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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.

2.8. Flow cytometry

To assess the effects of ipriflavone treatment on erythropoiesis, flow cytometry was performed to determine the percentage of mature and immature erythrocytes in the spleen and bone marrow using the method as described [5] with the following antibodies, APC-conjugated anti-Ter119 (#557909, BD Biosciences, San Jose, CA) and PE-conjugated anti-CD71 (#AB22393, Abcam, Cambridge, MA). Cells were counted and analyzed using a Gallios flow cytometer and the Kaluza acquisition and analysis programs (Beckman Coulter, Inc., Indianapolis, IN).

2.9. Transferrin-bound iron uptake assay

Human hepatoma cells, HepG2 (American Type Tissue Collection, Manassas, VA), were plated at a density of 10^6 cells/well in 2 mL of α-minimal essential medium/10% endotoxin-free fetal bovine serum/1% penicillin/streptomycin (Life Technologies, Grand Island, NY) at 37 °C, 5% CO_2. Twenty-four hours after plating, the media was changed to α-MEM/1% FBS/1% penicillin/streptomycin. The following day, the media was supplemented with 6 nM ^{55}Fe-transferrin and vehicle only (1% DMSO) or 1–100 μM ipriflavone. ^{55}Fe transferrin-bound iron uptake assay was performed as described [12].

2.10. Statistical analysis

Data shown are means ± standard errors unless otherwise indicated. The Kruskal-Wallis method was used to generate a global *p*-value using Prism 6.0c (GraphPad, San Diego, CA). Where the global *p*-value was < 0.05, pairwise Student's *t*-tests were performed. $p < 0.05$ was considered a significant result on the Student's *t*-test.

3. Results

3.1. Ipriflavone increases serum hepcidin levels in wild-type mice

We had previously identified ipriflavone as a potent inducer of *hepcidin* transcript levels in vitro, but had not evaluated its effect on iron metabolism in vivo. To avoid the confounding effects of soy-derived flavones in standard mouse diets, we used the soy-free diet, AIN 93G (Bioserv). To evaluate ipriflavone's short-term effects, we equilibrated 4-week old wild-type, C57/BL6 mice, to an iron-sufficient AIN 93G diet containing 35 mg of iron per kg of food for 1 week and then delivered ipriflavone (120 mg/kg of body weight) or vehicle alone by oral gavage. Four hours later (Fig. 1A), we measured serum hepcidin levels and found an increase in hepcidin levels in the ipriflavone-gavaged mice (550.3 ± 51.1 vs. 348.8 ± 83.79, $p = 0.05$). We then treated the mice for 7 days on the iron-sufficient diet supplemented with 500 mg ipriflavone/kg of food. At the end of 7 days, the mice exhibited a significant increase in serum hepcidin levels (351.7 ± 44.2 vs 151.4 ± 16.1, $p = 0.001$), however there was no significant change in liver *hepcidin* transcript levels, liver, or spleen iron stores (Fig. 1B-D).

3.2. Ipriflavone effectively reduced nonheme iron level in the livers of wild-type mice

We were particularly interested in whether longer-term treatment of the mice would prevent hepatic iron loading. To that end, we fed wild-type or Th3 +/− mice diets including higher amounts of

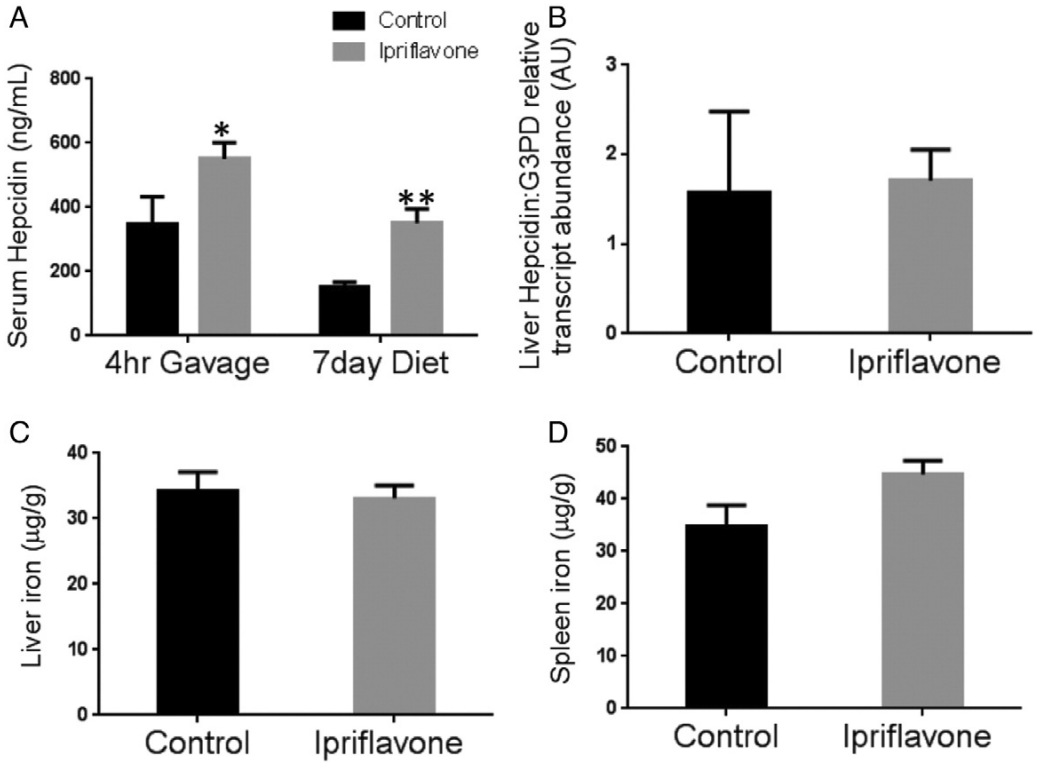

Fig. 1. Treatment with ipriflavone increases serum hepcidin levels in wild type mice. Four-week old C57/BL6 mice received an iron-sufficient diet (35 mg of iron/kg of food) for 1 week and then underwent a single gavage of ipriflavone (120 mg/kg in 90% PBS/10% DMSO) or vehicle alone. The mice then continued the iron-sufficient diet with or without ipriflavone (500 mg/kg of food) for 7 days. (A) Serum hepcidin levels of WT mice after a single gavage treatment or after 7 day dietary treatment with ipriflavone. (B-D) Evaluation of RNA transcript levels for liver *hepcidin* (B) and nonheme iron quantification of the liver (C) and spleen (D) after 7 days of dietary supplementation with ipriflavone (500 mg/kg of food). $N = 7$–8 mice per group. * signifies $p = 0.05$, while ** signifies $p = 0.001$ by Student's *t*-test compared to 0 mg/kg control group.

elemental iron (220 mg iron/kg food), supplemented with 0, 250, 500, and 750 mg ipriflavone/kg food. The WT mice achieved mean daily doses of 0, 56.30 ± 2.71, 104.0 ± 6.28, and 186 ± 14.4 mg of ipriflavone per kg of body weight, while the Th3 +/− mice, which ate more than the WT mice but gained less weight, ingested mean doses of 0, 108.9 ± 3.25, 151.4 ± 7.07, or 322.3 ± 10.3 mg of ipriflavone per kilogram of body weight (Supplementary Table 2).

We found that dietary supplementation with ipriflavone decreased liver nonheme iron stores (Fig. 2A, B) in WT mice. We observed a significant decrease in hepatic iron stores of WT mice treated with ipriflavone at 500 mg/kg or at 750 mg/kg, compared to those that did not receive ipriflavone supplementation (39.2 ± 2.06 and 44.1 ± 2.58 μg iron/g tissue, respectively, compared to 57.8 ± 3.39 μg iron/g tissue, $p = 0.003$ and $p = 0.012$, respectively). In contrast, spleen iron concentration did not change significantly with ipriflavone treatment. Blinded evaluation of diaminobenzidine (DAB)-enhanced Perls' staining (Fig. 2C, D), also revealed a decrease in hepatic iron stores in WT mice treated with ipriflavone 500 mg/kg or 750 mg/kg.

To evaluate whether ipriflavone is effective at relieving iron overload in a mouse model of thalassemia, we treated Th3 +/− mice with the same regimens that we had used in the WT mice. The untreated Th3 +/− mice exhibited levels of liver and spleen iron of 286.8 ± 41.24 and 1565 ± 86.87 μg iron/mg of tissue, respectively (Fig. 3A, B), which were nearly 5-fold greater than untreated WT liver iron and nearly 6-fold greater than WT spleen iron levels. Treatment with ipriflavone at 250–750 mg/kg failed to reduce significantly the mean level of iron loading in the liver ($p = 0.379$) or spleen ($p = 0.824$) or the extent of liver iron staining (Fig. 3C, D).

Th3 +/− mice have previously been shown to exhibit high levels of intestinal fpn [5], which contributes to the development of iron overload. In fact, we found that Th3 +/− mice exhibit high levels of fpn expression even in the distal small intestine, where fpn expression is usually low (Supplementary Fig. 1, Fig. 4A, B). Enterocyte fpn expression in WT mice declined with increasing ipriflavone dosage (60% of mouse intestines were strongly stained at 0 mg/kg, compared to 33% at 500 mg/kg and 20% at 750 mg/kg), while the intensity of fpn staining in Th3 +/− mice decreased slightly at 500 mg/kg and 750 mg/kg, but remained much higher than in WT mice treated with equivalent doses of ipriflavone (Fig. 4A, B).

3.3. Ipriflavone's effect on erythropoiesis

To evaluate the effect of ipriflavone on erythropoiesis in WT and Th3 +/− mice, we measured the hemoglobin level, hematocrit, mean

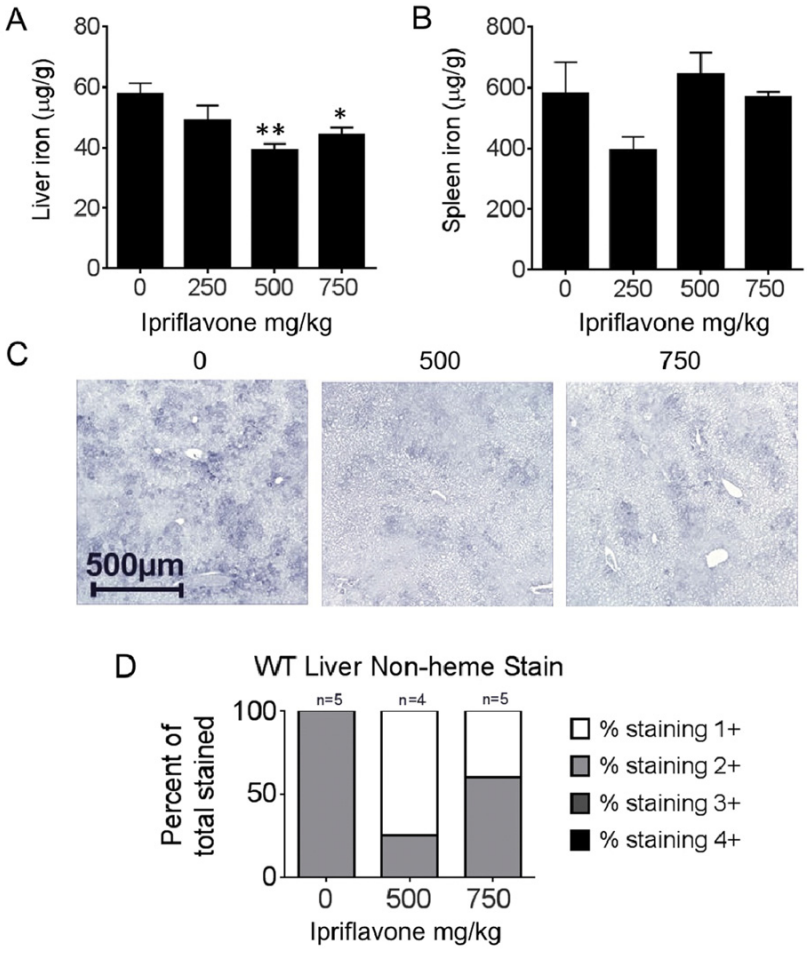

Fig. 2. Dietary supplementation with ipriflavone decreased hepatic iron stores in wild type mice. C57/BL6 mice received diets supplemented with ipriflavone in mg/kg of food at the following doses: 0 mg/kg ($n = 5$), 250 mg/kg ($n = 5$), 500 mg/kg ($n = 4$), and 750 mg/kg ($n = 5$) starting at 5 weeks of age for a total of 50 days followed by tissue harvest for quantification of nonheme iron levels in the liver (A) and spleen (B), reported as μg iron per g of tissue. Data shown are means ± standard errors. By Kruskal-Wallis test, $p = 0.027$ for liver comparisons and 0.099 for spleen comparisons. * signifies $p < 0.05$, while ** signifies $p < 0.01$ by Student's t-test compared to 0 mg/kg controls. (C) Diaminobenzidine (DAB)-enhanced Perls' staining for nonheme iron in liver tissue. (D) Blind analysis was used to score the staining levels of each slide from 1+ (weak) to 4+ (strong).

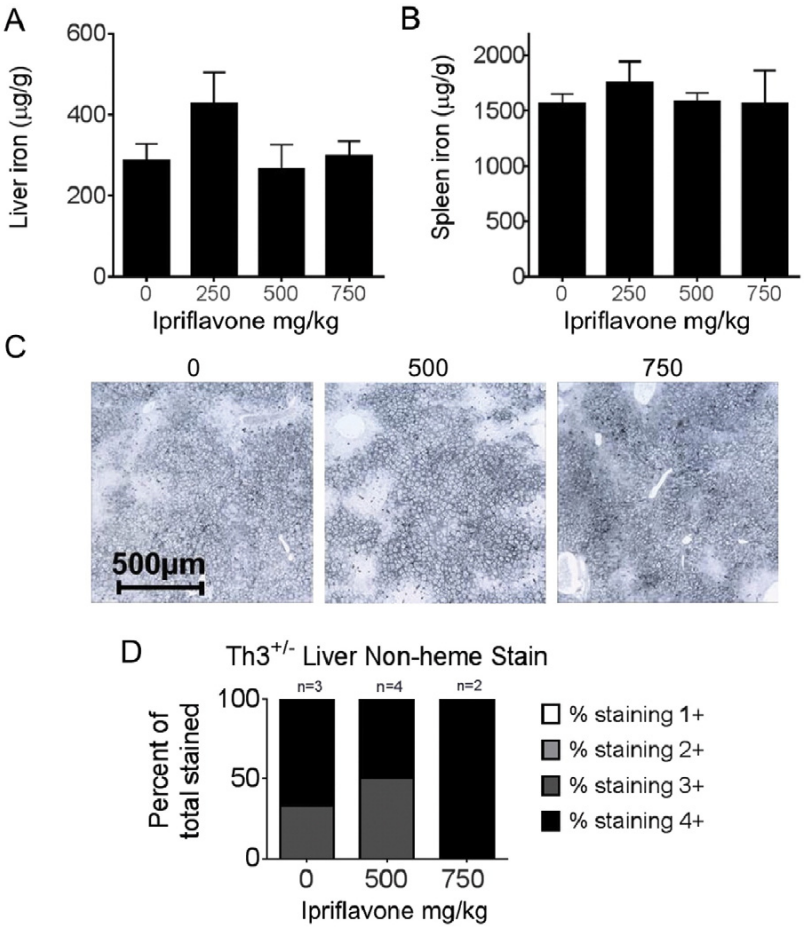

Fig. 3. Dietary supplementation with ipriflavone did not decrease iron stores in the livers or spleens of Th3 +/− mice. Th3 +/− mice on a C57/BL6 background received diets supplemented with ipriflavone in mg/kg of food at the following doses: 0 mg/kg ($n = 3$), 250 mg/kg ($n = 3$), 500 mg/kg ($n = 4$), and 750 mg/kg ($n = 2$) starting at 5 weeks of age for a total of 50 days followed by tissue harvest for quantification of nonheme iron levels in the liver (A) and spleen (B), reported as µg iron per g of tissue. Data shown are means ± standard errors. $p = 0.379$ by Kruskal-Wallis for liver comparisons and 0.824 for spleen comparisons. (C) Diaminobenzidine (DAB)-enhanced Perls' staining for nonheme iron in liver tissue. (D) Blind analysis was used to score the staining levels from 1+ (weak) to 4+ (strong).

corpuscular volume (MCV), and reticulocyte percentage (Fig. 5A-D). Interestingly, in WT mice, the mean MCV decreased significantly with ipriflavone treatment at all doses, from 59.86 ± 0.29 untreated, to 58.7 ± 0.41 at 250 mg/kg, 56.83 ± 0.67 at 500 mg/kg, and 58.26 at 750 mg/kg ($p = 0.049$, 0.003, and 0.019, respectively, in comparison to untreated). The reticulocyte percentage decreased significantly at the highest dose of ipriflavone (750 mg/kg) from 3.1 ± 0.16 to 1.9 ± 0.15 ($p = 0.001$), suggesting a toxic effect at this high dose. We found that Th3 +/− mice exhibited a phenotype consistent with ineffective erythropoiesis that was not altered by ipriflavone treatment. The Th3 +/− mice exhibited lower hemoglobin and hematocrit levels and erythrocyte mean corpuscular volumes (MCVs) than WT mice, while their reticulocyte percentages were consistently higher than WT mice (Fig. 5A-D).

To determine whether ipriflavone has any effect on the distribution of erythroid progenitors in Th3 +/− mice, we performed fluorescence-activated cell sorting (FACS) for CD71 and Ter119 to identify immature and mature erythroid populations in the bone marrow and spleens. We found no significant effect of ipriflavone treatment on the percentage of erythroid progenitor cells or mature erythrocytes in the bone marrow or spleen in Th3 +/− mice (Supplementary Fig. 2).

3.4. Ipriflavone's effect on hepcidin, DMT1, fpn, and erythroferrone transcript levels and transferrin-dependent iron uptake

To assess the effect of ipriflavone on gene transcription, we evaluated liver hepcidin transcript levels and intestinal transcript levels of the iron transporters, *DMT1* and *fpn*. The liver *hepcidin:G3PD* transcript levels did not change significantly between treated and untreated mice. The Th3 +/− mice exhibited higher *hepcidin* transcript levels than WT mice whether treated with ipriflavone or not (Fig. 6A). Treatment with ipriflavone 250 or 500 mg/kg, however, significantly increased the ratio of hepatic *hepcidin* transcript levels to liver iron stores in WT mice (Fig. 6B) (0.024 ± 0.003 and 0.02 ± 0.002, respectively, compared to 0.017 ± 0.001, $p = 0.049$ and $p = 0.021$, respectively). Transcript levels for intestinal *DMT1* decreased significantly at 750 mg/kg in WT mice (0.632 ± 0.04 vs 1.04 ± 0.11, $p = 0.017$), but were otherwise unaffected in both WT and Th3 +/− mice at all doses of ipriflavone. To determine if ipriflavone affected the transcript levels of the recently identified hormone, *erythroferrone*, we measured transcript levels in the spleens of the treated and untreated Th3 +/− mice, but found no significant difference in expression ($p = 0.96$) (Supplementary Fig. 3). We also considered that ipriflavone may be lowering hepatic iron stores

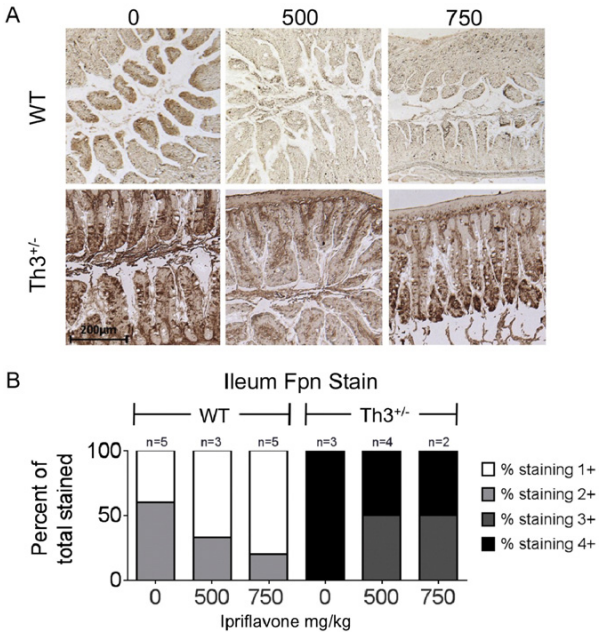

Fig. 4. Dietary supplementation with ipriflavone decreased intestinal fpn protein expression in WT mice. A. Representative immunohistochemical staining for fpn in cross-sections of intestine from WT and Th3 +/− mice treated with ipriflavone: 0, 500, or 750 mg/kg of food. B. Blinded evaluation of staining intensity from 1+ (weak) to 4+ (strong) staining.

by interfering with transferrin-dependent iron uptake by hepatocytes. To evaluate this hypothesis, we co-treated human hepatocytes, hepG2 cells, with ipriflavone and ^{55}Fe-transferrin for 4 h. We found no significant difference in ^{55}Fe-transferrin uptake in ipriflavone-treated cells compared to cells treated with vehicle only ($p = 0.46$) (Supplementary Fig. 4).

4. Discussion

In this study we evaluated the in vivo effects of ipriflavone, an orally available synthetic flavone, that we recently identified as a potent inducer of *hepcidin* expression in a high throughput screen in human hepatocytes (HepG2 cells) for small molecules that regulate *hepcidin* expression [11,12]. In the current study, we found that oral administration of ipriflavone by gavage or dietary supplementation increased serum hepcidin levels (Fig. 1). We also found that dietary supplementation with ipriflavone for 50 days lowered hepatic iron stores and increased *hepcidin* transcript levels relative to liver iron stores in WT, but not Th3 +/− mice, which are a mouse model of thalassemia intermedia. The Th3 +/− mice actually ingested more ipriflavone per kg of body mass than WT mice (Supplementary Table 2), but also ingested more iron per kg from their diet. Furthermore, baseline hepatic *hepcidin* expression was higher in the Th3 +/− mice than the WT mice, thus the Th3 +/− mice may require a stronger *hepcidin* inducer than ipriflavone.

The decrease in intestinal fpn protein expression in WT mice treated with ipriflavone 500 mg/kg may be secondary to the increase in the ratio of *hepcidin* transcript level to iron stores, however recent studies indicate that fpn down-regulation can occur in a hepcidin-independent manner. In mice, stimulation of toll-like receptors (TLR2 or 6) reduces *fpn* transcript and protein expression in macrophages, liver, and spleen without increasing macrophage *hepcidin* expression [17]. However, unlike the model of TLR-stimulation, we did not observe a decrease in intestinal *fpn* transcript levels with ipriflavone treatment (Fig. 6), thus we do not think that ipriflavone's effect is caused by TLR-stimulation. We did observe a decrease in intestinal *DMT1* expression

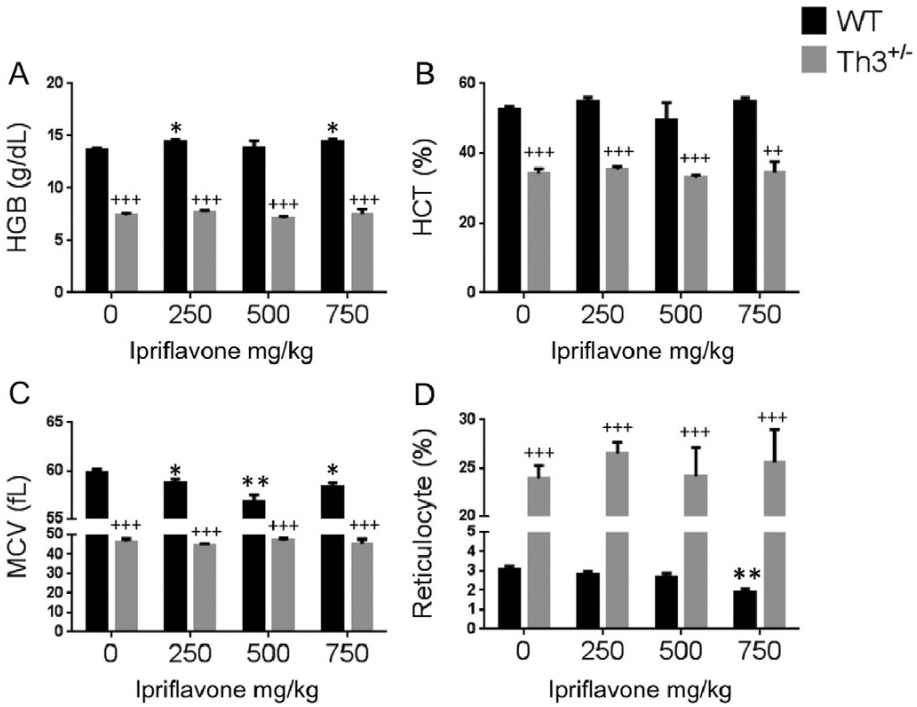

Fig. 5. Ipriflavone treatment decreased mean corpuscular volume (MCV) in wild type mice. Erythrocyte indices for WT and Th3 +/− mice after 50 days of treatment with ipriflavone 0–750 mg/kg of food. A. Hemoglobin measurements in g/dL, B. Hematocrit, C. MCV, D. Reticulocyte percentage. * denotes $p < 0.05$ and ** $p < 0.01$ for WT treated vs WT untreated. ++ denotes $p < 0.01$ and +++ denotes $p < 0.001$ for Th3 +/− vs WT untreated.

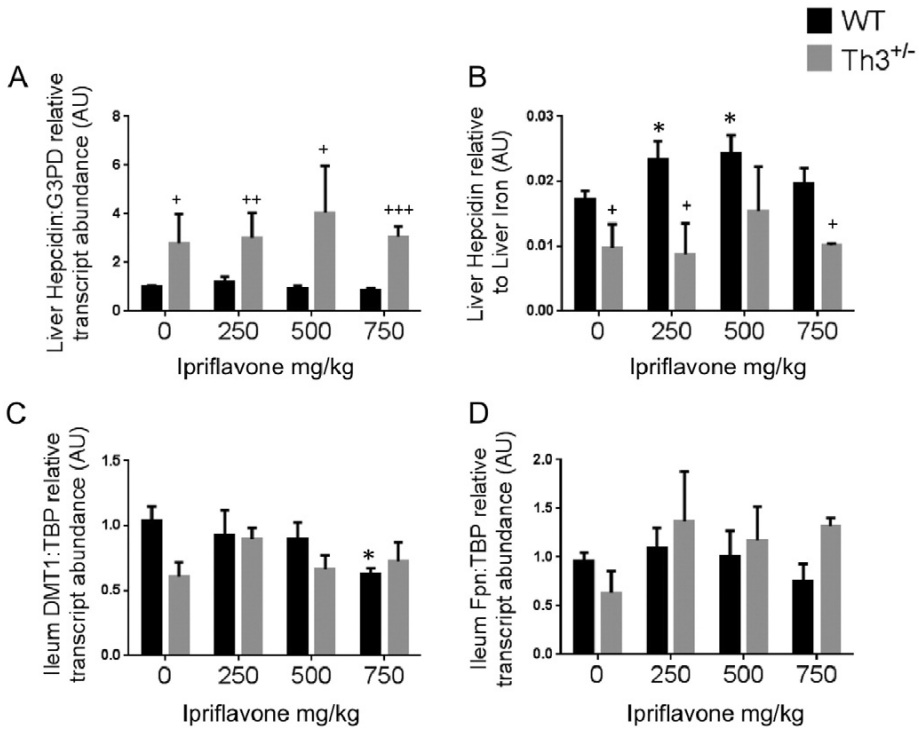

Fig. 6. Ipriflavone treatment increased the ratio of hepcidin transcript level to liver iron level in WT mice. Evaluation of RNA transcript levels for liver hepcidin (A), ratio of liver hepcidin to liver non-heme iron stores (B), intestinal *DMT1* expression (C), and intestinal *fpn* expression (D) for mice treated for 50 days with ipriflavone 0–750 mg/kg of food. * denotes $p < 0.05$ for WT treated vs WT untreated. + denotes $p < 0.05$, ++ $p < 0.01$, and +++ denotes $p < 0.001$ for Th3+/− vs WT untreated.

in WT mice treated with ipriflavone at the 750 mg/kg dose. This may have helped to limit intestinal iron absorption in these animals.

While ipriflavone structurally resembles estradiol, ipriflavone was effective in WT male mice (Figs. 1, 2), thus it does not appear that ipriflavone requires feminine gender for its effect. Ipriflavone is known to have effects that are independent of estrogen-receptor, as it has been shown to inhibit osteoclast activity by increasing intracellular calcium levels [18] in an effect that is not antagonized by estradiol treatment [19]. We have previously shown that estradiol does not induce *hepcidin* expression in vitro [12], while others have shown that estradiol inhibits *hepcidin* transcription [20], thus we think it is unlikely that ipriflavone is increasing *hepcidin* expression in an estrogen-receptor dependent manner.

Other investigators have been seeking to treat iron overload in mouse models by daily injection of minihepcidins, chemically stabilized derivatives of hepcidin, in hepcidin-knockout mice [8]. Injection of a minihepcidin, PR65, in hepcidin knockout mice caused a more profound reduction in liver iron stores than we observed with ipriflavone treatment in WT mice. Treatment with PR65 was accompanied by a decrease in hemoglobin level and an increase in splenic iron, which is consistent with iron-restricted erythropoiesis [8]. While ipriflavone treatment in WT mice did not produce anemia or a significant increase in splenic iron, we found that it was associated with a modest decrease in erythrocyte mean corpuscular volume (MCV) (Fig. 5C) that could be associated with mildly iron-restricted erythropoiesis. Interestingly, pre-loading *hepcidin*-knockout mice with iron and then treating with minihepcidins was less effective at reducing liver iron stores than treating the mice while they simultaneously received the high iron diet [8]. By 5 weeks of age, when we started ipriflavone as a *hepcidin* inducing treatment, Th3 +/− mice may already be "pre-loaded" with iron, as well.

We are intrigued by the fact that ipriflavone did not significantly increase the absolute *hepcidin* transcript levels or reduce the hepatic iron stores in the Th3 +/− mice. The hormone, erythroferrone, has recently been identified as an erythropoietic iron sensor that suppresses *hepcidin* expression in the Th3 +/− mouse model of thalassemia [21]. We found that erythroferrone (*ERFE*) transcript levels did not change in Th3 +/− mice in response to ipriflavone treatment (Supplementary Fig. 3). It may be necessary to find a small molecule or combination of molecules that can suppress ERFE activity in order to increase hepcidin expression further in Th3 +/− mice. Ideally, a small molecule treatment for iron overload would confer antioxidant effects, as retinoids appear to do [22], suppress intestinal expression of hypoxia inducible factor 2 alpha (HIF2α), which can stimulate intestinal iron absorption in mouse models of thalassemia [23], and not disrupt the transcriptional program that is characteristic of enterocyte differentiation [24]. In future experiments, we hope to identify more potent, yet nontoxic, small molecule hepcidin inducers that could be used in combination with iron chelators to treat thalassemia-related iron overload more effectively.

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