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Hemochromatosis: Evaluation of the dietary iron model and regulation of hepcidin

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ABSTRACT

Our knowledge of iron homeostasis has increased steadily over the last two decades; much of this has been made possible through the study of animal models of iron-related disease. Analysis of transgenic mice with deletions or perturbations in genes known to be involved in systemic or local regulation of iron metabolism has been particularly informative. The effect of these genes on iron accumulation and hepcidin regulation is traditionally compared with wildtype mice fed a high iron diet, most often a 2% carbonyl iron diet. Recent studies have indicated that a very high iron diet could be detrimental to the health of the mice and could potentially affect homeostasis of other metals, for example zinc and copper. We analyzed mice fed a diet containing either 0.25%, 0.5%, 1% or 2% carbonyl iron for two weeks and compared them with mice on a control diet. Our results indicate that a 0.25% carbonyl iron diet is sufficient to induce maximal hepatic hepcidin response. Importantly these results also demonstrate that in a chronic setting of iron administration, the amount of excess hepatic iron may not further influence hepcidin regulation and that expression of hepcidin plateaus at lower hepatic iron levels. These studies provide further insights into the regulation of this important hormone.

1. Introduction

Animal models of disease have helped us understand how mutations in disease-implicated genes contribute to the development of pathology. Most life forms require iron for their proper physiological functioning. Animal models of iron disorders have been instrumental in extending our knowledge of iron regulation and the flux of iron within the body. Transgenic and knockout mice with mutations or deletions in genes that are associated with regulation of iron metabolism are important tools. Studies using these transgenic mice have traditionally compared them to wildtype mice with either diet-induced iron overload [1–4] or anemia [4,5], to better understand the molecular functions of the affected genes. Most studies have used mice fed with a diet containing 2% carbonyl iron for one or two weeks [1–4] as a means of inducing dietary iron overload.

An increase in body iron levels is known to increase levels of the liver-expressed peptide, hepcidin, in mice and humans. Hepcidin, a 25-amino acid peptide, is also known as the master regulator of iron homeostasis, and its synthesis is regulated by many factors (reviewed in [6,7]) besides iron. The 2% carbonyl iron diet increases hepcidin levels not only in the livers of wildtype mice, but also in transgenic mice which have mutations in the genes proposed to be involved in the iron-mediated regulation of hepcidin, namely *Hfe* [3], *Tfr2* [3] and *Hjv* [8]. This suggests that there may be additional pathways that are activated in the presence of excess iron and hence lead to increased hepcidin expression in the livers.

In addition, it has been shown that exposure to a high-iron diet may affect the metabolism of other metals including copper [9,10]. There is also evidence showing that high-iron levels interferes with zinc absorption, where an increase in iron levels may result in a decrease in zinc absorption in both humans and rats [11,12]. A prolonged exposure to a high-iron diet has been shown to be detrimental to the health of rats [13]. Rats fed a high-iron diet had significantly lower body weights and increased liver and heart weights, as compared to controls. In addition, rats fed a high-iron diet also exhibited signs of cardiac hypertrophy and copper deficiency-induced anemia [13]. It was shown that in mice fed a high-iron diet, systemic copper homeostasis is disturbed [10]. Mice fed a high-iron diet had different tissue copper distribution leading to changes in the bioavailability of copper. These changes translate to symptoms of severe copper deficiency [10]. Together these observations suggest that a high-iron diet model may not be the best suited either to the health of the mice or the experimental design.

In order to determine the effect of varying iron levels on the hepcidin response and the optimum concentrations to be used in studies of

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dietary iron overload, we fed mice a range of diets containing between 0.25 and 2% carbonyl iron. These mice were fed the diets for two weeks as previous studies have established that hepatic hepcidin and serum iron levels saturate relatively quickly [4,14]. We examined iron parameters and expression of genes involved in the systemic regulation of iron metabolism. Our results indicate that the 0.25% carbonyl iron diet is sufficient to induce a hepcidin response and the regulatory system appears to be saturated at this iron level.

2. Methods

2.1. Animals and diets

Wildtype male C57BL6/J mice were purchased from the Animal Resource Centre (Murdoch, Western Australia). Animal experimentation was performed as per the guidelines and approval of the QIMR Berghofer (QIMRB) Animal Ethics Committee. The mice were housed under a 12 h light/dark cycle, were provided food and water ad libitum. Mice (n = 5) at the age of 4 weeks were fed either a control diet (AIN93G, Specialty Feeds), or a high-iron diet containing either 0.25%, 0.5%, 1% or 2% carbonyl iron for two weeks. The diets (Specialty Feeds, Glen Forest, Western Australia) were prepared by mixing 2% carbonyl iron diet (SF07-082, Specialty Feeds) with iron-deficient diet (SF01-017, Specialty Feeds). The 1% carbonyl iron diet was composed of 1:1 ratio of 2% carbonyl iron diet (SF07-082, Specialty Feeds) and iron-deficient diet (SF01-017, Specialty Feeds). This was then used to make 0.5% and 0.25% through serial dilution. At the end of two weeks the animals were sacrificed and tissues collected for further analysis.

2.2. Measurement of iron indices

Iron parameters (serum iron concentrations, hepatic iron concentration (HIC), splenic iron concentration (SIC), cardiac iron concentration (CIC), duodenal iron concentration (DIC) and pancreatic iron concentrations (PIC)) were measured as described previously [15,16].

2.3. Real-time quantitative PCR

Total RNA isolated from the liver, spleen and duodenum was used to prepare cDNA using a SensiFAST™ kit, (Bioline, Sydney, NSW, Australia). Real-time quantitative-PCR (qPCR) was performed using SensiFAST™ SYBR No-Rox (Bioline) as described previously [5].

2.4. Perls' staining

Tissues were fixed in 10% formalin and processed and sectioned by the Histology Facility at QIMRB. Perls' Prussian blue staining for iron was performed on the tissues as described by McDonald et al. [17]. The slides were scanned using the Aperio AT Turbo (Aperio, Vista, CA) at 40× magnification and scans were analyzed using Imagescope software (Aperio).

2.5. Western blotting

Liver tissue (100 mg) was homogenized in a lysis buffer containing phosphatase inhibitors [18] using the Precellys Evolution tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). Protein lysate (20 μg) was electrophoresed on 10% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane using the Trans-Blot Turbo apparatus (Bio-Rad Laboratories, Gladesville, NSW, Australia). After blocking with 10% non-fat milk for 2 h at room temperature, the membranes were incubated with primary antibodies (anti-Tfr2 [19] 1:10,000); anti-pSmad (Cell Signalling Technology, Danvers, MA) 1:1000, anti Smad1 (Cell Signalling Technology) 1:1000 and anti-β actin (Sigma-Aldrich 1:20,000) overnight at 4 °C. The membrane was then washed and incubated with anti-rabbit IgG-horseradish peroxidase (1:10000, Invitrogen, Life Technologies) diluted in 10% non-fat milk for 1 h at room temperature. The excess secondary antibody was washed off and the blots were incubated with chemiluminescent substrate (Lumina Forte; Merck Millipore, Kilsyth, Victoria, Australia) for 5 min at room temperature. The blots were then exposed to X-ray film (Fujifilm, Brookvale, NSW, Australia). Films were developed using the Minolta film processor (Konica Minolta Medical and Graphic, Tokyo, Japan).

2.6. Immunohistochemistry

Formalin-fixed paraffin-embedded liver sections were deparaffinised in xylene and rehydrated in graded ethanol. Following heat-induced epitope retrieval (10 mM Tris/1 Mm EDTA pH 9.0 solution), sections were blocked with 20% heat-inactivated fetal bovine serum for 1 h. Sections were incubated with anti-4 hydroxynonenal (4HNE) (1:250; Abcam, Cambridge, MA) overnight at 4 °C and staining detected using the Dako EnVision+ Dual Link System-HRP (DAB+) kit (Agilent Technologies, Santa Clara, CA). Images were acquired at 10× magnification using the Nikon Eclipse TS2 microscope (Nikon Australia, Rhodes, Australia).

3. Results

3.1. Differences in body weights and oxidative stress in mice fed varying concentrations of iron

The first observation that we made was that the mice fed the diet with high concentrations of iron i.e. 1% and 2%, had a lower body weight than mice fed lower iron concentrations (Fig. 1A). No significant differences in the liver and spleen weights of the mice were observed (data not shown). The decrease in the body weights of the mice fed iron diets with higher iron concentrations could be due to either the mice eating less or the increased iron in the diet being directly detrimental to health. An increase in iron levels is also known to result in an increase in reactive oxygen species leading to oxidative stress and associated DNA, lipid and protein damage [20]. Thus, we examined the mRNA levels of two markers of oxidative stress, superoxide dismutase 1 and 2 (*Sod1* and *Sod2*) in the livers of these mice (Fig. 1B and C). Interestingly, the mRNA levels of both *Sod1* and *Sod2* were significantly higher in the livers of mice fed a diet containing 1% or 2% carbonyl iron. We next measured the levels of oxidative damage in the livers of these mice by staining with 4-hydroxy-2-nonenal (4-HNE). 4-HNE is a known marker of oxidative damage, and lipid peroxidation. We did not observe any differences in the 4-HNE staining in the livers of these mice (Supplementary Fig. 1). In the absence of any detectable oxidative damage, it is more likely the weight loss is due to the mice eating less of the high-iron diet.

3.2. Iron parameters in the mice fed an increasingly iron-rich diet

We measured the iron parameters including tissue iron concentrations for liver (HIC), spleen (SIC), heart (CIC), duodenum (DIC) and pancreas (PIC). We also measured the total serum iron and transferrin saturation in the mice fed diets with different concentrations of iron. We saw a gradual and significant increase in the HICs (Fig. 2A) with increasing percentage of carbonyl iron in the diets. Interestingly, the SIC (Fig. 2B), total serum iron (Fig. 2F) and transferrin saturation (Fig. 2G) reached maximal levels at 0.25% of carbonyl iron in the diet. The level of iron in the heart (Fig. 2C), duodenum (Fig. 2D) and pancreas (Fig. 2E) increased significantly in the mice fed a diet containing 1.0% or more iron as compared to the controls. This gradual increase in the amount of iron being accumulated in the liver was also reflected in the Perls' staining for iron (Fig. 3). We examined the localization of iron in the duodenum, heart, liver, pancreas and the spleen of these mice (Fig. 3). There were no differences in the localization of iron in the