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Iron overload induces hypogonadism in male mice via extrahypothalamic mechanisms

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ABSTRACT

Introduction: Iron overload leads to multiple organ damage including endocrine organ dysfunctions. Hypogonadism is the most common non-diabetic endocrinopathy in primary and secondary iron overload syndromes.
Aim: To explore the molecular determinants of iron overload-induced hypogonadism with specific focus on hypothalamic derangements. A dysmetabolic male murine model fed iron-enriched diet (IED) and cell-based models of gonadotropin-releasing hormone (GnRH) neurons were used.
Results: Mice fed IED showed severe hypogonadism with a significant reduction of serum levels of testosterone (−83%) and of luteinizing hormone (−86%), as well as reduced body weight gain, body fat and plasma leptin. IED mice had a significant increment in iron concentration in testes and in the pituitary. Even if iron challenge of *in vitro* neuronal models (GN-11 and GT1-7 GnRH cells) resulted in 10- and 5-fold iron content increments, respectively, no iron content changes were found *in vivo* in hypothalamus of IED mice. Conversely, mice placed on IED showed a significant increment in hypothalamic GnRH gene expression (+34%) and in the intensity of GnRH-neuron innervation of the median eminence (+1.5-fold); similar changes were found in the murine model $HFE^{-/-}$, resembling human hemochromatosis.
Conclusions: IED-fed adult male mice show severe impairment of hypothalamus-pituitary-gonadal axis without a relevant contribution of the hypothalamic compartment, which thus appears sufficiently protected from systemic iron overload.

1. Introduction

Iron is an essential metal for fundamental biochemical activities such as oxygen transport and energy metabolism. It is also required for proper brain development during embryogenesis, and for brain function in the early neonatal period and adult age (Radlowski and Johnson, 2013). On the other hand, iron overload is prone to trigger Fenton chemistry resulting in cellular oxidative stress and generation of highly reactive radicals (Chevion, 1988).

Pathological iron overload conditions have been associated with dysfunctions of endocrine organs (Brissot et al., 2016). In individuals with primary (hereditary hemochromatosis) and secondary (transfusional and dietary) iron overload syndromes, iron plays a relevant causative role in several clinical manifestations, including diabetes mellitus, insulin resistance (IR) and non-alcoholic fatty liver disease (Datz et al., 2013; Simcox and McClain, 2013). The dysmetabolic iron overload syndrome (DIOS),

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also referred to as IR-associated hepatic iron overload (Mendler et al., 1999), corresponds to mild hepatic iron excess in the context of various features of metabolic syndrome (Dongiovanni et al., 2011). Notably, the pathogenesis of iron accumulation in DIOS has been related to altered iron trafficking associated with steatosis, hepatic inflammation, and IR (Dongiovanni et al., 2013).

Hypogonadism is the most common non-diabetic endocrinopathy both in primary (McDermott and Walsh, 2005) and secondary (Al-Rimawi et al., 2005) iron overload syndromes. Indeed, the clinical picture of hereditary hemochromatosis is associated to impotence in males (Niederau et al., 1994). Hypogonadism in men results from failure of the testes to produce physiological levels of testosterone (T) with a normal number of spermatozoa due to disruption of one or more levels of the hypothalamic-pituitary-testicular axis (Bhasin et al., 2006). Hypogonadal subjects are routinely classified into those with primary hypogonadism (testicular failure) characterized by low serum T and elevated gonadotropins (luteinizing hormone -LH-, follicle-stimulating hormone -FSH-) or those with secondary hypogonadism (hypothalamic-pituitary failure) with low serum T and low or normal gonadotropins (Tajar et al., 2010).

Hypogonadism related to hemochromatosis has been demonstrated to have a central origin, although there is unclear evidence of the specific contribution of hypothalamus vs. pituitary impairment (Duranteau et al., 1993; Piperno et al., 1992). The same mechanism has also been proposed in thalassaemic patients in whom the hypothalamus-pituitary axis is affected by iron in a dose-dependent manner (Chatterjee and Katz, 2000). Conversely, consistent with the effect of human chorionic gonadotropin (hCG) administration, testicular function does not seem to be affected by iron overload (Duranteau et al., 1993; Piperno et al., 1992).

Due to a lack of evidence clarifying whether the major driver of iron-induced hypogonadism is hypothalamus or pituitary, by using a murine model of dietary iron overload induced by iron-enriched diet (IED) and suitable cell models of GnRH neurons, the present study aimed at dissecting the molecular basis and the neuroendocrine involvement of iron overload-induced hypogonadism, with a specific focus on hypothalamic derangements.

2. Materials and methods

2.1. Animals

Five-week-old male C57BL/6J mice were purchased from Charles River Laboratories (Calco, Italy), housed at constant room temperature (RT, 23 °C), under 12-h light/dark cycles, with *ad libitum* access to tap water and food, in compliance with the European Union guidelines. The investigation conforms to the European Commission Directive 2010/63/EU. Animals were fed either standard iron concentration diet containing 180 mg/kg (control group, CTR; n = 15) or an iron-enriched diet with 3% carbonyl-iron (IED group; n = 15) for 11 weeks (wks) (Dongiovanni et al., 2013). Body weight and food ingestion were monitored weekly. At the end of treatment, animals were sacrificed by decapitation under anesthesia (isoflurane/oxygen mixture) between 10:00 a.m. and 12:00 p.m. to avoid circadian variations. Trunk blood was collected and serum, separated by centrifugation, was stored at −20 °C until assayed. Hypothalamus, pituitary and testes were dissected and either flash frozen in liquid nitrogen for RNA extraction or fixed in 4% paraformaldeide for immunohistochemistry procedures. Testicular weight and long diameter and perigonadal fat pad weight were measured before freezing or fixation. Hypothalamus from $HFE^{-/-}$ mice, a model of human genetic hemochromatosis (Zhou et al., 1998), has been used as a positive control.

2.2. Reproductive hormone assays

LH and T levels were evaluated by radioimmunoassay (RIA) (Pineda et al., 2010). Serum LH levels were determined in a volume of 50 μL using a double-antibody method and RIA kits supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 was labelled with 125I using Iodogen tubes, following the instructions of the manufacturer (Pierce, Rockford, IL). Hormone concentrations were expressed using reference preparation, LH-RP-3 as standard. Intra- and inter-assay coefficients of variation were less than 8 and 10%. The sensitivity of the assay was 5 pg/tube for LH. All samples were

Table 1
Primer sequences.

Gene	Primers	
	Forward primer (5′-3′)	Reverse primer (5′-3′)
CHOP	GTCCCTAGCTTGGCTGACAGA	TGGAGAGCGAGGGCTTTG
FSHβ	ATGGATTGTTCCAGGCAGAC	TCACTGCATGTGAGGGAAAG
FtH	CGAGATGATGTGGCTCTGAA	GTGCACACTCCATTGCATTC
GnRH	GGCCGGCATTCTACTGCTG	CTGCCTGGCTTCCTCTTCA
Gpr54	CAGTCCCAGGACACAATCCT	ACCAATGAGTTTCCGACCAG
Kiss1	AGCTGCTGCTTCTCCTCTGT	GCATACCGCGATTCCTTTT
LHβ	TGGCCGCAGAGAATGAGTTC	CTCGGACCATGCTAGGACAGTAG
SOD2	TCTGGCCAAGGGAGATGTTA	CCTCCAGCAACTCTCCTTTG
TfR	TCGCTTATATTGGGCAGACC	CCATGTTTTGACCAATGCTG
XBP-1	TGAGAACCAGGAGTTAAGAACACGC	TTCTGGGTAGACCTCTGGGAGTTCC
18S	CTCGCTCCTCTCCTACTTGG	CCATCGAAAGTTGATAGGGC
	Applied Biosystems® Custom TaqMan® 5′ FAM − 3′ MGB Probes *Assay ID*	
GnRH1	Mm01315605_m1	
IL-6	Mm00446190_m1	
Kiss1	Mm03058560_m1	
NPY	Mm03048253_m1	
18S	Hs99999901_s1	

FAM: 6-carboxy-fluorescein (reporter fluorescent dye).
MGB: minor-groove-binder moiety.

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