SMALL INTESTINE

Duodenal mucosal reductase in wild-type and Hfe knockout mice on iron adequate, iron deficient, and iron rich feeding

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Background: Genetic haemochromatosis is a common hereditary iron loading disorder in humans. The disease is associated with loss of function mutations in the HFE gene. This is thought to change iron stores via increased iron absorption.

Aims: In this study we investigated how adaptation of mucosal reductase activity is engaged in this process and how the changes compare with adaptation seen when an iron deficient diet is fed.

Methods: Duodenal mucosal surface reductase was measured with nitroblue tetrazolium in age matched groups of male Hfe knockout mice (Hfe) and wild-type mice fed a purified diet containing normal (iron adequate), high (iron rich), or low (iron deficient) iron concentrations.

Results: Reductase activity increased when mice were fed an iron deficient diet and decreased when they were fed an iron rich diet. Total villus activity, as measured by the average area under the activity curve along the crypt-villus axis, was increased 2.8–2.9-fold by iron deficiency in both genotypes. Approximately half of this difference was attributable to the significantly increased length of the villi in mice on an iron deficient diet ($p < 0.05$). Hfe knockout did not affect villus length but increased mucosal reductase activity near the villus tips. Similar increases (1.3–1.6-fold) were seen on all diets but the increase was significant for iron deficient and iron loaded diets only ($p < 0.05$).

Conclusion: Hfe gene product and dietary iron downregulate villus reductase activity in mice.

Genetic haemochromatosis is a common hereditary defect in humans that is characterised by massive tissue iron loading with associated pathology.¹ In humans, 80% of genetic haemochromatosis is related to the C282Y mutation in the HFE gene although null mutations show similar pathological features.² Accordingly, C282Y insertion and Hfe knockout (KO) both cause iron loading in mice but the latter gives a more severe defect in iron metabolism.³ The latter mice therefore provide an appropriate animal model for elucidating Hfe function in iron metabolism.⁴ Iron concentrations in mammals are normally tightly controlled by regulation of intestinal iron absorption. Haemochromatosis is characterised by a large increase (4–5-fold) in total body iron that is caused by inappropriately high levels of intestinal iron absorption.⁵ Therefore, defective iron absorption regulation is the focus of studies of HFE function.

Duodenal mucosal surface reductase activity is associated with intestinal iron absorption.⁶ Reductase activity can be measured with electron acceptors such as ferricyanide and ferric complexes.^{6–7} The membrane impermeant single electron acceptor nitroblue tetrazolium (NBT) also reacts with this reductase, allowing localisation of activity along the crypt-villus axis.⁸ As current hypotheses on regulation of iron absorption are based on changes associated with enterocyte maturation,^{9–12} it is important to compare the distribution of reductase activity to the distribution of iron uptake along the crypt-villus axis.¹³ It is also important to understand how much of the changes in such duodenal functions are due to changes in villus size, as opposed to specific upregulation of functional genes in enterocytes.

Ferric reductase is reported to increase in haemochromatosis¹⁴ but few data are available for the mouse Hfe KO model. Griffiths and colleagues¹⁵ were unable to detect an increase in mucosal ferric reductase in their Hfe KO mice. Data of Dupic and colleagues¹⁶ however suggest that Hfe KO in mice leads to upregulation of the putative ferric reductase

gene Dcytb although the magnitude of this effect seems to depend on the background mouse strain. In the light of these contradictory findings, we set out to compare mucosal reduction along the duodenal crypt-villus axis in Hfe KO and wild-type (WT) mice fed different concentrations of dietary iron.

METHODS

Mice (129yOla-C57BLy6 mixed background strain) with a 2 kb pgk-neor gene flanked by loxP sites replacing a 2.5 kb BgIII fragment (see Bahram and colleagues⁴ for details) were used as Hfe KO. Heterozygotes were mated and WT and homozygote Hfe KO littermates identified at 4–5 weeks of age. Mice were allocated to one of three diet feeding groups at seven weeks of age and given an iron deficient diet (6 mg/kg Fe), an iron adequate diet (180 mg/kg), or an iron rich diet (20 g/kg added carbonyl iron) ad libitum for five weeks. Details of diet source and composition have been reported previously.⁴ All experiments were carried out under the authority of the UK Home Office.

Haemoglobin, tissue non-haem iron concentrations, and in vivo iron absorption were determined as described previously.¹⁷ NBT was used to determine distribution of reductase activity along the crypt-villus axis. We have shown that both ferrireductase and NBT reductase activity correlates with and is blocked by anti-Dcytb in mouse duodenum.⁸ Therefore, NBT reduction is a measure of the activity of the putative duodenal iron regulated ferrireductase Dcytb. Mice were killed by pentobarbital overdose and the duodenum or whole small intestine was rapidly removed. Duodenal fragments were prepared by opening the duodenum longitudinally along the pancreatic-duodenal junction, rinsed in

Abbreviations: KO, knockout; WT, wild-type; NBT, nitroblue tetrazolium.

Table 1 Tissue iron and haemoglobin concentrations in mice					
Group	Liver (nmol/mg)	Pancreas (nmol/mg)	Spleen (nmol/mg)	Kidney (nmol/mg)	Hb (g/dl)
WT iron deficient	0.50 (0.04)	0.58 (0.08)	1.08 (0.10)	0.65 (0.10)	15.4 (0.4)
WT iron adequate	1.08 (0.09)	0.62 (0.11)	5.32 (0.58)	0.79 (0.09)	15.7 (0.3)
WT iron rich	16.5 (0.6)	0.93 (0.03)	10.44 (0.97)	1.68 (0.11)	16.8 (0.4)
Hfe iron deficient	2.25 (0.22)	1.11 (0.14)	2.46 (0.15)	0.92 (0.13)	16.9 (0.4)
Hfe iron adequate	6.07 (0.53)	1.04 (0.11)	6.72 (0.50)	1.39 (0.14)	17.2 (0.3)
Hfe iron rich	24.3 (1.10)	2.13 (0.30)	12.35 (1.23)	2.30 (0.27)	16.8 (0.4)
ANOVA					
Effect of diet	<0.001	<0.001	<0.001	<0.001	0.254
Hfe KO	<0.001	<0.001	<0.01	<0.001	<0.01

Mouse groups are: WT, wild-type; Hfe, Hfe knockout.
Data are mean (SEM) for n=10–55 mice.
The bottom rows show the p values for the effects of diet or Hfe knockout (KO) by analysis of variance (ANOVA).

Figure 1 Nitroblue tetrazolium (NBT) staining of duodenal villi in wild-type and Hfe knockout (KO) mice. Duodenal slices were incubated for five minutes with NBT 1 mM (see methods), washed in 150 mM NaCl, and imaged.

warm (37°C) 150 mM NaCl, blotted, and gently wiped to remove mucus and food particles. The duodenum adjacent to the pylorus was cut transversally to give a 1–2 mm wide slice. Duodenal fragments were incubated in vitro with 1 mM NBT in 125 mM NaCl, 16 mM HEPES-Na, pH 7.4, 3.5 mM KCl, and 10 mM glucose for five minutes at 37°C, rinsed in 150 mM NaCl, imaged (Polaroid Microcam, dissecting microscope), and scanned with a Biorad densitometer (Molecular Analyst software). Five well orientated representative villi were scanned in each piece of tissue.

Statistics

Data were averaged to give a villus length and optical density profile for each mouse. Data for groups of mice were then averaged in a similar manner. Results are given as mean (SEM). Multiple group comparisons were performed by ANOVA after testing for normal distribution. Two corresponding groups were compared by unpaired Student’s *t* test.

RESULTS

Mice are well able to regulate iron absorption to maintain iron homeostasis in the face of wide variations in dietary iron concentration.¹⁸ The data suggest that this is also true in Hfe KO mice. In contrast, there were significant differences in tissue iron load between WT and Hfe KO mice. Deletion of the

Hfe gene protected mice against iron deficiency when they were fed an iron deficient diet (table 1). On the other hand, different dietary iron levels led to significant differences in tissue iron concentrations. The dietary effects were similar in WT and Hfe KO animals however (table 1). NBT staining due to deposition of the reduced product NBT formazan on duodenal villi was measured. Representative samples from some of the experimental groups are shown in fig 1. Figure 2 shows NBT staining data averaged for 7–13 WT mice on the three different diets. Reductase activity increased on an iron deficient diet and decreased on an iron rich diet. A significant increase in villus length was noticeable in iron deficient diet fed mice (table 2). There was a tendency for villus length to decrease with iron rich diet feeding but this did not reach significance. Iron deficient diet feeding produced large increases in reductase activity. Total villus activity, as measured by the average area under the activity curve, was increased 2.8–2.9-fold by iron deficiency in both genotypes. Approximately half of this difference was attributable to the increased length of the villi (*p*<0.05) (see fig 2, table 2), seen when an iron deficient diet was fed.

Figure 3A–C shows increased activity near the villus tips when Hfe is knocked out. Similar (1.3–1.6-fold) increases were seen on all diets but the increase was significant for iron deficient and iron loaded diets only (*p*<0.05). Hfe KO did not significantly affect villus length (table 2). The increase in

Figure 2 Villus reductase activity in mice. Nitroblue tetrazolium (NBT) stained villi were scanned for NBT formazan. Optical density profiles (in arbitrary units (au)) were obtained by averaging five villi per mouse, then 7–13 mice per experimental group. Profiles show mean (SEM) optical density at the given points along the villus. $p < 0.01$, iron deficient diet versus iron adequate diet (area under the curve); $p < 0.05$, iron rich diet versus iron adequate diet.

Table 2 Villus length in mouse duodenum

Group	Villus length (μm)
WT iron deficient	393 (12) (13)*
WT iron adequate	317 (24) (8)
WT iron rich	286 (12) (8)
Hfe iron deficient	404 (28) (10)*
Hfe iron adequate	331 (15) (8)
Hfe iron rich	313 (17) (7)

Mouse groups are: WT, wild-type; Hfe, Hfe knockout.

Data are mean (SEM).

* $p < 0.05$ versus appropriate iron adequate diet fed mice. ANOVA showed that there was a significant effect of diet ($p < 0.001$) but not of Hfe status ($p = 0.346$).

reductase seen along the lower 0.3 mm of villi was similar when dietary iron was increased or decreased or when the Hfe gene was knocked out, in that a broadly similar proportional increase was observed at all points along the villus. Iron deficient diet feeding had an additional effect of increasing villus length with an associated further increase in total reductase activity.

DISCUSSION

Hfe KO mice have been validated previously as a model of genetic haemochromatosis.^{4 15 19} Increased duodenal iron uptake and absorption of dietary iron is strongly implicated as the main cause for increased iron storage in Hfe KO mice.^{4 15 20} Previous studies have provided contradictory findings as to whether mucosal surface ferric reductase activity is increased when HFE gene function is compromised.^{14 15} Dupic and colleagues¹⁶ showed that expression of the putative ferric reductase gene *Dcytb* is increased in Hfe KO mice but their study did not investigate how these changes are distributed along the villus.

The present work is the first attempt to quantify changes in villus reductase along the duodenal crypt-villus axis. We have shown that the maximum activity of reductase occurs near the tip—that is, further from the crypt than the maximum level of iron uptake¹³ (Debnam E *et al*, unpublished observations in mice). The difference in distribution of the two activities reflects a difference in distribution of the proteins DMT1 and *Dcytb*.^{8 21} This distribution may make sense considering that trivalent dietary iron will pass through the unstirred water

Figure 3 Effect of Hfe knockout (KO) on villus reductase activity. (A) Iron adequate diet, (B) iron deficient diet, and (C) iron rich diet. Optical density (in arbitrary units (au)) was significantly raised ($p < 0.05$) in the region 0.29–0.37 mm of the iron deficient diet Hfe KO villi compared with wild-type mice and between 0.25–0.30 mm in iron rich diet Hfe KO villi versus wild-type mice. *Significantly increased ($p < 0.05$).

layer at the tip where it is reduced to Fe^{2+} and can be taken up by the DMT-1 carrier function further down the villus.

The increase in reductase activity begins low on the villus in iron deficient diet fed mice, with a significant increase detected within 50 μm of the villus base. The decrease in activity seen with an iron rich diet also extends towards the villus base. This is consistent with the prevailing hypothesis regarding control of iron absorption by iron stores—that is, that changes in iron requirements are detected in the crypt, resulting in maturing villus enterocytes that display altered gene expression. The consequential increased activity of iron absorption genes causes increased iron absorption after a lag time necessary to repopulate the villus with these adapted enterocytes.^{9 12}

Hfe KO did not alter the pattern of change seen in iron deficient mice. Similarly, changes in dietary iron concentration did not alter the effect of Hfe KO, although the effect of Hfe KO on mucosal reductase only achieved statistical significance in mice fed iron deficient or iron rich diets. The study of Griffiths and colleagues¹⁵ failed to find a significant increase in reductase activity in Hfe KO mice, consistent with our findings in mice fed an iron adequate diet. The fact that only small increases in reductase activity were seen (less than twofold) is likely due to the use of the B6 background strain in our study. This background strain has been shown to give a smaller effect of Hfe KO than other strains.^{16 22} The use of this strain has the advantage however that massive changes in iron stores are avoided, thus they can be more useful for mechanistic studies of Hfe function.

Iron deficient diet feeding was found to increase villus length, as has been shown in rats.¹³ Feeding an iron rich diet caused a small non-significant reduction in villus length. Oates and colleagues²³ reported that iron rich diets such as we used can cause intestinal hypertrophy in rats. We did not observe this. However, our study was of limited duration (five weeks of diet feeding as opposed to 8–10). We found that the increased villus length of iron deficiency was associated with much of the increase in mucosal reductase activity in iron deficient mice. Changes in villus length were not seen to be caused by Hfe KO although iron deficient feeding induced changes to the same extent in Hfe KO mice as in WT mice. This observation suggests that selective effects on gene expression are caused by Hfe gene deletion and these effects differ, at least in part, from the effects of iron deficient diet feeding. Furthermore, the Hfe gene is not required for the adaptive changes in reductase activity and villus length seen when an iron deficient diet is fed.

CONCLUSION

The Hfe gene product and dietary iron downregulate villus reductase activity in mice. Adaptation in mucosal reductase activity in iron deficient diet fed mice involves increased villus length as well as increases in cellular reductase activity and these changes do not require the Hfe gene.

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