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Transport and intracellular accumulation of vitamin C in endothelial cells: relevance to collagen synthesis

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Abstract

Endothelial cells preserve vascular integrity in part by synthesizing type IV collagen for the basement membrane of blood vessels. Vitamin C, which at physiologic pH is largely the ascorbate mono-anion, both protects these cells from oxidant stress and is required for collagen synthesis. Therefore, cultured endothelial cells were used to correlate intracellular concentrations of ascorbate with its uptake and ability to stimulate collagen release into the culture medium. The kinetics and inhibitor specificity of ascorbate transport into EA.hy926 endothelial cells were similar to those observed in other cell types, indicative of a specific high affinity transport process. Further, transport of the vitamin generated intracellular ascorbate concentrations that were 80–100-fold higher than concentrations in the medium following overnight culture, and transport inhibition with sulfinpyrazone and phloretin partially prevented such ascorbate accumulation. On the other hand, low millimolar intracellular concentrations of ascorbate impaired its transport measured after overnight culture. Synthesis and release of type IV collagen into the culture medium was markedly stimulated by ascorbate in a time-dependent manner, and was saturable with increasing medium concentrations of the vitamin. Optimal rates of collagen synthesis required intracellular concentrations of the vitamin up to 2 mM. Since such concentrations can only be generated by the ascorbate transporter, these results show the necessity of transport for this crucial function of the vitamin in endothelium.

Keywords: Ascorbate; Type IV collagen; GSH; Transport-metabolism coupling; EA.hy926 endothelial cells

Many of the important functions of vitamin C, which exists largely as the ascorbate mono-anion at physiologic pH [1], occur within cells rather than in plasma or in the extracellular space. These functions include its role as an essential co-factor for various dioxygenases, such as enzymes mediating collagen hydroxylation and carnitine biosynthesis, as well as its ability to protect cells against oxidant stress [2]. Since ascorbate concentrations in many tissues (0.5–4 mM) are 10- to 80-fold higher than in plasma (~50 μM), it seems likely that these functions require relatively high intracellular concentrations of the vitamin. However, there is little evidence correlating intracellular ascorbate concentrations with non-antioxidant functions of the vitamin. Most nucleated cells concentrate ascorbate against a gradient across the plasma membrane. This ability has been attributed to an energy- and sodium-dependent transporter that has a high affinity (25–100 μM) and specificity for ascorbate [3,4]. Two isoforms of the transporter have been described, both of which, when expressed in *Xenopus laevis* oocytes, have the kinetic features and dependence on the trans-membrane sodium gradient expected for ascorbate transport in mammalian cells [3]. Whereas the two transporter isoforms have similar kinetics, they have different tissue distributions. The SVCT1 isoform (*SLC23A1*) is distributed primarily in intestine, liver, and kidney, whereas the SVCT2 isoform (*SLC23A1*) is found in brain, placenta, cardiac muscle, and most other tissues [3,5].

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The two-electron oxidation product of vitamin C, dehydroascorbate (DHA)[1] can also enter cells, but does so by facilitated diffusion on the GLUT-type glucose transporter [6]. Once inside the cell, DHA is rapidly reduced to ascorbate, and can at least transiently accumulate to low millimolar concentrations [7,8]. However, DHA concentrations are low in plasma [9] and probably also in the interstitium. Thus, in cells that are unable to synthesize the vitamin, most of the trans-plasma membrane ascorbate gradient is likely to be maintained by transport of ascorbate.

Even in mammals that can synthesize ascorbate, synthesis is limited to liver and kidney. All other cells must derive it from the circulation, which in most tissues means that it must cross the barrier formed by endothelial cells. For this reason, and because endothelial cells are exposed to oxidant stress generated by vascular inflammation in a variety of diseases, endothelial cells might be expected to have efficient ascorbate uptake. This has been confirmed for endothelial cells derived from human umbilical vein [10,11], and for microvascular endothelial cells prepared from rat skeletal muscle [12]. Wilson et al. [12] showed that endothelial cell ascorbate transport was sodium-dependent, distinct from hexose transport, and able to maintain relatively high steady-state intracellular ascorbate concentrations. Subsequent studies from our group in EA.hy926 endothelial cells confirmed the concentrative uptake of ascorbate, and also correlated intracellular ascorbate concentrations with defense against oxidant stress induced by the redox cycling agent menadione [11]. However, the extent to which intracellular ascorbate correlates with a non-antioxidant function of the vitamin in endothelial cells remains to be determined. Since ascorbate is required for collagen synthesis in endothelial cells [13,14], in this work we studied ascorbate transport and intracellular accumulation in EA.hy926 cells, and correlated this with the ability of ascorbate to stimulate synthesis and release of type IV collagen into the culture medium.

Materials and methods

Materials

Analytical reagents including sulfinpyrazone, phlorizin, phloretin, and the cytochalasins were purchased from Sigma–Aldrich Chemical (St. Louis, MO). These hydrophobic reagents were initially dissolved in dimethyl sulfoxide, such that the final dimethyl sulfoxide concentration in the incubation was never above 0.2% (v/v). Perkin–Elmer Life and Analytical Sciences, (Boston, MA) supplied the L-[1-^{14}C]ascorbic acid, which was dissolved in deionized water containing 0.1 mM acetic acid and stored in multiple aliquots at −20 °C. L-[1-^{14}C]DHA was prepared by bromine oxidation of L-[1-^{14}C]ascorbic acid just before use [15]. Protein was measured by a kit (Bio-Rad Laboratories, Hercules, CA) using the Bradford method.

Cell culture and preparation for assays

EA.hy926 cells were kindly provided by Dr. Cora Edgell (University of North Carolina, Chapel Hill, NC). This a permanent cell line derived from human umbilical vein endothelial cells that has been shown to express factor VIII antigen [16], oxidatively modify human LDL [17], and to exhibit calcium-dependent stimulation of endothelial nitric oxide synthase [11]. The cells were cultured in Dulbecco's minimal essential medium that contained 20 mM D-glucose, 5 mM hypoxanthine, 20 μM aminopterin, and 0.8 mM thymidine, and 10% (v/v) fetal bovine serum. Cells were seeded onto 6- or 12-well plates and cultured to confluence at 37 °C under 5% CO_2. Just before use, they were rinsed three times in 2 ml Krebs–Ringer Hepes (KRH) buffer at 37 °C. The latter consisted of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 128 mM NaCl, 5.2 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $MgSO_4$, and 1.4 mM $CaCl_2$, pH 7.4.

Assay of ascorbate and DHA transport

Following treatments as noted, confluent EA.hy926 cells in 12-well plates were incubated at 23 °C in KRH that contained 5 mM D-glucose and either 0.05 μCi L-[1-^{14}C]ascorbate or 0.05 μCi L-[1-^{14}C]DHA at a final concentration of 9 μM, except where noted. Ascorbate transport was carried out in the presence of 0.5 mM GSH, unless noted otherwise. After 30 min of incubation, the supernatant was aspirated, and the cells were rinsed twice in 2 ml ice-cold KRH. The cell mono-layer was solubilized with the addition of 1 ml of 0.05 N NaOH, the cells were scraped from the plate, and the combined extract was added to 5 ml Ecolume liquid scintillation fluid (ICN, Costa Mesa, CA) and mixed. The radioactivity of the samples was measured in a Packard CA-2200 liquid scintillation counter, after allowing at least 1 h for decay of chemiluminescence. Results are expressed relative to the protein content of the cells.

Assay of type IV collagen

Release of type IV collagen into the culture medium was measured by a competitive ELISA. Standards or aliquots of the culture medium (0.1–0.2 ml) were incubated either for 2 h at room temperature or overnight at 3 °C in

[1] *Abbreviations used:* BSO, L-buthionine-[*S*,*R*]- sulfoxime; DHA, dehydroascorbic acid; DIDS, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid; GSH, reduced glutathione; Hepes, (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid); KRH, Krebs–Ringer Hepes; PBS, phosphate-buffered saline.