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ORIGINAL CONTRIBUTION

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Ascorbic acid uptake affects ferritin, Dcytb and Nramp2 expression in Caco-2 cells

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Abstract *Background* Ascorbic acid (vitamin C) enhances iron uptake in human intestinal cells. It is commonly believed that the enhancement is due to the capacity of ascorbic acid to reduce ferric iron to ferrous iron. Other suggestions have recently been made about the effects of ascorbic acid on the cellular metabolism of iron. These effects must be investigated for several reasons. One important issue is to study whether ascorbic acid has effects on iron metabolism in the absence of extracellular iron in the intestinal lumen. *Aim of the study* The aim of this investigation was to determine whether cellular uptake of ascorbic acid affects iron acquisition in the Caco-2 cell line. The possible event was investigated by studying the expression of the iron storage protein ferritin, the iron uptake protein Nramp2 and a duodenal ferric reductase Dcytb after incubating ascorbic acid deficient or ascorbic acid fed cells with iron and/or ascorbic acid. *Methods* The above stated interactions were studied in the human Caco-2 cell model. Cell lysates were collected and subjected to SDS-PAGE and Western blotting. The blotted samples were stained with specific antibodies (Rabbit α-human-Nramp2 and Goat α-human Dcytb) against the respective proteins and the bands achieved were analysed by reflective density measurements. The cellular ferritin content was analysed with a commercial kit and the intracellular ascorbic acid concentration was measured by HPLC. *Results* The results indicate that ascorbic acid uptake induces both iron independent and iron dependent ferritin formation, but the effect on iron dependent ferritin expression was significantly greater (470% compared to 19%). Western Blot analyses revealed a long term down-regulating effect of ascorbic acid on iron independent and iron dependent Nramp2 and Dcytb expression. However, the down-regulation of Dcytb was in general more extensive than that of Nramp2 (31–50% compared to 8–29%). In a second study of short term Nramp2 and Dcytb expression, the results suggested that both proteins were significantly up-regulated by ascorbic acid, regardless of intracellular ascorbic acid status. However, the impact of iron alone on Nramp2 up-regulation seems to be greater in the absence of ascorbic acid. *Conclusions* The influence of intracellular ascorbic acid status on ferritin formation must be considered in iron uptake studies in Caco-2 cells. This could be a cause of diverging inter-

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laboratory results. The long term down-regulation of Nramp2 and Dcytb seems to correlate with results of human studies, where long term ascorbic acid supplementation does not affect iron status. Similarly, the short term up-regulation of Nramp2 and Dcytb seems to agree with the improvement in iron uptake shown in humans when single doses of ascorbic acid were administrated. These results are important for the understanding of the impact of ascorbic acid on iron status and will hopefully lead to further investigations on the matter.

Key words ascorbic acid – ferritin – Dcytb – Nramp2 – Caco-2 cells

Introduction

It is well established that ascorbic acid (or vitamin C) promotes iron uptake both *in vivo* [10] and *ex vivo* [20] in human cells. It is also commonly believed that food that contains iron should be consumed in the *presence* of ascorbic acid in order to enhance the bioavailability of non-heme iron. These issues raise the question: at what intestinal level does ascorbic acid act, in the lumen and/or in the enterocytes? This consideration is important and gives rise to other questions such as how to administrate ascorbic acid to maximize iron bioavailability. Another important and relevant issue is the need to consider ascorbic acid status in order to conduct iron bioavailability studies in humans or human cell lines *ex vivo*.

There are indications in the literature that ascorbic acid is able to reduce Fe^{3+} to Fe^{2+}, which is considered to promote iron uptake [17]. It is also suggested that ascorbic acid acts as a chelating agent [16] which may protect iron from oxidation on its way to the site of absorption. Another factor that might influence iron absorption is that ascorbic acid is actively transported across the enterocyte membrane. The uptake is mediated by a sodium-dependent antiport protein (SVCT1) [4]. This transporter is regulated by the luminal abundance of ascorbic acid [11]. These relationships between iron and ascorbic acid suggest the possible existence of intracellular effects.

An extensively used and validated cell model for iron uptake and transport studies is the human colon adeno-carcinoma cell line Caco-2. Caco-2 cells spontaneously differentiate into duodenal-like cells when grown confluent for a certain time. When fully differentiated, the cells express a variety of proteins involved in iron absorption, making them an excellent choice for *ex vivo* studies of iron uptake [6]. Cellular ferritin formation is widely used as an indirect measurement of iron uptake [9] as it has been shown to correlate with iron absorption.

It has been shown by Toth et al. [19] that continuously cultured human hepatoma and leucemia cells are ascorbic acid deficient and that the presence of ascorbic acid induces iron dependent ferritin formation in these cell lines. This suggests that the level of ascorbic acid could affect the outcome of iron uptake studies in other human cell lines, such as the Caco-2 cells, but this has not been studied to date. However, if this is the case, differences in the ascorbic acid status of the cells would affect the outcome of iron uptake studies when using ferritin levels as an indicator of iron absorption.

The purpose of the present study was to examine how ascorbic acid *uptake* affects iron uptake in Caco-2 cells by means of measuring intracellular ferritin and ascorbic acid levels. The response of the Caco-2 cells has been shown to closely resemble the human response to the studied substrates [20]. Furthermore, we investigated whether expression of the human iron uptake protein Nramp2 or the membrane bound ferric reductase Dcytb was also affected by the uptake of ascorbic acid. Attention must be paid as to whether the regulation of Nramp2 and Dcytb in Caco-2 cells is to be affected by ascorbic acid.

Nramp2/Dmt1/Dct1 is an integral membrane protein localized at the brush border membrane of intestinal enterocytes [18]. The function of this protein is to transport divalent cations such as Fe^{2+}, Mn^{2+}, Zn^{2+} and Ca^{2+} across the duodenal apical membrane in a proton dependent manner. Nramp2, the human homologue to Dmt1 or Dct1 in rats, has been demonstrated to show a preference for Fe^{2+} compared to other divalent cations. Nramp2 mRNA expression is regulated by iron uptake as shown in the Caco-2 cell model [13] where a high dose of iron (24 h of incubation) caused a decrease in expressed mRNA. Moreover, mutations of the gene coding for the rat and mice homologues for Nramp2 have been shown to induce microcytic anemia [7, 8]. These two statements indicate the importance of Nramp2 in iron absorption.

Another important duodenal membrane protein is Dcytb. The function of Dcytb in iron metabolism is to reduce Fe^{3+} to Fe^{2+} in order to make iron available for uptake via Nramp2. The possible effect of ascorbic acid uptake on Dcytb expression is particularly interesting because of its proposed mechanism of action. McKie and co-workers have shown that Dcytb in mice is a homolog to Cytochrome b561 [14]. Cytochrome b561 shuttles electrons between the cytosol and chromaffin vesicles of the adrenal glands

[2]. Ascorbic acid acts as the reducing factor in the cytosol and semidehydroascorbic acts as an electron acceptor in the vesicles. According to McKie et al. ferric iron serves as the electron acceptor from Dcytb and ascorbic acid as the electron donor, in mouse duodenal enterocytes. These studies in mice also indicate that Dcytb is regulated by luminal iron availability. In this case the expression of Dcytb was down-regulated in response to an iron challenge. It has also been shown that iron deficient humans have a higher Dcytb activity than iron replete subjects [1]. With this in mind, it is tempting to suggest that cellular ascorbic acid status could have some impact on Dcytb expression in human cells.

Materials and methods

Reagents

Polystyrene materials for cell cultivation were purchased from Corning, USA. Media, supplements and other reagents for culture maintenance were produced by PAA. All equipment and materials for electrophoresis and Western Blot analysis were purchased from Bio-Rad, Sweden, unless otherwise stated.

Antibodies

Rabbit α-human-Nramp2 with the IRE region was purchased from Alpha diagnostics (San Antonio) and used according to the distributors' guidelines. The specificity of the Nramp2 antibody was tested extensively by Moos & Morgan, who used this antibody in Western Blot analyses of human brain homogenates [15]. Our Western Blot data revealed one single band at approximately 65 kD, which is consistent with the literature on the size of the human Nramp2 protein [18]. Goat α-human Dcytb was bought from Novus Biologicals, Littleton. The specificity of α-Dcytb was tested in human colon lysates by the distributor, who observed three bands using Western Blot analyses, one band at approximately 35 kD, which corresponds to Dcytb, and a double band at 26 + 28 kD. All three bands were blocked by incubation with the immunizing peptide. The additional bands could not be identified by BLAST searches of the immunizing peptide which further supports the specificity of the used α-Dcytb antibody. Western Blot analyses done in our laboratory yielded the expected band at approximately 32 kD and the doublet as well. This doublet band as quantified by reflective density measurements has been found to have the same relative densities when compared to the control as the 32 kD band belonging to Dcytb.

Both secondary anti-bodies were conjugated to alkaline phosphatase (AP). Rabbit α-goat IgG-AP was purchased from Delta Biolabs, California, and Goat α-rabbit IgG-AP was distributed by Bio-Rad, Sweden.

Cell line

Caco-2 cell culture (HTB-37) was obtained from the American Type Culture Collection (Rockville, MD) at passage 19. Stock cultures were maintained in Dubellco's modified α essential medium (DMEM) supplemented with FBS (16%), non-essential amino acids (1%), penicillin (100 units/l) and streptamycin (10 mg/ml). The cells were cultured at 37°C in 95% humidified air and 5% CO_2. The medium was changed every second or third day and the cells were passaged at 80–90% confluence. After passage, the cells were re-seeded at a density of 11,000 cells cm^{-2}. For experiments, cells were seeded at passage 31–36 in 12-well plates at a density of 250,000 cells/well. All experiments were carried out 13–14 days post-seeding.

Ascorbic acid and iron uptake experiments

10 days post-seeding, the medium was exchanged for α-modified Minimal Essential Medium, MEM (PAA, E15-832), containing penicillin (100 units/l) and streptamycin (10 mg/ml). The medium was analysed for ascorbic acid to verify its absence. The medium used for experiments differed from the maintenance medium in order to control the medium composition, especially with respect to ascorbic acid and iron content.

On day 12, wells in triplicates were treated with MEM, unsupplemented (experiments 1 and 4) or supplemented (experiments 2, 3 and 5) with ascorbic acid (150 μmol/l). The concentration of ascorbic acid was chosen according to Toth et al. [19] and falls in the range of the concentration in human plasma. A comprehensive description of the various combinations of ascorbic acid and iron (exp. 1–5) is presented in Table 1. The iron uptake experiments were performed

Table 1 Description of the experimental setup

		t (h)			
Additions to MEM	(1) Baseline	(2)	(3)[a]	(4)	(5)[b]
Ascorbic acid 150 μmol/l	–	0–16	0–16	–	0–14
Iron 20 μmol/l	–	–	14–16	14–16	14–16
No additive	0–38	16–38	16–38	16–38	16–38

Study I was performed from time $t = 0$ to $t = 38$ h, experiment 1–5. In study II, the setup of experiment 1–4 was similar, but the experiments were terminated at $t = 16$ h
[a]Extracellular and intracellular ascorbic acid
[b]Intracellular ascorbic acid only

on day 13 when the ascorbic acid supplementation had lasted for 14 h (exp. 3 and 5). The medium was exchanged for freshly prepared MEM, unsupplemented or supplemented with ascorbic acid and/or iron (20 μmol/l). The iron concentration was chosen to fall in the range of linear uptake (1–100 μM). The integrity of the monolayer was closely followed by optical microscopy during all trials to verify that the cells tolerated the treatments. Triplicates of ascorbic acid treated cells (exp. 5) were washed carefully to remove all extra-cellular ascorbic acid prior to iron incubation experiments. After 2 h of incubation in a rotary shaker (35 rpm) at 37°C, the test solutions were discarded and new medium (MEM unsupplemented) was added. The cells were brought back to the incubator for another 22 h before harvest. Two parallel trials were performed each time, where each trial was exposed to one of the lysis methods described. This procedure was used because of the lability of ascorbic acid, which demanded the use of a suitable lysis buffer.

Harvest of Caco-2 cells for protein analysis

Prior to harvest, the medium was aspirated and the cells were washed in PBS. Lysis was achieved 22 h after the removal of test solutions by treatment with cold RIPA-buffer (Sigma-Aldrich, Sweden) containing EDTA-free protease inhibitor cocktail at 40 μl/ml of lysis buffer (Roche, Switzerland). After this step, all treatments were carried out on ice or by other means in a cold environment. The lysates were divided into aliquots and frozen at −80°C for later analysis. Ferritin analyses were made using a Coat-A-Count ferritin IRMA kit (DPC, Denmark) and counted for 20 min in a CG400 γ-counter (Intertechnique, France). Total cellular protein content was estimated by bicinchoninic acid (BCA) assays (Pierce, Sweden).

Harvest of Caco-2 cells for ascorbic acid analysis

A special lysis method was developed to detect ascorbic acid in the cell lysates. Lysis was obtained by adding a solution of ice cold m-phosphoric acid (3%) and dithiothreitol (0.1%) combined with incubation at 4°C for 10 min. The cells were scraped into Eppendorf tubes and sonicated for 15 min. Unfortunately, the lysis solution interfered with the BCA protein assay. The problem was circumvented by a higher dilution of the samples and by applying a blank sample containing the lysis solution which was subtracted from the measurements.

Ascorbic acid analyses were made using HPLC as described by Margolis et al. [12]. A Jasco system with a Jasco As-2080 Plus pump and autosampler Jasco As-2057 was used. Separation was done using an Aquasil C18, 3 μm column (Thermo Electron Corporation) in phosphate buffer pH 2.8. Electrochemical detection was carried out using a Decade II system (AntecLeyden) and the signals were evaluated using Jasco ChromPass software.

Protein separation and western blot analysis

Samples were added 2x Laemmli sample buffer with β-mercaptoethanol and boiled at 95°C for 5 min to disrupt protein-protein interactions. Samples containing 50 μg of protein were loaded on a precast 12% tris-HCl polyacrylamide gel and electrophoresed with Tris/Glycine/SDS buffer at 80 V, constant voltage. The Precision Plus Protein Dual Color ladder was used as a molecular weight standard. After electrophoresis, the gel was blotted at 350 mA for 30 min constant current to a polyvinyl difluoride (PVDF) membrane pre-soaked in 100% methanol. The gel and membrane were equilibrated in running buffer for 15 min prior to blotting. The proteins of the samples were separated and transferred to a membrane (blotted) in order to stain each sample for human Nramp2 and Dcytb proteins respectively. Samples were normalized against the total protein of each sample. The blotted bands were then relatively quantified on each blot against the band of the control sample. This procedure served as an internal loading control as well as the method for relative quantification of changes in protein expression.

Dcytb staining

After blotting, the PVDF membrane was immersed in TBS and put into blocking solution (Sigma-Aldrich, Sweden) for 45 min at room temperature (RT) to minimize unspecific binding. The membrane was washed in TBS tween 20 for 7 min on a rotary shaker. The blot was then incubated over night at 4°C with primary antibody (Goat α-human Dcytb) in blocking/probing solution at a concentration of 0.1 μg/ml. The next day, the membrane was carefully washed in PBS tween 20 (2 × 7 min) at RT prior to incubation with the secondary antibody. The secondary antibody (α-goat IgG-AP) was added at a concentration of 0.5 μg/ml. After 1 h the membrane was washed in TBS tween 20 (2 × 7 min) and finally dispersed in TBS to eliminate tween residues. The stained blot was developed by 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT) (Sigma-Aldrich, Sweden) for 15 min, and the reaction was stopped by deionised and distilled H_2O (ddH_2O). The blot was scanned with a densitometer (Bio-Rad, densitometer GS800) and

band intensities (reflective density, RD/mm^2) were analysed using Quantity One software (Bio-Rad). The RD of the baseline band (exp.1) was set to 100% and differences between bands were calculated as negative or positive changes from that origin.

Nramp2 staining

Staining for the Nramp2 protein was done using components from an immuno-blot assay kit, except for the first antibody and the probing/blocking buffer. Prior to incubation with the primary antibodies, the membrane was immersed in TBS and then put into blocking solution (Sigma-Aldrich, Sweden) for 45 min at RT and gentle agitation in order to block interactions with the membrane. After blocking, the membrane was washed in TBS tween 20 for 7 min. The membrane was then incubated with primary antibody (rabbit α-human-Nramp2) in blocking/probing solution (Sigma-Aldrich, Sweden) for 1 h at RT. The blot was washed in TBS tween (2 × 7 min) before incubation with secondary antibody (rabbit α-goat IgG-AP), which lasted for 1 h at RT. The stained blot was washed in TBS tween (2 × 7 min) and in TBS (5 min). The color was developed by a three-component AP development buffer for 20 min at RT. The reaction was stopped by agitating the blot in ddH_2O. The blot was scanned with a densitometer (Bio-Rad, densitometer GS800) and band intensities (RD/mm^2) were analysed using Quantity One software (Bio-Rad). The density of the control band (exp.1) was set to 100% and differences between bands were calculated as negative or positive changes from that origin (baseline).

Statistics

Cell experiments were performed in triplicates within trials and every trial was repeated on three different occasions. Calculated values for ferritin and total protein are means of 9 individual samples ± SEM. The baseline ferritin concentration was subtracted from ferritin values within experiments in order to correct differences in iron status between experiments. This was considered to be the correct treatment. However, if a mean value of the baseline from 3 separate trials was to be calculated, all samples would still remain within ±2 SD as all other calculated mean values. Samples from all 3 trials were analysed by electrophoreses and Western Blotting several times to make sure that the Dcytb and Nramp2 expression was consistent. The results for Dcytb and Nramp2 protein expression are presented as percentage reflected density (RD/mm^2) compared to the baseline on each blot. The values are means ± SEM (n = 3). The significance of differences between control samples and/or different treatments was analysed by Student's unpaired t test. Differences were considered to be significant at $P < 0.05$.

Results

Ascorbic acid uptake and ferritin formation

Uptake of ascorbic acid in the absence of iron (exp. 2) resulted in a 19% increase in ferritin formation (0.42 ± 0.06 ng/mg protein after baseline correction, $P < 0.016$) as compared to the ferritin content of the control cells (Fig. 1). The increase in ferritin response when ascorbic acid deficient cells were incubated with iron (20 μmol/l) for 2 h was 160% greater than the ferritin response of control cells as indicated by experiment 4 (3.7 ± 0.59 ng/mg protein, $P < 0.023$).

To study the ferritin response when extracellular ascorbic acid was absent while intracellular ascorbic acid was present, triplicates of cells were pre-incubated with ascorbic acid for 14 h. The medium containing ascorbic acid was removed by aspiration and cells were carefully washed in PBS to eliminate all remaining extracellular ascorbic acid. Iron uptake experiments were then performed with iron (20 μmol/l) for 2 h (exp. 5). Ferritin analyses showed an increase in ferritin formation by 600% (14.67 ± 0.95 ng/mg protein, $P < 0.013$). The results of this experiment (exp. 5) indicated that the effect of ascorbic acid on ferritin formation was not due to the extracellular presence of ascorbic acid.

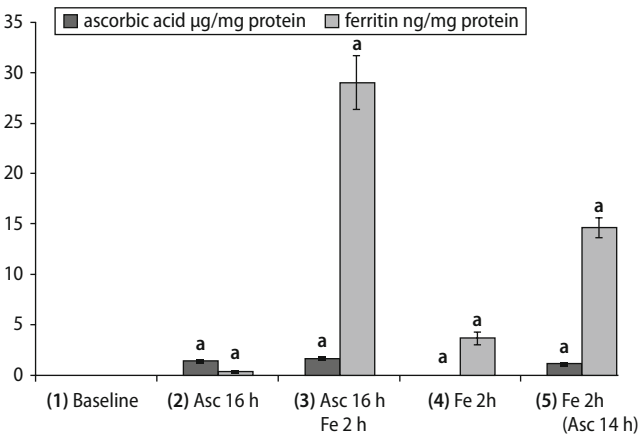

Fig. 1 Intracellular ascorbic acid and ferritin levels in Caco-2 cells. Ascorbic acid and ferritin content of Caco-2 cell lysates as measured 22 h after the uptake experiments. Base line corrected ferritin values are means ± SEM (n = 9). Ascorbic acid content is shown as means ± SEM (n = 3). All values reported are within ±2 SD (95% CI). Differences between iron and/or ascorbic acid treated cells and baseline expression are statistically significant ($P < 0.03$) as indicated by the letter *a*

When the cells were incubated with ascorbic acid (from t = 0–16 h) and iron (from t = 14–16 h) at the same time (exp. 3), the ferritin formation was 1,300% more efficient compared to baseline (29 ± 2.65 ng/mg protein, $P < 0.025$). Thus, about 50% of the increase in iron dependent ferritin formation was caused by the presence of intracellular ascorbic acid only.

Long term Dcytb expression trials

The Western Blot analyses indicate a down-regulation of the Dcytb protein in the Caco-2 cells after exposure to ascorbic acid for 14 and 16 h (Fig. 2). 22 h after the removal of ascorbic acid, the expression level of ascorbic acid treated cells compared to the control cells was down-regulated by 29 ± 2.9%, $P < 0.01$ (exp. 2). When cells were treated with ascorbic acid together with iron or iron alone (both ascorbic acid deficient and fed; exp.3-5), Dcytb expression was down-regulated by 46–50 ± 2.9%, $P < 0.004$. The difference between 3 iron treatments for Dcytb at hour 38 was $P > 0.05$, which is not a significant difference. However, the very similar levels of down-regulation for these 3 combinations coupled with the response to ascorbic acid alone indicated that ascorbic acid and iron effects on Dcytb expression were not synergetic, at least not on a long term basis (exp. 2–5, Fig. 2). In comparison, the effect of iron and ascorbic acid on ferritin formation was clearly synergetic (exp. 2 and 4 compared to 3 and 5, Fig. 1).

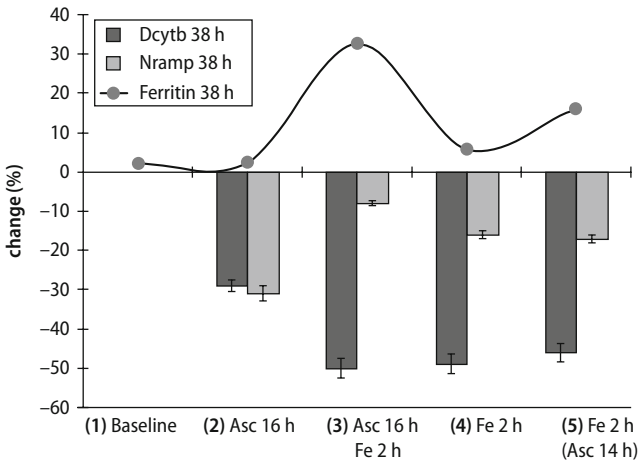

Fig. 2 Down-regulation of Dcytb and Nramp2, 22 h after iron and ascorbic acid uptake trials (study I, exp. 1–5). The cell lysates were analysed by SDS-page and Western Blotting. The values of ferritin up-regulation (in ng/mg protein) were included for comparative reasons. Data are presented as means ± SEM (n = 3). The letter *a* indicates statistically significant differences compared to control data ($P < 0.02$). There were no significant differences in Dcytb expression between the iron treated samples ($P > 0.05$) indicated by the letter *b*

Long term Nramp2 expression trials

22 h after the removal of ascorbic acid, the levels of Nramp2 were diminished by 31 ± 3.5%, $P < 0.013$, as can be seen in experiment 2, Fig. 2. Iron treatment (without simultaneous addition of ascorbic acid, experiments 4 and 5) did not affect Nramp2 expression to the same extent as it affected Dcytb. However, there was a down-regulation by 16–17 ± 2.9% ($P < 0.005$) for cells treated with iron for 2 h, irrespective of their intracellular ascorbic acid status (exp. 4 and 5). An interesting effect, which was first believed to be an artefact, was the much weaker down-regulation of Nramp2 (exp 3) when incubated with ascorbic acid and iron at the same time (8 ± 1.7%, $P < 0.01$). The observed effect might suggest that ascorbic acid could elicit an extracellular response on the expression of Nramp2 not seen for Dcytb.

Short term Dcytb and Nramp2 expression

In an attempt to find clues as to why ferritin expression increases as a result of the presence of ascorbic acid while the expression of uptake proteins decreases, a second study (study II) was conducted to investigate the status of the expression levels at an earlier stage. In this study, Dcytb and Nramp2 expression was studied directly after the uptake experiments (at t = 16 h) instead of waiting another 22 h for ferritin formation to occur. In fact, the results indicated that, immediately after 2 h of iron and 16 h of simultaneous ascorbic acid incubation (exp. 3), the expression of Dcytb was up-regulated by 24 ± 3.5%, $P < 0.02$ compared to the control cells (Fig. 3). An

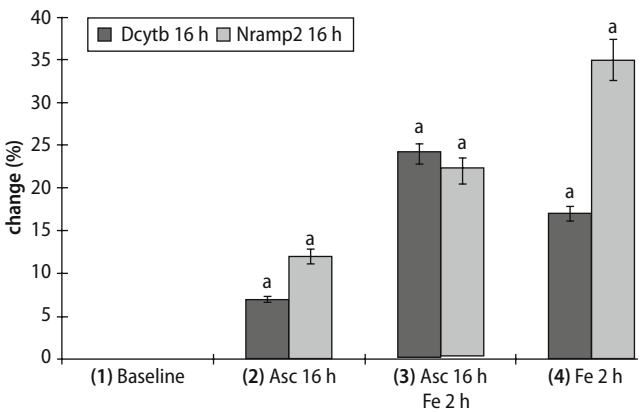

Fig. 3 Short term up-regulation of Nramp2 and Dcytb expression in Caco-2 cells (study II, exp.1–4). Lysis was achieved immediately after the iron uptake experiments (at t = 16). Values are shown as percentage ±SEM of the Nramp2 and Dcytb expression in the control cells (n = 3). The letter *a* indicates that the values reported are statistically significant ($P < 0.03$)

increase in Dcytb expression by iron incubation (exp. 4) was also indicated, although was less pronounced than the effect induced by ascorbic acid in combination with iron (17 ± 2.9%, $P < 0.028$). However, there was also a small increase in Dcytb formation (7 ± 1.7%, $P < 0.016$) when exposed to ascorbic acid alone (exp. 2). This could indicate that the effects of iron and ascorbic acid on short term up-regulation of Dcytb potentiate one another.

Regarding the short term regulation of Nramp2, the blots indicated that Nramp2 expression increased when the protein levels were analysed 22 h earlier than in study I (t = 16 h according to Table 1). After 16 h of ascorbic acid incubation, the Nramp2 level was raised by 12 ± 2.9% ($P < 0.015$) (exp. 2) which was comparative to the rise in Dcytb levels (7 ± 1.7%) which did not differ significantly. When addition of ascorbic acid and iron was combined (exp. 3) the levels of Nramp2 increased by 22 ± 3.5% ($P < 0.0032$), which was similar to the increase in Dcytb expression. However, when the cells were incubated with iron only (exp. 4), the Nramp2 levels increased even more (35 ± 2.9%, $P < 0.007$) and this was not expected. At present, we have no explanation for this increase.

Discussion

There is great interest in the enhancing effects of ascorbic acid on iron bioavailability. The most obvious reason is the importance for human nutrition. In this study we demonstrate that intracellular ascorbic acid status affects the protein levels of ferritin, Nramp2 and Dcytb in Caco-2 cells. These results lead to the conclusion that the influence of intracellular ascorbic acid status on ferritin, Nramp2 and Dcytb formation must be considered in iron uptake studies in Caco-2 cells since this could be a cause of diverging inter-laboratory results. In fact, the effect of ascorbic acid status on ferritin formation is an important result since ferritin formation has previously been shown to correlate with iron absorption, and is widely used as an indirect measurement of iron uptake [9].

In the first part of these studies intracellular ferritin and ascorbic acid levels were measured. The results of ferritin measurements, experiments 1–4 (Fig. 1), correlated with the study of Toth et al. [19] with the exception for experiments with ascorbic acid without iron, where the authors do not consider the measured change in ferritin expression as significant. In the study of Toth et al. the experiments were conducted on human hepatoma and leucemia cells. The experimental conditions of the ascorbic acid trials were comparable with incubation times of 16 h and at 150 μM. In our study, the up-regulation of ferritin expression was 19% when cells were treated with ascorbic acid without iron. The validity of this result is supported by the accompanied change in Nramp2 and Dcytb expression. Unfortunately, we are unable to compare our data from trials with only intracellular ascorbic acid (exp. 5) with literature. According to our knowledge, there are no literature data on intracellular ascorbic acid concentrations during cell trials or studies where the authors have excluded extracellular ascorbic acid while keeping the interior of the cell replete.

To summarize the general changes in long term regulation of Nramp2 and Dcytb expression, the Western Blot analyses indicate a down-regulation of both proteins when exposed to iron for 2 h. This result is consistent with mRNA data on mice [5] and for Nramp2 mRNA data in Caco-2 cells [13]. The expression data also show that there is a significant down-regulation of both Nramp2 and Dcytb when exposed to ascorbic acid without addition of iron. Regarding the short term regulation of Dcytb and Nramp2, the results indicate that Dcytb protein expression is up-regulated in the simultaneous presence of ascorbic acid and iron while the Nramp2 protein expression is enhanced by the presence of iron alone. This interpretation might suggest that the expression of Dcytb is more important than Nramp2 for the increase in iron uptake caused by the presence of ascorbic acid.

The literature reports that long term ascorbic acid supplementation in humans does not show any effect on long term iron status in contrast to single doses of ascorbic acid [3]. One of the reasons for this might be a long term down-regulation of both Dcytb and Nramp2 caused by ascorbic acid uptake. However, a short term up-regulation of both Dcytb and Nramp2 proteins could partially explain why single doses of ascorbic acid improve iron status. This new knowledge about the regulation of iron uptake might be important when planning and evaluating supplementation with ascorbic acid *in vivo* in order to decrease the prevalence of iron deficiency. Further studies must be performed in order to verify the correlation between *ex vivo* and *in vivo* data. The duration of the up-regulation/down-regulation of Dcytb and Nramp2 proteins must be estimated in order to reach the optimal time between administrated doses to achieve a maximal effect on iron status.

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