, including the liver, heart, and pancreatic tissues (Ramey et al., 2007; Masaratana et al., 2011). The iron storage proteins are ferritin, the primary intracellular iron-storage protein, and hemosiderin, a pigment protein produced by ferritin degradation (Iancu, 2011).

Although it is well known that hepcidin is mainly synthesized in hepatocytes (Park et al., 2001), several studies have identified and characterized its synthesis in extra hepatic tissues, such as the pancreas and kidney (Kulaksiz et al., 2008; Veuthey et al., 2008).

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Previously, the indirect relationship between hepcidin and DMT1 in duodenal and renal tissue has been demonstrated (Veuthey et al., 2008; Mena et al., 2008; Chung et al., 2009). Moreover, the role of the hepcidin–ferroportin axis in the regulation of systemic iron balance is well established (De Domenico et al., 2011; D'Anna and Roque, 2013).

Recent studies have suggested that iron balance is maintained, not only by this systemic regulation, but also by mechanisms that regulate iron balance at the cellular level (Finberg, 2011). For example, mice harboring an intestinal-specific deletion of the HIF-2α subunit were found to exhibit decreased serum and liver iron levels, even though their hepatic hepcidin expression was markedly decreased (Mastrogiannaki et al., 2009). Studies by Vanoaica et al. (2010) using mice with intestine-specific deletion of ferritin H demonstrated that not only Hepcidin-DMT1 axis is important to regulate duodenal iron uptake, but also ferritin expression is essential to prevent excessive iron absorption.

It is well known that pancreatic tissue is particularly susceptible to iron-related disorders such as hemochromatosis and secondary iron overload (Beutler, 2006). Furthermore, previous studies have found that key proteins of iron balance, such as hepcidin, DMT1 and ferritin, are expressed in the pancreas (MacDonald et al., 1994; Koch et al., 2003; Kulaksiz et al., 2008). However, the regulation and function of these crucial proteins of iron cycle in the pancreas are not entirely clear.

The aim of this study was to clarify the effects of iron excess on pancreatic tissue, in terms of regulation of iron proteins, and its relation to tissues most studied such as duodenum and liver. Thus, the aim of the present study was to determine prohepcidin expression using an *in vivo* model of iron overload and its relationship with DMT1 and ferritin.

Materials and methods

Animals

The studies have been carried out with CF1 mice, a strain with preserved genetic variability and phenotypic stability (Oyarzabal and Rabasa, 1999). CF1 female mice (25 ± 5 g; three months old) were bred at the animal facility of the Universidad Nacional del Sur. The animals were kept in cages at controlled room temperature and humidity under standard conditions (12 h light-dark period) and were fed throughout on a standard diet with access to water *ad libitum*. The animals selected for the experiment were distributed in cages 10 days prior to the beginning of the study. According to the paired sample design used, the body weight of each mice pair was similar at the beginning of the study and was controlled throughout the study. The procedures followed are in line with the Guide for the Care and Use of Laboratory Animals of NIH (Committee on Care and Use of Laboratory Animals, 1996). Prior to the initiation of this study, the protocol was approved by the Institutional Committee on Experimental Animal Use and Care of the Universidad Nacional del Sur (ICEAUC).

Experimental design

Adult CF1 female mice were divided into two groups (9 mice per group) and were selected in pairs according to body weight as variable following a paired-sample design: (1) iron overloaded mice group; mice received an intraperitoneal injection every 2 days with 500 μl (dose 333.3 mg/kg) iron saccharate (catalog no. 311: Rivero Laboratory, Buenos Aires, Argentina) during 18 days (overall dose 3000 mg/kg) and (2) iron-adequate mice group; mice received an intraperitoneal (500 μl) injection with saline solution (0.9% NaCl) (Sanadrog, Buenos Aires, Argentina). 5 days prior to iron saccharate or saline solution administration, a blood sample (80 μL) was collected from each mouse to determine baseline hematology. Animals were anesthetized with isoflurane for blood collection (day 20) to determine hematological parameters and plasma iron.

Antibodies

Primary antibodies: rabbit anti-mouse DMT1 (Canonne-Hergaux et al., 1999) kindly provided by François Canonne-Hergaux, INSERM, France. Rabbit anti-mouse L-chain ferritin (RbαMoLF) (Santambrogio et al., 2000) kindly provided by Paolo Santambrogio from Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Milan, Italy. Prohepcidin antibody (Valore and Ganz, 2008) was kindly provided by Tomas Ganz, UCLA, Los Angeles, CA, USA. Goat anti-mouse actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies: Donkey anti-goat labeled HRP (Santa Cruz Biotechnology); Goat anti-rabbit labeled HRP (Alpha Diagnostics, Owings Mills, MD, USA).

Immunohistochemical and histochemical technique

The animals were sacrificed by cervical dislocation (day 20). The duodenum, pancreas and liver were removed under sterile conditions by abdominal incision. Tissues samples from mice were fixed by immersion in fresh fixative solution (10% neutral buffered formalin, pH 7) (catalog no. 2000170200; Biopack, Buenos Aires, Argentina) and embedded in paraffin wax. Sections of 5 μm were cut and mounted on glass slides. Before labeling, sections were deparaffinized in xylene and rehydrated in a graded series of ethanol baths. Endogenous peroxidase activity in deparaffinized sections was blocked with 3% H_2O_2 (catalog no. 7258; Anedra, Buenos Aires, Argentina). Sections were then incubated in phosphate buffered saline (PBS), pH 7.1, for 10 min at room temperature. Incubation with the primary antibody diluted in PBS (pH 7.1) was carried out in a humid chamber for 1 h at room temperature for DMT1, and overnight at 4 °C for prohepcidin and L-ferritin immunodetection. Dilutions of antibodies were as follows: anti-DMT1 (pancreas 1:250 and gut 1:50), anti-prohepcidin (1:1000) and anti-L-ferritin (1:500). Following incubation with the primary antibody, tissues were washed with PBS, incubated with goat anti-rabbit IgG peroxidase coupled secondary antibody for 1 h at room temperature, and then re-washed with PBS. Subsequent localization of proteins was revealed by reaction with 3′-diaminobenzidine tetrahydrochloride (DAB) in solution (catalog no. K3468; Dako, Glostrup, Denmark). Immunostained sections were followed by Prussian blue staining for iron, counterstaining with nuclear red to identify immunostained proteins separately from hemosiderin (double staining). Finally, sections were dehydrated in ethanol and xylene and mounted with coverslips. Negative controls included incubation with PBS for anti-DMT1 and anti-L-ferritin detection or pre-immune serum for anti-prohepcidin detection instead of the primary antibody, in each experiment and tissue studied. Immunostaining was analyzed using an Olympus BX51 microscope, equipped with ×10, ×20 and ×40 dry objectives, and an ×100 oil immersion objective. Digital images were obtained with an Olympus C7070 camera.

Prussian blue staining for iron was performed on sections of pancreas, duodenum and liver previously treated and non-treated with primary antibodies. Deparaffinized tissue sections were incubated in 2% HCl (catalog no. 6050: Anedra, Buenos Aires, Argentina) containing 10% potassium ferrocyanide for 15 min (catalog no. 104984: Merck Millipore, Buenos Aires, Argentina), washed and counterstained with nuclear red before visualization (catalog no. 1159390025: Merck Millipore, Buenos Aires, Argentina). We carried out the double staining (immunohistochemistry followed by