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Dietary supplementation with ipriflavone decreases hepatic iron stores in wild type mice

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ABSTRACT

Hepcidin, a peptide produced in the liver, decreases intestinal iron absorption and macrophage iron release by causing degradation of the iron exporter, ferroportin. Because its levels are inappropriately low in patients with iron overload syndromes, hepcidin is a potential drug target. We previously conducted a chemical screen that revealed ipriflavone, an orally available small molecule, as a potent inducer of hepcidin expression. To evaluate ipriflavone's effect on iron homeostasis, we placed groups of 5-week old wild type or thalassemia intermedia ($Hbb^{Th3+/-}$) mice on a soy-free, iron-sufficient diet, AIN-93G containing 220 mg iron and 0–750 mg ipriflavone/kg of food for 50 days. Ipriflavone 500 mg/kg significantly reduced liver iron stores and intestinal ferroportin expression in WT mice, while increasing the ratio of hepcidin transcript levels to liver iron stores. Ipriflavone supplementation in $Hbb^{Th3+/-}$ mice failed to alleviate iron overload and was associated with a milder reduction in intestinal ferroportin and a failure to alter the ratio of hepcidin transcript levels to liver iron stores or splenic expression of the hepcidin-regulatory hormone, erythroferrone. These data suggest that dietary supplementation with ipriflavone alone would not be sufficient to treat iron overload in thalassemia intermedia.

1. Introduction

As humans do not actively excrete iron, maintaining iron homeostasis requires modulation of intestinal iron uptake to reflect the body's iron stores. Hepcidin is a transcriptionally regulated peptide hormone [1] that is produced primarily in the liver and excreted in the urine. Hepcidin transcription increases in response to inflammation [2,3] or iron overload [4] and decreases in the setting of increased erythropoiesis, iron deficiency, or hypoxia [2]. Hepcidin regulates iron uptake by enterocytes and iron delivery to erythrocytes by binding the iron exporter ferroportin1 (fpn), which results in internalization and degradation of fpn [5].

In patients with iron overload disorders such as β-thalassemia and hereditary hemochromatosis, regulation of iron absorption and metabolism is disrupted, resulting in accumulation of iron in body tissues such as the liver and spleen. Thalassemias are inherited hemoglobinopathies resulting in impaired synthesis of hemoglobin and are among the most common genetic diseases in the world [1,2]. Patients with thalassemia major (TM), most commonly those homozygous for β-globin gene deletions (β^0-thalassemia), exhibit severe anemia resulting in dependence on blood transfusions to maintain normal development and limit progression of liver and spleen enlargement and skeletal deformities caused by extramedullary hematopoiesis [1]. Patients with thalassemia intermedia (TI) have less severe abnormalities in globin production and do not usually require blood transfusions, although they are prone to increased intestinal iron absorption [1]. While life expectancy for thalassemia major (TM) patients has improved in recent decades, heart failure remains the leading cause of mortality [3,4]. Improved control of iron overload, as assessed by serum ferritin levels, correlates with a lower probability of heart disease and death [4].

Hbb^{Th3}, subsequently referred to as Th3, mice have a targeted deletion in the mouse β^{minor} and β^{major} gene. Th3 homozygote mice, a model of thalassemia major, lack all β globin genes and are nonviable unless rescued by blood transfusions. Th3 heterozygotes (Th3 +/−), a model for thalassemia intermedia, are viable without blood transfusions, but exhibit moderate anemia, ineffective erythropoiesis, and iron overload, particularly in the liver and spleen [5]. Th3 +/− exhibit increased intestinal fpn expression and inappropriately low hepatic transcript levels of hepcidin [5].

Because its levels are inappropriately low in patients with iron overload syndromes [6] and experimental over-expression of *hepcidin* or

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injection of synthetic hepcidin improves iron overload in mouse models [7,8], hepcidin is a potential drug target for the treatment of tissue iron accumulation. While patients with hereditary hemochromatosis may be treated with therapeutic phlebotomy, individuals with iron-loading anemias, such as thalassemia rely on chelation therapy rather than phlebotomy. Despite the widespread use of chelators, the majority of thalassemia major patients die of iron-related organ failure, which is incompletely resolved by chelation therapy [9,10], thus we would like to identify small molecules that modify hepcidin expression, reduce tissue iron overload, and may synergize with iron chelators.

We recently conducted a chemical screen in human hepatocytes (HepG2 cells) for small molecules that regulate hepcidin expression [11,12]. We first identified genistein as a flavone molecule that increased hepcidin expression in human hepatocytes in vitro and in zebrafish embryos in vivo [12]. On further screening [11], we identified ipriflavone, an isoflavone with estrogenic properties, as an upregulator of *hepcidin* expression that increases hepcidin luciferase activity and *hepcidin* mRNA transcript abundance at 1 μM concentration, which is ten-fold more potent than genistein. Ipriflavone increased expression of the BMP-dependent gene *ID3* [11], suggesting it activates the BMP-6 pathway to modulate *hepcidin* transcription.

Because ipriflavone is a relatively nontoxic, orally available small molecule that has been approved as a drug for osteoporosis in Europe and Japan, we hypothesized that it could be useful in the treatment of iron overload syndromes in vivo. Ipriflavone is a synthetic isoflavone that is derived from daizdein, a molecule that is abundant in soybeans. While ipriflavone has estrogenic effects, it is less stimulatory to the endometrium than estradiol [13]. Ipriflavone, taken as an oral supplement, has been shown to increase bone mineral density in postmenopausal women [14] and to have antioxidant properties [15].

In this study we investigated the effect of dietary supplementation with ipriflavone on iron homeostasis in wild type (WT) mice and in a mouse model of thalassemia intermedia, $Hbb^{Th3+/-}$ (Th3+/−). We found that short-term gavage or dietary supplementation with ipriflavone increased serum hepcidin levels in WT mice. Long-term dietary supplementation with ipriflavone significantly reduced liver iron stores in WT, but not in Th3+/− mice. Furthermore, dietary supplementation with ipriflavone at 500 mg/kg decreased intestinal *fpn* expression in WT and Th3+/− mice and significantly increased the ratio of *hepcidin* transcript levels to liver iron stores in WT mice.

2. Materials and methods

2.1. Mouse strains and diet

All mouse strains were maintained on the C57BL/J6 background and included WT C57BL/J6 (Jackson Laboratories, Bar Harbor, ME) and a thalassemia intermedia model ($Hbb^{Th3+/-}$ (Th3+/−) a gift of Dr. Seth Alper). In the experiments, WT mice were males, while Th3+/− mice included both males and females, housed separately. All experiments were approved by the Animal Care and Use Committee of Beth Israel Deaconess Medical Center. At 4 weeks of age, the mice were equilibrated for 1 week on a soy-free rodent diet (AIN-93G with 2.5 mg of iron per kg of food, BioServ, Flemington, NJ) that was supplemented with ferrous gluconate hydrate (Sigma Aldrich, St. Louis, MO) to achieve a total iron content of 35 mg of elemental iron per kilogram of food for short-term experiments or 220 mg of elemental iron per kilogram of food, which is typical for a laboratory mouse diet, for long-term experiments. For 4-hour experiments, ipriflavone (#I6068, LKT Laboratories, St. Paul, MN) dissolved in 90% phosphate buffered saline/10% DMSO was administered by oral gavage at 120 mg/kg body weight for one dose followed by nonterminal blood collection 4 h later. The diet was then supplemented with 0, 250, 500, or 750 mg ipriflavone/kg of food and maintained for 7 or 50 days. All groups were fed and watered ad libitum and food intake and body weight were monitored throughout. Animals were maintained on a 12 h light-dark schedule. After 7 or 50 days, the mice were anesthetized by intraperitoneal injection of tribromoethanol and underwent terminal cardiac exsanguination and tissue harvest. Genotypes of the Th3+/− animals were confirmed by PCR amplification using the primers in Supplementary Table 1.

2.2. Hematologic analysis

Blood was collected from the heart using a heparinized sterile 1 ml tuberculin syringe and 25-gauge needle and then transferred to a test tube with 3.6 mg EDTA. Complete blood counts, white blood cell differential, and reticulocyte counts were performed in the Boston Children's Hospital Department of Laboratory Medicine on an Advia 120 (Siemens Healthcare, Malvern, PA).

2.3. Quantitative real time RT-PCR and quantitation of serum hepcidin levels

Fresh tissues were immediately stabilized in RNAlater solution (Thermo Fisher, Cambridge, MA) and stored at 4 °C. Thirty milligram sections of tissue were removed from the stabilization reagent, flash frozen in liquid nitrogen, and disrupted by grinding with a mortar and pestle. RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. cDNA was generated and quantitative real time RT-PCR was performed according to the method as described [12]. Serum hepcidin protein level was measured using the hepcidin Murine-Compete ELISA Kit (Intrinsic LifeSciences, La Jolla CA).

2.4. Non-heme tissue iron assay

Fresh aliquots, 200 mg each, of liver and spleen were weighed and frozen at −20 °C. Nonheme iron content was determined using the bathophenanthroline method, as described [16]. Data shown are μg iron per gram of tissue. All standards and samples were evaluated in triplicate.

2.5. Histology and immunohistochemistry

The ileum and a lobe of the liver from each mouse were fixed in phosphate buffered paraformaldehyde (4%, pH 7.4) and embedded in paraffin, sectioned, and mounted on glass slides.

2.6. Diaminobenzidine (DAB)-enhanced Perls' staining for nonheme iron

Deparaffinized and rehydrated liver and ileum slides were incubated in 1% potassium ferrocyanide/0.12 M HCl for 30 min, washed 3 times in PBS, quenched in 0.3% H_2O_2/Methanol for 20 min, washed 3 times in PBS, and incubated in 3.3′-Diaminobenzidine tetrahydrochloride solution (SIGMA *FAST*™ DAB with Metal Enhancer Tablet, #D0426, 1 tablet set per 5 ml solution, Sigma Aldrich) for 30 min. Slides were then washed 3 times in PBS, prior to dehydration and placement of a cover slip.

2.7. Immunohistochemistry

Immunohistochemical staining of ileum slides with an anti-ferroportin (anti-fpn) antibody (#MTP11-A, Alpha Diagnostic International, San Antonio, TX) was performed using the Dako EnVision + System-HRP (#K4010, Dako, Carpinteria, CA). Control slides were stained using a mouse fpn control peptide (MTP11-P, Alpha Diagnostic International) with 10 μg/ml antibody in 0.05 M Tris-HCl-1% BSA, or 0.05 M Tris-HCl-1% BSA alone.