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Biochimica et Biophysica Acta 1712 (2005) 212 – 221

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Chondrocyte transport and concentration of ascorbic acid is mediated by SVCT2

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Received 28 February 2005; received in revised form 15 April 2005; accepted 18 April 2005
Available online 11 May 2005

Abstract

Collagen II is the major protein component of articular cartilage and forms the collagen fibril network, which provides the tensile strength of cartilage. Collagen II synthesis is enhanced by ascorbic acid (vitamin C) at both a transcriptional and post-transcriptional level. While the importance of ascorbic acid in the synthesis of collagen has been established, the mechanism by which this essential nutrient is transported into chondrocytes has not been investigated previously. We have characterized the transport of the reduced form of ascorbic acid in passaged primary human chondrocytes to discern the physiologically relevant pathways of ascorbic acid transport in cartilage. We have found that chondrocytes are robust concentrators of ascorbic acid, capable of transporting the reduced form, and concentrating total ascorbic acid, in the reduced form and its metabolites, 960-fold over the concentration in the extracellular milieu. Chondrocyte transport of ascorbic acid was sodium and temperature dependent, stereoselective for the L-forms, and inhibited by the anion transport inhibitor, sulfinpyrazone. Chondrocytes preferentially expressed the full-length and functional isoform of sodium-dependent vitamin C transporter 2 (SVCT2). When this transcript was suppressed with sequence-specific siRNAs, the active transport component of ascorbic acid was abolished. Thus, we provide the first evidence that SVCT2 mediates the secondary active and concentrative transport of ascorbic acid in human chondrocytes.

Keywords: Ascorbic acid; Sodium-dependent vitamin C transporter; Dehydroascorbate; Glucose transporter; Chondrocyte; Vitamin C

1. Introduction

Articular cartilage is a compressive material that distributes load over bone surfaces and in conjunction with the synovial fluid, provides a low-friction surface for joint movement [1]. These unique biomechanical properties are due to the specific arrangement and interactions of chondrocytes with cartilage matrix macromolecules. As the major constituent of the extracellular matrix, type II collagen forms a fibrillar network that maintains the volume, shape, and tensile strength of articular cartilage.

Type II collagen biosynthesis is a multi-step process that is dependent on ascorbic acid (AA or vitamin C) at both a transcriptional and post-transcriptional level. At a transcriptional level, AA promotes collagen II and prolyl 4-hydroxylase expression [2,3]. At a post-transcriptional level, AA reduces iron to the ferrous state, which is necessary for the hydroxylation reactions of proline and lysine in type II collagen [4]. Hydroxyproline residues form non-covalent interchain crosslinks, which are required for collagen triple helix formation. Hydroxylysine residues promote non-covalent crosslinks between triple helices necessary for collagen fibril formation [5]. Collagen hydroxylation in the endoplasmic reticulum appears to be tightly linked to collagen secretion from cells. In turn, these AA-dependent hydroxylation events enhance productive collagen synthesis by promoting collagen secretion from cells [6].

While the importance of AA in the biosynthesis of collagen II has been established, the mechanism by which this essential nutrient is transported into chondrocytes has

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doi:10.1016/j.bbamem.2005.04.009

not yet been investigated. Two distinct pathways of ascorbate transport across cellular membranes have been discovered to date, the sodium-dependent vitamin C transporters (SVCTs) [7], and the glucose transporters (GLUTs) [8,9]. The GLUTs are ubiquitous in expression. To date, a total of 13 members of the GLUT family have been described [10,11]. GLUT1 and GLUT3 are expressed in human articular chondrocytes [12] and are known to transport AA in other cell types, but only in its oxidized state, dehydroascorbate (DHA) [8,9].

On the other hand, in its reduced form, AA is transported by the SVCTs [7]. The SVCTs exist in two isoforms, SVCT1 and SVCT2, which are discretely distributed. The SVCT1 isoform is expressed in epithelial systems, such as the intestine, liver, kidney [7], thymus, prostate [13], and hepatocytes [14]. The SVCT2 isoform is expressed in more metabolically active and specialized tissues, such as the brain [7,15], adrenal, osteoblasts [7], placenta [16], neurons [17], lens [18], endothelial cells [19], hepatocytes [14], hypothalamic glial cells [20], spleen [21], and skeletal muscle [21]. SVCT2 expression appears to be localized coordinately with a strong requirement for AA and coincident with AA concentrating ability. For instance, the adrenal, which requires large amounts of AA for catecholamine synthesis, concentrates AA 236-fold, over plasma levels [22].

We have reported that cartilage may be a storage site for AA, having shown that cartilage concentrations of AA are 3–8 times higher than levels in synovial fluid [23]. This is consistent with the essential role of AA in type II collagen extracellular matrix synthesis. However, it was not determined whether the AA was concentrated inside the chondrocytes or trapped in the cartilage extracellular matrix. Therefore, in this study, we have examined the ability of chondrocytes to concentrate AA. We have also evaluated the mechanism of transport of the reduced form of AA in primary human chondrocytes to discern the physiologically relevant pathways of AA transport in cartilage. Our data represent the first evidence that chondrocytes concentrate AA and that this concentrative transport is mediated by SVCT2.

2. Materials and methods

2.1. Chondrocyte isolation and cell culture

Articular cartilage was obtained from human knee surgical waste tissues at the time of knee replacement surgery ($n=7$). Primary human chondrocytes were isolated from cartilage specimens, which were harvested from non-lesioned areas. For each specimen, the cartilage was minced and the chondrocytes isolated by enzymatic digestion, similar to methods published previously [24]. Cells were given fresh media every 3 days and passaged upon confluence, approximately once every 10 days. The cells were cultured for no longer than eight passages. Every experimental treatment was performed at least one time in duplicate within the first seven passages. We verified that cells within the first seven passages consistently expressed aggrecan and collagen II. Due to limited availability of primary chondrocytes and to further confirm the results obtained from earlier passages, subsequent competition and inhibition experiments were performed in passage eight cells. No difference was observed between earlier and later passages in either expression or activity of the SVCTs.

2.2. Explant isolation and culture

Cartilage explants were isolated from human knee articular cartilage surgical waste tissues at the time of knee replacement surgery ($n=5$). Cartilage was harvested from non-lesioned areas, using a 2-mm diameter micro dissecting trephine (Biomedical Research Instruments, Rockville, MD). A group of five cartilage plugs was pooled to constitute one sample. Each sample was paired with a corresponding sample from the same specimen and the same sites. The cartilage plugs were weighed and then transferred to a 24-well plate, containing 1 ml wash media (described in the chondrocyte isolation procedure). The cartilage plugs were incubated in 1 ml DMEM/F12 (Gibco, Grand Island, NY) with 10% FCS (Gibco) and no AA for 48 h at 37 °C/5% CO_2, prior to the procedures below.

2.3. Ascorbic acid concentration in cartilage explants

AA transport by live cartilage explants was compared with uptake by dead explants to differentiate the potential for active transport from passive interaction of AA with the cartilage extracellular matrix.

2.3.1. Live/dead generation

The cartilage explants were washed with PBS and half of the samples were transferred to 1.5 ml centrifuge tubes with a drop of PBS. The tubes were frozen in liquid nitrogen for 3 min and thawed at 37 °C for 5 min. This freeze/thaw procedure was performed for a total of three cycles. The dead explants were transferred back to the 24-well plates.

2.3.2. Cell viability assay

Cartilage plugs were harvested as described above and then washed three times in transport buffer (see AA Transport Assay in Isolated Primary Human Chondrocytes). Explants were cut in half and cell viability was assessed with the Live–Dead assay (Molecular Probes, Eugene, OR), according to the manufacturer's protocol. The explants were then viewed with the Zeiss LSM 510 (Carl Zeiss Inc., Thornwood, NY) to verify the live or dead state of the chondrocytes within the explants.

2.3.3. Explant ascorbic acid transport assay

Explants were washed three times in transport buffer (see AA Transport Assay in Isolated Primary Human Chondrocytes). A stock solution of L-[^{14}C]-ascorbic acid (L-^{14}C-Asc, 4 mCi/mmol, NEN, Boston, MA) in 0.4 mM DL-homocysteine (Sigma, Saint Louis, MO) was diluted to a final concentration of 50 μM L-^{14}C-Asc. Each sample was incubated with 1 ml of 50 μM L-^{14}C-Asc for 21–22 h at 37 °C/5% CO_2. The samples were then washed four times with ice-cold PBS to remove extracellular L-^{14}C-Asc. Samples were processed to isolate the cells from the matrix fraction (see Intracellular versus extracellular ascorbic acid) or counted for radioactivity by transferring the whole explants to glass scintillation vials and solubilizing the samples overnight at room temperature in 0.5 ml Solulene 350 (Perkin-Elmer Life and Analytical Services, Boston, MA). Hionic Fluor scintillation fluid (5 ml, Perkin-Elmer Life and Analytical Services) was added, and radioactivity was counted in the live and dead explants, using a scintillation counter.

2.3.4. Intracellular versus extracellular ascorbic acid

Explant isolation, explant treatments, and the explant AA transport assay (through the PBS wash step) were performed, followed by the chondrocyte isolation procedure described above. After the cell fraction was isolated from the matrix, the cells were washed, then 15 μl of the cells were counted on a hemacytometer, in order to determine the total number of cells in the cell fraction. The remaining cell fraction was lysed in 300 μl Puregene Cell Lysis Solution (Gentra Systems, Minneapolis, MN) and scintillation counting was performed in Uniscint BD (National Diagnostics, Atlanta, GA). The total fmol of L-^{14}C-Asc inside the chondrocytes was calculated using the total dpm (disintegrations per minute) of L-^{14}C-Asc measured and the specific activity of the radiolabel. The average fmol of L-^{14}C-Asc inside each chondrocyte was calculated by dividing the total fmol L-^{14}C-Asc in the cell lysate by the total number of cells in the lysate. The concentration inside each chondrocyte was calculated, using the data for the fmol L-^{14}C-Asc per cell and the average human chondrocyte (from non-eroded articular cartilage) total cell volume of 550 μm^3 (mathematically equivalent to 1.34×10^{-12} L) [25]. Bush and Hall have shown that the volume of chondrocytes in non-degenerate human cartilage increases from the superficial to the deep zones of cartilage, ranging from 396 μm^3 to 590 μm^3 [25]. Our full thickness cartilage explants contained cells from the superficial, middle, and deep zones of cartilage and thus our results depict the possible range of intracellular AA concentrations, depending on the location of the chondrocyte in the cartilage extracellular matrix. The fold concentration inside the chondrocytes was determined by comparing the intracellular concentration of L-^{14}C-Asc to the concentration in the media (50 μM).

2.4. Ascorbic acid transport assay in isolated primary human chondrocytes

AA transport was measured using a modified version of the uptake assay published by Wilson and Dixon [26]. Primary human chondrocytes were seeded at a density of 4.5×10^5 cells/well on a 6-well plate (or 2.25×10^5 cells/well on a 12-well plate for the kinetic experiments). Prior to the transport assay, cells were plated for 48 h in DMEM/F12, containing 10% FCS but no AA. Cells were washed in transport buffer containing: 134 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 10 mM D-glucose, 20 mM HEPES, pH 7.3 with NaOH. AA uptake was assessed by incubating the chondrocytes in transport buffer containing 189 μM L-^{14}C-Asc. Immediately before use, the L-^{14}C-Asc was solubilized in 0.4 mM DL-homocysteine (Sigma) to prevent AA oxidation. The standard AA transport assay conditions were 37 °C/5% CO_2 for 10 min. AA uptake was terminated by washing the cells four times in ice-cold PBS. The cells were lysed with Puregene Cell Lysis Solution (Gentra Systems) and scintillation counting was performed in Uniscint BD (National Diagnostics). The total pmol of L-^{14}C-Asc transported was calculated using the total dpm of L-^{14}C-Asc transported and the specific activity of the radiolabel.

We performed AA transport assays under various conditions designed to augment or inhibit the function of the AA transporters, including variations of the following: time; ±134 mM Na^+; ±10 mM D-glucose; temperature (4 °C or 37 °C); 100 μM Cytochalasin B (Sigma), 100 μM Cytochalasin E (Sigma), and 100 μM Sulfinpyrazone (Sigma); 2 mM unlabeled L-ascorbic acid (L-Asc, Sigma), L-DHA, D-isoascorbic acid (D-Asc, Aldrich), Na L-ascorbate (Na L-Asc, Sigma), and Na D-isoascorbate (Na D-Asc, Aldrich); increasing concentrations of unlabeled L-Asc; and 0-500 μM L-^{14}C-Asc. In experiments with sodium-free transport buffer, iso-osmotic concentrations of LiCl were substituted for NaCl. Sulfinpyrazone is an anion transport inhibitor [27], while cytochalasin B inhibits transport via the GLUTs [28] and also inhibits actin polymerization. Cytochalasin E has no known inhibitory effects on transport mechanisms but also inhibits actin polymerization and thus is a control for the actin effects of cytochalasin B. The properties of the AA forms used in these experiments were discussed in detail previously [2]. Stock solutions of DHA were generated immediately before use by incubating 2 mM L-Asc in 0.4 mM homocysteine with an AA oxidase spatula (Roche Diagnostics, Mannheim, Germany) for 15 min, stirring at room temperature. Detailed HPLC analyses (see below) of the DHA generated from L-Asc, using AA oxidase, revealed that on average, the oxidation process resulted in conversion of all of the L-Asc to DHA (41±6%) or metabolites of DHA (59%). Thus, the "2 mM" DHA stock solutions contained approximately 0.82 mM DHA.

2.5. High performance liquid chromatography (HPLC)

The integrity of the L-^{14}C-Asc stocks utilized in these experiments was determined by measuring the proportion of AA and DHA by HPLC. AA was measured with an electrochemical detector (EC), using the method of Lee et al. [29]. The amount of AA oxidation to DHA in the stocks and samples was determined by reducing the DHA to AA, as previously described [30].

2.6. RNA isolation

Monolayer cells were grown to confluency on 35 mm plates and the cells were lysed, using 1 ml of Trizol reagent (Gibco). The RNA extraction procedure was carried out according to the manufacturer's protocol through the phase separation step. The aqueous phase was transferred to a new tube and 10 μg tRNA (Sigma) was added. Then 0.5 ml isopropanol was added and the sample was frozen overnight at −80 °C. The RNA was pelleted at 13,000 rpm for 20 min at 4 °C, the liquid phase was discarded, and the pellet was air dried. The RNA was then processed according to the manufacturer's protocol for the Qiagen RNeasy kit (Qiagen, Valencia, CA).

2.7. RT-PCR

Total RNA was reverse transcribed into complementary DNA (cDNA), using Superscript II Reverse Transcriptase (Gibco) and random hexamer primers. Multiple Choice human liver cDNA (Origene, Rockville, MD) was used as a positive control for SVCT1 expression. Intron spanning primers were designed for actin (5′GACTACCTCATGAAGATCCT3′ and 5′ATCCACATCTGCTGGAAGGT3′), SVCT1 (5′GCCCCTGAACACCTCTCATAT3′ and 5′ATGGCCAGCATGATAGGAAA3′), and SVCT2 (5′AAGCACTGGGGCATTGCCAT3′ and 5′GTAATTCCCAAAACTCCAAT3′), corresponding to the human sequences available in Genbank. The following PCR primers were also generated to distinguish the full-length SVCT2 transcript from the short form (splice variant) of SVCT2, identified recently by Lutsenko et al. [31]: 5′GGGGCTACAGCACTACCTG3′ and 5′GGATGGCCAGGATGATAG3′. Primers specific for alpha-1 type II collagen and aggrecan were generously provided by Dr. Carl Flannery. Annealing temperatures were 55 °C for alpha-1 type II collagen, 61.3 °C for aggrecan, 50 °C for actin, 64 °C for SVCT1, and 55 °C for SVCT2. Standard PCR procedures were used with AmpliTaq Gold DNA polymerase (Roche).

2.8. Functional analysis of SVCT2

2.8.1. RNA interference (RNAi)

The RNAi transfection procedure was performed using Amaxa's Nucleofector device (Gaithersburg, MD), the primary human chondrocyte kit (Amaxa) with the corresponding manufacturer's protocol and a total of 3 μg short interfering RNAs (siRNAs). The cell/siRNA mixture was transfected using program U24. The samples in these experiments were transfected with the following siRNAs: (1) 3 μg Silencer Negative Control #1 siRNA (Ambion, Austin, TX), to control for nonspecific effects, or (2) a pool of three different human SVCT2 specific siRNAs, 1 μg each of siRNA ID #15859, #15950, and #15765 (Ambion). All of the SVCT2-specific siRNAs targeted both the short- and full-length isoforms of the SVCT2 mRNA.

The cells were incubated at 37 °C/5% CO_2 for 68 h after the transfection to allow the suppression of the SVCT2 mRNA expression and the turnover of pre-existing SVCT2 proteins. At this time, the samples were utilized in the AA transport assay (described above) and in parallel, samples were treated with Trizol to isolate the RNA (described above).

2.8.2. Real time RT-PCR

The cDNA generated from the RNAi experiments was subjected to real time RT-PCR in order to quantify the changes in gene expression that occurred upon suppression of the SVCT2 transcript levels. The ABI Prism 7000 Sequence Detection System instrument and relative quantification software (Applied Biosystems, Foster City, CA) were utilized for the real time analyses. The SVCT2 primer/probe set was designed, using the ABI Prism software and the sequences available in GenBank. The following primers and probe were custom synthesized by Applied Biosystems: primer SVCT2-229F (5′GGCTTCTATGCTCGCACAGAT3′), primer SVCT2-303R (5′AAATGGGTATGGAACCTTAAACCA3′), and SVCT2-253T probe (6FAM-AGGCAAGGCGTGCTTCTGGTAGCC-TAMRA). The real time reactions were each performed in quadruplicate in a final volume of 25 μl. The 18 S rRNA reactions were set up according to the manufacturer's instructions and the SVCT2 mRNA reactions each contained 900 nM of each primer and 250 nM probe. Expression levels of SVCT2 were compared between chondrocytes from the same specimen, transfected with either the negative control siRNA or the SVCT2 siRNAs. The cDNA samples were normalized for comparison by determining 18 S rRNA levels by real time RT-PCR, using the 18 S-PDAR primer and probe set (Applied Biosystems). Relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ formula, in which ΔC_T equals the difference between C_T (cycle threshold) values for negative control and SVCT2 transfected cells [32]. The data are expressed as a percentage of the mean fold change in mRNA levels for the experimental samples (SVCT2 siRNAs) as compared to the calibrator (negative control siRNA).

2.8.3. Protein assay

The concentration of proteins in the cell lysates from the RNAi experiments was determined using the Detergent

Compatible (DC) Protein Assay (Bio-Rad, Hercules, CA) and a microplate reader at 750 nm.

2.9. Statistical analyses

Statistical computations were performed using GraphPad Prism version 3.00 (GraphPad, San Diego, CA) and the data analysis feature of Microsoft Excel. Descriptive statistics, sample means, and standard error for all values were calculated for subgroups of interest. For descriptive purposes, pairwise comparisons between subgroups of interest were performed using analysis of variance (ANOVA) and the Newman–Keuls post hoc test or the paired t-test. A P value of <0.05 was considered significant. Kinetic analyses were performed using the GraphPad Prism software and the enzyme kinetic template.

3. Results

3.1. Chondrocyte expression of SVCT2

The primary human chondrocytes expressed transcripts for SVCT2 but not SVCT1 (Fig. 1, top panels). Fig. 1A (top panel) demonstrates expression of SVCT1 (360 bp fragment) in human liver but not in primary human chondrocytes. The full-length SVCT2 isoform (646 bp fragment) and the short isoform (301 bp fragment), which contains a 345-bp deletion in the transcript that arises due to alternative splicing, were both expressed by primary human chondrocytes (Fig. 1B, top panel). However, the predominant isoform expressed by the chondrocytes was the full-length SVCT2. Actin was successfully amplified from each sample confirming the use of equivalent amounts of intact cDNA for these comparisons (Figs. 1A and B, bottom panel). The chondrocytic phenotype of these cells was confirmed by RT-PCR for the major protein components in cartilage, type II collagen and aggrecan. Both were expressed through the seventh passage, in all of the chondrocytes used in these experiments (data not shown).

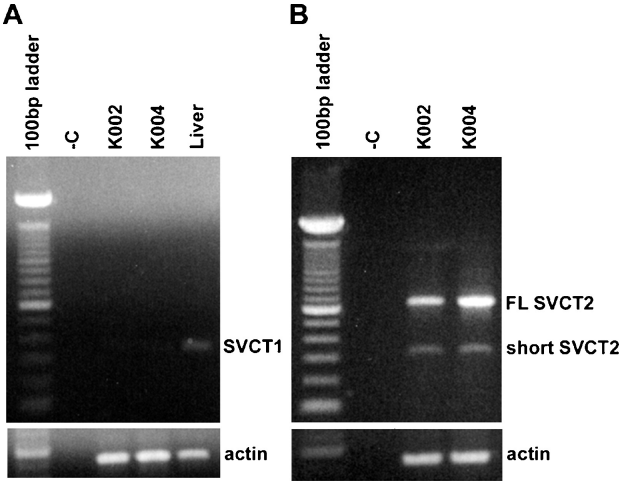

Fig. 1. Chondrocyte expression of SVCT2 but not SVCT1. (A) RT-PCR amplification of SVCT1 (top panel) and actin (bottom panel) from two different lines of passaged primary human chondrocytes (K002 and K004) and human liver. (B) RT-PCR amplification of SVCT2 (top panel) and actin (bottom panel) from two different lines of passaged primary human chondrocytes (K002 and K004). The 646-bp fragment represents the full-length (FL) SVCT2 PCR product and the 301-bp fragment represents the short SVCT2 isoform. The negative control (-C) contained dH_2O instead of cDNA. The 100 bp ladder was used as a size reference.

3.2. Concentration of AA by chondrocytes in the context of cartilage explants

Chondrocytes, in the context of human articular cartilage explants, are able to transport and concentrate L-^{14}C-Asc intracellularly. The uptake of L-^{14}C-Asc over 21 h by cartilage explants was determined for whole explants that were either live or dead. There was 2-fold more L-^{14}C-Asc found in the live explants than the dead explants ($P=0.05$), demonstrating active transport of AA by explants with live cells. Whole live explants contained 16.56 ± 4.22 pmol L-^{14}C-Asc/mg tissue and dead explants contained 7.77 ± 3.03 pmol L-^{14}C-Asc/mg tissue. After being cultured in the presence of L-^{14}C-Asc for 21 h, chondrocytes were isolated from the cartilage matrix, to further distinguish AA concentrated in chondrocytes from AA trapped in the extracellular matrix. The calculated average intracellular concentration of total AA (the radiolabeled reduced form and its metabolites) was 48 ± 5 mM (67 mM in superficial zone cells, 51 mM in middle zone cells, and 45 mM in deep zone cells). When compared with the media concentration of 50 μM L-^{14}C-Asc, on average chondrocytes concentrated total AA, in the reduced form and its metabolites, 960-fold.

3.3. Time dependence of AA transport in primary human chondrocytes

The transport of L-^{14}C-Asc in primary human chondrocytes is dependent on both time and the presence of extracellular sodium (Fig. 2). In the presence of sodium, L-^{14}C-Asc transport increased significantly over a 12-h time course. However, the measured L-^{14}C-Asc content inside the cells decreased slightly by 21 h. This corresponded with the HPLC measurements of the AA concentration in the transport buffer, which indicated that only 16% of the initial AA concentration remained in the transport buffer at the 12-h time point (data not shown). At all time points, the transport of L-^{14}C-Asc was independent of glucose.

3.4. Sodium-dependence and temperature-dependence of AA transport

To assess the sodium dependence of AA transport and to identify the active component of AA transport, the amount of L-^{14}C-Asc transported into primary human chondrocytes was assessed at either 37 °C or 4 °C. There was approximately 3-fold more L-^{14}C-Asc transported in the presence

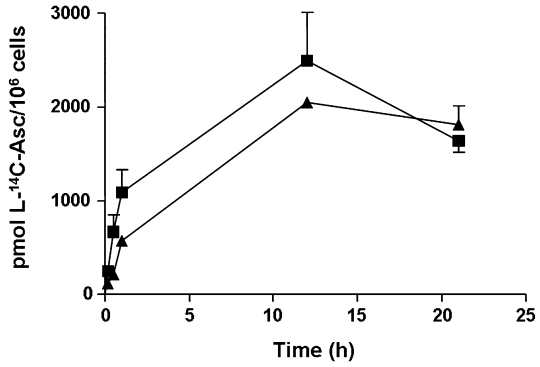

Fig. 2. Time dependence of AA transport in passaged primary human chondrocytes. L-^{14}C-Asc uptake was measured over time in the presence of sodium (Na^+) and presence or absence of D-glucose (Glc). The data points are expressed as the mean pmol L-^{14}C-Asc/ 10^6 cells ± standard error (n = 2–8 for each treatment). The transport buffer contained the following: ■ = 134 mM Na^+ and 10 mM glucose; ▲ = 134 mM Na^+ and no glucose.

of sodium than in the absence of sodium (Fig. 3; $P < 0.001$). Additionally, in the presence of sodium, there was 3.8-fold more L-^{14}C-Asc transported at 37 °C than at 4 °C ($P < 0.001$). Interestingly, the amount of L-^{14}C-Asc uptake at 4 °C was nearly equivalent to the amount transported in the absence of sodium at 37 °C, suggesting that under these conditions, about 45 pmol L-^{14}C-Asc/10^6 cells diffuses passively into human chondrocytes in 10 min. HPLC measurements were also performed to verify that the cell lysates contained AA and not simply the ^{14}C radiolabel alone. The AA concentration in the lysates measured by HPLC reflected that which was measured by radioactivity (data not shown).

3.5. Sulfinpyrazone inhibition of AA transport

The transport of L-^{14}C-Asc into primary human chondrocytes was inhibited approximately 65% by the anion transport inhibitor sulfinpyrazone ($P < 0.001$; Fig. 4).

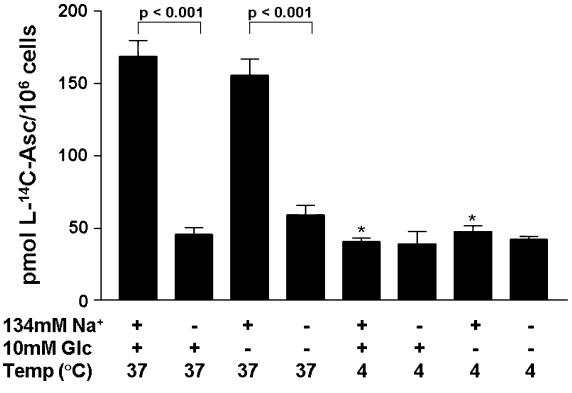

Fig. 3. Sodium-dependence and temperature-dependence of AA transport in passaged primary human chondrocytes. L-^{14}C-Asc uptake was measured after 10 min at either 37 °C or 4 °C in the presence and absence of 134 mM sodium (Na^+) and/or 10 mM D-glucose (Glc). The bars indicate the mean pmol L-^{14}C-Asc/ 10^6 cells ± standard error (n = 3–16 for each treatment). *$P < 0.001$ compared to the same treatment at 37 °C.

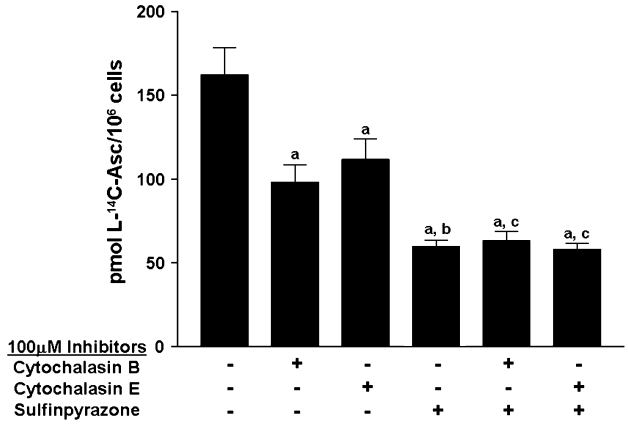

Fig. 4. Sulfinpyrazone inhibition of AA transport in passaged primary human chondrocytes. L-^{14}C-Asc uptake was measured after 10 min in the presence of 100 μM cytochalasin B, cytochalasin E, and/or sulfinpyrazone. The bars indicate the mean pmol L-^{14}C-Asc/ 10^6 cells ± standard error (n = 8–12 for each treatment). [a]$P < 0.001$ compared to the sample without inhibitor; [b]$P < 0.05$ compared to the samples with cytochalasin B or cytochalasin E; [c]$P < 0.05$ compared to the corresponding samples with cytochalasin B or cytochalasin E alone.

Cytochalasin B, a specific inhibitor of the GLUTs and of actin polymerization, decreased the transport of L-^{14}C-Asc by 40% ($P < 0.001$). However, cytochalasin E, a control for the actin effects of cytochalasin B, suppressed L-^{14}C-Asc transport to a similar degree (35%, $P < 0.001$). The treatment of chondrocytes with either cytochalasin B or cytochalasin E in combination with sulfinpyrazone did not suppress the level of L-^{14}C-Asc uptake beyond the level achieved with sulfinpyrazone alone. Thus, the effects of the cytochalasins can be attributed to an alteration in chondrocyte actin polymerization rather than an inhibition of transport via the GLUTs. In contrast, sulfinpyrazone significantly decreased the uptake of L-^{14}C-Asc when compared to either of the cytochalasins ($P < 0.05$). In fact, the sulfinpyrazone treatment decreased the uptake of L-^{14}C-Asc to the level of L-^{14}C-Asc uptake observed at 4 °C and in the absence of sodium (Fig. 3), representing passive uptake alone. These results taken together suggest that transport of AA by human chondrocytes is mediated by an anion transporter.

3.6. Stereoselective transport of the L-forms of AA

To determine the stereospecificity of AA uptake by chondrocytes, the L-^{14}C-Asc uptake assay was performed in the presence of various AA forms (Fig. 5). Only the L-forms of AA were able to effectively compete with L-^{14}C-Asc transport. L-Asc was able to suppress L-^{14}C-Asc transport by approximately 60%, as compared to the sample with no competitor ($P < 0.001$). Furthermore, Na L-Asc was able to decrease the transport of L-^{14}C-Asc by 75% ($P < 0.001$). On the other hand, the oxidized form of AA, DHA, and the D-forms of AA, D-Asc, and Na D-Asc were unable to effectively compete with L-^{14}C-Asc for transport into the cells. These results demonstrate stereospecificity of AA transport by chondrocytes.

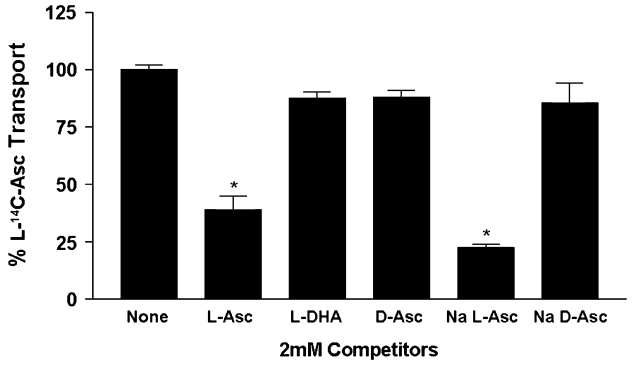

Fig. 5. Stereoselective transport of the L-forms of AA in passaged primary human chondrocytes. L-^{14}C-Asc uptake was measured after 10 min in the presence of 2 mM of the following unlabeled competitors: no competitor; L-ascorbic acid (L-Asc); dehydroascorbic acid (L-DHA); D-isoascorbic acid (D-Asc); Na L-ascorbate (Na L-Asc); or Na D-isoascorbate (Na D-Asc). The bars indicate the mean percentage of L-^{14}C-Asc transported ± standard error (n = 9–11 for each treatment). The amount of L-^{14}C-Asc transported in the samples without competitor was set to 100%. *P < 0.001 compared to the sample with no competitor.

3.7. AA transport kinetics

The transport of L-^{14}C-Asc was measured in the presence of increasing concentrations of unlabeled L-Asc in order to assess the saturability of the transport mechanism (Fig. 6A). The addition of unlabeled L-Asc strongly competed for the transport of L-^{14}C-Asc until approximately 1000 μM L-Asc had been added. At concentrations above 1000 μM, the transporter was fully saturated and there was no further decrease in the amount of L-^{14}C-Asc that was transported into the cells. In order to determine the kinetic properties of the active AA uptake mechanism, chondrocytes were incubated with increasing concentrations of L-^{14}C-Asc at 37 °C and 4 °C. Fig. 6B is a representative Michaelis–Menton plot that shows an increase in AA uptake with increasing concentrations of L-^{14}C-Asc. The vMax for AA transport in chondrocytes was 142 ± 49 pmol L-^{14}C-Asc/ 10^6 cells/ 10 min and the K_m was 62 ± 25 μM.

3.8. Suppression of SVCT2 expression by RNAi

To assess the role of the SVCT2 transporter in mediating AA transport in primary human chondrocytes, we suppressed the expression of SVCT2, with sequence specific siRNAs (Fig. 7). The SVCT2 mRNA levels were decreased approximately 80% by the SVCT2 siRNAs, as compared to the negative control (Fig. 7A). As shown in Fig. 7B, this decrease in mRNA levels resulted in a 75% decrease in the transport of L-^{14}C-Asc in the SVCT2 siRNA treated samples (P = 0.007). Thus, suppression of SVCT2 alone fully inhibited the active component of L-^{14}C-Asc transport and reduced L-^{14}C-Asc uptake to levels attributable to passive diffusion (as shown in Fig. 3 and indicated by the dashed line in Fig. 7B). These results demonstrate that SVCT2 mediates and fully accounts for the active component of AA transport in primary human chondrocytes.

4. Discussion

We have shown that chondrocytes are robust concentrators of AA, capable of transporting and concentrating total ascorbic acid, the reduced form and its metabolites, 960-fold over the concentration in the extracellular environment. The average intracellular total AA concentration in chondrocytes, based on uptake of radiolabeled AA, was calculated to be 48 mM, when the physiologic synovial fluid concentration of 50 μM AA [23] was included in the solution bathing the articular cartilage explants. Other cells that require large quantities of AA for cellular function have also been shown to concentrate AA intracellularly. Neurons, which rely on AA as an antioxidant, enzyme cofactor, and

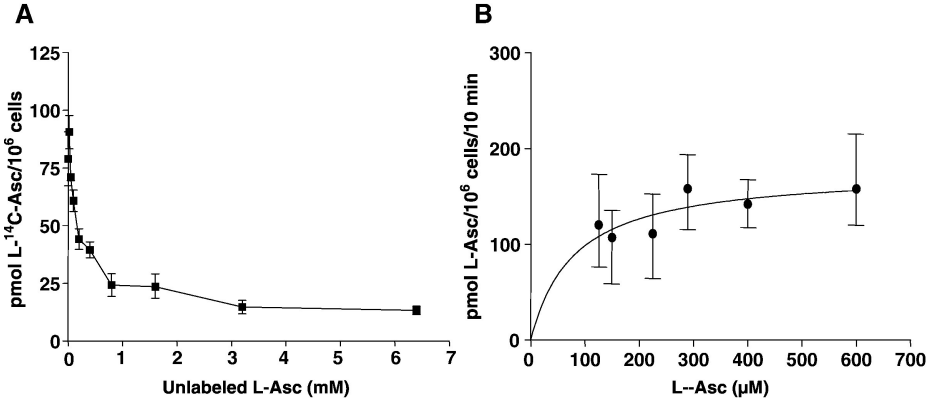

Fig. 6. AA transport kinetics in passaged primary human chondrocytes. (A) Saturability of active AA transport. The intracellular uptake of 200 μM L-^{14}C-Asc was measured after 10 min in the presence of increasing concentrations of unlabeled L-ascorbic acid (L-Asc). The data points indicate the mean pmol L-^{14}C-Asc/ 10^6 cells ± standard error (n = 3 for each treatment). (B) Michaelis–Menton kinetics of AA transport. The intracellular uptake of 0–600 μM L-Asc (0–500 μM L-^{14}C-Asc in the presence of 100 μM unlabeled L-Asc) was measured after 10 min. The data points indicate the mean velocity (pmol L-Asc/10^6 cells/10 min) at the given concentration of L-Asc. This is a representative experiment where each condition was performed in triplicate and the bars indicate the range of velocities for each condition.

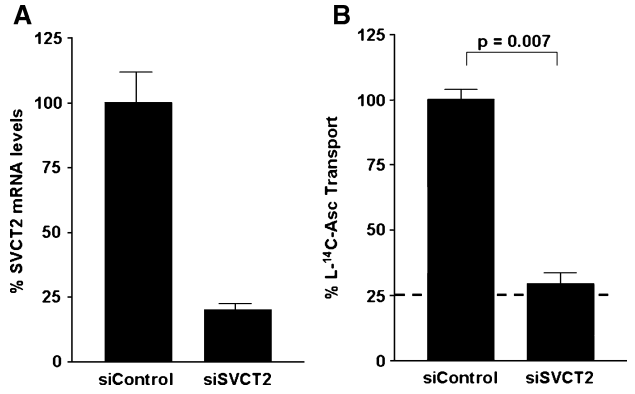

Fig. 7. Suppression of SVCT2 expression by RNAi. Passaged primary human chondrocytes were transfected with a negative control siRNA (siControl) or siRNAs specific for SVCT2 (siSVCT2). SVCT2 steady-state mRNA levels and AA uptake were assessed in these cells 68 h post-transfection. (A) SVCT2 mRNA levels by real-time RT-PCR in siRNA transfected cells. The bars indicate the mean SVCT2 mRNA level ± the 95% confidence level ($n=4$ for each treatment), expressed as a percentage of the negative control (the mRNA level in cells transfected with the negative control siRNA and designated as 100%). (B) AA transport in siRNA transfected cells. The bars indicate the mean L-^{14}C-Asc transport level (±standard error) expressed as a percentage of the negative control (the amount of L-^{14}C-Asc transported in cells transfected with the negative control siRNA and designated as 100%). The level of transport that is assumed to occur by passive diffusion in primary chondrocytes is indicated by a dashed line. This is a representative experiment where each condition was performed in duplicate.

neuromodulator, concentrate this vitamin 25- to 50-fold from the brain extracellular fluid [33]. The average AA concentration in neurons is estimated to be 10 mM. AA is also required for type I collagen production by osteoblasts and induction of the osteoblastic phenotype during differentiation. Franceschi et al. have demonstrated that a preosteoblast cell line is capable of concentrating AA 100-fold in culture, resulting in an average intracellular concentration of 10 mM AA [27]. Our data from human chondrocytes are consistent with the fact that chondrocytes require AA for type II collagen synthesis and cartilage extracellular matrix formation.

This is the first evidence that chondrocytes are concentrators of AA. This storage of AA by chondrocytes may serve as a buffer to variations in the dietary intake of AA. However, scurvy-related symptoms and depletion of AA plasma levels are known to occur after only 35–40 days exposure to a diet devoid of AA [34]. Furthermore, within just 2 weeks of weaning, rats that are unable to synthesize AA and that are maintained on an AA free diet show chondrocyte disorganization and developmental abnormalities [35]. These observations suggest that the chondrocyte supply of stored AA suffices for less than 2 weeks to maintain normal chondrocyte function during development, and less than 6 weeks to maintain normal extracellular matrix homeostasis in an adult.

We have demonstrated that primary human chondrocytes express transcripts for both the short and the full-length isoforms of SVCT2. However, the full-length isoform is the predominant transcript expressed by these cells. Lutsenko et al. have shown that the short form of SVCT2 results in a non-functional transporter protein that inhibits the AA transport activity of the full-length SVCT2 protein [31]. They demonstrated that cells that produce transcripts for SVCT2, but do not transport AA, express predominantly the short isoform, while cells that transport AA express predominantly the full-length SVCT2 isoform. The preferential expression of the full-length SVCT2 transcript by chondrocytes is consistent with our data demonstrating robust AA transport and concentrating ability by chondrocytes.

SVCT2 expression may decrease with aging, as shown in rat hepatocytes [14], or with the inflammatory cytokines IL-1 β and TNF-α, as shown in human endothelial cells [19]. We used cartilage from middle aged to older adults and from non-lesioned areas. However, the cartilage as a whole was exposed to an inflammatory milieu given that it came from osteoarthritic knees. On the other hand, over the course of a 21-h incubation with L-^{14}C-Asc, 40% of the intracellular AA concentration in isolated chondrocytes existed as the reduced form of AA, and 60% existed as metabolites of AA. Thus, the concentration gradient of AA may have diminished over time, facilitating the continued secondary active transport of AA. This would correspond to an average intracellular AA concentration of 19 mM (ranging from 27 mM in the superficial zone cells to 18 mM in the deep zone cells), corresponding to a 380-fold concentration of AA relative to the concentration in the extracellular environment. Additionally, we, along with others [27], have shown that the AA concentration in the culture medium is decreased substantially by 12 h, and thus the cartilage explants were exposed to less than 50 μM AA during the 21-h incubation. This would suggest that the ability of chondrocytes to concentrate AA may be underestimated in our study.

The mechanism by which AA is transported into chondrocytes is sodium dependent and glucose independent. The dependence of AA uptake on external sodium in primary human chondrocytes suggests that the transport is mediated by the SVCTs. The transport of AA through the SVCTs is driven by a sodium electrochemical gradient [7], with a stoichiometry of two sodium ions to one AA molecule [13,16]. Interestingly, in the cartilage extracellular matrix, there is a very high concentration of sodium (240–350 mM) due to the high concentration of negatively charged proteoglycans [36]. This establishes a large inward concentration gradient that can be exploited for the secondary active transport of nutrients, including AA.

The independence of AA transport on glucose indicates that the transport of AA is not occurring via the GLUTs. However, DHA transported into cells via the GLUTs can be rapidly converted intracellularly to the reduced form of AA [37], therefore, this pathway is also a potential means by which AA may be concentrated within chondrocytes. The oxidized form of AA, DHA, accounts for only about 10% of

the total AA in plasma [38] and the physiological glucose concentrations significantly inhibit GLUT-mediated DHA transport [9]. This suggests that AA transport via SVCT2 is a significant source of intracellular AA. The selective inhibition of AA transport by sulfinpyrazone and not cytochalasin B further supports the conclusion that SVCT2 mediates concentrative transport of AA. Finally, the suppression of SVCT2 mRNA levels and resultant decrease in AA uptake to passive levels proves that SVCT2 is mediating the concentrative transport of AA in primary human chondrocytes.

The L-forms of AA are transported efficiently into primary human chondrocytes, however, DHA and the D-forms of AA are not efficiently transported by this mechanism of uptake. The SVCTs from rat are also stereospecific for L-Asc with transport of D-Asc being only 30% as efficient, and transport of DHA being only 5% as efficient [7]. The transport of the L-forms of AA into chondrocytes, and the inefficiency of transport of the D-forms of AA to compete with L-^{14}C-Asc appears to explain the nutritive properties of this vitamin. D-Asc has only 5% of the nutritive (anti-scorbutic) properties of L-Asc [39], which reflects the ability of the different AA forms to be transported into cells.

The mechanism of AA transport in chondrocytes is a carrier-mediated process that can be saturated at high concentrations of the substrate and can be described by Michaelis–Menton kinetics. The K_m value of 62 μM measured in the primary human chondrocytes is similar to previously published values for SVCT2 ranging from approximately 13 μM to 125 μM [17,19,20,26,27,40,41]. The lowest K_m value of 13 μM was determined in human endothelial cells [19]. The highest value of 125 μM was for a high affinity AA transporter in bovine retinal pigment epithelial cells but this was prior to the identification and characterization of the SVCTs [41]. A K_m value of 30 μM for osteoblasts is the closest to chondrocytes for cells clearly shown to solely express SVCT2 [26,27].

In summary, we provide the first evidence that human chondrocytes express SVCT2 and also that this transporter on chondrocytes is capable of concentrating AA against a concentration gradient to extremely high intracellular levels. The SVCT2-mediated AA transport and concentration in human chondrocytes is commensurate with the importance of AA to chondrocyte function and cartilage extracellular matrix homeostasis.

Acknowledgements

This work was supported by a National Science Foundation Graduate Fellowship (A.L.M.) and the NIH Program Project Grant in OA P50 AR049056. We thank Dr. Farshid Guilak for advice regarding the AA concentration experiments and Thomas Stabler for technical assistance with the HPLC. We also thank Dr. Brice Weinberg for providing the nucleofector device for the RNAi experiments.

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