Duodenal Reductase Activity and Spleen Iron Stores Are Reduced and Erythropoiesis Is Abnormal in Dcytb Knockout Mice Exposed to Hypoxic Conditions\textsuperscript{1–3}

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Abstract

Duodenal cytochrome b (Dcytb, Cybrd1) is a ferric reductase localized in the duodenum that is highly upregulated in circumstances of increased iron absorption. To address the contribution of Dcytb to total duodenal ferric reductase activity as well as its wider role in iron absorption, we first measured duodenal ferric reductase activity in wild-type (WT) and Dcytb knockout (Dcytb\textsuperscript{−/−}) mice under 3 conditions known to induce gut ferric reductase: dietary iron deficiency, hypoxia, and pregnancy. Dcytb\textsuperscript{−/−} and WT mice were randomly assigned to control (iron deficiency experiment, 48 mg/kg dietary iron; hypoxia experiment, normal atmospheric pressure; pregnancy experiment, nonpregnant animals) or treatment (iron deficiency experiment, 2–3 mg/kg dietary iron; hypoxia experiment, 53.3 kPa pressure; pregnancy experiment, d 20 of pregnancy) groups and duodenal reductase activity measured. We found no induction of ferric reductase activity in Dcytb\textsuperscript{−/−} mice under any of these conditions, indicating there are no other inducible ferric reductases present in the duodenum. To test whether Dcytb was required for iron absorption in conditions with increased erythropoietic demand, we also measured tissue nonheme iron levels and hematological indices in WT and Dcytb\textsuperscript{−/−} mice exposed to hypoxia. There was no evidence of gross alterations in iron absorption, hemoglobin, or total liver nonheme iron in Dcytb\textsuperscript{−/−} mice exposed to hypoxia compared with WT mice. However, spleen nonheme iron was significantly less (6.7 ± 1.0 vs. 12.7 ± 0.9 nmol · mg tissue\textsuperscript{−1}; \textit{P} < 0.01, \textit{n} = 7–8) in hypoxic Dcytb\textsuperscript{−/−} compared with hypoxic WT mice and there was evidence of impaired reticulocyte hemoglobinization with a lower reticulocyte mean corpuscular hemoglobin (276 ± 1 vs. 283 ± 2 g · L\textsuperscript{−1}; \textit{P} < 0.05, \textit{n} = 7–8) in normoxic Dcytb\textsuperscript{−/−} compared with normoxic WT mice. We therefore conclude that DCYTB is the primary iron-regulated duodenal ferric reductase in the gut and that Dcytb is necessary for optimal iron metabolism.


Introduction

Iron is indispensable in most biological systems, because it is used by many key enzymes essential for life and both iron deficiency and iron overload have deleterious consequences. Iron absorption is mediated by mature duodenal enterocytes and first involves the reduction of dietary ferric iron to ferrous iron. The importance of ferric iron reduction in dietary nonheme iron uptake has been known for a long time. Iron absorption from ferric complexes is abolished by adding ferrozine or ceruloplasmin into proximal intestine segments of iron-deficient rats, indirectly suggesting the presence of ferric reduction prior to transport (1,2). A subsequent study showed that intestinal iron-reducing activity parallels the changes in duodenal iron uptake rate in hypoxic or iron-deficient mice (3). With the discovery of the critical role of divalent metal transporter 1 in iron absorption, it was apparent that dietary ferric iron must be reduced to the ferrous form to be absorbed from the lumen into the mucosa (4,5).

Subsequently, an iron-regulated duodenal ferric reductase, Dcytb (Cybrd1, duodenal cytochrome b), was identified in a cDNA screen using hypotransferrinemic mice (6). Dcytb is very highly expressed in duodenum and the protein is localized at the apical brush border membrane of mature enterocytes. Dcytb expressed in Xenopus oocytes or human cells demonstrated ferric reductase activity (6). Numerous studies have reported

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increased Dcytb expression in iron deficiency, pregnancy, hypotransferrinemia, hemolytic anemia, or hemochromatosis in humans, rats, or mice (6–15). In many of these studies, Dcytb was the most highly iron-regulated mRNA among iron absorption genes. Furthermore, it was recently shown that Dcytb expression in Caco-2 cells stimulates iron uptake, the magnitude of which increased further after cotransfection with divalent metal transporter 1 (16).

In 2005, Gunshin et al. (17) generated a Dcytb knockout (Dcytb−/−) mouse in which Dcytb was deleted in all tissues. They observed no significant change in iron stores when mice were fed purified diets containing either 380 or 2–3 mg/kg iron (17). The lack of effect of loss of Dcytb on iron status in normal mice suggested that other ferric reductases [e.g. six-transmembrane epithelial antigen of prostate 1-4 (STEAP1-4)] might be able to compensate for Dcytb loss or that ferric reductase was not necessary for normal rates of iron absorption (18). The latter could occur because the diet contained sufficient ferrous iron or contained reducing agents that produced sufficient ferrous iron in the duodenal lumen. Therefore, the present study investigated ferric reductase activity in Dcytb−/− mice under various circumstances of enhanced iron absorption to determine whether other iron-regulated ferric reductases were present in duodenum.

Another point of interest is whether Dcytb is important only in circumstances of enhanced iron absorption or in tissues other than duodenum. We therefore also investigated tissue iron levels in mice subjected to hypoxia, a driver for increased erythropoiesis.

Materials and Methods

**Animal care.** Dcytb−/− mice have been previously described (17). Dcytb−/− mice on a homogenous 129S6/SvEvTac background were transported to King’s College London then mated with 129S2/Sv mice (Harlan). Dcytb−/− heterozygotes were identified (Supplemental Methods) and mated to produce wild-type (WT) and Dcytb−/− males and females that were then mated to produce WT and knockout (Dcytb−/−, knockout) mice for experimental study. Although the background strain was a 129S6 strain deriving from LC Stevens at the Jackson Laboratory in 1953 [information provided by Harlan], as for the original knockout mouse work of Gunshin et al. (17), it should be noted that there is reported to be variability between such 129 strains (19). All mouse procedures were approved by the UK Home Office.

**Diets.** Mice were maintained with a standard, laboratory mouse, nonpurified diet containing 159 mg/kg iron [Diet RM1, SDS; manufacturer’s composition data are as follows: 14.4% protein, 2.7% oil, 17.05% fiber, 6.0% ash, 45% starches, 4.05% sugars, 10% water (20)], purified iron-deficient diet (TD 80393, 2–3 mg/kg iron, Harlan Teklad), or the same diet containing 48 mg/kg additional iron. Treated groups were exposed to iron-deficient dietary stress by feeding purified diet without added iron for 2, 3, or 6 wk. Mice were killed, the duodenum was removed for ferric reductase or NBT assay, and/or tissue samples were taken for nonheme iron assays or blood counts as described above.

**Expt. 1: dietary iron deficiency.** Experimental groups of WT (A,B) and Dcytb−/− (C,D) mice were maintained with nonpurified diet until 4–7 wk of age (depending on duration of treatment, longer treatments started with younger mice). Mice were randomly assigned to control (A,C) or treatment groups (B,D). Control groups were fed a purified diet with 48 mg/kg added iron. Treated groups were exposed to iron-deficient dietary stress by feeding purified diet without added iron for 2, 3, or 6 wk. Mice were killed, the duodenum was removed for ferric reductase or NBT assay, and/or tissue samples were taken for nonheme iron assays or blood counts as described above.

**Expt. 2: hypoxic treatment.** Experimental groups of WT (E,F) and Dcytb−/− (G,H) mice were fed nonpurified diet until 6–7 wk of age. Mice were then either left at normal pressure (groups E,G) or exposed to hypoxia in a hypobaric chamber set at 33.3 kPa (0.5 × atmospheric pressure) (groups E,H) for 3 d as previously described (20). Mice were fed nonpurified diet during hypoxic or control treatment. Mice were killed, the duodenum was removed for ferric reductase or NBT assay, and/or tissue samples were taken for nonheme iron assays or blood counts as described above.

**Expt. 3: effect of pregnancy on ferric reductase.** Female WT and Dcytb−/− mice (13 wk old) were mated and then killed on d 20 of gestation. Mice were fed nonpurified diet throughout. The duodenum was removed and ferric reductase activity measured as described above.

**Statistical analysis methods.** Values are expressed as means ± SEM. Two-way ANOVA was used to test significance of treatment effects (feeding low-iron diet in Expt. 1, hypoxia in Expt. 2) and genotype. ANOVA were performed with SPSS version 17 (IBM) to test differences between individual groups. Where significant interactions were found, post hoc t tests with the Bonferroni correction were performed to identify significant differences between groups. P < 0.05 was accepted as significant. In Expt. 3, the effect of pregnancy was tested by t test.

Results

**Inducible ferric reductase.** DCYT B protein levels were higher in duodenum of low-iron WT mice compared with control WT
mice but was absent from low-iron Dcytb+/− mice fed diet for 2 or 6 wk and from hypoxic Dcytb+/− mice (Supplemental Fig. 1). Ferric reductase levels in control Dcytb+/− mice were not significantly different from those of control WT mice (Fig. 1A). Nevertheless, low-iron WT mice had greater reductase activity compared with control WT mice (P = 0.003), whereas no effect was observed in low-iron Dcytb+/− mice. Low-iron WT mice had higher levels of reductase activity than low-iron Dcytb+/− mice (P = 0.003). Hypoxic treatment had a similar response. Two-way ANOVA showed effects of genotype (P = 0.0028), hypoxic treatment (P = 0.0049), and a significant interaction (P < 0.05). There was greater ferric reductase activity in hypoxic WT mice compared with hypoxic Dcytb+/− mice (P = 0.02). We found pregnant Dcytb+/− mice had lower reductase activity than pregnant WT mice (Fig. 1C) (P = 0.002).

In accordance with lower ferric reductase activity in the Dcytb+/− mice in a hypoxic environment or after low-iron diet feeding noted above (Fig. 1A,B), apical staining for reductase activity was lower in the Dcytb+/− duodenum in both treatments (Fig. 2, note abundance of well-stained villi in treated WT duodenum, while WT control or Dcytb+/− duodenum had fewer stained villi; also see Fig. 3). Quantification of staining density is shown in Figure 3, where 2-way ANOVA revealed an effect of genotype (P = 0.0015) and hypoxia (P = 0.0154) with no significant interaction (P = 0.13). Overall, these data imply that there is no redundancy in duodenal ferric reductase enzymes, at least under conditions of increased demand for iron.

**Hematology and tissue iron concentrations.** The liver nonheme iron concentration did not differ in WT and Dcytb+/− mice when fed standard laboratory nonpurified diet, even under hypoxic treatment conditions (Fig. 4A). The liver nonheme iron concentration was greater in hypoxic mice of both genotypes compared with normal controls. Two-way ANOVA revealed an effect of hypoxic treatment (P < 0.001), but no significant effect of genotype or interaction between factors was found. Spleen nonheme iron was affected by genotype (Fig. 4B). Two-way ANOVA revealed an effect of genotype (P = 0.01) and an interaction between genotype and hypoxia (P = 0.004) on spleen nonheme iron concentrations. We observed a significantly lower spleen nonheme iron concentration in hypoxic Dcytb+/− mice compared with hypoxic WT mice (Fig. 4B). No significant effects of hypoxia or genotype on serum iron were seen in mice fed 159 mg Fe/kg standard laboratory nonpurified diet with or without hypoxia (data not shown). We measured RBC counts, hemoglobin, and hematocrit in these mice to examine whether reduced spleen nonheme iron concentrations were associated with hematological changes. As expected, hypoxic treatment significantly elevated all variables in hypoxic WT and hypoxic Dcytb+/− mice compared with normoxic controls, but RBC count and hemoglobin did not differ between Dcytb+/− and WT mice (data not shown). Reticulocyte indices, however, showed significant genotype effects. In particular, reticulocyte mean corpuscular hemoglobin was significantly lower in normoxic control Dcytb+/− mice compared with normoxic control WT mice (Fig. 5). Two-way ANOVA revealed effects of genotype (P = 0.003) and hypoxic treatment (P = 0.006) and an interaction (P = 0.015).

**Expression of STEAP family reductases.** We found that overall expression levels of Steap 1, 2, 3, and 4 mRNA in duodenum were low compared with Dcytb in WT mice (Supplemental Fig. 2). We also found no difference in Steap 1, 2, 3, and 4 mRNA levels between the genotypes (Supplemental Figs. 2, 3). To test whether duodenal STEAP reductases might be iron regulated, we interrogated the publicly available geo profile datasets (24) to assess mRNA signal levels. In accordance with our data above, we noted that Steap 1, 2, and 3 mRNA signals were all much lower than Dcytb in mouse duodenum (GDS521, 522, and 524). We also examined array work carried out by Collins et al. (14) comparing duodenal gene expression in rats fed a low- or high-iron diet at various age points (GDS1054 and GDS1055). Their data showed no significant overall differences in Steap 1, 2, 3, and 4 signals in response to dietary iron. If anything, feeding a low-iron diet slightly reduced Steap 1 and 2 signal levels compared with feeding a high-iron diet. On the other hand, the Dcytb signal was highly inducible by low levels of iron.

**FIGURE 1** Duodenal ferric reductase activity in mice with increased iron demand due to low-iron diet feeding (A), hypoxia (B), or pregnancy (C). Mice were fed a purified diet containing 48 mg Fe/kg [control: WT (n = 9) and Dcytb+/− (n = 8)] or 2–3 mg Fe/kg [treated: WT (n = 10), Dcytb+/− (n = 9)] for 3 wk. (B) Mice (7–13 wk old) were fed a normal nonpurified diet containing 159 mg Fe/kg at normal atmospheric pressure [control: WT (n = 5), Dcytb+/− (n = 5)] or in a hypoxic chamber at 53.3 kPa for 3 d [treated: WT (n = 5), Dcytb+/− (n = 6)]. (C) WT and Dcytb+/− pregnant female mice at 20 d of gestation [WT (n = 4), Dcytb+/− (n = 4)]. Values are mean ± SEM. Comparisons shown in the graphs were made using post hoc t tests with the Bonferroni correction: means without a common letter differ, P < 0.05. KO, knockout; WT, wild type.
dietary iron. In addition, it is noteworthy that a list of genes altered by more than 2-fold in iron-deficient mouse duodenum published by Taylor et al. (25) showed that no Steaps were altered above this threshold, whereas Dcytb was increased 5.9-fold.

**Mucosal iron uptake was not affected by loss of Dcytb.** No significant effects of Dcytb knockout on mucosal iron uptake (measured in tied-off segments of duodenum, in vivo) were observed (Supplemental Fig. 4). In separate experiments, no effect of Dcytb knockout on mucosal iron uptake was found in mice fed either a purified or iron-deficient diet (data not shown).

**Discussion**

In WT mice, DCYTB protein was increased in hypoxic and iron-deficient duodenum and this was associated with an increase in duodenal ferric reductase activity. There was no detectable duodenal DCYTB protein in Dcytb$^{2/2}$ mice with or without treatment, and ferric reductase activity was not stimulated by iron-deficient dietary stress, hypoxia, or pregnancy. STEAP3 is expressed in many tissues, including spleen, duodenum, bone marrow, and liver, and plays a key role in ferric reduction in erythroid cells (18). Hence, it is possible that STEAP3 and/or other STEAP family members could be involved in ferric reduction in duodenum. We therefore tested whether loss of Dcytb may trigger increased duodenal expression of Steap 1-4 by comparing duodenal mRNA expression levels of WT and Dcytb$^{2/2}$ mice fed a purified, 48-mg Fe/kg diet. We found that overall duodenal expression of Steap family reductases at the mRNA level was low in mouse duodenum and was not increased in Dcytb$^{2/2}$ mice. In addition, data by Collins et al. (14) showed that these reductases were not induced by low-iron diet feeding in rat duodenum. These findings, combined with our reductase data, suggest that Steap 1, 2, 3, and 4 are not iron regulated and do not compensate the loss of Dcytb and therefore are unlikely to be major duodenal ferric reductases. A low level of ferric reductase activity was found to be present in control Dcytb$^{2/2}$ mice; therefore, it is possible that STEAP proteins or other non-iron-regulated reductases contribute to this basal activity. It also remains possible that reducing agents generated by food digestion and/or ascorbic acid present in mouse mucosa may contribute to this basal level of reductase activity. Regardless, we found no evidence that other iron-regulated ferric reductases contributed to mucosal surface ferric reductase activity in duodenum of mice with enhanced iron absorption rates due to hypoxia, low-iron diet feeding, or pregnancy. The evidence suggests that duodenal mucosal reductase activity in mice is in considerable excess over iron absorption rates [see corrigendum to ref (3)], thus allowing some loss of activity without affecting iron absorption. Note, however, that reduction rates measured in the presence of ferrozine may be higher than rates in the absence of this Fe(II) chelator, thus overestimating reductase activity.

The original report on Dcytb$^{2/2}$ mice found little or no difference in iron status and metabolism between the knockout and WT mice (17). In that study, neither feeding a purified diet with 380 mg/kg iron nor the same diet containing 2-3 mg/kg iron for as long as 12 wk showed any difference between the 2 genotypes in terms of liver or spleen iron concentration. We used
a 129 background strain similar to that used by Gunshin et al. (17) (mixed 129S6SvEvTac/129S2Sv vs. homogenous 129S6SvEvTac) and in preliminary studies with a similar purified diet containing 48 mg Fe/kg, we found no consistent effects of $Dcytb^{−/−}$ on tissue iron levels or hematological indices (data not shown). Feeding mice a low-iron diet stresses iron metabolism, but because there is very little iron in the diet, iron absorption is unlikely to be a major factor in determining tissue iron levels. We therefore set out to study a stress to iron metabolism in other ways that did not require alteration of dietary iron levels. Hypoxia is one such stress. Liver and spleen nonheme iron levels were measured as markers of iron stores in mice exposed to hypoxia. Compared with WT mice, $Dcytb^{−/−}$ mice had no significant reduction in spleen nonheme iron when fed a standard laboratory nonpurified diet (Fig. 4B). Hypoxic treatment increased spleen iron in WT mice, but $Dcytb^{−/−}$ mice maintained a lower spleen nonheme iron concentration. This might indicate that compared with WT mice, the $Dcytb^{−/−}$ mice started utilizing splenic iron more readily, perhaps to compensate for a reduced iron supply when fed a standard laboratory nonpurified diet. In addition to a reduced spleen nonheme iron concentration, $Dcytb^{−/−}$ mice had significantly reduced reticulocyte hemoglobin ($P = 0.003$). $DCTYB$ protein is highly expressed in mature RBC of scorbatic species such as humans and guinea pigs but not in mice or rats (26). In mice, $DCTYB$ protein was found to be highly expressed in developing erythroblasts and early reticulocytes, with expression decreasing thereafter (26). The function of $DCTYB$ in mature RBC of scorbatic species [i.e., species such as humans or guinea pigs that require dietary ascorbic acid (vitamin C), because they cannot synthesize it] or in developing erythroblasts in mice is not yet known; however, our data would suggest that $Dcytb$ loss may impair RBC hemoglobin synthesis or maturation.

Tissue iron concentrations reflect the balance between iron absorption and body iron losses; furthermore, food and gastrointestinal secretions may contain reducing agents that negate the effects of loss of $Dcytb$ in knockout mice. We therefore investigated in vivo iron absorption from a ferric complex, FeNTA$_2$, but could not demonstrate a major effect of $Dcytb^{−/−}$, confirming that any effect of $Dcytb$ loss on iron absorption in 129 strain mice is minor. Note that mice strains vary in their tissue iron concentrations and responses to variation in dietary iron and loss of iron metabolism genes (9,27).

In summary, we conclude that $DCTYB$ is the only major iron-regulated reductase in duodenal mucosa and that loss of $DCTYB$ leads to significantly lower spleen iron and hemoglobin synthesis in developing reticulocytes under specific conditions of enhanced erythropoiesis. However, these effects did not result in significant alterations in RBC numbers or hemoglobin concentrations. We therefore conclude that under certain conditions, $Dcytb$ is necessary for optimal iron metabolism.

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Literature Cited


