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Short communication

Decreased expression of the vitamin C transporter SVCT1 by ascorbic acid in a human intestinal epithelial cell line

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Vitamin C (ascorbic acid) is an essential nutrient that is involved in a number of cellular processes. However, unlike most mammals, man is unable to synthesize vitamin C and it must therefore be acquired from the diet. Absorption of vitamin C is achieved by two transporters, SVCT1 and SVCT2, recently cloned from rat and human kidney. SVCT1 is thought to be the predominant transporter in the intestine. Vitamin C supplements are increasingly common, thus contributing to an increased dietary load, and therefore the aim of the present study was to investigate the effect of high doses of ascorbic acid on SVCT1 expression. Using the Caco-2 TC7 cell model of small intestinal enterocytes, we measured the effects of ascorbic acid (4·5 mg/ml culture medium) on L-[^{14}C]ascorbic acid uptake and SVCT1 expression (determined by reverse transcription-polymerase chain reaction). Ascorbic acid uptake was decreased significantly in Caco-2 TC7 cells exposed to ascorbate for 24 h (−50 %, $P<0·0005$). Expression of SVCT1 was also significantly reduced by exposure to elevated levels of ascorbate for 24 h (−77 %, $P<0·005$). Taken together these results suggest that high-dose supplements might not be the most efficient way of increasing the body pool of vitamin C.

Ascorbic acid: Absorption: Transporters: supplements

Vitamin C is an essential nutrient that is involved in a number of cellular processes. However, unlike most mammals, man is unable to synthesize vitamin C as he lacks L-gulonolactone oxidase, the final enzyme in the biosynthetic pathway from hexose sugars to vitamin C. Thus man must acquire vitamin C from the diet to satisfy the body's requirements (UK reference nutrient intake for vitamin C is 40 mg/d for adults (Department of Health, 1991)). There are two major forms of vitamin C present in the diet, L-ascorbic acid, which is a physiologically important reducing agent (Englard & Seifter, 1986; Padh, 1991), and L-dehydroascorbic acid. Both forms of vitamin C are available for absorption from the diet. L-Dehydroascorbic acid, the oxidized form of the vitamin, is thought to be absorbed by facilitated transport via the GLUT family of transporters (Vera *et al.* 1993; Rumsey *et al.* 1997). However, ascorbic acid is not a substrate for these transport proteins and recently, two Na^+-dependent vitamin C transporters (SVCT1 and SVCT2) have been isolated and cloned from rat (Tsukaguchi *et al.* 1999) and human (Daruwala *et al.* 1999) kidney cDNA libraries. Northern blotting to investigate tissue distribution of these transporters suggests that SVCT1 is the most abundant in the intestine (Tsukaguchi *et al.* 1999), and recent studies have shown that SVCT1 is functionally expressed in the human intestinal cell line Caco-2 (Wang *et al.* 1999).

Vitamin C supplements are becoming increasingly common, adding greatly to the dietary load. However, little is known regarding the effects of vitamin supplements on intestinal physiology. There are reports that taking high doses of vitamin C can reduce the percentage absorption of the vitamin and that the unabsorbed fraction is retained within the intestine, producing mild diarrhoea (for review see Bates, 1997). Therefore, the aim of our current study was to investigate the effect of high doses of ascorbic acid on SVCT1 expression, using the Caco-2 cell model of small intestine enterocytes.

Abbreviation: PCR, polymerase chain reaction.

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Experimental methods

All experiments were performed using Caco-2 TC7 cells, maintained in air–CO_2 (95:5, v/v) atmosphere in Dulbecco's modified Eagle's minimal essential medium, supplemented with heat-inactivated fetal bovine serum (200 ml/l). Studies were carried out 21 d post-seeding on cells, between passage numbers 30–40, grown on Anapore permeable supports (pore size 0·2 μm; Life Technologies, Paisley, UK). Culture medium was supplemented with 45 μg–4·5 mg ascorbic acid/ml for the final 24 h prior to experimentation. This time period was chosen to maximize changes in uptake and mRNA expression.

Ascorbic acid uptake was measured (3 min at 37°C) by addition of 100 μM L-ascorbic acid (containing 3·7 kBq L-[^{14}C]-ascorbate/ml; NEN Life Science Products, Hounslow, Middlesex, UK) in Hepes buffered saline (pH 7·5; 140 mM-NaCl, 5 mM-KCl, 1 mM-Na_2HPO_4, 1 mM-$CaCl_2$, 0·5 mM-$MgCl_2$, 5 mM-glucose, 10 mM-Hepes, bovine serum albumin (2 g/l)). The transport buffer was supplemented with 1 mM-dithiothreitol to maintain ascorbate in the reduced form. Cell monolayers were washed with ice-cold transport medium, solubilized overnight with 200 mM-NaOH and cell-associated radioactivity determined by liquid scintillation counting.

To investigate the effects of ascorbic acid on SVCT1 expression, total RNA was isolated from Caco-2 cells using Trizol reagent (Life Technologies) and stored at −70°C in ethanol (750 ml/l) until required. Reverse transcription-polymerase chain reaction (PCR) was performed in a single step reaction, using Ready-to-go reverse transcription-PCR Beads (Amersham Pharmacia Biotech, Amersham, Bucks., UK) on total RNA samples (1 μg/tube) using the following primer sequences: SVCT1 5′ ATA CGG GAG GTC CAG GGT 3′ (forward), 5′ GCC CCA GCG GTA GAC AGG 3′ (reverse); β-actin (control) 5′ CCA AGG CCA ACC GCG AGA AGA TGA C 3′ (forward), 5′ AGG GTA CAT GGT GGT GCC GCC AGA C 3′ (reverse). The cDNA transcript was produced by incubation at 42°C for 30 min. PCR was performed by twenty-eight cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 1 min. This was followed by a final step at 72°C for 10 min in a Hybaid Omn-E thermal cycler (Hybaid Ltd, Ashford, Middlesex, UK). PCR products were stained with ethidium bromide on a 2·5 % agarose gel and visualized using Fluor-S MultiImager (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts., UK), and bands were analysed using Scion Image analysis software (Frederick, MD, USA).

Data are presented as the mean values with their standard errors and were analysed using Student's unpaired *t* test. Differences between groups were considered significant at $P<0·05$.

Results

Preliminary studies revealed that Na^+-dependent ascorbic acid uptake by Caco-2 TC7 cells displayed typical exponential time course kinetics and had a rate constant (k) of 0·33 min^{-1} and a half-time of 2·1 min. Pre-incubation of Caco-2 TC7 cells for the final 24 h of the culture period with ascorbic acid (4·5 mg/ml) significantly reduced

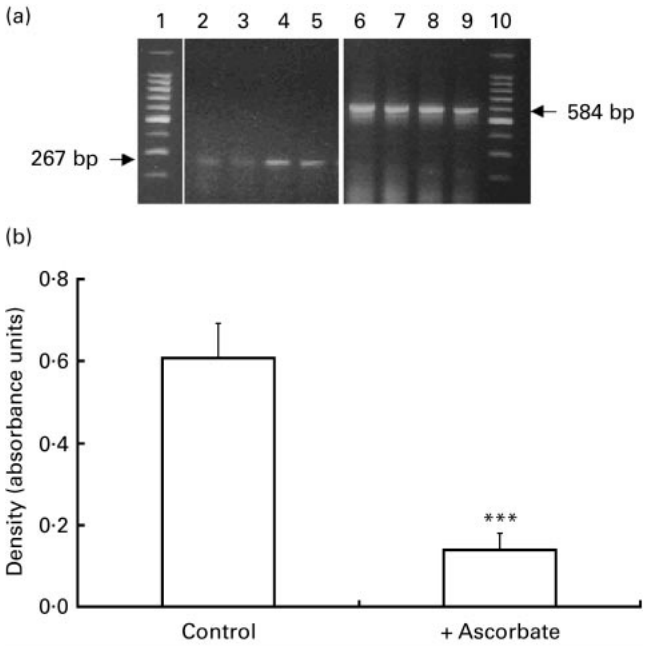

Fig. 1. (a) Agarose gel showing reverse transcription-polymerase chain reaction (RT-PCR) products using specific primers for SVCT1 (lanes 2 and 3, 4·5 mg ascorbate/ml; lanes 4 and 5, control) and β-actin (lanes 6 and 7, 4·5 mg ascorbate/ml; lanes 8 and 9, control). Calibration ladders are shown in lanes 1 and 10. Analysis of all samples yielded one band, at 267 bp for SVCT1 and 584 bp for β-actin. (b) Semi-quantification of RT-PCR products showed that exposure to ascorbate for 24 h significantly reduced SVCT1 mRNA levels (normalized to β-actin mRNA levels) by 77 % (***$P<0·005$). Values are means for four samples in each group with standard errors shown by vertical bars. For details of procedures, see p. 98.

Na^+-dependent L-ascorbic acid uptake (control 0·37 (SE 0·03) nmol/cm^2 per min, n 6; + ascorbate 0·18 (SE 0·01) nmol/cm^2 per min, n 6; $P<0·0005$). Lower concentrations of ascorbate (45 and 450 μg/ml respectively) did not alter uptake significantly (results not shown).

To investigate whether the decrease in ascorbic acid uptake occurred as a consequence of changes in transporter expression, the effect of ascorbic acid (4·5 mg/ml) on SVCT1 mRNA expression was determined using reverse transcription-PCR. SVCT1 and β-actin specific primers gave single PCR products of 267 and 584 bp respectively (Fig. 1(a)), which corresponded with the predicted size based on the published mRNA sequences. In all cell samples, SVCT1 mRNA, expressed as SVCT1:β-actin ratio to correct for fluctuation in RNA integrity and content, was significantly reduced following ascorbate treatment (−77 %, $P<0·005$; Fig. 1(b)). Levels of β-actin mRNA were unaffected by ascorbic acid treatment. Epithelial monolayers integrity appeared to be unaffected by high levels of ascorbate since transepithelial resistance (measured before and after the incubation period) and [^{14}C]-mannitol permeability were not different in the ascorbate-treated or control groups.

Discussion

Man, like other higher primates, has to rely on dietary intake of vitamin C to satisfy the body's daily requirements. The

recent cloning and characterization of two transporters, SVCT1 and SVCT2 (Daruwala *et al.* 1999; Tsukaguchi *et al.* 1999), has provided an insight into the mechanisms involved in vitamin C assimilation from the diet. In terms of intestinal transport, tissue distribution suggests that SVCT1 is the most important transporter (Tsukaguchi *et al.* 1999). Previous studies have shown that SVCT1 is highly selective for ascorbic acid and requires that Na^+ be present (Tsukaguchi *et al.* 1999). Using the Caco-2 cell line, which has been shown to express SVCT1 (Wang *et al.* 1999), we have demonstrated that ascorbic acid uptake across the apical membrane of Caco-2 cells is Na^+-dependent and follows an exponential time course (results not shown). Previous studies measuring the kinetics of ascorbate transport by SVCT1 have revealed a range of values for k_t from 30 μM for rat SVCT1 expressed in *Xenopus* oocytes (Tsukaguchi *et al.* 1999) and 75 μM for human SVCT1 expressed in Human retinal pigment epithelial cells (Wang *et al.* 1999) at the low end of the scale up to 200 μM for uptake in human brush border membrane vesicles (Malo & Wilson, 2000) and 250 μM for human SVCT1 in *Xenopus* oocytes (Daruwala *et al.* 1999). For our present uptake studies we chose to use 100 μM-ascorbic acid, which is the middle of this range.

Daily intakes of vitamin C from dietary sources (i.e. fruits and vegetables) could be as high as 210–280 mg (Levine *et al.* 1999), which could lead to intestinal lumen concentrations as high as 500 μM. This compares with a UK reference nutrient intake of 40 mg/d (Department of Health, 1991) and the new recommended dietary allowance of 90 mg/d for the USA and Canada (Food & Nutrition Board, 2000). However, there is an increasing trend towards the consumption of commercial vitamin supplements, which may increase daily intake by more than 10-fold, leading to intralumenal concentrations in excess of 10 mM. The effects of dietary vitamin C deficiency in man have been known for many years, with a gradual decrease in the body pool leading to scurvy. However, there is little information regarding the effects of excessive intakes of vitamin C on intestinal physiology. Bioavailability and absorption of vitamin C at doses up to 200 mg is nearly complete (Levine *et al.* 1996; Graumlich *et al.* 1997), whereas at higher doses the percentage absorption drops dramatically: ingestion of 3–5 g vitamin C results in only 20–30 % being absorbed (Hornig *et al.* 1980). Indeed at steady state, vitamin C doses >500 mg have virtually no impact on the body pool and are almost totally excreted in the urine (Levine *et al.* 1996). In our present study, we pre-incubated cells with a high dose of ascorbic acid (4·5 mg/ml) 24 h prior to measurement of Na^+-dependent ascorbate uptake across the apical membrane of Caco-2 cells. This time point was chosen to maximize the differences between the control and ascorbate-treated groups. Uptake of 100 μM-ascorbic acid was significantly reduced (-50 %) in cells exposed to ascorbate for 24 h prior to experimentation. We postulated that the cellular effects of ascorbate might be on the SVCT1 transporter itself. To test this hypothesis we carried out reverse transcription-PCR on total RNA isolated from control cells or cells exposed for 24 h to ascorbic acid. In ascorbate-treated cells SVCT1 mRNA levels were reduced by some 77 %. Given the magnitude of the changes in both ascorbate uptake and SVCT1 expression, a significant reduction in the function and expression of the transporter is likely to be evident within a more physiologically relevant time scale. The mechanisms involved in this dramatic reduction in SVCT1 expression are unknown at this time but may involve activation of transcriptional repressor elements, which could either bind in the 5′ promoter region of the SVCT1 gene blocking transcription or, alternatively, might decrease SVCT1 mRNA stability. These areas will be the subject of further investigation.

In conclusion, the results presented here provide the first evidence that vitamin C levels in the intestinal lumen might regulate the expression of the specific vitamin C transporter SVCT1. Furthermore, the use of Caco-2 cells may provide a useful tool, along with studies in human subjects, for developing recommendations for vitamin C intakes. Previous studies have shown that high doses of vitamin C result in a decrease in the percentage absorption of this essential micronutrient (Hornig *et al.* 1980; Levine *et al.* 1996; Graumlich *et al.* 1997). Our results provide a cellular mechanism for these earlier findings and taken together suggest that large single doses of vitamin C, rather than restoring or maintaining the body vitamin C pool, have a deleterious effect on the expression of the transport machinery.

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