

Duodenal ascorbate and ferric reductase in human iron deficiency¹⁻³

Bisera D Atanasova, Andy CY Li, Ingvar Bjarnason, Kamen N Tzatchev, and Robert J Simpson

ABSTRACT

Background: The first step in iron absorption requires the reduction of ferric iron to ferrous iron, a change that is catalyzed by duodenal ferric reductase. Iron deficiency is associated with high iron absorption, high ferric reductase activity, and high duodenal ascorbate concentrations in experimental animals, but it is not known whether a relation between reductase and ascorbate is evident in humans.

Objective: The objective of the study was to assess the relation between ferric reductase activity in human duodenal biopsy specimens and ascorbate concentrations in iron-replete and iron-deficient subjects.

Design: Patients and control subjects were overnight-fasted adults presenting sequentially for upper gastrointestinal endoscopic investigation. Ferric reductase activity in duodenal biopsy specimens was assayed by using nitroblue tetrazolium. Ascorbate was assayed in duodenal biopsy specimens and plasma.

Results: Iron-deficient patients had significantly higher reductase activity ($n = 6-9$; $P < 0.05$) and duodenal ($n = 20$; $P < 0.001$) and plasma ($n = 6$; $P < 0.001$) ascorbate concentrations than did control subjects. Incubation of biopsy specimens with dehydroascorbate (to boost cellular ascorbate) increased reductase activity in the tissues that initially had normal activity ($n = 9$; $P < 0.01$) but inhibited reductase activity in the tissues that already had high reductase activity ($n = 13$; $P < 0.001$).

Conclusions: Iron deficiency in humans is associated with increased duodenal ascorbate concentrations. This finding suggests that increased reductase activity is partly due to an increase in this substrate for duodenal cytochrome b reductase 1. *Am J Clin Nutr* 2005; 81:130-3.

KEY WORDS Iron absorption, iron nutrition, vitamin C

INTRODUCTION

Studies in both humans (1, 2) and mice (3-5) have implicated duodenal ferric reductase [catalyzed by duodenal cytochrome b (Dcytb) or cytochrome b reductase 1 (Cybrd1)] activity as an important factor in the regulation of intestinal iron absorption in both healthy subjects and those with altered iron metabolism. Duodenal ferric reductase activity is increased in persons with iron deficiency (1) and hemochromatosis (1, 2). We showed in mice that Dcytb activity is partly dependent on the concentration of its intracellular substrate, ascorbate (3, 6) and that concentrations of duodenal ascorbate are higher in mice with iron deficiency than in those that are iron replete (7). It follows from this that intracellular concentrations of ascorbate in the duodenal epithelium may be a factor in controlling intestinal iron absorption in humans. We therefore set out to investigate the alterations

of ascorbate concentrations and ferric reductase activity in duodenal biopsy specimens from control subjects and patients with iron deficiency.

SUBJECTS AND METHODS

Subjects

Overnight-fasted adults (mixed group of males and females; age range: 25-91 y) presenting sequentially for upper gastrointestinal endoscopic investigation were the subjects for study. Duodenal biopsy specimens ($n = 3$ from second part of duodenum) were obtained during routine gastroscopy. One specimen was sent for routine histologic tests, and the other 2 specimens were used for 1 of 3 studies. In all patients, blood samples were taken for serum ferritin and hemoglobin assays.

Written informed consent was obtained from all subjects. Ethical approval for the study protocol was obtained from the ethics committees of St Thomas' Hospital and King's College Hospital.

Study design

In study 1, biopsy specimens were taken for immediate assay of reductase activity (reductase activity is labile on storage of samples) as described below. In study 2, biopsy specimens were homogenized in 5% ice-cold metaphosphoric acid by using a high-speed Ultra Turrax homogenizer (IAK Werke; Kanke & Kunkel BmbH & Co KG, Stauffen, Germany) and analyzed for ascorbate acid (AA) and dehydroascorbate acid (DHA) concentrations. Plasma was obtained by whole-blood centrifugation with the use of EDTA as an anticoagulant and assayed for AA and DHA concentrations. In studies 1 and 2, the iron status of each subject was not known to the investigators until all analyses were completed, at which point subjects were allocated to the iron-deficient (ferritin $< 30 \mu\text{g/L}$) or the control (ie, iron-replete;

¹ From the Department of Clinical Laboratory and Clinical Immunology, Medical University, Sofia, Bulgaria; the Division of Life Sciences, King's College London, London; the Gastrointestinal Laboratory, The Rayne Institute, St Thomas' Hospital, London; and the Department of Medicine, GKT School of Medicine, King's College, London.

² Supported by the International Federation of Clinical Chemistry-Professional Scientific Exchange Programme and the Medical Research Center of the United Kingdom.

³ Reprints not available. Address correspondence to RJ Simpson, Division of Life Sciences, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NN, United Kingdom. E-mail: robert.simpson@kcl.ac.uk.

Received August 16, 2004.

Accepted for publication September 30, 2004.

TABLE 1
Hemoglobin and ferritin concentrations in subject groups in study 1¹

Subject group	Hemoglobin	Serum ferritin
	g/L	μg/L
Control (n = 9)	144 ± 9	93 ± 47
Iron-deficient (n = 6)	113 ± 8	15 ± 10
P	<0.001	<0.002

¹ All values are $\bar{x} \pm SD$.

ferritin > 40 μg/L) group or were excluded on the grounds of excessive C-reactive protein concentrations or erythrocyte sedimentation rates or incomplete data. Study 3 was intended to investigate the effect of supplementing the in vitro incubation medium with DHA to increase tissue ascorbate concentrations. We previously showed that the incubation of duodenum with DHA results in increased tissue ascorbate (7). In study 3, one biopsy specimen was incubated as in study 1 for reductase assay, and a second biopsy specimen was incubated in parallel in the same medium supplemented with DHA and assayed for reductase. The samples for study 3 came from subjects who were not separated on the basis of iron status, and that group included some subjects with incomplete data sets for iron status.

Reductase activity assay

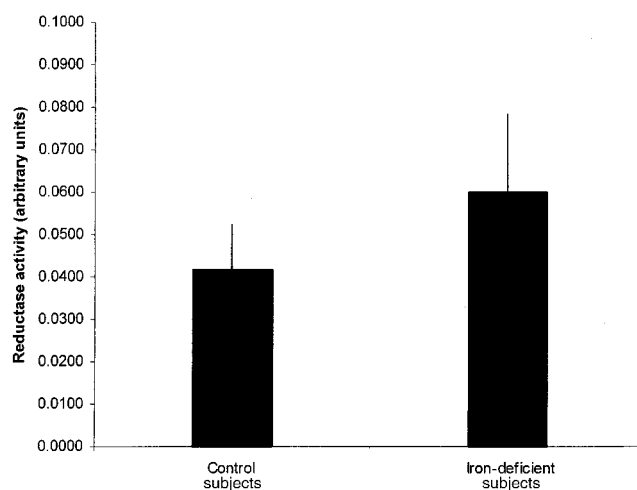
Freshly taken biopsy specimens from iron-deficient or control subjects were incubated for 5 min at 37 °C in buffer [125 mmol NaCl/L, 16 mmol HEPES-Na/L (pH 7.4), 3.5 mmol KCl/L, and 10 mmol glucose/L]. Biopsy specimens were then incubated for 10 min at 37 °C in the same buffer supplemented with 1 mmol nitroblue tetrazolium/L (NBT; Sigma Chemical Co, Poole, United Kingdom), rinsed in 0.15 mol NaCl/L, and imaged; then the formazan deposition was semiquantified by densitometry as described previously (8). The surface reduction of NBT by duodenal tissue was shown by us (3) and others (1) to be blocked by anti-Dcytb antibodies and to closely correlate with duodenal ferric reductase activity (3). In study 3, one of the 2 biopsy specimens was incubated as above, and the other was incubated with 1 mmol NBT/L, which was supplemented with 10 mmol freshly dissolved DHA/L, and analyzed in the same way.

Ascorbate assays

Ascorbate was assayed as described previously (7). The spectrophotometric determination was modified from an assay described by Kampfenkel et al (9) and based on the reduction of Fe³⁺ to Fe²⁺ by AA and on spectrophotometric detection of Fe²⁺

TABLE 2
Hemoglobin and ascorbate concentrations in control and iron-deficient subject groups in study 2¹

Subject group	Hemoglobin	Serum ferritin	Duodenal ascorbate	Plasma ascorbate
	g/L	μg/L	μmol/g	μmol/L
Control	135 ± 15	89 ± 49	1.04 ± 0.3	36 ± 18
n	23	13	24	16
Iron-deficient	111 ± 18	15 ± 7	1.90 ± 0.42	76 ± 12
n	17	15	17	5
P	<0.001	<0.001	<0.001	<0.001

¹ All values are $\bar{x} \pm SD$.**FIGURE 1.** Duodenal nitroblue tetrazolium reductase activity in control and iron-deficient humans. Duodenal biopsy specimens were incubated with 1 mmol nitroblue tetrazolium/L in physiologic buffer, and formazan deposition was quantified by densitometry (Student's *t* test, *P* = 0.026).

complexed with ferrozine. DHA was reduced to AA by incubation of the samples with dithiothreitol. Excess dithiothreitol was removed with ethylmaleimide, and total AA was determined by the same procedure. The concentration of DHA was then calculated from the difference between total ascorbic and AA (9). Plasma AA and DHA concentrations were analyzed according to a modification of the method of Ocamura (10).

Statistical analysis

All results are expressed as means ± SDs. Differences between groups were analyzed by using paired or unpaired Student's *t* test as appropriate (MICROSOFT EXCEL 2002; Microsoft Corp, Redmond, WA).

RESULTS

All subjects had normal results on duodenal histologic tests. The results of ferritin and hemoglobin assays in the subject groups for studies 1 and 2 are shown in **Tables 1** and **2**. Iron-deficient patients had significantly lower hemoglobin and serum ferritin concentrations (both: *P* < 0.001) than did iron-replete subjects in both studies. The values were similar in the corresponding groups in both studies.

Reductase activity was measured with NBT reduction (study 1) and was found to be higher in iron-deficient subjects than in control subjects (**Figure 1**; *P* < 0.05). AA concentrations in both plasma and duodenum (study 2) were higher in duodenal biopsy specimens from subjects with iron deficiency than in those from iron-replete subjects (Table 2). DHA was below the detection limit (0.1 nmol/mg or 5 μmol/L) in most biopsy and plasma samples from both subject groups and overall did not differ significantly (*P* > 0.4) from zero in either group (data not shown).

In addition, we found (study 3) that, when reductase activity was low in biopsy specimens, the inclusion of DHA, which is taken up by cells and converted into AA (7, 11), tended to increase reductase activity (**Figure 2**; *P* < 0.01). Paradoxically, DHA had an inhibitory effect on reductase activity in samples

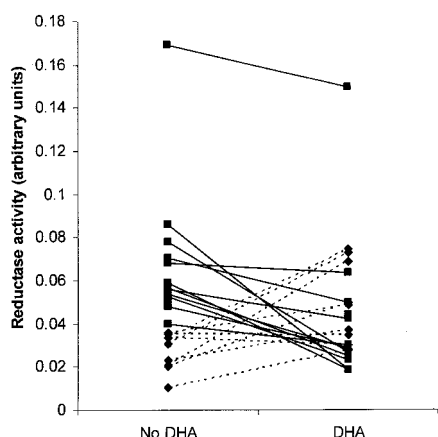



FIGURE 2. Effect of incubation with dehydroascorbate (DHA) on reductase activity. Reductase activity was measured with nitroblue tetrazolium in 2 biopsy specimens from each patient: one in the presence of 10 mmol DHA/L (right column) and the other without added DHA. Data from patients with low activity (<0.04 ; \blacklozenge) was increased by DHA ($P < 0.01$), whereas activity from biopsy specimens with high reductase (>0.04 ; \blacksquare) was decreased by DHA ($P < 0.001$).

with higher initial activity (Figure 2; $P < 0.001$), which suggests a threshold for the effect.

DISCUSSION

The present data support the findings of Zoller et al (1) that duodenal ferric reductase activity [here measured with the chromogenic substrate NBT (1, 3, 7)] is higher in persons with iron deficiency than in those who are iron replete. Zoller et al showed that iron-deficient patients have significantly higher expression of Dcytb than do iron-replete patients (1). We have further investigated the basis for increased reductase activity and found that iron-deficient subjects have greater concentrations of the putative intracellular substrate for ferric reductase—namely, ascorbate—than do iron-replete subjects (6, 7). The present data also show that, when reductase activity is initially low, in vitro incubation of tissue with DHA leads to an increase in reductase activity. Incubation with DHA enhances intracellular AA concentrations (7, 11), which suggests that those concentrations can be limiting for reductase activity in human duodenum. It is interesting that, when reductase (and presumably endogenous AA) concentrations were initially high, incubation with DHA led to an inhibition in reductase activity. AA feeding has previously been reported to enhance mucous secretion by rat stomach, presumably by sparing glutathione and protecting cells from oxidative damage (12). Our own studies (not shown) in which biopsy specimens from mouse intestine were incubated with 1 mmol AA/L showed that such high concentrations of medium AA provoked mucous hypersecretion, which could prevent the access of NBT to the biopsy specimen's surface and the deposition of NBT-formazan there. We therefore hypothesize that, when AA concentrations exceed a threshold, mucous secretion can be stimulated, and that would result in reduced deposition of NBT-formazan.

These findings provide an additional mechanism (ie, supplementing increases in the reductase protein, Dcytb; 1) by which human duodenal ferric reductase activity can be increased. In particular, the concentration of the intracellular substrate for Dcytb, ascorbate, is higher in persons with iron deficiency than

in those who are iron replete. Other effects such as mucous secretion may, however, limit the increase in reductase, at least in in vitro studies. In vivo effects of AA on mucous secretion are likely governed by complex factors, including AA transport, oxygen concentrations, antioxidant defenses, and additional regulators of mucous secretion. Moreover, in vivo effects of AA on overall iron absorption will include ascorbate's well-known iron-solubilizing and iron-reducing effects (13), which tend to counteract inhibitory effects due to increased mucous secretion. The finding that duodenal AA is higher in iron-deficient subjects than in those who are iron replete is in keeping with studies showing that iron concentrations generally are inversely related to tissue AA concentrations (14). However, epidemiologic studies found a positive correlation between dietary AA intake and iron stores (15). The relation between dietary AA intake and iron status has proved more complex than expected from studies with test meals (13, 16, 17). Whether dietary AA intake is the main regulator of ferric reductase activity requires further investigation, which will demand studies in which the subjects are strictly controlled for dietary AA intake as well as other factors that influence AA concentrations, such as smoking (18). The present findings provide an additional mechanism by which iron absorption can be enhanced by AA, namely, the enhancement of ferric reductase activity. 

We are grateful to Richard P Thompson of St. Thomas' Hospital, London, and the staffs of the endoscopy units of both St. Thomas' and King's College hospitals for help with provision of human samples.

BDA carried out ascorbate assays, assisted with study design, and collated data; ACYL and IB supplied biopsy specimens and collated data; KNT assisted with study design; and RJS carried out reductase assays, assisted with study design, and collated data. None of the authors had a conflict of interest.

REFERENCES

- Zoller H, Theurl I, Koch RO, Mckie AT, Vogel W, Weiss G. Duodenal cytochrome b and hephaestin expression in patients with iron deficiency and hemochromatosis. *Gastroenterology* 2003;125:746–54.
- Raja KB, Pountney DJ, Bomford A, et al. A duodenal mucosal abnormality in the reduction of Fe(III) in patients with genetic haemochromatosis. *Gut* 1996;38:765–9.
- Mckie AT, Barrow D, LatundeDada GO, et al. An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* 2001; 291:1755–9.
- Dupic F, Fruchon S, Bensaid M, et al. Inactivation of the hemochromatosis gene differentially regulates duodenal expression of iron-related mRNAs between mouse strains. *Gastroenterology* 2002;122:745–51.
- Muckenthaler M, Roy CN, Custodio AO, et al. Regulatory defects in liver and intestine implicate abnormal hepcidin and Cybrd1 expression in mouse hemochromatosis. *Nat Genet* 2003;34:102–7.
- LatundeDada GO, Van Der Westhuizen JP, Vulpe CD, Anderson G, Simpson RJ, Mckie AT. Molecular and functional roles of duodenal cytochrome B (Dcytb) in iron metabolism. *Blood Cells Mol Dis* 2002; 29:356–60.
- Atanasova B, Mudway IS, Laftah AH, et al. Duodenal ascorbate levels are changed in mice with altered iron metabolism. *J Nutr* 2004;134: 501–5.
- Simpson RJ, Debnam E, Beaumont N, Bahram S, Schumann K, Srail S. Duodenal mucosal reductase in wild type and HFE knock-out mice on iron-adequate, iron-deficient and iron-rich feeding. *Gut* 2003;52:510–3.
- Kampfenkel K, Van Montagu M, Inze D. Extraction and determination

- of ascorbate and dehydroascorbate from plant tissue. *Anal Biochem* 1995;225:165-7.
10. Okamura M. An improved method for determination of L-ascorbic acid and L-dehydroascorbic acid in blood plasma. *Clin Chim Acta* 1980;103:259-68.
 11. Goldenberg H, Schweinzer E. Transport of vitamin C in animal and human cells. *J Bioenerg Biomembr* 1994;26:359-67.
 12. Hung CR, Neu SL. Acid-induced gastric damage in rats is aggravated by starvation and prevented by several nutrients. *J Nutr* 1997;127:630-6.
 13. Lynch SR, Cook JD. Interaction of vitamin C and iron. *Ann N Y Acad Sci* 1980;355:32-44.
 14. Roeser HP. The role of ascorbic acid in the turnover of storage iron. *Semin Hematol* 1983;20:91-100.
 15. Fleming DJ, Jacques PF, Dallal GE, Tucker KL, Wilson PW, Wood RJ. Dietary determinants of iron stores in a free-living elderly population: the Framingham Heart Study. *Am J Clin Nutr* 1998;67:722-33.
 16. Reddy MB, Hurrell RF, Cook JD. Estimation of nonheme-iron bioavailability from meal composition. *Am J Clin Nutr* 2000;71:937-43.
 17. Cook JD, Reddy MB. Effect of ascorbic acid intake on nonheme-iron absorption from a complete diet. *Am J Clin Nutr* 2001;73:93-8.
 18. Schectman G, Byrd JC, Gruchow HW. The influence of smoking on vitamin C status in adults. *Am J Public Health* 1989;79:158-62.