

Ascorbic Acid Mediated Iron Toxicity in Caco-2 Cells: Effects of Different Iron Species

Bettina Zödl¹, Susanne Humpeler¹, Michaela Zeiner², Claudia Gundacker³, Ilse Steffan², Cem Ekmekcioglu¹

¹Center for Physiology and Pharmacology, Department of Physiology, Medical University of Vienna, Schwarzspanierstr. 17, A-1090 Vienna, Austria

²Department of Analytical Chemistry, University of Vienna, Währingerstrasse 38, A-1090 Vienna, Austria (at time of research)

³Center for Public Health, Dept. Ecotoxicology, Medical University of Vienna, Währinger Strasse 10, A-1090 Vienna, Austria

ABSTRACT

Aim of the presented study was to investigate whether ascorbic acid enhances iron uptake and/or toxicity in Caco-2 cells. Cells were incubated with 1.5 mM of different iron species combined with increasing doses of ascorbic acid (1-3 mM). Thereafter viability, membrane damage, lipid peroxidation, and intracellular iron concentrations were measured. No effects on iron-induced damage to cells were found regarding viability. Lipid peroxidation significantly increased when ascorbic acid was added to cells incubated with FeSO₄. In contrast lactate dehydrogenase (LDH)-release decreased by addition of ascorbic acid in Caco-2 cells treated with FeCl₃: nitrilotriacetic acid (NTA) and FeCl₃: citric acid. Iron uptake was enhanced by ascorbic acid, when FeCl₃: citric acid (1:2) or FeSO₄ were used. We suggested that, especially dependent on the iron species used, a combination of ascorbic acid and iron favours lipid peroxidation in Caco-2 cells. But on the other hand we also found beneficial effects of this type of co-treatment, like an enhanced iron uptake and decreased plasma membrane damage.

Keywords: oxidative stress, lipid peroxidation, ascorbic acid, intestine, membrane damage

ABBREVIATIONS

LDH : Lactate dehydrogenase
MDA : Malondialdehyde

ROS : Reactive oxygen species
TBA : Thiobarbituric acid

INTRODUCTION

In a healthy organism iron is an essential micronutrient which is involved in oxygen transport and energy metabolism. However there are diseases like hereditary hemochromatosis, a very common autosomal recessive disorder with an inappropriately increased iron absorption leading to iron loading of parenchymal cells in liver, pancreas, heart and other organs, with resultant damage of their structure and impairment of their function (Limdi and Crampton 2004). Free iron is detrimental to cells, as it is involved in forming reactive oxygen species (ROS) and therefore in inducing oxidative stress (Nunez et al., 2001, Crichton et al., 2002, Zödl et al., 2004). Regarding hemochromatosis, which is caused by a mutation in the HFE gene, it was shown that iron caused oxidative damage in the colon of HFE-knockout mice, indicating detrimental effects of iron overload on intestinal cells in vivo (Stevens et al., 2003).

Ascorbic acid and ascorbate on the one hand acts as an antioxidative, scavenging ROS in the cytosol of cells (Ozaki et al., 1995, Chaudiere and Ferrari-Iliou 1999). On the other hand it can reduce Fe³⁺ to Fe²⁺, which is the more catalytically active metal ion in the contribution of oxidative stress by the formation of free radi-

cals in the Fenton reaction (Carr and Frei 1999).

Previous papers postulated an ascorbic acid dependent effect on iron mediated cytotoxicity (Courtois et al., 2000) and iron uptake (Garcia et al., 1996, Glahn et al., 1998, Garcia-Casal et al., 2000). FeCl₃ uptake was enhanced 8 fold in human intestinal Caco-2 cells, when iron was complexed to ascorbic acid in a molar ratio of 1:20 compared to FeCl₃ complexed either to citrate or nitrilotriacetic acid (NTA) (Glahn et al., 1998). Additionally, lipid peroxidation, initiated by free radicals, was promoted when Caco-2 cells were incubated with Fe²⁺-ascorbate (molar ratio 1:10) and not by iron or ascorbate alone (Courtois et al., 2000). In healthy volunteers oxidative DNA damage products in white blood cells were elevated in the group with the higher mean initial level of plasma ascorbic acid after co-supplementation of FeSO₄ and ascorbate (Rehman et al., 1998).

We were especially interested in the effects of ascorbic acid in a situation where iron per se is already toxic. For that we used the relatively high iron concentration of 1.5 mM, which showed significant detrimental effects to Caco-2 cells in preliminary tests as well as in our previous study (Zödl et al., 2004).

First aim of the presented study was to

investigate the effect of increasing amounts of ascorbic acid on iron uptake and to find out whether it exerts pro- or antioxidant properties on highly iron burdened cells. Additionally we investigated if different iron species cause different results. Therefore we treated confluent (differentiating) Caco-2 cells with FeCl_3 :citric acid (molar ratio 1:2) and FeSO_4 as physiologically iron forms as well as with FeCl_3 :NTA (molar ratio 1:2), which is a form of iron that is normally not present in the human gut (Beard 2000), but used in many in vitro studies describing toxicity and uptake of iron (Nunez et al., 2001, Zodl et al., 2004).

Materials and Methods

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville MD) and were used between passage 20 and 39. The cells were maintained in 175 cm² flasks in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 20 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin G and 100 µg/mL streptomycin. After being nearly confluent, the cells were washed with phosphate buffered saline to remove any unattached cells. The remaining cells were then harvested using a trypsin/EDTA solution and seeded in 24 well plates at a density of approximately 250000/mL. Postconfluent (8 day old) cells were incubated for 22 hrs in the culture medium containing 1.5 mM of different species of iron and/or increasing amounts of ascorbic acid. Cytotoxicological assessments and iron uptake were carried out on the cells and/or culture medium using different markers of cellular damage.

Preparation of iron/ ascorbic acid solutions

Different species of iron (see below) with or without increasing amounts of ascorbic acid were dissolved in 0.1 N HCl or (if they were insoluble in 0.1 N HCl) in deionized water [FeCl_3 :NTA (1:2)] and sterilized using a 0.22 µm filter. The solutions as well as the controls were diluted with the medium (1 in 20) to obtain a final iron concentration of 1.5 mM and 0, 1, 2, 3 mM of ascorbic acid.

Iron species

- Ferric chloride (FeCl_3) and nitrilotri-

acetic acid (NTA) (1:2)

- Ferric chloride (FeCl_3) and citric acid (1:2)
- Ferrous sulphate (FeSO_4)

Controls

- 0.1 N HCl
- Deionized water

HCl, NTA, FeCl_3 and all other chemicals for cell culture were obtained from Sigma, (Austria, Vienna); tissue flasks, filters and wells from Corning, (Austria, Vienna); citric acid, ascorbic acid as well as FeSO_4 from Merck, (Austria, Vienna).

Viability Index

After collection of the cells with trypsin/EDTA, cells were counted by using a hemocytometer. Viability index was calculated as the ratio of living cells to the total number of cells according to the trypan blue exclusion method (JRH Biosciences, Austria). Dead cells were detected by a blue staining counted in a microscope (Reichert, Austria).

LDH-Release

LDH-activity was measured in an aliquot of the culture medium according to the method of Guzzie (Guzzie 1994). The assay is based on oxidation of NADH (Sigma, Austria) in the presence of pyruvate (Sigma, Austria). The change in absorbance at 340 nm was measured using a Hitachi U-2000 spectrophotometer (Inula, Austria).

Lipid peroxidation

Polyunsaturated fatty acids generate malondialdehyde (MDA) upon oxidative decomposition. The MDA produced in cultured cells reacts with thiobarbituric acid (TBA) to form a pink chromogen (MDA:TBA adduct), which can be separated from other interfering substances on a reversed phase HPLC (BioRad, Austria; column: C18, Supelco, Austria). After elution, the MDA:TBA adduct is quantified via calibration curve produced by tetramethoxypropane (Sigma, Austria), which undergoes hydrolysis to MDA under the same test conditions. The MDA-TBA adduct was then detected using a UV/VIS detector (BioRad, Austria) at 532 nm. Measurement of MDA-concentration was

modified according to the method of Wong et al., (Wong et al., 1987). Limit of detection was 2.6 nM.

Intracellular iron

Cellular iron uptake was measured using electrothermal atomic absorption spectrometry (ET-AAS) with Zeeman background correction (Perkin Elmer 4100 ZL, Austria). Cells were lysed and completely dissolved in DMSO (Sigma, Austria). A volume of 20 μ L of each solution was introduced to the graphite tube. All measurements were performed in triplicate. ET-AAS determination of iron in the organic solvent was optimized establishing the proper temperature program and applying the standard addition method. Optimized temperature program was: 600°C for preashing, 1200°C for pretreatment, 2100°C for atomization (Zeiner et al., 2005). Limit of Detection was under of 1.3 μ g/L.

Statistical analysis

Results are presented as mean \pm SEM (Standard Error of the Mean). Statistical analysis to compare co-treatment of increasing amounts of ascorbic acid and 1.5 mM of each species of iron in the culture medium was performed using univariate ANOVA with Dunnett Post Hoc test or for lipid peroxidation with t-test. Malondialdehyde values below the detection limit were calculated by linear extrapolation of the peak area. A p-value less than 5% ($p < 0.05$) was considered significant. The number of cases was $n = 6-12$ for each concentration of iron and/or ascorbic acid.

Results

Cytotoxicological assessment

Cell viability assessments showed no reduction in the presence of increasing amounts of ascorbic acid co-treated with different species of iron. (Figure 1)

Plasma membrane damage in Caco-2 cells treated with 1.5 mM of iron decreased by addition of increasing amounts of ascorbic acid (as assessed by LDH-release). Significant reduction in LDH-release was found in cells treated with FeCl₃:NTA (1:2) and 2 mM and more of ascorbic acid ($p=0.006$). The least (significant) damage to the plasmamembrane among all iron species tested was found in cells incubated with FeCl₃:citric acid (1:2) and 1 mM or above

of ascorbic acid ($p < 0.001$). Severe membrane damage was found in cells treated with 1.5 mM of FeCl₃:NTA (1:2) alone. (Fig. 2)

An unexpectedly high (about 22 fold) significant increase of lipid peroxidation was detected after incubation with ascorbic acid and FeSO₄. (Figure 3) Lipid peroxidation increased up to 45% when the cells were treated with 1.5 mM of FeCl₃:citric acid (1:2) and ascorbic acid and up to 35% after incubation with 1.5 mM of FeCl₃:NTA (1:2) and ascorbic acid. MDA levels in Caco-2 cells treated with iron, except FeSO₄, were much higher than in controls. (Fig. 3)

Intracellular iron uptake

Iron uptake was enhanced by ascorbic acid in the presence of all species of iron tested (significant with FeCl₃:citric acid and FeSO₄). Enhancement of iron uptake was dose dependent with increasing amounts of ascorbic acid as shown with FeCl₃:citric acid and FeSO₄. Lowest intracellular iron concentration (21.56 μ g/g) was found when the cells were incubated with 1.5 mM FeSO₄. Iron uptake increased about 2

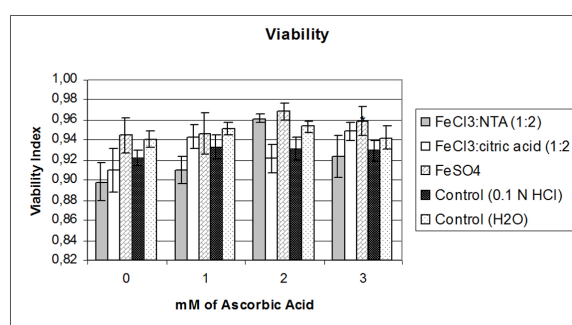


Figure 1. Viability (trypan blue exclusion) in Caco-2 cells after exposure to 1.5 mM of iron and increasing amounts of ascorbic acid (mean \pm SEM). Differences in mean values between 0 mM and increasing amounts of ascorbic acid within the different iron species were not significant.

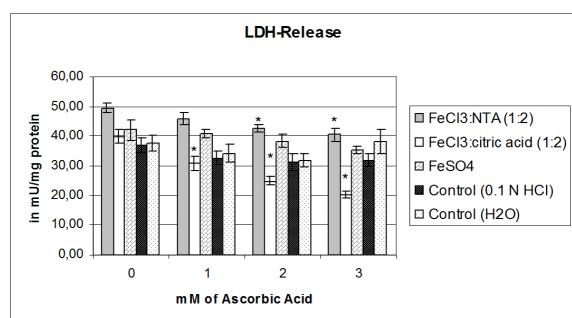


Figure 2. LDH-release in Caco-2 cells after exposure to 1.5 mM of iron and increasing amounts of ascorbic acid. Values are indicated as mUnit/ mg Protein (mean \pm SEM).

* Significantly different from 0 mM ascorbic acid.

fold after treatment with 1.5 mM FeCl₃:citric acid (1:2) or FeSO₄ and 3 mM ascorbic acid ($p < 0.01$). The lowest, not dose dependent and not significant, enhancement of iron uptake due to co-administration with ascorbic acid was found in cells incubated with FeCl₃:NTA (1:2) (about 1.2 fold in addition with 1 mM ascorbic acid). (Figure 4)

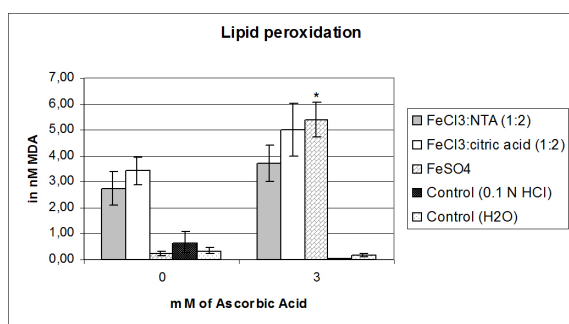


Figure 3. Lipid peroxidation in Caco-2 cells after exposure to 1.5 mM of iron and increasing amounts of ascorbic acid. Values are indicated as nM MDA (mean \pm SEM).

*Significantly different from 0 mM ascorbic acid.

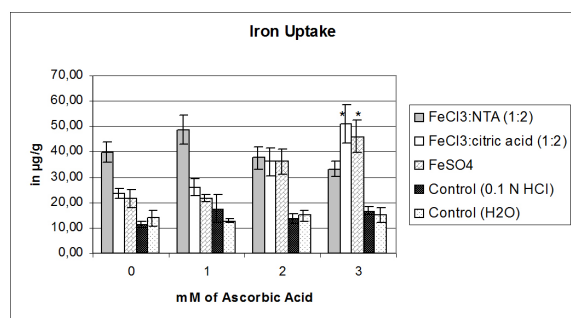


Figure 4. Intracellular iron uptake in Caco-2 cells after exposure to 1.5 mM of iron and increasing amounts of ascorbic acid (mean \pm SEM).

*Significantly different from 0 mM ascorbic acid.

Discussion

Increasing evidence has demonstrated the important impact of metal-mediated oxidative stress on human health. Significant increases in markers of oxidative stress (TBARS and protein carbonyls) were found in Fe-depleted female WKY rats after feeding with 8 mg FeSO₄ alone or especially in the presence of 24 mg ascorbic acid (Srigiridhar and Nair 2000). However it has to be mentioned that rats possibly are not an ideal model of studying effects of ascorbic acid as they can produce ascorbic acid endogenously (Carr and Frei 1999). Therefore various cell lines were used to study oxidative stress by iron and ascorbate (Rego and Oliveira 1995,

Bernotti et al., 2003). In our study lipid peroxidation increased about 22 fold in Caco-2 cells after addition of 3 mM ascorbic acid to 1.5 mM of FeSO₄. Additionally a moderate increase in the MDA concentrations of the Caco-2 cells was found after co-treatment with ascorbic acid and 1.5 mM FeCl₃:NTA (1:2) (up to 35 %) or with 1.5 mM FeCl₃:citric acid (1:2) (up to 45%). Lower increase of MDA concentrations might be due to the valency of the iron.

An increase of TBARS, including MDA, by Fe²⁺/ascorbate was also shown in fully differentiated Caco-2 cells as well as in primary cultures of chick retinal cells (Rego and Oliveira 1995, Bernotti et al., 2003). These authors used iron doses in the physiologically range. Additionally Courtois et al., exposed differentiated Caco-2 cells to iron and ascorbate (0.2 mM each) and found a fourfold increase of MDA levels (Courtois et al., 2002). However in contrast to our study others who described Fe²⁺/ascorbate induced lipid peroxidation, did not investigated the effects of iron alone (Rego and Oliveira 1995, Courtois et al., 2002, Bernotti et al., 2003).

Viability seem to be a much less sensitive biomarker of toxicity of co-treatment of ascorbic acid and iron than the raise of TBARS. For example lipid peroxidation was induced by iron/ascorbate but no effects were found regarding cell viability or morphology of mice hepatocytes (Rush et al., 1985). In the presented study as well, no effects of co-administration of iron and ascorbic acid on viability of Caco-2 cells were detected. Similar results have been found when postconfluent Caco-2 cells were incubated with Fe²⁺-ascorbate (0.2 mM/2 mM) whereby DNA and protein content as well as cell viability were not affected at all (Courtois et al., 2000).

Surprisingly we found a clear decrease of LDH-release, which is a parameter for cell membrane damage, after treatment of Caco-2 cells with iron/ascorbic acid although this combination had induced lipid peroxidation. This might be probably due to the different fatty acid composition of membranes as polar lipids are more susceptible to lipid peroxidation than are neutral lipids (Catala and Cerruti 1997). Differences in lipid peroxidation between biomembranes of different intracellular compartments (Catala and Cerruti 1997) or tissues (Pushpendran et al., 1998, Gutierrez et al., 2002) were shown previously. It might be possible that intracellular

biomembranes (like mitochondrial) are affected earlier by iron/ ascorbic acid induced lipid peroxidation than the plasma membrane. In the meantime the anti-oxidant properties of ascorbic acid could protect the plasma membrane. LDH-release decreased in a concentration dependent manner with increasing amounts of ascorbic acid. This observation is not in accordance with previous results (Rego and Oliveira 1995, Cardoso et al., 1999). Since a loss of membrane integrity, as assessed by an increase of LDH-leakage, was observed in peroxidized synaptosomes from rat brain cortex homogenates treated with iron-ascorbate (Cardoso et al., 1999) as well as in primary chick retinal cells (Rego and Oliveira 1995). However these authors used lower iron doses and there is lack of data regarding effects of iron on membrane integrity without ascorbate (Rego and Oliveira 1995, Cardoso et al., 1999).

Our findings showed that iron uptake was improved by ascorbic acid in Caco-2 cells as also shown by others (Garcia et al., 1996, Glahn et al., 1998, Garcia-Casal et al., 2000). On the one hand the valency of iron seems to have no effect on ascorbic acid stimulated iron uptake, possibly due to the reducing properties of this vitamin (Garcia et al., 1996, Glahn et al., 1998, Garcia-Casal et al., 2000). On the other hand the binding of the iron seems to be quite important as shown with FeCl_3 :NTA and FeCl_3 :citric acid. We suggest the higher the iron

is complexed the lower is the increase in iron uptake due to ascorbic acid. Increasing amounts of ascorbic acid enhanced intracellular iron uptake in a dose dependent manner only if the cells were treated with the physiologically iron forms (FeCl_3 :citric acid or FeSO_4).

In conclusion we suggest that administration of ascorbic acid together with iron might have membrane protecting properties although this combination obviously favours oxidative stress. These effects might be linked very much to the complexing agent of the iron. Additionally lipid peroxidation depends strongly on the valency of the iron. This finding might have practical relevance to therapeutic treatment regarding intoxication via accidental ingested iron or via diseases like hemochromatosis. FeSO_4 and ascorbic acid highly favours lipid peroxidation, whereas other combinations do not have such strong effects. This could have also relevance for nutritional supplements or iron therapy. Nevertheless further studies regarding ascorbic acid mediated effects with lower doses of iron and other iron forms are necessary to draw a conclusion to a dietary recommendation.

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