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Lactic Acid Decreases Fe(II) and Fe(III) Retention but Increases Fe(III) Transepithelial Transfer by Caco-2 Cells

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Lactic acid (LA) has been proposed to be an enhancer for dietary iron absorption, but contradictory results have also been reported. In the present study, fully differentiated Caco-2 cell monolayers were used to evaluate the effects of LA (1−50 mmol/L) on the cellular retention and transepithelial transport of soluble non-heme iron (as ferric nitrilotriacetate). Our data revealed a linear decline in Fe(III) retention with respect to the concentration of LA added. In the presence of 50 mmol/L LA, retention of Fe(III) and Fe(II) decreased 57% and 58%, respectively. In contrast, transfer of Fe(III) across the cell monolayer was doubled, while Fe(II) transfer across the cell monolayer decreased 35%. We conclude that LA reduces cellular retention and transepithelial transport of Fe(II) by Caco-2 cells in a dose-dependent manner. However, while LA also reduces retention of Fe(III) by Caco-2 cells, the transfer of Fe(III) across cell monolayers is enhanced, possibly due to effects on paracellular transport.

KEYWORDS: Lactic acid; ascorbic acid; iron absorption; Caco-2 cells

INTRODUCTION

Iron is an essential element in man. It provides the oxygen-carrying capacity of heme and acts as a cofactor in many enzymatic reactions involved in the mitochondrial respiratory chain. Iron homeostasis is strictly regulated by duodenal absorption since active excretion pathways do not exist. Various dietary factors are considered to modify iron absorption by influencing the valence state of iron and/or its solubility at physiological pH or by competing with iron for uptake by the enterocyte. Examples of dietary modifiers include ascorbic acid, phytate, certain organic acids, and polyphenols (*1*−*7*). Lactic acid (LA) is a widely used food acidulate and preservative that occurs in some fruits such as apple, pear, and grape. Apart from its presence in foods, LA is also commonly used in pharmaceutical applications as an acidulate. It is the major end product in LA-fermented foods and has been suggested to contribute to the improved iron bioavailability in such foods by forming soluble iron ligands and lowering pH (*8*, *9*). However, in a human study by Baynes et al. (*10*), LA did not affect iron absorption. In studies of the effects of dietary factors on iron absorption, several aspects such as valency, solubility, pH, oxidation, chelation, and interactions between iron and other food compounds must be considered (*11*). This is especially true for organic acids that can chelate both Fe(III) and Fe(II) in soluble complexes via carboxyl and hydroxyl groups at neutral pH. Previous work in our laboratory indicated that increased iron absorption from $FeCl_3$ in the presence of organic acids may be a result of their solubilizing and pH-lowering properties (*12*, *13*). Whether the iron from its soluble complex is easily taken up by the brush border or there is direct influence exerted by LA on iron uptake has not yet been clarified. This is of interest since the pH microclimate close to the epithelial cells at the duodenal mucosa has been suggested to be stable and independent of pH changes in the lumen (*14*). The present work was designed to study the influence of LA on the cellular retention and transepithelial transfer of iron at near-physiological conditions, apart from its influence on improved iron solubility due to soluble complex formation and pH-lowering effects. To this end, the influence of LA on Caco-2 cell iron transport properties was characterized.

MATERIALS AND METHODS

Reagents. All reagents were from GTF (Göteborg, Sweden) unless otherwise noticed. The radioisotope $^{55}FeCl_3$ was purchased from Perkin-Elmer (Billerica, MA).

Caco-2 Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used for experiments at passages 20−35. Stock cultures were maintained in Dulbecco's modified α essential medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/L penicillin G, and 100 mg/L streptomycin at 37 °C in a humidified atmosphere of 95% air−

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5% CO_2. The growth medium was changed every second to third day. Cells were split at ~80% confluence using 0.5 g/L trypsin with 0.5 mmol/L EDTA in Dulbecco's phosphate-buffered saline (PBS).

Prior to the experiments, 50000 cells in 0.5 mL of supplemented DMEM were seeded on 0.4 μm microporous polycarbonate membrane inserts (1 cm^2 Transwell inserts; Corning, Acton, MA). The basolateral chamber contained 1.5 mL of supplemented DMEM. The medium on both sides of the filter insert was changed every 2−3 days. Transepithelial electrical resistance (TEER) was monitored to assess the formation of a tight cell monolayer, and the formation of a fully differentiated cell monolayer was typically established by day 10 (data not shown). All iron retention and transfer experiments were performed 22 days postseeding.

Assay for Cellular ^{55}Fe Retention and Transfer across Monolayers. Fresh supplemented DMEM was provided to the cells 1 day prior to the retention and transfer assays. To study Fe(III) and Fe(II) retention and transepithelial transfer by Caco-2 cells, sample solutions containing various concentrations of LA with or without ascorbic acid (AA) (0.2 mmol/L) were freshly prepared in uptake buffer (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $MgSO_4$, 5 mmol/L glucose, 50 mmol/L HEPES, pH 7), according to Han et al. (*15*). The uptake buffer containing LA and/or AA was adjusted to pH 7 with 1 mol/L NaOH before addition of $^{55}Fe^{III}(NTA)_4$ (pH 7) (NTA = nitrilotriacetate) to a final concentration of 10 μmol/L. Sample solutions in volumes of 0.5 mL were placed on the apical side of Caco-2 cells, while the basolateral chamber contained 1.5 mL of DMEM. Cells were incubated at 37 °C in a humidified atmosphere of 95% air−5% CO_2. Transfer of iron across the cell monolayer was monitored by withdrawing a 0.2 mL aliquot from the basolateral chamber every hour, and this volume was immediately replaced by an equivalent amount of DMEM. All transfer rates were found to be linear from 1 to 4 h on the basis of linear regression analysis ($P \leq 0.05$). After a 4 h incubation, the cells were washed three times with ice-cold wash buffer (150 mmol/L NaCl, 10 mmol/L HEPES, 1 mmol/L EDTA, pH 7) and homogenized in PBS with 1 mmol/L EDTA and 0.2% Triton X-100 added. ^{55}Fe transferred to the basolateral chamber or associated with the Caco-2 cell lysates was measured by liquid scintillation counting; protein determinations were accomplished by the Bradford assay (Bio-Rad, Sundbyberg, Sweden). The integrity of the cell monolayers was monitored before and after the assays by measuring TEER, and leaky cell monolayers (with reduced TEER value) were discarded. On the basis of LA levels measured for fermented food in our previous study (*16*), and in consideration of a 2−3-fold dilution of the food by gastric juice and intestinal secretions, physiological concentrations of up to 80 mmol/L LA may be found in the intestinal lumen. Preliminary studies indicated that concentrations of LA higher than 100 mmol/L in the in vitro experiments were problematic, since TEER values demonstrated a loss of monolayer integrity. Thus, concentrations of LA (1−50 mmol/L) were tested in this study.

Determination of the Valence State of Iron in the Test Solution. The valence state of iron in the sample solutions was determined as previously described (*17*). Briefly, test solutions were incubated with $Fe^{III}(NTA)_4$ in uptake buffer as described above for the transport assay. After a 1.5 h incubation, a 10 min colorimetric reaction of 0.95 mL samples with 0.05 mL of 3.34 mg/mL bathophenathrolinesulfonate (BPS) was carried out to determine the formation of Fe^{II}(BPS) spectrophotometrically at 535 nm.

Statistics. All variables were tested in triplicate within experiments that were repeated on at least two different occasions. Values presented are means ± SD. Effects from treatment were analyzed by ANOVA using the Tukey post-hoc test using SYSTAT software (SYSTAT Inc., Evanston, IL). Differences are considered significant if $P \leq 0.05$.

RESULTS

Lactic Acid Does Not Affect the Valence States of Iron in the Sample Solutions. When the effect of LA on both Fe(III) and Fe(II) absorption by Caco-2 cells was examined, the valency of iron in the test solution was determined spectrophotometrically by the formation of Fe^{II}(BPS). As shown in **Table 1**, Fe(III) was reduced to Fe(II) in the presence of 0.2 mmol/L AA.

Table 1. LA Does Not Influence Reduction of Iron by AA As Indicated by Formation of Fe^{II}(BPS)[a]

	AA (0.2 mmol/L)/LA (50 mmol/L)			
	–/–	–/+	+/–	+/+
absorbance (535 nm)	0.012 ± 0.000	0.013 ± 0.001	0.220 ± 0.011[b]	0.240 ± 0.019[b]

[a] Data are means ± SD of two identical experiments. Uptake solution containing 10 μmol/L $Fe(NTA)_4$, with (+) or without (–) 0.2 mmol/L AA and/or 50 mmol/L LA was adjusted to pH 7 and incubated for 1.5 h. Reduction of iron was determined at $OD_{535\ nm}$ after a 10 min colorimetric reaction with BPS. [b] Significant difference ($P \leq 0.05$).

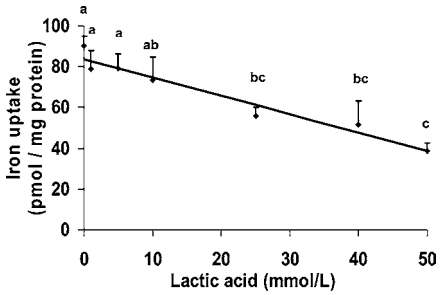

Figure 1. Effect of LA on retention of Fe(III) by Caco-2 cell monolayers. Uptake solutions containing 10 μmol/L $^{55}Fe(NTA)_4$ and various concentrations of LA were adjusted to pH 7 for iron uptake studies using fully differentiated (22 days postconfluent) cells. After a 4 h incubation, nonspecifically bound iron was washed from the apical membrane, and the amount of ^{55}Fe assimilated by the cells was quantified by liquid scintillation. Cell-associated radioactivity was normalized to the protein content measured for each insert. Data are means ± SD of three wells from one experiment representative of similar experiments performed on at least three occasions. Measurements without common letters are considered significantly different ($P \leq 0.05$). The regression line indicates the inverse linear correlation between Fe(III) retention and concentration of LA ($R^2 = 0.95$).

The ability of AA to reduce iron was not affected by the presence of 50 mmol/L LA ($P \leq 0.05$).

Effect of LA on Cellular Retention and Transepithelial Transport of Fe(III). The dose response of iron retention to LA (at concentrations of 1, 5, 10, 25, 40, and 50 mmol/L) was evaluated on fully differentiated Caco-2 cells (**Figure 1**). After a 4 h incubation with 10 μmol/L $^{55}Fe(NTA)_4$, 25, 40, and 50 mmol/L LA significantly reduced iron retention from 90 to 56, 52, and 38.5 pmol/mg of protein, respectively. Regression analysis revealed an inverse linear correlation between the level of assimilated iron in Caco-2 cells and the concentration of LA ($P \leq 0.05$).

Transfer of iron across the Caco-2 cell monolayer was also measured in the presence of LA (**Figure 2**). Transport rates were calculated on the basis of linear regression ($P \leq 0.05$) as shown in the inset. Surprisingly, while the cell content of iron was reduced by 57% in uptake buffer containing 50 mmol/L LA, the amount of iron transferred across the cell monolayer was doubled (47.4 versus 22.8 pmol/mg of protein).

Effect of LA on Cellular Retention and Transepithelial Transport of Fe(II). To study the influence of LA on ferrous iron retention and transport, assays were performed in the presence of 0.2 mmol/L AA (**Figures 3** and **4**). Under these conditions, LA does not interfere with the reduction of Fe(II) by AA as shown in **Table 1**. In the absence of LA, addition of AA increased cellular iron retention 3-fold (**Figure 3**) and nearly doubled the transport rate across the Caco-2 cell monolayer. In

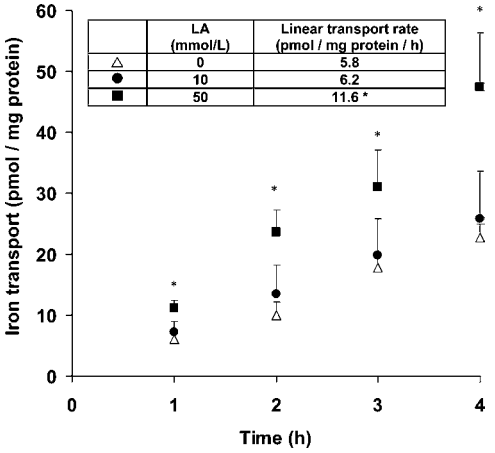

Figure 2. Effect of LA on transepithelial transport of Fe(III) across Caco-2 cell monolayers. Uptake solutions containing 10 μmol/L ^{55}Fe(NTA)$_4$ and various concentrations of LA were adjusted to pH 7, and transepithelial transport was determined by incubating fully differentiated Caco-2 cells for 4 h. Transported iron was monitored every hour by withdrawing 0.2 mL aliquots from the basolateral chamber as described in the Materials and Methods. ^{55}Fe in the aliquots was quantified by liquid scintillation. Transport was normalized to the protein content measured for each insert. All transport measurements fit well to linear regressions within the 4 h time frame, and the transport rates were calculated and are shown in the table. The asterisks indicate that each value of transported iron and the transport rate from 50 mmol/L LA are significantly different from those without LA. Data are means ± SD of three wells from one representative experiment repeated at least twice.

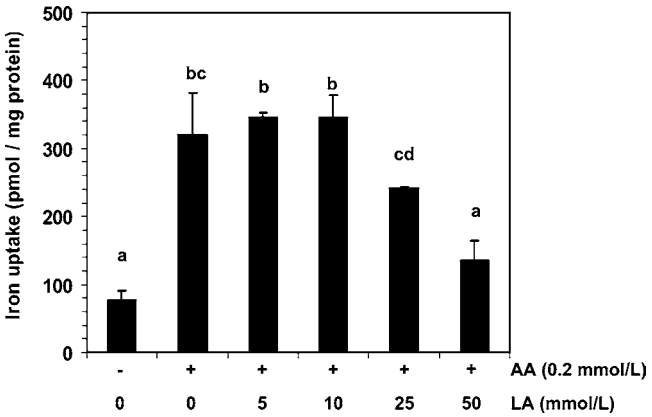

Figure 3. Effect of LA on retention of Fe(II) by Caco-2 cell monolayers. Uptake solutions containing 10 μmol/L ^{55}Fe(NTA)$_4$ with or without 0.2 mmol/L ascorbic acid and various concentrations of LA were adjusted to pH 7 and then incubated with fully differentiated Caco-2 cells for 4 h. After removal of nonspecifically bound iron on the apical membrane, the cell-associated ^{55}Fe was quantified by liquid scintillation. Retention was normalized by the protein content for each insert, and the data shown are the means ± SD of three wells from one representative experiment that was repeated at least three times. Measurements without common letters are considered significantly different ($P \leq 0.05$).

the experiments shown, ferrous iron retention and transepithelial transfer increased from 76 to 321 pmol/mg of protein and from 5.9 to 11.7 pmol/(mg of protein h), respectively. However, under these conditions the cellular retention of iron was inhibited by the presence of LA (**Figure 3**). LA at concentrations of 25 and 50 mmol/L significantly decreased the amount of assimilated ^{55}Fe by Caco-2 cells by 25% and 58%, respectively. LA also inhibited Fe(II) transfer across the cell monolayer (**Figure 4**). Addition of 25 and 50 mmol/L LA resulted in ~12% and 35%

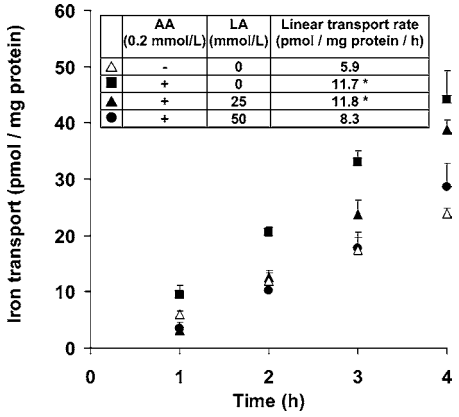

Figure 4. Effect of LA on transepithelial transport of Fe(II) across Caco-2 cell monolayers. Uptake solutions containing 10 μmol/L ^{55}Fe(NTA)$_4$ with or without 0.2 mmol/L ascorbic acid and various concentrations of LA were adjusted to pH 7 and incubated with fully differentiated Caco-2 cells for 4 h. Transported iron was monitored every hour by withdrawing 0.2 mL aliquots from the basolateral chamber as described in the Materials and Methods, and ^{55}Fe in these aliquots was quantified by liquid scintillation. Transport was normalized by the protein content for each insert. All transport measurements fit well to linear regression within the 4 h time frame ($P \leq 0.05$), and the calculated transport rates are shown in the inset. Data are means ± SD of three wells from one representative experiment repeated at least twice.

decrements in the amount of ^{55}Fe(II) transferred across the cell insert at 4 h, respectively, although the effect of 25 mmol/L LA did not achieve statistical significance ($P \leq 0.05$). Noticeably, the enhancement of iron assimilation and transport across the Caco-2 cells in the presence of 0.2 mmol/L AA was reversed by addition of 50 mmol/L LA ($P \leq 0.05$).

DISCUSSION

The results of our study demonstrate that LA inhibits the retention of both Fe(II) and Fe(III) by Caco-2 cell monolayers. It has been reported that reduction of Fe(III), mediated by either AA or a cellular ferrireductase, is necessary for its uptake by Caco-2 cells (*15*, *18*). The effect of LA on cellular retention of Fe(III) is unlikely to be due to interference of the reduction of Fe(III) to Fe(II) since our study shows that LA does not affect iron valency under the experimental conditions employed in our assay system. We speculate that the reduced amount of Fe(III) retained by Caco-2 cells in the presence of LA may be due to direct inhibition of the uptake of Fe(II). This model is supported by the results showing that LA inhibited retention of Fe(III) and Fe(II) by Caco-2 cell monolayers in a similar fashion. For example, we found that 50 mmol/L LA decreased the amount of Fe(III) and Fe(II) associated with cell monolayers by 58% and 57%, respectively.

Studies on citric acid suggested the possibility that iron-chelating ligands act as iron uptake inhibitors by competing with absorptive proteins on the apical membrane (*19*, *20*). In fact, using a different approach, Narasinga Rao and Subba Rao (*7*) found decreased uptake was associated with an increased ligand (citric acid)/iron molar ratio. A simple explanation for our data is that LA has a similar influence on the uptake of iron by Caco-2 cells. However, Gorman and Clydesdale (*21*) showed that the kinetics of iron transfer to apotransferrin at pH 7.5 from lactate and citrate (at an iron:acid ratio of 1:1 and a concentration of 0.1 mol/L) are quite different, suggesting that further work is necessary to explore this model.

It is also possible that the decreased iron uptake observed in the presence of LA might be a consequence of its influence on the pathway of iron uptake. Transport of LA across the enterocyte membrane has been suggested to involve an anion antiport system and a proton cotransporter (*22*). It has been reported that uptake of iron by Caco-2 cells involves a proton-dependent mechanism, accompanied by intracellular acidification (*23*). Therefore, the decrease in iron uptake observed in our study may be due to a change of proton efflux stimulated by LA. Divalent transporter 1 (DMT1) is a proton-coupled Fe(II) transporter that is expressed by Caco-2 cells and thought to be the major mediator of intestinal iron absorption. Preliminary Western blot data indicated that LA does not directly influence the total amount of Caco-2 cell DMT1, but it is possible that LA may alter the functional activity of this protein.

In contrast to the inhibitory effect of 50 mmol/L LA on the transfer of Fe(II) across the Caco-2 monolayer, transfer of Fe(III) was stimulated. One speculative explanation for the effect is that paracellular iron transport by Caco-2 cells may be altered by LA. Iron has been reported to stimulate LA-induced hyperpermeability of Caco-2 cells by increasing reactive oxygen metabolite-mediated damage (*24*). Droke et al. (*25*) also suggested that some fatty acids may improve iron absorption by interfering with paracellular transport. However, in the latter study both iron retention and transepithelial transport by Caco-2 cell monolayers were enhanced. TEER did not decline during incubation of the cells with LA up to 50 mmol/L (data not shown). However, we noticed a tendency of TEER to be reduced for cell monolayers incubated with 100 mmol/L LA. It is worth mentioning that a 0.2-fold increase of TEER was normally observed directly after addition of LA, suggesting that a more critical evaluation of cell integrity based on TEER measurements may be necessary in the presence of organic acids. An alternative hypothesis may be that LA influences cell signaling to stimulate the exit of Fe(III) from cells. However, relatively little is known about how luminal factors may influence the level and activity of the basolateral iron transporter ferroportin-1. Ferroportin-1 is expressed by Caco-2 cells (*15*), but its differential contributions to the egress of iron entering the intestine via different uptake mechanisms remains unknown.

While a complete explanation for how lactic acid-fermented food improves iron absorption remains to be established, it is well-known that some dietary inhibitors such as phytate and polyphenols chelate iron in an insoluble and unavailable form for uptake, and these may be degraded during LA fermentation (*16*, *26*). However, degradation of such inhibitors may not be the only reason for the enhanced iron absorption by fermented foods (*27*). As the major end product in LA fermentation, LA has been proposed to be at least partially responsible for enhanced iron absorption (*28*). Noticeably, all of the above studies were all conducted by combining LA with food matrixes. Whether and how such effects are generated by interaction of food matrixes with LA−iron complexes remain unknown. Even though LA has the potential to increase iron solubility, it has been suggested that success in solubilizing iron in complexes by iron-binding ligands does not necessarily ensure effective donation of the chelated iron to the brush border (*7*). Miller and Berner (*19*) have proposed a theory that citric acid may act either as an enhancer when the major limitation for the iron absorption is solubility or as an inhibitor when soluble iron is sufficient through competition for iron with the cellular receptors. The same theory could also apply to the effects of LA on iron absorption. This idea is supported by the fact that LA decreased iron absorption when the iron was supplied soluble as a complex with NTA in the present study, but increased absorption when the iron was supplied as $FeCl_3$ as noted by Salovaara et al. (*12*).

To summarize, our investigation establishes a dose-dependent inhibitory effect of LA on Fe(II) and Fe(III) retention by Caco-2 cell monolayers. While Fe(II) transfer across the cells was also decreased by LA, Fe(III) transfer was increased, suggesting that LA may stimulate paracellular transfer of Fe(III).

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