

### Mice are poor heme absorbers and do not require intestinal *Hmox1* for dietary heme iron assimilation

Dietary iron absorption offsets non-specific iron losses and is crucial for systemic iron balance. Inadequate iron acquisition leads to iron deficiency, a condition associated with anemia, poor pregnancy outcome and impaired cognitive and motor development.<sup>1</sup> Heme derived from hemoglobin (Hb) and myoglobin of meat products is an important nutritional iron source.<sup>2</sup> It is more bioavailable compared to inorganic iron, although less abundant in mixed diets. Thus, two-thirds of body iron stores are estimated to originate from heme, which accounts for only one-third of total dietary iron content in Western populations.<sup>3</sup>

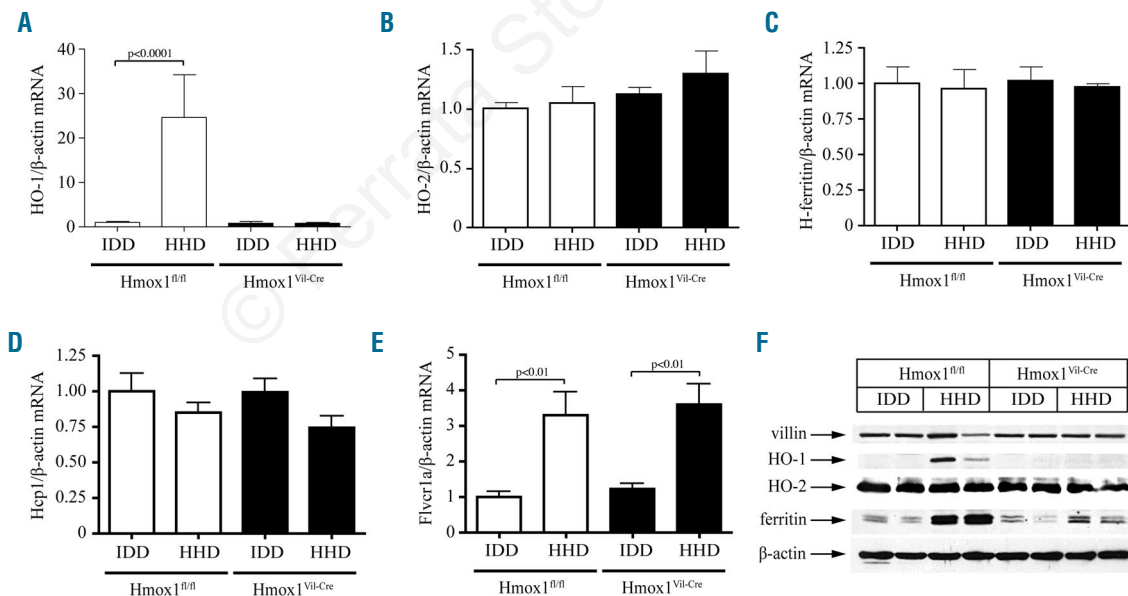
The mechanism for inorganic iron absorption has been clarified from mouse studies.<sup>4</sup> Ingested Fe(III) is reduced to Fe(II) in the intestinal lumen and then internalized by enterocytes *via* the apical transporter DMT1. The basolateral transporter ferroportin mediates Fe(II) efflux to the bloodstream.

Heme is absorbed by enterocytes as an intact molecule *via* receptor-mediated endocytosis or direct transport.<sup>3,5</sup> The underlying mechanisms are not completely understood. Early studies suggested that internalized heme is catabolized to liberate Fe(II), which follows the fate of dietary Fe(II), although direct transport of intact heme to the plasma has also been reported.<sup>6</sup> Enterocytes express heme-catabolic enzymes (heme oxygenases), the heme-

inducible HO-1 and its constitutive homolog HO-2,<sup>7</sup> as well as the low-affinity heme transporter HCP1 and the heme exporter FLVCR1.<sup>5</sup>

We generated intestinal-specific *Hmox1*<sup>-/-</sup> mice (*Hmox1*<sup>Vil-Cre</sup>) to dissect the role of HO-1 in heme iron assimilation (*Online Supplementary Appendix*). Because high-iron diets promote iron overload in mice,<sup>8</sup> we reasoned that a high-heme diet (HHD) might elicit similar effects, or at least increase body iron stores in *Hmox1*<sup>fl/fl</sup> controls. Conversely, *Hmox1*<sup>Vil-Cre</sup> mice should be either spared and accumulate excess heme in enterocytes, or export heme to the bloodstream. To address these hypotheses, *Hmox1*<sup>fl/fl</sup> and *Hmox1*<sup>Vil-Cre</sup> mice were fed for two weeks with an iron-deficient diet (IDD) or a HHD.

Surprisingly, the HHD did not cause any appreciable increases in serum or hepatic iron in both genotypes. Likewise, mRNA levels of the iron regulator hepcidin were largely unaffected (*Online Supplementary Figure S1*). The absence of iron overload in HHD-fed control animals was not associated with inflammation or alterations in duodenal epithelial architecture (*Online Supplementary Figure S2*), suggesting that heme may be a poor dietary iron source for mice. Nevertheless, the HHD intake promoted a profound increase of intestinal HO-1 in *Hmox1*<sup>fl/fl</sup> mice, consistent with hemin-mediated induction (Figure 1A and F). Thus, intestinal enterocytes are capable of internalizing a fraction of dietary heme. Importantly, intestinal HO-1 expression was undetectable in *Hmox1*<sup>Vil-Cre</sup> mice and did not respond to HHD intake, demonstrating efficient tissue-specific ablation of the *Hmox1* gene. The HHD did not affect expression of



**Figure 1.** Effects of dietary heme excess in expression of intestinal heme and iron metabolism genes. 6-week old male *Hmox1*<sup>fl/fl</sup> and *Hmox1*<sup>Vil-Cre</sup> mice (n=5 for each genotype) were fed for two weeks with an IDD (containing only 2-6 ppm iron) or a HHD (IDD supplemented with 30 mmol/kg hemin, equivalent to 2% carbonyl iron in terms of iron quantity). At the end point the animals were sacrificed and duodenal RNA and protein extracts were prepared for qPCR and Western blot analysis, respectively. (A) The HHD promoted 23-fold induction of intestinal HO-1 mRNA in control *Hmox1*<sup>fl/fl</sup> but not in *Hmox1*<sup>Vil-Cre</sup> mice. (B-D) Intestinal HO-2 (B), H-ferritin (C) and Hcp1 (D) mRNA levels were not significantly affected by the diets and the genotypes. Constitutive expression of HO-2 mRNA was similar to that of HHD-induced HO-1 mRNA in *Hmox1*<sup>fl/fl</sup> mice. (E) The HHD stimulated intestinal Flvcr1a mRNA expression in both *Hmox1*<sup>fl/fl</sup> and *Hmox1*<sup>Vil-Cre</sup> mice. qPCR data in (A-E) are presented as the mean  $\pm$  SEM. Statistical analysis was performed by one-way ANOVA. (F) The HHD induced intestinal HO-1 expression in control *Hmox1*<sup>fl/fl</sup> mice and intestinal ferritin expression in both *Hmox1*<sup>fl/fl</sup> and *Hmox1*<sup>Vil-Cre</sup> mice. Expression of HO-2, villin (intestinal marker) and  $\beta$ -actin (loading control) was not affected by the diets and the genotypes. Data from 2 representative mice are shown. Similar results were obtained with samples from the other mice used in the experiment.

housekeeping HO-2 (Figure 1B and F), which was equal between Hmox1<sup>fl/fl</sup> and Hmox1<sup>Vil-Cre</sup> mice.

Hcp1 mRNA was not significantly affected by the HHD in either genotype (Figure 1D). Flvcr1a mRNA did not differ substantially between Hmox1<sup>Vil-Cre</sup> and Hmox1<sup>fl/fl</sup> mice, but was similarly induced by HHD (Figure 1F), as reported in hemin-treated hepatocytes.<sup>9</sup> This may indicate increased heme efflux independently of HO-1 expression. However, the lack of hemopexin or HO-1 mRNA induction in the liver and spleen (Figure 2A-C) suggests that heme export is negligible.

Heme catabolism liberates Fe(II), which in turn post-transcriptionally up-regulates ferritin for iron storage.<sup>10</sup> Intestinal ferritin (but not H-ferritin mRNA) was induced in HHD-fed Hmox1<sup>fl/fl</sup> mice (Figure 1C and F), presumably following enzymatic degradation of internalized heme by HO-1. A similar response, although quantitatively less pronounced, was observed in HHD-fed Hmox1<sup>Vil-Cre</sup> mice. Considering that the HHD has virtually no inorganic iron, this indicates partial enzymatic degradation of internalized heme by HO-2.

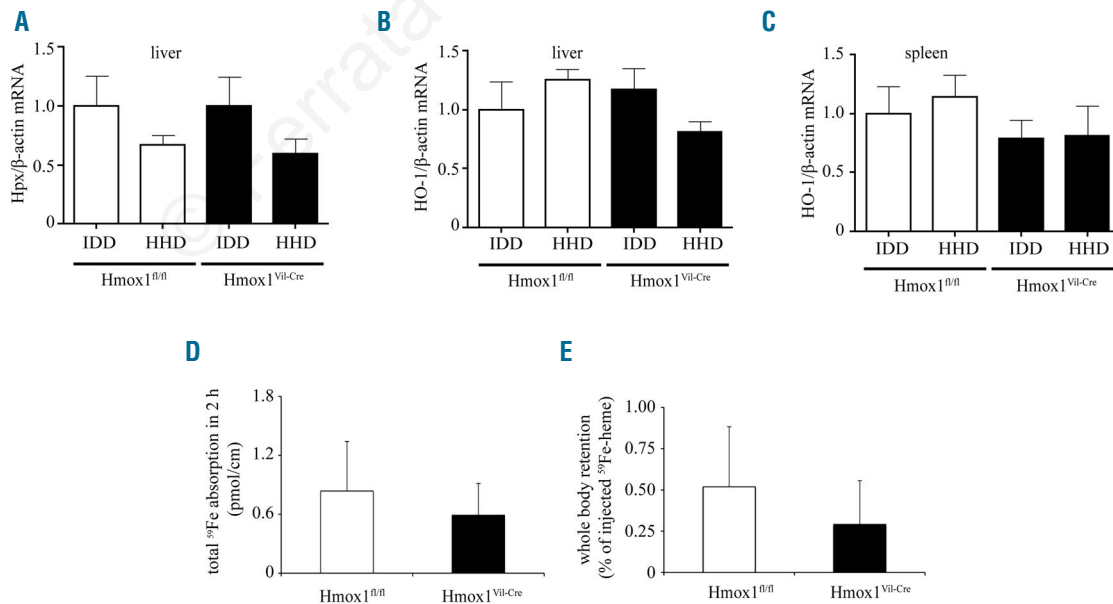
Conceivably, the failure of the HHD to elevate systemic iron levels is related to reduced bioavailability of chemically prepared heme, which may polymerize and form aggregates.<sup>6</sup> Therefore, we used a murine hemolysate containing <sup>59</sup>Fe-Hb as physiological heme source. This was administered to ligated intestinal loops for 2 h; a prolonged incubation period to maximize signal-to-noise ratio. <sup>59</sup>Fe absorption was slightly lower in Hmox1<sup>Vil-Cre</sup> mice but the difference was insignificant (Figure 2D). Due to limitations in the amount of <sup>59</sup>Fe-Hb, we were not able to determine linear <sup>59</sup>Fe absorption or calculate <sup>59</sup>Fe lumen-to-body transfer rates. Assuming that plateau was not reached before 30 min, we estimate that iron absorption from <sup>59</sup>Fe-Hb is at least 100-fold less

efficient than from <sup>59</sup>Fe-nitriloacetate.<sup>11</sup> Consistently, there was very little whole body <sup>59</sup>Fe retention (Figure 2E). These data reinforce the concept that mice cannot efficiently assimilate heme iron, independently of intestinal HO-1.

Since iron absorption is enhanced by erythropoietic drive,<sup>4</sup> we examined heme bioavailability under anemia. Wild-type mice were subjected to a 4-week dietary iron restriction during their rapid growth period, immediately after weaning. Subsequently, they either remained on IDD, or switched to IDD supplemented with 50 ppm FeSO<sub>4</sub> or hemin for three weeks (Figure 3A). The diets did not affect body weights (*Online Supplementary Figure S3*). At the end point, the IDD-fed mice exhibited reduced hematologic parameters and serum iron levels, consistent with iron deficiency anemia (Figure 3B-E). The switch to IDD+FeSO<sub>4</sub> efficiently corrected anemia and increased serum iron. Even though the switch to IDD+hemin tended to slightly improve hematologic parameters and to increase serum iron, statistical significance was only reached for MCV values.

Furthermore, FeSO<sub>4</sub> intake significantly increased total hepatic and splenic iron, while the effects of hemin intake were marginal (Figure 3F,G). Nonetheless, both hemin and FeSO<sub>4</sub> supplementation regimens promoted hepcidin mRNA induction, yet the hemin effect was weaker (Figure 3H). These findings further support the concept that heme is an inferior dietary iron source for mice, even under high iron demand. Notably, only FeSO<sub>4</sub> but not hemin supplementation, significantly increased total intestinal iron content (Figure 3I). Thus, the limiting step for dietary heme iron acquisition is the transport of heme from the intestinal lumen across the apical membrane of brush border enterocytes.

We demonstrate that intestinal HO-1 is not essential



**Figure 2.** Effects of dietary heme excess on expression of hepatic and splenic heme metabolism genes, and analysis of intestinal heme transport. (A-C) Hepatic and splenic RNA was prepared from the mice described in Figure 1 and used for qPCR analysis. Dietary heme excess did not induce hemopexin (Hpx) mRNA in the liver (A), and HO-2 mRNA in the liver (B) or spleen (C). qPCR data are presented as the mean ± SEM. Statistical analysis was performed by one-way ANOVA. (D and E) Disruption of intestinal HO-1 did not affect absorption of <sup>59</sup>Fe from <sup>59</sup>Fe-Hb. A murine hemolysate containing <sup>59</sup>Fe-Hb was administered to ligated intestinal loops from Hmox1<sup>fl/fl</sup> (n=5) and Hmox1<sup>Vil-Cre</sup> mice (n=6) for 2 h. (D) Total intestinal absorption of <sup>59</sup>Fe. (E) Retention of <sup>59</sup>Fe in the whole carcass (excluding the intestine). Data are presented as the mean ± SEM. Statistical analysis was performed by two-tailed Student's t test.

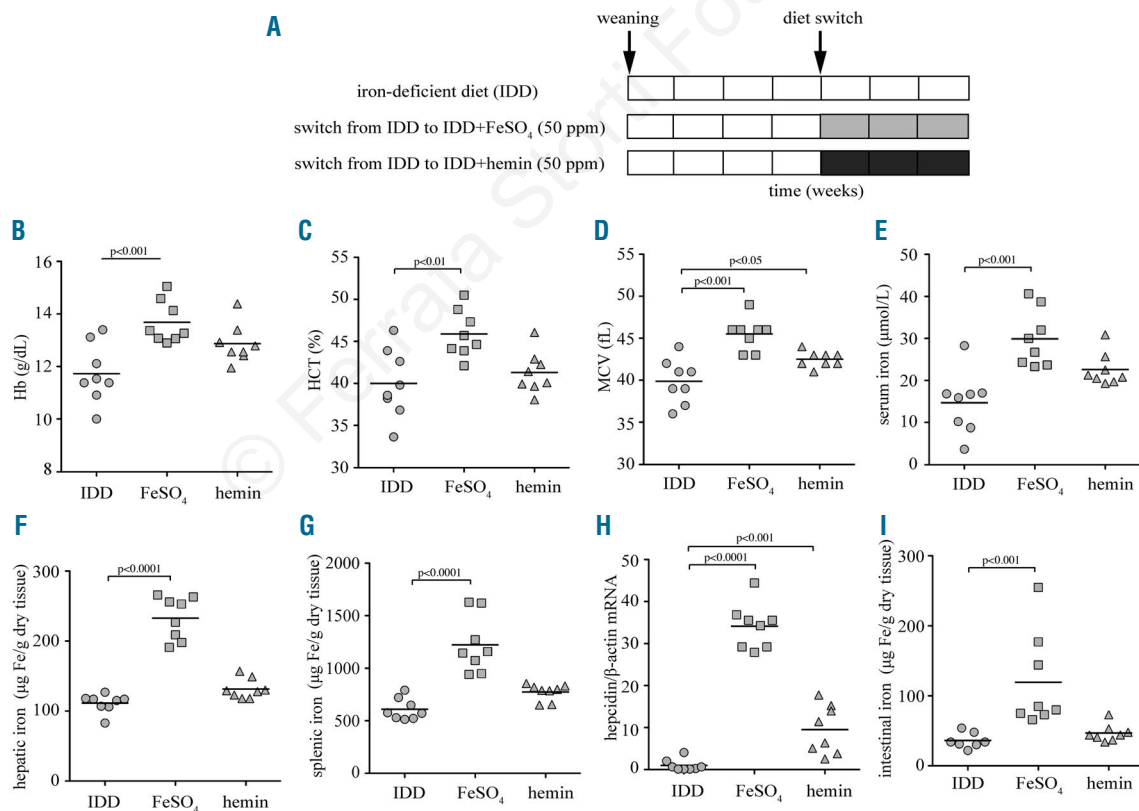
for dietary heme iron assimilation in mice. Thus,  $Hmox1^{Vil-Cre}$  mice do not exhibit any appreciable handicap in heme absorption relative to controls. The capacity of mice to acquire dietary heme iron is extremely low compared to inorganic iron sources. This holds true even under iron deficiency anemia, where iron absorption is stimulated to meet the high erythropoietic needs. The only limitation is in the apical transport of heme across the intestinal epithelium. This defect may be intrinsic to rodents, considering that also rats inefficiently absorb Hb iron.<sup>12</sup> Our findings indicate that the mouse is not an appropriate model organism for intestinal heme transport studies. Given the established bioavailability of heme iron in humans, we speculate that carnivores and omnivores, including humans who became active predators in response to climate changes some 2 million years ago,<sup>13</sup> have evolved more efficient mechanisms for heme absorption than mice, which feed mostly on grains, with some carrion if available.

We provide evidence that enzymatic heme degradation is obligatory for iron utilization in mice, and exclude significant export of intact heme. The potent induction of intestinal HO-1 and upregulation of ferritin in HHD-fed mice indicate a contribution of HO-1 in heme degradation. The persistence of ferritin upregulation, albeit rela-

tively blunted, suggests partial compensation of HO-1 by HO-2.

The apparent functional redundancy of HO-1 and HO-2 in dietary heme processing is unexpected.  $Hmox1^{-/-}$  and  $Hmox2^{-/-}$  mice have distinct phenotypes, suggesting diverse physiological functions of HO-1 and HO-2.  $Hmox1^{-/-}$  mice manifest severe pathological features including anemia, defective erythrophagocytosis, iron overload and inflammation, while  $Hmox2^{-/-}$  mice exhibit sensitivity to hyperoxia.<sup>5</sup> Importantly, HO-1 and HO-2 are expressed in distinct intracellular compartments. HO-1 is evenly distributed in rat enterocytes even following heme absorption, while HO-2 co-localizes extensively with endosomes.<sup>7</sup> These data support the hypothesis that dietary heme is internalized *via* receptor-mediated endocytosis and undergoes degradation within endosomes *via* HO-2, without HO-1 involvement.<sup>7</sup>

This model would provide an attractive mechanism for basal heme absorption. Nonetheless, the heme-mediated induction of intestinal HO-1 and subsequent upregulation of ferritin in control  $Hmox1^{fl/fl}$  mice indicate a physiological function of both HOs in heme iron acquisition. We propose that HO-2 suffices to handle basal heme levels, while HO-1 is required for higher heme loads. Hence, HO-1 may be essential for efficient heme iron assimila-



**Figure 3.** Iron deficiency anemia is efficiently corrected by a diet supplemented with inorganic iron but not heme, due to low luminal heme transport in the gut. 3-week old female C57BL/6 mice ( $n=24$ ) were fed with an IDD for four weeks. One group of the animals ( $n=8$ ) remained on this diet for another three weeks; a second group ( $n=8$ ) was switched to IDD supplemented with 50 ppm FeSO<sub>4</sub> and a third group ( $n=8$ ) was switched to IDD supplemented with 50 ppm hemin. At the end point, blood was prepared and used for hematologic and serum iron analysis, hepatic RNA was prepared for qPCR analysis, and tissues were used for assessment of total (non-heme and heme) iron content. (A) Schematic representation of experimental design. (B) Hb concentration. (C) Hematocrit (HCT). (D) Mean corpuscular volume (MCV). (E) Serum iron. (F) Hepatic iron. (G) Splenic iron. (H) Hepcidin mRNA was induced 34-fold by dietary FeSO<sub>4</sub> and 9.5-fold by dietary hemin supplementation. (I) Intestinal iron levels were increased 3.3-fold following dietary FeSO<sub>4</sub> supplementation and were not significantly affected by dietary hemin. All data are presented as the mean  $\pm$  SEM. Statistical analysis was performed by one-way ANOVA.

tion in carnivores/omnivores, even though it is dispensable in mice, which are poor heme absorbers. It is tempting to speculate that HO-2 may degrade basal heme internalized via receptor-mediated endocytosis; conversely, HO-1 may process excessive heme directly transferred via a transporter. While mice express Hcp1<sup>5</sup>, it is likely that carnivores/omnivores express high-affinity heme transporter(s), which still have to be identified.

An important ramification of our findings is that the clinical use of HO-1-specific inhibitors would not affect basal HO-2-mediated heme iron absorption. This may prevent iron deficiency anemia, a side-effect of heme malabsorption in patients with Crigler-Najar type I syndrome subjected to prolonged therapy with tin-mesoporphyrin.<sup>14</sup> Such non-selective HO substrate analogs have also received wider attention in the context of cancer therapy because HO-1 exerts anti-apoptotic and cytoprotective functions.<sup>15</sup> However, these drugs diminish intestinal heme iron absorption and, in the long run, may promote iron deficiency anemia analogous to that observed in Crigler-Najar type I patients.

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