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Ascorbic acid transport in brain microvascular pericytes

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ABSTRACT

Intracellular vitamin C, or ascorbic acid, has been shown to prevent the apoptosis of cultured vascular pericytes under simulated diabetic conditions. We sought to determine the mechanism by which ascorbate is transported into pericytes prior to exerting this protective effect. Measuring intracellular ascorbate, we found that pericytes display a linear uptake over 30 min and an apparent transport K_m of 21 μM, both of which are consistent with activity of the Sodium-dependent Vitamin C Transporter 2 (SVCT2). Uptake of both radiolabeled and unlabeled ascorbate was prevented by inhibiting SVCT2 activity, but not by inhibiting the activity of GLUT-type glucose transporters, which import dehydroascorbate to also generate intracellular ascorbate. Likewise, uptake of dehydroascorbate was prevented with the inhibition of GLUTs, but not by inhibiting the SVCT2, indicating substrate specificity of both transporters. Finally, presence of the SVCT2 in pericytes was confirmed by western blot analysis, and immunocytochemistry was used to localize it to the plasma membrane and intracellular sites. Together, these data clarify previous inconsistencies in the literature, implicate SVCT2 as the pericyte ascorbate transporter, and show that pericytes are capable of concentrating intracellular ascorbate against a gradient in an energy- and sodium-dependent fashion.

1. Introduction

Pericytes surround the endothelium of venules, post-capillary venules, and capillaries [1]. They are smooth muscle-derived cells that interact with endothelial cells to regulate blood flow and to tighten endothelial barrier permeability [2–5]. Particularly in the brain and retina, pericytes help to maintain a tight blood–brain barrier and preserve vascular integrity. For example, dropout of pericytes is one of the earliest changes of diabetic retinopathy [6–8], leading to endothelial cell dysfunction and subsequent extravasation of serum proteins into the retinal interstitium [9–12].

We recently evaluated human brain pericytes exposed to a diabetic milieu of high glucose-induced oxidative stress, mediated largely by activation of the Receptor for Advanced Glycation End-products (RAGE). With the daily addition of 100 μM ascorbate, an increase in intracellular ascorbate from 0.8 mM to 2–3 mM was shown to prevent apoptosis in these cultured pericytes [13]. This suggests that intracellular ascorbate accumulated against a concentration gradient, but the mechanism was not evaluated.

In contrast, a previous study using primary bovine retinal pericytes did not find that 5 μM radioactive ascorbate was concentrated against a gradient [14]. This was surprising because most non-epithelial cultured cells transport ascorbate in a sodium- and energy-dependent manner using the Sodium-dependent Vitamin C Transporter 2 (SVCT2) [15,16]. This co-transporter imports ascorbate against a gradient by coupling its entry with sodium influx, thus maintaining electroneutrality and utilizing energy derived from the inward-to-outward sodium gradient generated by the trans-membrane Na/K ATPase [17,18]. The SVCT2 shows saturable, high-affinity ascorbate uptake (apparent K_m 20–50 μM). It is inhibited by removal of extracellular sodium, by energy depletion with ouabain, and by several anion transport inhibitors, such as sulfinpyrazone [16]. Ascorbate uptake on the SVCT2 is not inhibited by *D*-glucose [19–21]. In contrast, pericyte ascorbate uptake was inhibited by *D*-glucose and its derivatives [14], which further brings into question how pericytes transport ascorbate.

Dehydroascorbate (DHA), the two-electron oxidized form of ascorbate, is a substrate for the ubiquitous GLUT-type facilitative transporters but not for the SVCT2 [22,23]. DHA uptake on GLUTs is

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DHA, dehydroascorbate; GLUT, facilitative glucose transporter; Hepes, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; KRH, Krebs-Ringer Hepes; NG2, neural/glial antigen-2; RAGE, receptor for advanced glycation end products; SVCT, sodium-dependent vitamin C transporter.

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rapid compared with that of ascorbate on the SVCT2 and is inhibited by glucose and its transported derivatives, but not by energy depletion or sodium removal [21]. Although not transported on the SVCT2, DHA has recently been shown to inhibit radioactive ascorbate uptake in several immortalized cell lines, an effect that is half-maximal at about 20 μM DHA [24]. The mechanism of this inhibition is unknown, but was also observed at low millimolar DHA concentrations in primary culture pericytes by Khatami [14]. Whether this effect persists at lower, physiologically relevant DHA concentrations remains to be determined.

To define the role of the SVCT2 in pericyte ascorbate transport, to resolve the discrepancy between Khatami's study and the established function of the SVCT2 in other cells, and to assess whether DHA inhibits ascorbate transport, we studied SVCT2 expression and ascorbate transport and accumulation in human brain microvascular pericytes.

2. Materials and methods

2.1. Materials

Sigma/Aldrich Chemical Co. (St. Louis, MO) supplied 3-*O*-methylglucose, ascorbate, ascorbate oxidase, *N*-2-hydroxyethylpiperazine N′-2-ethanesulfonic acid (Hepes), ouabain and sulfinpyrazone. Perkin–Elmer Life and Analytical Sciences, Inc. (Boston, MA) supplied the [1-^{14}C]ascorbic acid (4.8 μCi/mmol).

2.2. Cell culture

Human brain vascular pericytes were obtained from ScienCell Research Laboratories (catalog #1200, Carlsbad, CA) and cultured in Pericyte Medium with included supplements (catalog #1201). Cells were cultured on plates coated with poly-*L*-lysine at 37 °C in humidified air containing 5% CO_2. Cells were used within 3–10 passages.

2.3. Assay of intracellular ascorbate

To measure intracellular pericyte ascorbate, near-confluent cells in 6-well plates were rinsed with Krebs-Ringer Hepes buffer (KRH; 20 mM Hepes, 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) and lysed with 25% metaphosphoric acid (w/v). Following neutralization with 3 volumes of 100 mM Na_2HPO_4 and 0.05 mM EDTA (pH 8.0), cells were scraped from the plate with a rubber spatula. Lysates were centrifuged at 3 °C for 1 min at 13,000 × *g*, and the supernatant was taken for assay of ascorbate. Assay of ascorbate was performed in duplicate by ion-pair high-performance liquid chromatography with electrochemical detection as previously described [25]. Intracellular ascorbate concentrations were calculated based on the intracellular distribution space of 3-*O*-methylglucose in pericytes, measured as previously described in endothelial cells [26]. This pericyte distribution space was 6.1 ± 1.6 μl/mg protein (N = 6 determinations).

2.4. Radioactive ascorbate uptake

Pericytes cultured to confluence in 12-well plates were treated as described for 30 min, followed by addition of up to 10 μM [1-^{14}C] ascorbate or [1-^{14}C]DHA. [1-^{14}C]DHA was generated by treating [1-^{14}C]ascorbate with 2 unit/ml ascorbate oxidase for 5 min. Following incubation with radioactive ascorbate or DHA for 30 min at 37 °C, buffer was removed and the cell layer was rinsed with ice-cold KRH. Cells were then treated with 1 ml of 0.05 N NaOH, scraped from the plate, and the extract was added to 5 ml Ecolume liquid scintillation fluid (catalog #882470 ICN, Costa Mesa, CA) and mixed. After ≥1 h, sample radioactivity was measured in duplicate with a Packard CA-2200 liquid scintillation counter.

2.5. Immunoblotting of the SVCT2

Near-confluent pericytes were lysed with RIPA Buffer (catalog #R0278 Sigma/Aldrich), and immunoblotting was performed as described previously [27]. Briefly, protein yield was quantified using a BCA assay (catalog # 23225, Pierce Biotechnology, Rockford, IL). Normalized samples were prepared with Laemmli sample buffer [28] containing 5% β-mercaptoethanol and electrophoresed on a 4–20% sodium dodecyl sulfate-polyacrylamide gel. Following transfer to polyvinylidene difluoride membrane, binding of antibodies against SVCT2 (catalog # 9926, Santa Cruz Biotechnology, Santa Cruz, CA; 1:900) and actin (catalog # 1616-R; 1:10,000) was detected with enhanced chemiluminescence reagent (catalog # NEL105001EA, Perkin Elmer) using 1:5000 horseradish peroxidase-conjugated secondary antibodies (catalog #W4011, Promega Corporation, Madison, WI; catalog # A5420, Sigma). As a negative control, anti-SVCT2 was pre-incubated overnight with its immunizing peptide at 5× the antibody concentration (catalog # 9926 P, Santa Cruz) before probing the membrane. Immunoblots were also carried out using a primary antibody against SVCT1 (catalog # 9924, Santa Cruz; 1:900).

2.6. Immunofluorescence microscopy

Cells were grown on glass coverslips coated with poly-*L*-lysine and fixed with 4% formaldehyde for 15 min. Cells were blocked with 10% donkey serum, permeabilized with 0.1% saponin, and probed for SVCT2 (Santa Cruz #9926; 1:200) and neural/glial antigen-2 (NG2, catalog # ab50009, Abcam, Cambridge, MA; 1:200). Following incubation with Alexa Fluor 488 and Alexa Fluor 555-conjugated secondary antibodies (catalog #s A11055 and A31570, Life Technologies, Carlsbad, CA; 1:500), nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were visualized using an Olympus FV1000 inverted confocal microscope (Olympus Corporation, Tokyo, Japan; Vanderbilt Cell Imaging Shared Resource).

2.7. Data analysis

Results are shown as mean + standard error. Statistical comparisons were made using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA). Differences between treatments were assessed by one-way ANOVA with replication with post-hoc testing using Tukey's or Dunnett's test, as appropriate.

3. Results

3.1. Transport of ascorbate and DHA by human brain microvascular pericytes

To determine whether ascorbate transport in pericytes reflects that expected of the SVCT2, transport kinetics and inhibitor studies were performed. The commercial culture medium initially contained 100 μM ascorbate, but this was depleted with storage of the medium for ~2 weeks at 3 °C before use in culture (cold-stored), resulting in low intracellular ascorbate concentrations at baseline (Fig. 1A and B). Brain microvascular pericytes readily took up added ascorbate (100 μM) with a linear time course over 30 min (Fig. 1A, circles). In contrast, uptake and reduction of 100 μM DHA to ascorbate was much more rapid than uptake of ascorbate, reaching a plateau beginning after 30 min of incubation (Fig. 1A, squares). Using the 30 min time point, addition of increasing amounts of