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Iron and cadmium uptake by duodenum of hypotransferrinaemic mice

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Abstract

Absorption from food is an important route for entry of the toxic metal, cadmium, into the body. Both cadmium and iron are believed to be taken up by duodenal enterocytes via the iron regulated, proton-coupled transporter, DMT1. This means that cadmium uptake could be enhanced in conditions where iron absorption is increased. We measured pH dependent uptake of ^{109}Cd and ^{59}Fe by duodenum from mice with an *in vitro* method. Mice with experimental (hypoxia, iron deficiency) or hereditary (hypotransferrinaemia) increased iron absorption were studied. All three groups of mice showed increased ^{59}Fe uptake ($p < 0.05$) compared to their respective controls. Hypotransferrinaemic and iron deficient mice exhibited an increase in ^{109}Cd uptake ($p < 0.05$). Cadmium uptake was not, however, increased by lowering the medium pH from 7.4 to 6. In contrast, ^{59}Fe uptake (from $^{59}FeNTA_2$) and ferric reductase activity was increased by lowering medium pH in control and iron deficient mice ($p < 0.05$). The data show that duodenal cadmium uptake can be increased by hereditary iron overload conditions. The uptake is not, however, altered by lowering medium pH suggesting that DMT1-independent uptake pathways may operate.

Introduction

Toxic metals such as cadmium (Cd) remain a significant environmental health hazard for humans (Friberg *et al.* 1986). The anthropogenic release of cadmium to the environment and consequently its serious health threat has been recognised for more than a century. Exposure of humans to Cd leads to toxicity to a number of tissues/organs, including liver, kidney, lung, bone and reproductive organs and the immune system (Zalups & Ahmad 2003). Exposure and absorption of cadmium in humans generally occurs via one of two main routes: inhalation or ingestion (Zalups & Ahmad 2003). Absorption of cadmium from ingested sources occurs via the gastrointestinal tract (Bressler *et al.* 2004). Early animal studies showed that maximal absorption of cadmium (Sorensen *et al.* 1993; Andersen *et al.* 1994) occurs in the upper small intestine by a carrier-mediated process (Foulkes 1979). The uptake of cadmium and its subsequent distribution to target tissues is dependent on the chemical form of cadmium presented to the intestinal epithelium (Zalups & Ahmad 2003).

Recent work has greatly increased understanding of the absorptive mechanism for iron (Miret *et al.* 2003). Iron is believed to be reduced by mucosal surface reductases such as Dcytb (Cybrd1), then transported into enterocytes by the divalent metal ion transporter DMT1 (Slc11a2). In enterocytes, DMT1 has been shown to be present in the lumenal or brush border membrane, where it is believed to serve as the major pathway for the absorption of dietary non-haem iron (Gunshin *et al.* 1997, 2005; Tandy *et al.* 2000). Iron absorption is enhanced when iron requirements

are increased as occurs in, for example, iron deficiency or hypoxia. Genetic diseases can also be associated with increased iron absorption, despite iron overload being present in, for example, hypotransferrinaemia. Duodenal enterocyte DMT1 levels are increased not only by iron deficiency (Gunshin *et al*. 1997), but also by other alterations in iron metabolism associated with increased iron absorption including hypotransferrinaemia (Canonne-Hergaux *et al*. 2001; Latunde-Dada *et al*. 2004).

Iron deficiency increases the gastrointestinal absorption of cadmium (reviewed in Zalups & Ahmad 2003), which is believed to be taken up by enterocytes via several pathways, the most important of which is likely to be via the iron-regulated metal transporter DMT1 (Bressler *et al*. 2004). Thus cadmium uptake by the enterocyte-like cell line, Caco2, is increased by lowering the pH, a characteristic of metal transport by DMT1 (Elisma & Jumarie 2001; Bannon *et al*. 2003). Furthermore, DMT1 expressed in *Xenopus* oocytes can transport not only Fe, but also Cd and other metal ions in a pH dependent fashion (Gunshin *et al*. 1997; Okubo *et al*. 2003). Disruption of DMT1 in Caco2-cells reduces cadmium uptake (Bannon *et al*. 2003). Moreover, Park *et al*. showed that increased DMT1 in iron deficient rats is closely associated with increased cadmium absorption (Park *et al*. 2002). Taken together these findings imply that intestinal cadmium uptake could be increased in a variety of disorders characterised by increased iron absorption (and therefore DMT1), including hereditary iron overload conditions. Interestingly, a study has shown increased blood cadmium levels in phlebotomised haemochromatosis patients (Akesson *et al*. 2000), while others have shown increased blood cadmium (Barany *et al*. 2005), or cadmium absorption (Flanagan *et al*. 1978), in human iron deficiency. Previous studies in animals have focussed on the effect of dietary iron levels on cadmium absorption (Ragan 1977; Flanagan *et al*. 1978; Park *et al*. 2002). The only study of cadmium absorption in animals with iron metabolism altered by non-dietary means being that of Flanagan *et al*. (1980), who looked at mice made iron deficient by bleeding.

The purpose of the present study was to investigate uptake of iron and Cd by duodenal fragments from mice with altered iron metabolism, namely, hypotransferrinaemic mice and those rendered hypoxic or iron deficient, conditions known to enhance intestinal absorption of iron. To gain further insight into the possible role of DMT1, we also measured pH-dependence of Fe and Cd^{2+} uptake.

Materials and methods

Animals

Male, CD1 strain, 6–8 week old (25–30) mice were obtained from Charles Rivers and maintained in the Comparative Biology Unit at King's College School of Medicine and Dentistry, Denmark Hill. The genetically hypotransferrinaemic mice originated from balbc/j background and were maintained in a closed colony. $Trf^{hpx/hpx}$ (homozygous mutant mice) or $Trf^{+/-}$ (normal littermates – a mixed group of heterozygotes and wild-type, as heterozygote mice have normal iron absorption rates (Raja *et al*. 1994)) were studied at 2–6 month of age. $Trf^{hpx/hpx}$ are phenotypically distinguishable at birth by their pale appearance and were maintained by weekly injections of mouse serum (150 μg to 1 mg transferrin) as described by Simpson *et al*. (1991).

Hypoxic and hypotransferrinaemic mice were fed on a commercial pelleted rodent diet, except on the day of experiment, while water was provided ad lib. Iron deficiency was induced by feeding CD1 mice an iron-free synthetic diet for 3 weeks from weaning. Controls were fed the same diet supplemented with iron (62 mg/kg) (see Simpson 1996 for details). Iron deficiency was confirmed by measuring liver non-haem iron as described by Simpson and Peters (1990). Animal experiments were conducted under the authority of licences issued by the UK Home Office.

To induce hypoxia, caged animals were placed in a steel chamber (80 cm diameter × 48 cm height) the pressure of which was lowered to 53.3 kPa (approximately 0.5 atm), and maintained at this pressure for 72 h. Food and water was freely provided during this period. The decompression corresponds to a simulated altitude of 15,000–16,000 feet above sea level (Frisancho 1975). Airflow through the chamber was fast enough to maintain CO_2 levels below 0.2% (determined using an infrared CO_2 analyser) (Raja *et al*. 1987b).

Reagents

Chemicals were purchased from BDH-Merck or Sigma Chemical Company Ltd. (Poole, Dorset). $^{109}CdCl_2$ and ^{57}Co-cyanocobalamin was purchased from Amersham International. $^{59}FeCl_3$ was purchased from NEN-Dupont, Stevenage, Herts, UK (specific activity 0.19–2.78 TBq/g).

In vitro *uptake of ^{59}Fe or ^{109}Cd*

An *in vitro* technique, described by Raja *et al.* (1987a) was used for determining rates of iron or cadmium uptake by duodenal fragments. Mice were anaesthetised with halothane and killed by cervical dislocation. Pieces of duodenum were removed and cleared of any adjoining tissue. The duodenum was then cut length wise and sectioned into fragments (2–6 mg wet weight). These were rinsed in warm (37 °C) oxygenated medium (125 mM NaCl, 3.5 mM KCl, 10 mM D-glucose, 8 mM MES, 8 mM Hepes, pH adjusted to 6.0 or 7.4 with NaOH/HCl as required) and incubated in a similar buffer containing 100 or 250 μM metal as either $FeNTA_2$, $FeSO_4$, Fe ascorbate (1:20) or $CdCl_2$ together with Co-cyanocobalamin (5 nM). The latter was used as an extracellular fluid (ECF) marker for adherent medium and non-specific permeation of the metals. To these solutions were added tracer doses of $^{59}FeCl_3$ or ^{109}Cd and ^{57}Co-cyanocobalamin. The pH of the medium was checked prior to incubations. Incubation was for 5 min after which time fragments were removed, blotted and rinsed in 1 ml of ice-cold buffer. After reblotting and weighing, the amount of ^{57}Co and ^{59}Fe or ^{109}Cd in the tissue samples and in an aliquot of the medium was measured by twin channel gamma counting (LKB Wallac 1280, Finland).

Iron reduction measurements were performed by including 1mM ferrozine in $FeNTA_2$-containing medium and removing aliquots for spectrophotometric determination of Fe^{2+} at 562 nm, as described previously (Raja *et al.* 1992).

Statistical analysis

The data are presented as mean ± SEM for (n) determinations. The Student's t-test was used (paired or unpaired as appropriate) where comparison was between one control and one test group. When appropriate, multiple groups were compared by ANOVA with the SPSS program, followed by 't' testing to identify individual group differences.

Results

Uptake of Fe and Cd was measured with duodenum from hypotransferrinaemic mice which have a genetic defect in iron metabolism leading to greatly enhanced rates of iron absorption. We found higher uptake of ferrous iron at pH 6.0 ($FeSO_4$, Fe ascorbate) than was seen with the ferric complex $FeNTA_2$ (Figure 1). This could be due to the fact that Fe in $FeNTA_2$ is chelated, thus decreasing its availability for transport, or alternatively, it could be that the ferric iron in $FeNTA_2$ has to be reduced by ferric reductases prior to transport, thus slowing the overall uptake rate. Clear increases in iron uptake were seen at both pH 6 and 7.4 in hypotransferrinaemic mice. Cd uptake paralleled the uptake rate for ferrous iron and was significantly increased in hypotransferrinaemic mice at pH 6.0. We also found evidence for increased cadmium uptake in hypotransferrinaemia at pH 7.4, however, this did not reach significance at the 0.05 level ($p = 0.052$).

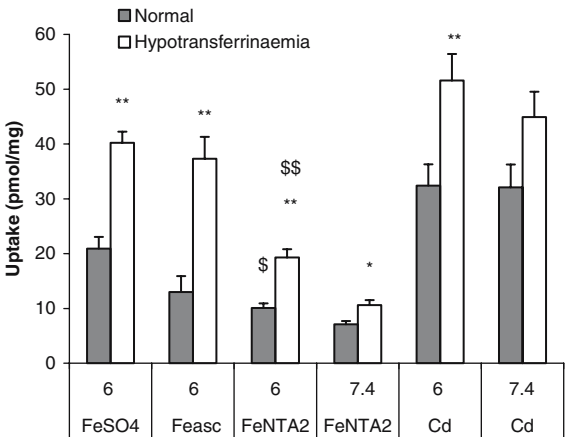

Figure 1. Iron and cadmium uptake by hypotransferrinaemic mice. Normal (balbc/j mice of wild-type or heterozygous genotype) and mutant ($trf^{hpx/hpx}$) mouse duodenum was incubated at pH 6 or 7.4 with 100 μM $FeSO_4$, $FeNTA_2$, Fe ascorbate or $CdCl_2$. 5–12 determinations were performed in each experimental group. Uptake of all metal complexes at pH 6 was increased by hypotransferrinaemia (**$p < 0.01$) as was uptake of iron from $FeNTA_2$ at pH 7.4 (*$p < 0.02$). Lowering the pH significantly increased uptake of $FeNTA_2$ in normal ($$p < 0.02$) and hypotransferrinaemic mice ($$$p < 0.001$).

There was also a clear increase in iron uptake from $FeNTA_2$ on lowering medium pH from 7.4 to 6.0 in both normal and hypotransferrinaemic mice, whereas no such effect was seen with Cd in normal mice. Cd uptake tended to be higher at pH 6 than at 7.4 in hypotransferrinaemic mice, but the effect did not approach statistical significance ($p = 0.33$). It was not possible to perform pH dependency studies with uncomplexed ferrous iron due to the instability of this ion in oxygenated buffers (Dorey *et al*. 1993).

Figure 2 displays the uptake of cadmium and iron in normal and hypoxic mice at pH 6.0 and 7.4. Iron uptake from $FeNTA_2$ showed an increase with both hypoxic exposure and with lowering medium pH from 7.4 to 6.0. Cadmium uptake showed no significant increases either with hypoxia or with lowering medium pH.

In iron deficiency, iron uptake was increased significantly at both pH 6.0 and 7.4 with $FeNTA_2$ complex as the iron source (Figure 3). Iron uptake from $FeNTA_2$ showed a tendency to increase on lowering the pH from 7.4 to 6.0, however, in this model, the effect did not reach statistical significant ($p = 0.064$). Cadmium uptake showed a significant increase with iron deficiency but not with lowering pH from 7.4 to 6.0.

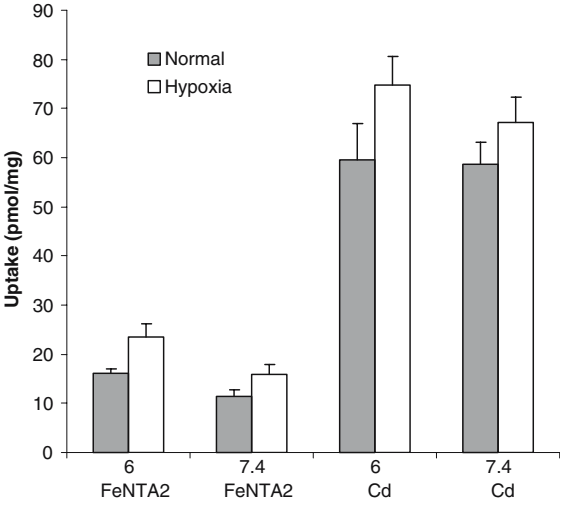

Figure 2. Iron and cadmium uptake in hypoxic mice. Normal (CD1 mice fed on standard lab diet) and hypoxic mice duodenum was incubated at pH 6.0 and 7.4 with 250 μM Fe or Cd. 8–12 determinations were performed in each experimental group. ANOVA revealed that uptake of $FeNTA_2$ was increased by hypoxia ($p < 0.05$) and by reduced pH ($p < 0.05$) although individual group comparisons did not reach significance. No significant effects were seen with cadmium.

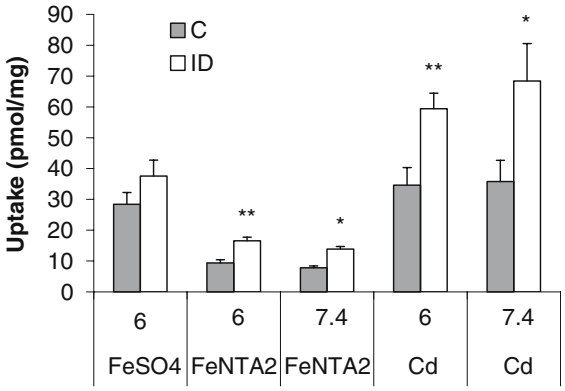

Figure 3. Iron and cadmium uptake in iron deficiency. Control (CD1 mice fed on iron supplemented diet, C) and Fe-deficient (ID) mice duodenum were incubated at pH 6.0 and 7.4 with 100 μM metal ion concentration. 9–17 determinations were performed in each experimental group. Uptake of all metal complexes except $FeSO_4$ at pH 6 was increased by iron deficiency (**$p < 0.01$) as was uptake of $FeNTA_2$ and Cd at pH 7.4 (*$p < 0.05$). Effects of pH did not reach statistical significance.

To further investigate the pH dependency of iron uptake, ferric reductase activity was assayed at pH 6.0 and 7.4 in control and iron deficient mice. Results show that reductase activity is increased by lowering medium pH and also by iron deficiency (Figure 4).

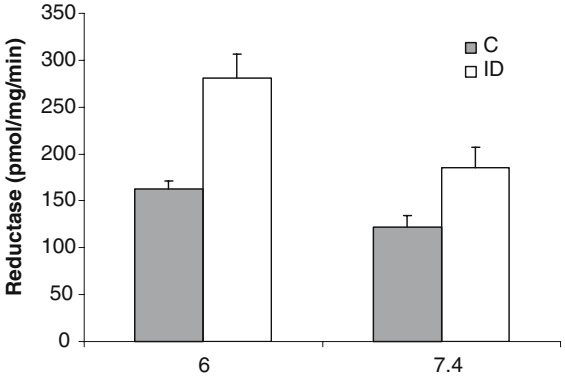

Figure 4. Reductase activity in duodenum from control and iron deficiency. Control (CD1 mice fed on iron supplemented diet) and iron deficient mouse duodenum was incubated at pH 6.0 and 7.4. 100 μM Fe was used. 6 determinations were performed in each experimental group. ANOVA revealed that both pH and iron deficiency significantly affected reductase activity ($p < 0.05$), although individual group comparisons did not reach significance.

Discussion

We used a well characterised *in vitro* method that measures initial rates of metal ion uptake by mouse duodenum (Raja *et al*. 1987a, 1987b), thus effects of basolateral transfer are presumed to be excluded in this system at these short incubation times. We have demonstrated that not only mucosal uptake of Fe but also Cd was increased by alterations in iron metabolism including a genetic anaemia (hypotransferrinaemia). Duodenal DMT1 levels have been shown to be increased by iron deficiency and especially hypotransferrinaemia (Canonne-Hergaux *et al*. 2001; Dupic *et al*. 2002). This is likely to be secondary to decreased hepcidin production (Weinstein *et al*. 2002). There were also some indications that cadmium uptake increased in hypoxic mice, but this did not reach significance, perhaps because the increase in duodenal DMT1 in these mice is modest (Miret S, Mckie, A, and Simpson, R, unpublished observation 2002). Overall these findings show that enhanced uptake of dietary cadmium could be significant in conditions of altered iron metabolism i.e., not only iron deficiency but also some iron overload conditions. It is noteworthy that previous studies showing increased cadmium absorption when iron absorption is increased have focussed on iron deficiency. This condition is associated with decreased dietary and/or mucosal iron levels (Pountney *et al*. 1999) while hypotransferrinaemic mice have normal dietary and mucosal iron levels (Pountney *et al*. 1999). Thus increased cadmium absorption in the latter case would not be attributable simply to decreased competition from iron.

The study of pH dependence of transition metal ion uptake in physiological systems is complicated by possible interactions of protons or hydroxide ions with the metals themselves or their complexing agents, where present. Cadmium can, however, exist as Cd^{2+} in the physiological pH range, therefore allowing study of pH dependence of Cd uptake. Our study is, we believe, the first to investigate pH dependence of cadmium uptake by intact duodenum. Previous studies of pH dependence of cadmium (or iron) uptake have employed cultured cells or DMT1-expressing *Xenopus* oocytes. In attempting to relate our findings to those previous studies, we must consider differences in the media and cadmium concentrations used and the responses of tissue to pH changes.

Elisma and Joumarie (2001) suggested that pH-insensitive chloride complexes of cadmium would dominate in typical uptake experiments with physiological media. Our physiological media are, however, gassed with 5% CO_2 therefore the highly pH sensitive $CdHCO_3$ may dominate Cd speciation. An additional complicating factor in comparing animal studies with cultured cell and *Xenopus* oocyte work is that the latter two systems reveal a K_m for Cd uptake of 1 μM, this being attributed to DMT1. pH sensitive Cd uptake seems to be restricted to this concentration range (Elisma & Jumarie 2001). We used a higher Cd concentration (100 μM or 250 μM) as in most other animal studies (e.g. Flanagan *et al*. 1980; Park *et al*. 2002) used 100 μM). Therefore although we have observed Cd uptake that increases in association with increased iron uptake, this may not all be mediated by DMT1. Non-DMT1 mechanisms for Cd membrane transport have been proposed (Foulkes 1991; Lou *et al*. 1991; Hinkle *et al*. 1993; Elisma & Jumarie 2001). Furthermore, data in (Savigni & Morgan 1998) show that rats with mutated DMT1 lack high affinity ($K_m < 1$ μM) Cd transport in reticulocytes but retain a low affinity mechanism that dominates at higher Cd concentrations and relates to the mechanism reported by (Lou *et al*. 1991) in erythrocytes. On the other hand, it may be that pH dependence effects on DMT1 are not observable in our system, however we also studied iron uptake and found that a pH dependence was demonstrable.

It is possible that the mouse duodenal fragments are able to maintain a constant low pH microclimate at the enterocyte surface (Lucas 1983) independent of the medium pH. Our *in vitro* system, however, involves removal of the mucous layer by blotting the tissue, as well as vigorous agitation of the tissue by bubbling to reduce unstirred layers.

Iron however, presents greater difficulties for pH dependence studies as the Fe^{2+} ion is liable to oxidation in a process that speeds up with increasing pH, while Fe^{3+} forms complexes with hydroxide ions. Additionally, many Fe^{3+} chelators (including NTA) bind protons in competition with Fe^{3+}. In general lowering medium pH will increase the stability and concentration of Fe^{2+} ions and if these are the species transported by e.g. DMT1, then there will be increased transport rates

independent of any pH effects on the transport protein itself. Previous studies of pH dependence of metal transport have focussed on cultured cell models or *Xenopus* oocytes and have used various medium metal concentrations (1–10 μM (Elisma & Jumarie 2001; Bannon *et al*. 2003); 100 μM; Tandy *et al*. 2000). All used Fe ascorbate solutions that are liable to oxidation (Dorey *et al*. 1993) at least at the higher Fe concentrations.

A pH dependence for Fe uptake was demonstrated with the $FeNTA_2$ complex, but as discussed above, this complex has pH dependent stability. A similar dependence was found by measuring ferric reductase activity (reduction of Fe^{3+} in the $FeNTA_2$ complex precedes Fe^{2+} transport by DMT1), suggesting that the pH dependence of uptake did not have to be due to pH dependence of DMT1. We were unable to provide support for a pH dependent mechanism for Cd uptake by mouse duodenal enterocytes, although uptake was clearly regulated by alterations in iron metabolism. Iron uptake from $FeNTA_2$ did show pH dependent uptake, but this could be explained by pH dependence of ferric reductase activity rather than of divalent metal transport.

Conclusions

Cadmium uptake by duodenum is increased by genetic hypotransferrinaemia; therefore increased cadmium uptake by duodenum can occur in hereditary iron overload conditions. We could not, however, demonstrate a pH dependency of cadmium uptake in mouse duodenum.

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