

A duodenal mucosal abnormality in the reduction of Fe(III) in patients with genetic haemochromatosis

K B Raja, D Pountney, A Bomford, R Przemioslo, D Sherman, R J Simpson, Roger Williams, T J Peters

Abstract

Background—Previous *in vitro* studies have shown that the uptake of Fe(III) by freshly isolated duodenal mucosal biopsy specimens is increased in patients with genetic haemochromatosis. Moreover, in the mouse it has recently been found that reduction of Fe(III) to Fe(II) is a prerequisite for iron uptake by the proximal intestine.

Aims/methods—This study used the *in vitro* technique to investigate the rates of reduction and uptake of $^{59}\text{Fe(III)}$ by duodenal mucosal biopsy specimens obtained at endoscopy from treated and untreated patients with genetic haemochromatosis.

Results—The rate of reduction of iron in the medium was proportional to the incubation time and was not caused by the release of reducing factors from the tissue fragments. Ferrozine, a specific Fe(II) chelator and ferricyanide, a non-permeable oxidising agent, inhibited uptake of ^{59}Fe showing that reduction of Fe(III) precedes uptake. The rates (all values given as pmol/mg/min) of reduction (152 (49) *v* 92 (23)) and uptake (8.3 (4.0) *v* 3.6 (1.3), mean (SD)), were significantly increased in biopsy specimens from the untreated group ($n=6$) compared with those from 10 control subjects ($p<0.04$). Furthermore, the reduction and uptake rates were still increased in five patients in whom iron stores were normal after venesection treatment.

Conclusions—These results show that there is a persistent abnormality in the reduction and uptake of iron by the intestine in genetic haemochromatosis.

(Gut 1996; 38: 765-769)

Keywords: Fe(III) reduction, genetic haemochromatosis, iron absorption, small intestine.

defect that leads to increased absorption of iron from a normal diet and the progressive deposition of iron in parenchymal tissue in this condition remains unclear. Abnormalities in the handling of iron by the intestine, liver or the reticuloendothelial system have all been implicated in the pathogenesis of GH.³⁻⁵ The frequency of GH in the Western world is higher than for the more familiar genetic conditions, cystic fibrosis and phenylketonuria⁶ and thus the understanding of the basis of the abnormality remains of paramount importance.

In vivo studies using whole-body counting⁷⁻¹⁰ suggest that an abnormality lies at the intestinal level. Although these studies have confirmed that iron absorption is inappropriately high in GH, this technique does not provide information about the site of absorption nor details of kinetic changes in the two phases of the iron absorption pathway, namely 'uptake' and 'transfer'.¹¹ It is now generally agreed that both these steps are capable of independent regulation¹² and it is feasible that either or both steps could be abnormal in GH. Indeed, Cox and Peters,¹³ using a well characterised *in vitro* method to investigate the rate of uptake of iron by mucosal biopsy specimens, have found evidence of increased rates in untreated and partially treated GH patients. However, the mechanism(s) of iron uptake and its control remains unclear.

We have recently shown in the mouse, a mucosal surface Fe(III) reducing activity in the proximal intestine. Mucosal Fe(III) reduction rates, though quantitatively higher than uptake rates,¹⁴ paralleled changes in the iron uptake rates induced by changes in body iron stores (iron deficiency) or by metabolic inhibitors.¹⁵ Overall, these results provide strong evidence for a reduction and uptake process operating at the intestinal level in the mouse.

The investigations reported here were carried out to ascertain whether such mucosal Fe(III) reducing activity was present in human tissue and whether the abnormalities in mucosal iron uptake reported previously in GH patients¹³ were reflected by changes in mucosal reducing activity.

Methods

Twenty one subjects were investigated: 11 with GH (six untreated and five fully treated) and 10 who acted as normal controls. The control group included two laboratory staff who had no symptomatic or clinical evidence of

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Accepted for publication
20 November 1995

Iron homeostasis is maintained primarily by controlling proximal intestinal absorption.¹ Under normal conditions, small daily losses of iron (1-2 mg) from the body (through desquamation of cells, urinary and biliary excretion, menstruation/parturition) are finely balanced by the regulated absorption of appropriate amounts of dietary iron. A reciprocal relation exists between body iron stores and iron absorption.² Although normal iron balance is known to break down in genetic haemochromatosis (GH), the underlying metabolic

TABLE I Biochemical data in control and GH patients

Group	Age (y)	Haemoglobin (g/dl)	Serum Fe (μM)	Transferrin saturation (%)	Serum ferritin ($\mu\text{g/l}$)
Controls	56.8 (19.2) (10)	13.5 (1.3) (8)	ND	ND	100 (58.5) (3)
Untreated GH	47.8 (10.9) (6)	14.4 (1.5) (5)	41.8 (3.3) (4)	96 (8.7) (4)	2503 (914) (6)*
Treated GH	62.6 (6.1) (5)	14.1 (0.7) (5)	22 (7.1) (4)	44 (15.7) (4)	35.5 (25.9) (4)
Reference range		>11	13-32	20-50	25-250

Date: mean (SD) for (n) determinations. ND=not determined. * $p < 0.001$ compared with control values.

gastrointestinal disease and eight patients who were undergoing upper gastrointestinal endoscopy for investigation of dyspeptic symptoms. Endoscopic examination and subsequent histological examination of the biopsy specimen obtained was normal in all 21 subjects. The diagnosis of GH was made on the basis of increased serum iron and ferritin, increased serum transferrin saturation, and evidence of increased hepatic storage iron.¹⁶ Liver iron, fasting serum iron and iron binding capacity, and serum ferritin were measured according to methods described previously.¹⁷ After diagnosis patients were treated by venesection therapy until iron stores were reduced to normal as confirmed by biochemical parameters and in some cases repeat liver biopsy. Maintenance venesection therapy (two to six units annually) was then performed to keep iron stores within normal limits. The study was performed in accordance with local ethical committee guidelines and was approved by the committee. Informed consent was obtained from all subjects who underwent peroral endoscopy after a 12 hour fast to obtain duodenal mucosal biopsy specimens.

Iron uptake/reduction

Mucosal fragments (3-4 pieces, 1.5-10 mg) were taken with standard biopsy forceps from the second part of the duodenum in subjects undergoing peroral endoscopic examination. The tissue samples were rinsed and briefly pre-incubated at 37°C in an oxygenated physiological buffer (125 mM NaCl, 3.5 mM KCl, 1 mM CaCl_2 , 10 mM MgSO_4 , 10 mM D-glucose in 16 mM HEPES/NaOH, pH 7.4). One to two fragments were thereafter

transferred and incubated for up to six minutes in a similar buffer containing ^{59}Fe (as a ferric chelate of nitrilotriacetate, $\text{Fe}=90 \mu\text{M}$; $\text{NTA}=180 \mu\text{M}$) and ^{57}Co -cyanocobalamin as the extracellular fluid marker. The iron concentration chosen has been reported by Cox and Peters¹³ to exhibit maximal difference in uptake between the control and GH groups. ^{59}Fe and ^{57}Co in the tissue samples and in an aliquot of the medium were separately counted in a LKB Wallac 1282 gammacounter. Uptake corrected for adherent medium and non-specific uptake (^{57}Co space) was then expressed as pmol/mg/min. This in vitro uptake method^{13 18} satisfies the criteria of Sallee *et al*¹⁹ for valid measurement of unidirectional nutrient uptake into intestinal tissue. For simultaneous determination of ferric iron reduction, ferrozine (1 mM final) was added to the incubation buffer containing Fe-NTA in either labelled or unlabelled form, just before the addition of the tissue. The rate of reduction of medium Fe(III) was ascertained by monitoring aliquots (100 μl) of medium in a Pye Unicam Model PU8600 spectrophotometer set at 562 nm for the detection of the stable, coloured, Fe(II)-ferrozine complex.²⁰

Statistical methods

Data are given as mean (SD) with median and interquartile ranges given where appropriate. Analysis of variance was performed by the Kruskal-Wallis method. Subsidiary Wilcoxon's *t* tests were used to test differences between individual groups.

Results

Table I depicts the laboratory data for the different study groups. Subjects with untreated GH were not anaemic and had considerably increased serum ferritin, transferrin saturation, and serum iron values compared with the control values and laboratory reference ranges. Liver iron analysis performed in three patients

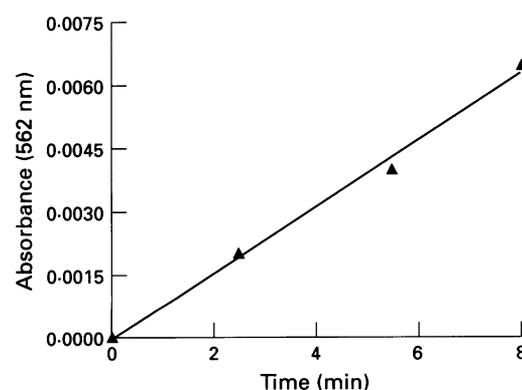


Figure 1: Time course for Fe(III) reduction by human duodenum. Duodenal biopsy tissue was incubated in physiological medium containing Fe(III)NTA₂ (90 μM) and ferrozine (1 mM). At various times aliquots of incubation medium were sampled for absorbance determinations. The increase in absorbance was linear with time ($r=0.99$; $p=0.003$).

TABLE II Reduction and uptake of iron by human duodenum

Patient group	n	pmol/mg/min	
		Reduction	Uptake
Normal controls	10	92.0 (23.4) (95.4; 69-111)	3.6 (1.3) (4.0; 2.9-4.4)
Untreated GH	6	152 (49)† (125; 119-201)	8.3 (4.40)‡ (7.2; 5.3-11.6)
Treated GH	5	148 (84)* (118; 87-224)	10.6 (4.1)‡ (11.9; 6.9-13.7)

Data: mean (SD) for (n) determinations. The median and interquartile range are given in parentheses. * $p=0.055$; † $p < 0.04$; ‡ $p < 0.008$ compared with normal controls. [Fe(III)]=90 μM as Fe^{3+} (NTA)₂.

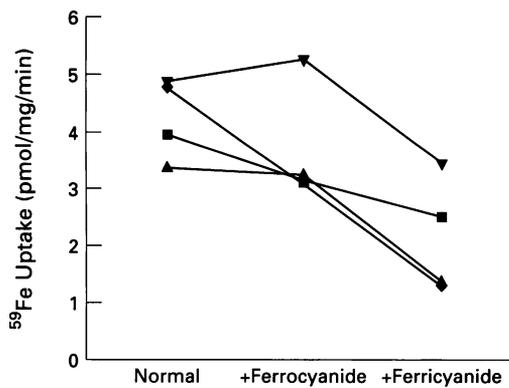


Figure 2: Effect of the presence of 1 mM ferro- or ferricyanide on ^{59}Fe uptake by duodenal mucosal tissue.

yielded values in the range 8.6–22.8 mg/g dry weight (normal values ≤ 1.5 mg/g). GH patients treated by multiple venesections (8–21 g of iron removed) were also not anaemic, and showed a pronounced reduction in all three parameters with almost all individual values falling within the normal range.

Characterisation of reductase activity in human mucosal tissue

Reduction of medium iron was ascertained spectrophotometrically by monitoring the formation of the stable, coloured Fe(II)-ferrozine complex. Fe(III) incubation medium showed no colour change when incubated at 37°C in the presence of freshly prepared ferrozine. Reduction of medium Fe(III) however, occurred after the addition of freshly isolated duodenal biopsy specimens and the rate of colour production was found to be linear with time of incubation (Fig 1). The proportion of total medium iron that was reduced during the incubations carried out in this study was $< 5\%$. The reduction of medium Fe(III) by mucosal tissue was found not to be influenced by the concentration of ferrozine in the medium (+5 mM, 109 (19%) (number of

determinations=4) of the values at 1 mM). Addition of ferrozine to media that had been pre-incubated with duodenal tissue for up to six minutes and then the tissue removed, failed to exhibit any significant colour change (ΔOD_{562} of ≤ 0.001) showing that reduction of medium iron was not caused by the release of iron reducing factors from the tissue fragments. Furthermore, duodenal mucosal biopsy specimens incubated in iron free medium containing ferrozine, and with the presence or absence of NTA, failed to exhibit any coloured complex formation.

The importance of mucosal Fe(III) reducing activity for iron uptake was further supported by the finding that the inclusion of the cell impermeable oxidising agent, ferricyanide,²¹ in the incubation medium resulted in a pronounced inhibition (49.1 (20.2)% (4)) in the uptake of ^{59}Fe from $^{59}\text{Fe}^{3+}(\text{NTA})_2$ by duodenal mucosal biopsy specimens. Ferrocyanide, on the other hand, had little effect (Fig 2).

Uptake/reduction of Fe(III) in genetic haemochromatosis

Duodenal tissue obtained from untreated GH patients exhibited higher rates of medium iron reduction compared with controls (Table II); the increases were paralleled by changes in the ^{59}Fe uptake rates. A significant correlation was evident between the rates of medium Fe(III) reduction and ^{59}Fe uptake rates by duodenal mucosal samples (Fig 3). GH patients whose total body iron stores were within normal limits after venesection treatment still exhibited increased duodenal reduction and uptake of iron. One GH patient studied before and immediately after the initial course of venesection (12.6 g of iron removed) showed an increase in the rates of both the mucosal ^{59}Fe uptake (before: 3.9; after: 14.3 pmol/mg/min) and reduction (before: 126; after: 279 pmol/mg/min). It is noteworthy that limited determinations of Fe(III) reducing activity of gastric antrum biopsy specimens obtained from GH patients yielded comparable values (76.8; 40.4 (2) pmol/mg/min) to that in control subjects (49.3 (0.8) (3)).

A decrease in ^{59}Fe uptake was seen when duodenal tissue obtained from either control subjects (27.7 (21.9)% (4)) or untreated patients with GH (48.2 (17.1)% (3)), was incubated in physiological medium containing 1 mM ferrozine, a specific Fe(II) chelator.²² In two experiments performed with biopsy samples from treated GH patients the inhibition of uptake was 53 and 70%, respectively. The ferrozine dependent inhibition of uptake in the GH groups is thus proportionally higher than in the control group.

Discussion

Environmental iron, which is abundant in the form of ferric ions requires solubilisation by reduction before the resulting Fe(II) is transported across the cell membrane.²³ The requirements of this system, a ferric reductase

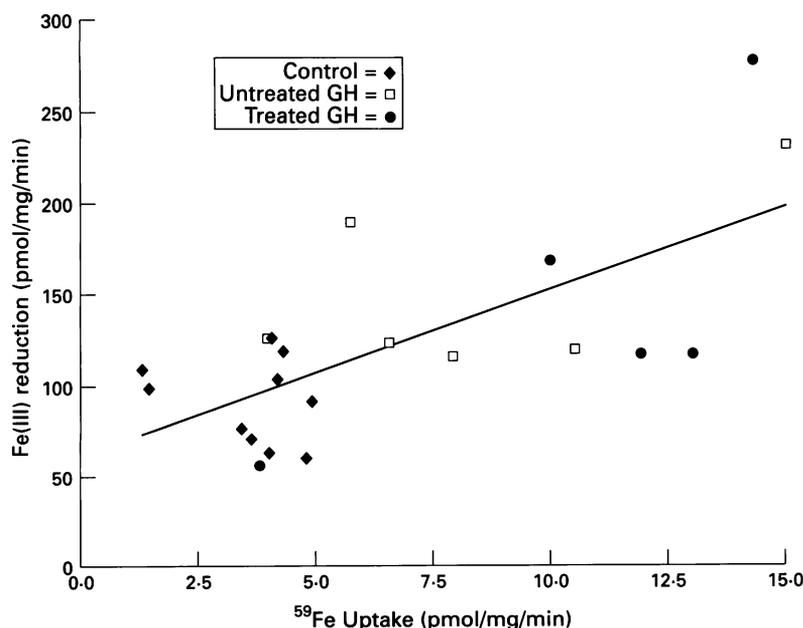


Figure 3: Correlation between reduction and uptake of medium iron. Data for all patient groups are included ($n=21$, $r=0.69$; $p<0.001$).

activity and an additional ferrous iron transport pathway have recently been detected in the yeast *Saccharomyces cerevisiae* and the genes encoding the proteins responsible for these processes have been cloned and sequenced.²⁴ The importance of a reductase component in intestinal iron uptake is shown by our recent studies, which showed that mouse duodenum has the capacity to reduce a variety of Fe(III) complexes and dyes (maltol, citrate, EDTA, nitroblue tetrazolium, 3(4,5 dimethyl thiazol-2yl) 2,5 diphenyl tetrazolium (MTT), cytochrome c, ferricyanide, and NTA^{15,25}) and that this ferri-reducing activity paralleled perturbations in iron uptake rates induced by changes in body stores (iron deficiency) or by the presence of metabolic inhibitors or modulators of membrane potential.¹⁵ These findings provide support for the concept of separate reduction and uptake processes operating at the level of the proximal intestine.

The results of this study confirm that freshly isolated human duodenal mucosal tissue possesses, as in the mouse, the ability to reduce Fe(III) added to the incubation medium. The duodenal reducing activity was detected by the increase in ferrozine-Fe(II)-chelatable iron in the incubation medium. The reaction was specific because ferrozine alone induced no change in colour in the medium in the presence or absence of iron, in support of the findings of Chindambaram *et al.*²⁶ A recent study²⁷ has suggested that ferrozine can promote non-specific reduction of Fe³⁺(NTA)₂ medium, although the rate of reduction is low and can be attributed to photoreduction reactions.²⁸ The colour change in the present experiments was not attributable to the release of reducing factors by the duodenal tissue, which is consistent with our previous study on the reduction of ferricyanide by mouse duodenum. Furthermore, in the present study, the presence of ferricyanide in the medium considerably affected the uptake of ⁵⁹Fe from ⁵⁹Fe³⁺(NTA)₂ by human duodenal tissue. As the mucosal surface presents a much larger functional area than the basal surface, the Fe(III) reducing activity is envisaged to be associated with the absorptive surface of the mucosal epithelium.

Patients with GH showed a significant increase in the duodenal mucosal Fe(III) reducing activity compared with normal controls. The increase seems to be site specific as no enhancement in reducing activity was seen with gastric samples. The ability to reduce medium Fe(III) must be inherent, as the tissue fragments were incubated in the medium in the absence of any hormonal or corporeal factors. Moreover, the increase in reducing activity correlated with the changes in ⁵⁹Fe uptake rates ($p < 0.05$). The increase in mucosal uptake in the group of untreated GH subjects at the iron concentration used, confirms previous findings¹³ and indicates changes in the duodenal mucosal avidity for iron. A persistent disorder in the regulation of iron uptake is further suggested by the fact that both reduction and uptake of iron by duodenal mucosa are increased in the group of GH

patients who have normal body iron stores after venesection treatment. The treated group had a mean (SD) age (62.6 (6.1) years) that was higher than in the untreated GH group (47.8 (10.9)), suggesting that the biochemical manifestations of the metabolic defect do not decrease with age, in agreement with previous clinical findings.^{7, 29}

Current evidence based on microscopical findings,³⁰ mucosal iron concentration,³¹ ferritin protein values,^{32, 33} and ferritin mRNA values³⁴ shows that in GH the duodenal enterocytes do not load up with iron despite considerably increased body iron stores. Thus, mucosal iron concentrations do not seem to govern the iron uptake/reduction rates in this clinical condition. A further line of evidence that supports this proposition is the finding that patients with anaemia of chronic disease, who have reduced duodenal mucosal iron concentrations,³⁵ failed to show an increase in the Fe(III) reducing activity (62.1 (51.7) (4) pmol/mg/min) compared with control values (Table II). Whether changes in mucosal transfer rates also contribute to the iron loading seen in the early stages of GH and after treatment, as suggested by a number of previous studies,^{8-10, 36} remains to be determined.

The inhibitory effect of ferrozine (a specific Fe(II) chelator) on in vitro ⁵⁹Fe uptake by human duodenal mucosal fragments, supports the proposition that reduction precedes uptake. Furthermore, the ferrozine dependent inhibition of uptake was proportionally higher in patients with GH compared with the control group, which is consistent with the abnormal defect in haemochromatosis being caused by a gain of function mutation. The incomplete abolition of ⁵⁹Fe uptake even in the presence of 5 mM ferrozine in the medium suggests that up to 30% of uptake occurs via a ferrozine insensitive pathway. As the dietary content of iron in GH patients does not differ appreciably from that in normal subjects³⁷ and as excretion of iron is limited, a regulatory defect in the reduction/uptake of iron by the proximal intestine is believed to play an important part in the considerable iron loading seen in GH. In yeast, the two processes of reduction and uptake are independently regulated under separate genetic control. As to whether the two processes work in unison or are separate entities, and whether the same regulatory principle as that operating in yeast controls mammalian intestinal iron uptake remains to be elucidated.

This work was funded by a project grant from the Wellcome Trust. This is a contribution from the King's College Centre for the study of Metals in Biology and Medicine.

This work was presented in part as an abstract at the Medical Research Society Meeting 1993.

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Gut 1996 38: 765-769
doi: 10.1136/gut.38.5.765

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