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Functional Characterization and Molecular Identification of Vitamin C Transporter (SVCT2) in Human Corneal Epithelial (HCEC) and Retinal Pigment Epithelial (D407) Cells

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Abstract

Purpose—The main goal of this study is to investigate the existence of sodium-dependent vitamin C transport system (SVCT2) and to define time-dependent uptake mechanism and intracellular regulation of ascorbic acid (AA) in human corneal epithelial (HCEC) and human retinal pigment epithelial (D407) cells.

Methods—Uptake of [¹⁴C] AA was studied in HCEC and D407 cells. Functional aspects of [¹⁴C] AA uptake were studied in the presence of different concentrations of unlabeled AA, pH, temperature, metabolic inhibitors, substrates and structural analogs. Molecular identification of SVCT2 was examined with reverse transcription–polymerase chain reaction (RT-PCR).

Results—Uptake of [¹⁴C] AA was observed to be sodium, chloride, temperature, pH and energy-dependent in both cell lines. [¹⁴C] AA uptake was found to be saturable, with K_m values of 46.14 ± 6.03 and 47.26 ± 3.24 μ M and V_{max} values of 17.34 ± 0.58 and 31.86 ± 0.56 pmol/min/mg protein, across HCEC and D407 cells, respectively. The process is inhibited by structural analogs (L-AA and D-Iso AA) but not by structurally unrelated substrates (glucose and PAHA). Ca^{++} /calmodulin and protein kinase pathways play an important role in modulating uptake of AA. A 626 bp band corresponding to a vitamin C transporter (SVCT2) has been identified by RT-PCR analysis in both the cell lines.

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Supplementary material available online

Supplementary Figures S1–S3

DECLARATION OF INTEREST

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Conclusion—This research article reports regarding the ascorbic acid uptake mechanism, kinetics and regulation by sodium dependent vitamin C transporter (SVCT2) in HCEC and D407 cells. Also, SVCT2 can be utilized for targeted delivery in enhancing ocular permeation and bioavailability of highly potent ophthalmic drugs.

Keywords

Ascorbic; acid kinetics; carrier-mediated; transport and nutrient; transporter; *in vitro* models; targeted; drug; delivery

INTRODUCTION

Ascorbic acid (AA, vitamin C) is an essential water-soluble vitamin required for various physiological and metabolic functions. It is a potent reducing agent that effectively quenches various reactive oxygen species (ROS), absorbs ultraviolet (UV) radiation and protects cornea, lens and other intraocular tissues against light-induced damage.^{1,2} Levels of antioxidant AA in aqueous humor plays an important role in the prevention of cataract. Human ocular tissues contain considerably higher amounts of ascorbic acid (1.33 mg/g wet weight in corneal epithelium, 0.20 mg/ml in aqueous humor, 23 mg/100 g in neural retina and 5 mg/100 mg in retinal pigment epithelium (RPE)/choroid).^{2,3} Concentration of ascorbic acid in human tear fluid (23 μ M) is responsible for rapid wound healing and tear stability during ocular infections. Protective role of AA against various ocular infections and UV-induced damage of ocular tissue suggests the need to scrutinize the mechanism of AA transport across ocular tissues (mainly, cornea and retina).²

Human and other primates cannot synthesize AA thus making this vitamin an essential dietary requirement. Therefore, AA is usually obtained from exogenous sources through the dietary intake.⁴ AA uptake has already been reported in intestine,⁵ brain,⁶ kidney,⁷ skin,⁸ eye^{2,9} and bone¹⁰ via specific transporter system. Human *SLC23A2* family consists of two isoforms of sodium-dependent vitamin C transporters (SVCT) namely SVCT1 and SVCT2.¹¹ Comparative analysis of two isoforms reveals that AA exhibits higher affinity towards SVCT2 than SVCT1. Both SVCT1 and SVCT2 express close sequence homology and functional similarity but vary in distribution.² Structural and functional studies show that transport of AA across various epithelial cells is mainly mediated via SVCT^{2,4}

In recent years, transporter-targeted drug delivery has been widely employed to improve the drug absorption across biological membranes. In order to enhance drug permeation and absorption, active agent is chemically modified with transporter-targeted moieties. Interaction between targeting moiety and the transporter will facilitate transport of conjugated drug across the cell membrane.¹² Many investigators have exploited SVCT2 as an important target for drug delivery across various membranes. AA-conjugated saquinavir shows higher absorptive permeability and metabolic stability compared to saquinavir alone.¹³ Conjugation of nipecotic, kynurenic and diclophenamic acids with AA has been utilized as an important tool in studying the mechanism of AA prodrugs interactions with vitamin C transporters across RPE cells. This approach has provided a new perspective for transporter-/receptor-targeted prodrug delivery by means of SVCT2 transporter system.¹⁴

Hence, SVCT-targeted drug delivery can be utilized as a vital strategy for enhancing intracellular accumulation of drugs in ocular complications. The aim of this study is to investigate the presence of a specialized carrier-mediated transport system (SVCT2) across human corneal (HCEC) and retinal (D407) cells.

The presence of SVCT on rabbit cornea and Madin–Darby canine kidney cells has been previously reported from our laboratory.^{2,4} Variation in structural and biological properties between the rabbit and human eye suggest that the expression of transporters and receptors can highly differ. These anatomical and functional disparities among various species make it difficult to determine their impact on ocular drug absorption in human.¹⁵ This compelled us to study the practicality of human-derived ocular cell lines as *in vitro* models for ocular drug absorption.

In the present study, we evaluated the ascorbic acid uptake process, kinetics as well as the expression, relative contribution and regulation of the SVCT2 in HCEC and D407 cells. No information currently exists with regard to a comprehensive study of AA carrier-mediated uptake across HCEC and D407 cells. Results obtained from this study may indicate involvement of a specific and high-affinity carrier transport system (SVCT2) for translocation of AA.

MATERIAL AND METHODS

Materials

[¹⁴C] Ascorbic acid ([¹⁴C] AA specific activity 8.5 mCi/mM) was procured from Perkin Elmer (Boston, MA). Unlabeled L-ascorbic acid, D-iso-ascorbic acid, dehydro-ascorbic acid (DHAA), glucose, para-amino hippuric acid (PAHA), sodium azide, ouabain, 2,4-dinitrophenol, choline chloride, HEPES, bovine insulin, human epidermal growth factor, Triton X-100, phorbol-12-myristate-13-acetate (PMA), bisindolylmaleimide I (BIS), 3-isobutyl-1-methyl-xanthine (IBMX), 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid (DIDS), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITC) and D-glucose were purchased from Sigma Chemical Co (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Atlanta biologicals (Lawrenceville, GA). Culture flasks (75 cm² growth area) and uptake plates (3.8 cm² growth area) were obtained from Corning Costar Corp. (Cambridge, MA). The buffers for cDNA synthesis and amplification (oligodT, dNTP, MgCl₂, M-MLV reverse transcriptase and Taq polymerase) were procured from Promega Corporation (Madison, WI). Light Cycler 480[®] SYBR I green master mix was obtained from Roche Applied Science (Indianapolis, IN). Qualitative and quantitative primers used in this study were custom-designed and obtained from Invitrogen Life Technologies (Carlsbad, CA). All other chemicals were obtained from Fisher Scientific Co. (Fair Lawn, NJ) and utilized without further purification.

Cell culture

HCEC and D407 cells were generous gifts from Dr Araki-Sasaki (Kinki Central Hospital, Japan) and Dr Richard Hunt (University of South Carolina, Columbia, SC, USA), respectively. HCEC cells were cultured according to previously published protocol.^{16–18} Cells of passage numbers between 25 and 30 were cultured at 37 °C, humidified 5% CO₂/95% air atmosphere in a DMEM/F-12 culture medium supplemented with 15% (v/v) FBS (heat inactivated), 15 mM HEPES, 22 mM NaHCO₃, 100 mg of penicillin and streptomycin each, 5 µg/ml insulin, and 10 ng/ml of human epidermal growth factor.

D407 cells (passage numbers between 75 and 80) were grown at 37 °C, humidified 5% CO₂/95% air atmosphere in a DMEM culture medium supplemented with 10% (v/v) FBS (heat inactivated), 29 mM NaHCO₃, 20 mM HEPES, 100 mg of penicillin and streptomycin each, and 1% non-essential amino acids at pH 7.4.^{19,20} The growth medium was changed every alternate day. Both HCEC and D407 cells were cultured in 75 cm² flasks, harvested at 80–90% confluency with TrypLE™ Express (Invitrogen, Carlsbad, CA). Cells were then plated in 24-well uptake plates at a density of 300,000 cells/well. Cells were grown in a similar way as mentioned above and utilized for additional studies.

Uptake studies

Confluent HCEC and D407 cells were utilized for uptake experiments. Following media removal, cells were rinsed thrice for 5 min each with 1–2 ml of Dulbecco's phosphate-buffered saline (DPBS) containing 130 mM NaCl, 0.03 mM KCl, 7.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgSO₄, 20 mM HEPES, and 5 mM glucose maintained at pH 7.4. Uptake studies were initiated by adding 250 µl of solution containing 0.25 µCi/ml of [¹⁴C] AA in the presence and absence of various competing substrates. Following incubation, the solution was removed and uptake was terminated with 2 ml of ice-cold stop solution containing 200 mM KCl and 2 mM HEPES. The cell monolayer was washed thrice, 5 min each and 1 ml of lysis buffer (0.1% Triton-X solution in 0.3% NaOH) was added to each well and plates were stored overnight at room temperature. Subsequently, the cell lysate (400 µl) from each well was transferred to scintillation vials containing 3 ml of scintillation cocktail (Fisher Scientific, Fairlawn, NJ). Samples were quantified by measuring the radioactivity using liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA; model LS-6500). Protein content of each sample was estimated by BioRad Protein Estimation Kit (BioRad Protein Estimation Kit, Hercules, CA).

Time and temperature dependency

Optimum time for [¹⁴C] AA uptake was determined by performing the uptake studies over various time points (5, 10, 15, 30, 45 and 60 min). The uptake study was conducted according to previously published protocol.^{16–18}

The effect of temperature on [¹⁴C] AA uptake was determined by carrying out the uptake study at different temperatures, i.e. 4 °C, 25 °C and 37 °C.

pH and ion dependency

The effect of pH on [^{14}C] AA uptake was observed by adjusting the buffer pH to 5, 6, 6.5, 7.4 and 8. For delineating the role of sodium ions on [^{14}C] AA uptake, sodium chloride (NaCl) and sodium phosphate dibasic (Na_2HPO_4) in DPBS were substituted with equimolar quantities of choline chloride and potassium phosphate dibasic (KH_2PO_4), respectively. Hill coefficient for AA uptake, as function of Na^+ was determined. In a similar way, buffer solution containing sodium (130 mM), potassium (0.03 mM), and calcium (1 mM) chlorides were replaced with equimolar quantities of sodium phosphate, potassium phosphate and calcium acetate, respectively.

In another study, cells were pre-incubated with 1 mM amiloride (sodium channel inhibitor) and the uptake study was carried out as mentioned earlier.

Concentration dependency

Several working concentrations of L-AA were prepared ranging from (0.12 μM –1000 μM) in DPBS (pH 7.4) spiked with [^{14}C] AA (0.25 $\mu\text{Ci/ml}$). The data were fitted to Michaelis–Menten equation as shown in Equation (2) and the maximum transport rate (V_{max}) and Michaelis–Menten constant (K_m) were calculated according to a non-linear least squares regression analysis program; GraphPad Prism version 5 (San Diego, CA, USA).

Role of metabolic and membrane transport inhibitors

For energy dependency, simultaneous incubation of [^{14}C] AA along with metabolic inhibitors, such as ouabain (Na^+/K^+ ATPase inhibitor), 2,4-dinitrophenol (intracellular ATP reducer) and sodium azide (oxidative phosphorylation inhibitor) was performed for 1 h. In order to examine the effect of anionic membrane transport inhibitors, cells were pre-incubated with 1 mM SITC, DIDS and probenecid for 1 h.

Substrate specificity

The substrate specificity for SVCT was delineated by carrying out [^{14}C] AA uptake in the presence of three different concentrations of (250, 500 and 1000 μM) unlabeled L-AA and structural analogs such as D-Iso AA and DHAA. A similar study was carried out at three different concentrations of (250, 500 and 1000 μM) various structurally unrelated analogs such as glucose (Glucose transporter/GLUT substrate) and PAHA (organic anion transporter/OAT substrate).

Intracellular regulation

The influence of various intracellular regulatory pathways such as Ca^{++} /calmodulin, PTK (protein tyrosine kinase), PKC (protein kinase C) and PKA (protein kinase A) pathway on [^{14}C] AA uptake was also investigated. Cells were pre-incubated with three different concentrations of modulators, such as calmidazolium (CaM) and KN-62 to delineate if the process is dependent on Ca^{++} /calmodulin pathway or not. In order to examine the role of PTK pathway in [^{14}C] AA uptake, cells were pre-incubated with three different concentrations of genistein and tyrphostin A25. For studying the effect of PKC pathway, cells were pre-incubated with three different concentrations of BIS and PMA. The effect of PKA

pathway was studied by incubating cells with three different concentrations of IBMX and forskolin. Uptake of [^{14}C] AA was then performed according to the procedure.

Reverse transcription–polymerase chain reaction

Reverse transcription–polymerase chain reaction (RT-PCR) analysis was employed to determine the expression of SVCT2 on HCEC and D407 at the molecular level. TRIzol[®] reagent (Invitrogen, USA) was used to carry out the cell lysis and chloroform was added to the lysate for phase separation. Following separation of aqueous phase containing RNA, isopropanol was added to precipitate RNA. Obtained RNA was rinsed twice with 75% ethanol followed by resuspension in RNase-DNase free water. The concentration and purity of RNA was determined using Nanodrop (Thermo Fisher Scientific, Wilmington, DE). RNA was reverse-transcribed to obtain cDNA using oligodT as a template and M-MLV reverse transcriptase. The conditions for reverse transcription were: denaturation of the template RNA for 5 min at 70 °C; reverse transcription for 60 min at 42 °C followed by a final extension at 72 °C for 5 min. cDNA obtained was then subjected to PCR for amplification of SVCT with specific primers. The primers (5' → 3') designed for SVCT2 were: forward: CCAGCGGTGAGCAGGACA AT, reverse primer: TAGGGCCACCGTGGGTGTAA. These primers correspond to a 626 base pair (bp) product in human SVCT2 cDNA. The conditions of PCR amplification were: denaturation for 30 s at 94 °C, annealing for 1 min at 56 °C, and extension for 1 min at 72 °C, for 45 cycles followed by a final extension for 5 min at 72 °C. PCR product was analyzed by gel electrophoresis on 1.5% agarose in TAE buffer and visualized under UV.¹⁵

Quantitative real-time PCR

Following reverse transcription, quantitative real-time PCR (qPCR) was performed with LightCycler[®] SYBR green technology (Roche). cDNA equivalent to 80 ng in each well was subjected to amplification with specific primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an internal control to normalize the amount of cDNA in each well. The sequences of real-time primers (5' → 3') used for SVCT2 were: forward: CCAGCGGTGAGCAGGA CAAT, reverse primer: TAGGGCCACCGTGGGTGT AA, GAPDH: forward—ATCCCTCCAAAATCAAG TGG and reverse—GTTGTCATGGATGACCTTGG. A preliminary experiment was performed to ensure that SVCT2 and GAPDH were amplified with equal efficiencies. The specificity of these primers was also confirmed with melting-curve analysis. The comparative threshold method was applied to calculate the relative amount of SVCT2 in HCEC and D407 cells.^{15,21}

Data analysis

Radioactive sample analysis—Uptake of [^{14}C] AA was calculated using disintegrations per minute (DPM) of sample and donor solutions as represented in Equation (1)

$$C_{\text{sample}} = \frac{DPM_{\text{sample}} * C_{\text{donor}}}{DPM_{\text{donor}}} \quad (1)$$

where DPM_{sample} and DPM_{donor} represent average values of DPM counts of sample and donor, respectively ($n = 4$); C_{donor} denotes concentration of donor used and C_{sample} represents the concentration of sample.

Calculation of Michaelis–Menten kinetic parameters

In order to determine K_m and V_{max} associated with [^{14}C] AA uptake, concentration-dependency data was fitted to a modified Michaelis–Menten equation [Equation (2)]

$$v = \frac{V_{\text{max}}[C]}{K_m + [C]} + K_d[C] \quad (2)$$

where v represents the total uptake, V_{max} stands for the maximum uptake rate for the carrier-mediated process, K_m is the Michaelis–Menten constant which represents the concentration at half saturation, K_d is a non-saturable diffusion rate constant and C is the substrate concentration. In Equation (2), $(V_{\text{max}} * C)/(K_m + C)$ represents carrier-mediated saturable process whereas $K_d(C)$ gives the non-saturable diffusion component. Data were fitted to a non-linear least-square regression analysis program (GraphPad Prism 5.0). The Michaelis–Menten kinetic parameters were calculated to determine saturable and non-saturable component of the total uptake.

Calculation of Hill's ratio

The $\text{Na}^+:\text{L-Ascorbic acid}$ coupling ratio was determined using the logarithmic form of the Hill equation [Equation (3)]

$$\text{Log} \left[\frac{v'}{V_{\text{max}}} - v' \right] = n \log(S) - \log K' \quad (3)$$

where v' denotes the initial velocity, V_{max} represents the maximal velocity, n is the number of substrate binding sites, S denotes the substrate concentration, and K' is a constant comprising multiple interaction factors and intrinsic dissociation constant.

Statistical analysis

All the experiments were conducted at least in quadruplicate ($n = 4$) and the outcomes were expressed as mean $\pm$ standard deviation (SD). To calculate statistical significance, Student's t -test was performed. Any difference between mean values is considered statistically significant for p value < 0.05 .

RESULTS

Time and temperature dependency

Time-dependent uptake of [^{14}C] AA in HCEC and D407 cells is depicted in Figure 1. Linear uptake of [^{14}C] AA was noticed up to 60 min of incubation period. Therefore, 30 min uptake time was selected for further uptake experiments.

Effect of temperature on the uptake of AA by HCEC and D407 cells was studied. Figure 2A clearly depicts that the uptake of [^{14}C] AA in HCEC and D407 cells was maximal at 37 °C. Uptake of [^{14}C] AA in HCEC shows 45 and 80% decrease when the temperature was reduced to 25 °C and 4 °C, respectively. In D407 cells, uptake was reduced to 60 and 20% when measured at 25 °C and 4 °C, respectively, suggesting that the process may be carrier-mediated in both cell lines. Activation energy (E_a) was calculated by plotting the temperature-dependent data, i.e. uptake rate $\ln(v)$ versus $1/T$ (Figure S1). E_a was calculated to be 7.80 kcal/mol and 7.23 kcal/mol in HCEC and D407 cells, respectively.

pH and ion dependency

In order to determine the role of an inward-driven proton gradient for [^{14}C] AA uptake, the study was carried out at different pH ranging from 5 to 8 in HCEC and D407 cells. Uptake of [^{14}C] AA elevated with a rise in extracellular pH from 5 to 8 in both cell lines. Relative to pH 7.4, uptake of [^{14}C] AA diminished to 40% and 50% at pH 5 and 6, respectively, in HCEC and D407 cells (Figure 2B). Based on these results, further uptake studies were carried out at pH 7.4 with both the cell lines.

Uptake of [^{14}C] AA was reduced to 35 and 50%, respectively, in HCEC and D407 cells, in the presence of sodium-free media (Figure 3A). Similarly uptake of AA was also diminished to 50 and 60% in HCEC and D407 cells, in the presence of amiloride (Na^+ transport inhibitor) indicating possible involvement of sodium ions in the translocation of AA (Figure 3A).

The effect of chloride ions was studied by replacing chloride ions with equimolar quantities of other monovalent cations in DPBS. A marked reduction (40 and 45%) in the uptake rate of [^{14}C] AA was observed in the absence of chloride ions in HCEC and D407 cells, respectively. This study clearly delineates the involvement of sodium and chloride ions in active transport of AA (Figure 3A).

Kinetics of AA in HCEC and D407 cells were also evaluated with various concentrations of sodium (0–140 mM) in the DPBS. Elevated uptake of [^{14}C] AA was observed with higher Na^+ concentrations. Uptake data demonstrated saturation kinetics displaying the saturation of [^{14}C] AA uptake at about 70 mM of Na^+ concentration in both HCEC and D407 cells (Figure 3B). Hill's transformation of the Na^+ saturation kinetics data showed 2:1 molar ratio of Na^+ :AA coupling in both cell lines (Figure S2).

Concentration dependency

The Michaelis–Menten saturation kinetics of AA uptake were investigated by incubating HCEC and D407 cells with various concentrations (0.12–1000 μM) of unlabeled AA for 30

min at 37 °C. The uptake process involves both the saturable carrier-mediated pathway and non-saturable diffusional process in both HCEC and D407 cells. Uptake study performed at 4 °C clearly delineated the passive diffusion component from the AA transport in both HCEC and D407 cells. The carrier-mediated process of AA uptake in both the cell lines was plotted as the difference of total uptake of AA at 37 °C and passive uptake of AA at 4 °C. Uptake of [¹⁴C] AA in HCEC cells was found to be concentration-dependent and saturable with K_m and V_{max} values of $46.14 \pm 6.03 \mu\text{M}$ and $17.34 \pm 0.58 \text{ pmol/mg protein/min}$, respectively (Figure 4A). The kinetic parameters (K_m and V_{max}) estimated for D407 cells were higher than those observed with HCEC, although similar saturation kinetics plot was observed in D407 cells. The K_m and V_{max} values obtained from the saturation kinetics plot for D407 cells were $47.26 \pm 3.24 \mu\text{M}$ and $31.86 \pm 0.56 \text{ pmol/mg protein/min}$, respectively (Figure 4B). The Lineweaver–Burk ($1/v$ versus $1/[S]$) plot, indicate the involvement of a single carrier in the uptake process of AA across HCEC and D407 cell lines (Figure S3).

Role of metabolic and membrane transport inhibitors

In order to delineate the effect of metabolic inhibitors on [¹⁴C] AA uptake in both HCEC and D407 cells, uptake studies were carried out in the presence of metabolic inhibitors, such as ouabain (Na^+/K^+ ATPase inhibitor), sodium azide (oxidative phosphorylation inhibitor) and 2,4-DNP (intracellular ATP reducer). In the presence of these metabolic inhibitors, a significant reduction (40 to 60%) in the uptake of [¹⁴C] AA was observed (Figure 5A).

Further investigations were carried out in HCEC and D407 cells to investigate the effect of membrane inhibitors (SITC, DIDS and probenecid) on [¹⁴C] AA uptake. AA uptake was reduced to 40% in the presence of SITC, DIDS and probenecid, showing the involvement of an anion exchanger in carrier-mediated transport of [¹⁴C] AA (Figure 5B).

Substrate specificity

With increasing concentration (250, 500 and 1000 μM) of AA structural analogs, [¹⁴C] AA uptake in HCEC and D407 cells was significantly inhibited in the presence of L-AA and D-Iso AA, whereas no significant change in the uptake of [¹⁴C] AA was observed in DHAA. Also, no significant change in the uptake of [¹⁴C] AA was observed in the presence of structurally unrelated analogs, i.e. glucose and PAHA (250, 500 and 1000 μM) in both the cell lines (Figure 6).

Intracellular regulation

The role of different cellular regulation pathways on [¹⁴C] AA uptake was also investigated. With increasing concentrations of calmidazolium and KN-62 (modulators of $\text{Ca}^{++}/\text{calmodium}$ ($\text{Ca}^{++}/\text{CaM}$) pathway), reduced uptake of [¹⁴C] AA ranging from 25–41% to 27–41%, respectively, in HCEC, and, 20–47% and 20–40%, respectively, in D407 cells were observed. PKC activator, PMA (10–100 μM), significantly inhibited uptake of AA in the HCEC and D407 cells. Inhibitory effect of PMA was muted in both the cell lines by BIS (25–100 μM). Involvement of a PKA-mediated pathway in the regulation of [¹⁴C] AA uptake was also examined by evaluating the effects of substrates at various concentrations. These compounds are known to induce intracellular cAMP levels (IBMX and forskolin), thus activating PKA in HCEC and D407 cells. Concentration-dependent effect on the

reduction of [^{14}C] AA uptake was evident in the presence of IBMX and forskolin, indicating the involvement of cAMP-regulated PKA pathways in AA transport. Similarly, we examined the contribution of the PTK pathway on [^{14}C] AA uptake by incubating cells with genistein and tyrphostinA25 for 1 h. Significant inhibition was observed in the uptake of AA with genistein and tyrphostinA25 (modulators of PTK-mediated pathway) across HCEC and D407 cells (Table 1).

Reverse transcription–polymerase chain reaction analysis

For confirmation of the existence of AA transport system (SVCT) in HCEC and D407 cells, RT-PCR analysis was carried out. Agarose gel electrophoresis using ethidium bromide was employed to analyze the PCR product. PCR amplification of cDNA produced from total RNA was done with primers specific for a human SVCT system. The PCR product obtained at 626 bp confirms the expression of the AA transport system (SVCT2) on HCEC as well as D407 cells (Figure 7A).

Quantitative real-time PCR analysis

RNA extraction and cDNA synthesis were carried out according to published protocol.²² Quantitative estimates of the relative abundance of SVCT2 mRNA were obtained with qPCR analysis. mRNA levels of SVCT2 were analyzed in HCEC and D407 cells. Expression of SVCT2 mRNA in D407 was significantly higher relative to HCEC (Figure 7B).

DISCUSSION

AA (vitamin C) is a vital cellular nutrient responsible for normal metabolic and physiological functions. Due to their biosynthetic limitation, distribution of AA between extra- and intra-cellular fluids in all cells is strictly reliant on absorption through functional vitamin C transporters.²³ AA is present in highest concentration in ocular tissues and aqueous humor to protect retinal cells and lens from the deleterious effects of sunlight.²⁴ AA is responsible for absorption of 99.96% of radiation even before it reaches the lens.¹ Anti-cataract role of ascorbic acid justifies the presence of high ascorbic acid levels in aqueous humor. All these biological progressions put emphasis on having a thorough understanding of the mechanism of AA transport in ocular tissues.^{25,26}

In the present study, we have evaluated the AA uptake process, as well as the expression, contribution and regulation of the SVCT2 across HCEC and D407 cells. Recently, *in vitro* culture models have been applied as prized tools to predict ocular drug permeation. These cell culture models have provided a platform for additional investigation on ocular drug delivery *in lieu* of *in vivo* studies. Evaluation of drug transport across ocular tissues has been traditionally done utilizing primary cultures of rabbit corneal (rPCEC) and human retinal pigment epithelial (ARPE-19).²⁷ Unlike primary cultures, immortalized cell cultures can be subcultured many times without losing their metabolic and physiological characteristics including enzymatic activity and cytoskeletal polarization. These immortalized human ocular cell culture models have been utilized for cell biology, toxicity, ocular irritancy, drug delivery, drug permeability and transport studies.^{15,27}

Time course of AA uptake showed the linear uptake till 60 min. Hence, an incubation time of 30 min is selected for further uptake studies in HCEC as well as D407 cells (Figure 1). AA uptake process appears to be temperature-dependent with significant rapid rate at physiological temperature (37 °C) in comparison to 25 °C and 4 °C (Figure 2A). Rise in pH results in enhanced uptake of AA. Since the pK_{a1} of AA is 4.17, it exists primarily in ascorbate form (–1 charge) above the pH range of 5.0.^{28,29} As a result, AA enhanced uptake with increase in pH may not be due to the ionic state of AA. It can be concluded from pH dependency results that SVCT2 shows higher affinity towards ascorbate at higher pH (Figure 2B). Ion–ion and/or ion–polarity interactions between SVCT and its substrates are the major forces responsible for the binding of SVCT2 with the ionized form of AA. At lower pH, protonation of histidine residues reduces the binding affinity of SVCT2 with its substrates.²⁸

In the absence of sodium and chloride ions, lower AA uptake was noticed confirming that SVCT2 transporter system may be highly sodium and chloride dependent (Figure 3A). Moreover, presence of amiloride (a Na^+ transport inhibitor) caused significant inhibition to AA uptake demonstrating that the transport system is highly sodium dependent (Figure 3A). Transmembrane sodium gradient as well as the membrane potential are responsible for the uphill transport of SVCT2 substrates.² Hence, we studied whether the carrier-mediated transport of AA via SVCT2 is coupled with the electrochemical gradient of Na^+ ions in HCEC and D407 cells. In both cell lines, elevated uptake of AA was observed with rising concentrations of Na^+ in the uptake buffer and was found to be saturated at higher concentrations. These results suggest that AA is coupled to Na^+ and transported directly via SVCT2 (Figure 3B). The Hill ratio analysis indicates that approximately two sodium ions (1.9 for HCEC and 2.3 for D407) are required for translocation of each AA molecule (Figure S2). In this study, we have utilized 1 mM concentration of amiloride. Also, in previously reported results, same concentration of amiloride has shown inhibition of Na ion channels.^{12,30} We performed our studies within same concentration. Absence of Na ions in uptake buffer significantly lowers AA uptake indicating that Na ions may be essential for functioning of SVCT2. The sodium dependency is additionally reinforced by inhibitory effects of amiloride (Na^+ channel blocker). These studies clearly show that AA uptake is coupled with an inwardly directed gradient of Na ions in ocular cells. The decrease in AA uptake in the absence of Cl^- ions or in presence of Cl channel blockers requires further investigation in order to delineate its effect on AA uptake process at molecular levels. Concentration dependency of AA uptake clearly reveals that the carrier-mediated process is saturable at higher concentrations of unlabeled AA. In order to distinguish the passive diffusion component from the active AA transport, uptake study was carried out at 4 °C in both HCEC and D407 cells. AA uptake was found to be saturable with K_m and V_{max} values of $46.14 \pm 6.03 \mu M$ and $17.34 \pm 0.58 \text{ pmol/mg protein/min}$, respectively, in HCEC cells and K_m and V_{max} values of $47.26 \pm 3.24 \mu M$ and $31.86 \pm 0.56 \text{ pmol/mg protein/min}$, respectively, in D407 cells (Figure 4A and B). The Michaelis–Menten kinetic parameters (K_m and V_{max}) are two important parameters responsible for defining the functional and kinetic constants of a transporter. K_m defines the measure of apparent binding affinity of a substrate, whereas V_{max} represents a measure of translocation capacity of the carrier-mediated process. On the basis of Michaelis–Menten kinetics, the K_m value of SVCT2 was

found to be similar in both HCEC and D407 cells, indicating a similar binding strength and affinity of AA with both the cell lines. However, comparison of $V_{\max}$ values of SVCT2 for both cell lines suggest that transport capacity of SVCT2 in D407 cells is higher relative to HCEC cells (31.86 ± 0.56 versus 17.34 ± 0.58 pmol/mg protein/min). Catalytic efficiency of SVCT2 was estimated by the ratio of Michaelis–Menten kinetic parameters ($V_{\max}/K_m$). A variation in $V_{\max}$ values results in higher transport efficiency ($V_{\max}/K_m$) of SVCT2 for D407 (0.67 μ l/mg protein/min) than HCEC (0.37 μ l/mg protein/min). These kinetic parameters indicate possible involvement of a carrier-mediated transport system for the translocation of AA in HCEC and D407 cell lines. When the kinetic data was plotted in the form of Lineweaver–Burk ($1/v$ versus $1/[S]$) plot, a single line was obtained for HCEC and D407 cell, respectively, which suggests the involvement of a single transporter for the translocation of AA across human corneal and retinal cells (Figure S3). These Michaelis–Menten kinetic parameters of SVCT2 in D407 cells were in accordance with previously published reports showing the presence of SVCT2 across human RPE cells.¹⁴

AA uptake was significantly inhibited in HCEC and D407 cells in the presence of metabolic inhibitors such as sodium azide (oxidative phosphorylation inhibitor), 2,4-dinitrophenol (intracellular ATP reducer) and ouabain, (a known Na^+/K^+ ATPase inhibitor). These results confirm that the uptake of AA via SVCT2 is highly dependent on energy and directly coupled to ATP energy sources (Figure 5A). Uptake of AA was also significantly inhibited in the presence of various membrane/anion inhibitors such as DIDS, SITS and probenecid (Figure 5B). Similar reports involving inhibition of AA uptake by membrane inhibitors has been published.^{4,10} These results indicate that uptake of AA is altered by the presence of specific anions and the site of the SVCT2 transport system may be the plasma membrane.^{4,10}

In HCEC and D407 cells, concentration-dependent inhibition in SVCT2-mediated AA uptake was revealed in the presence of structural analogs of AA, i.e. L-AA and D-Iso AA. No significant inhibition in the SVCT2-mediated AA uptake was observed in the presence of DHAA (structural analog and GLUT substrate), glucose (GLUT substrate) and PAHA (OAT substrate) (Figure 6). Similar results with respect to substrate specificity have been published from our laboratory.^{4,31} These results in rabbit corneal epithelial cells corroborate our findings in HCEC and D407 cells. Due to a lack of 3D structure of SVCT2 transporter, it is difficult to postulate about structural requirements for the binding of SVCT2 with its substrates at this time and further investigations are required to address the issue.

Several intracellular regulatory pathways, such as Ca^{++} /calmodulin, PKA, PTK and PKC are involved in the regulation of expression sodium-ascorbate cotransporters.⁴ Five putative PKC phosphorylation sites in hSVCT1 and hSVCT2 and one additional PKA site in hSVCT1 have been identified based on the analysis of deduced primary amino acid sequence of SVCT.²⁸ It has also been reported that the uptake of AA mediated by SVCT2 expressed in COS-1 and MDCK-MDR1 cells was under the regulation of PKC-mediated pathway.^{4,32} For this reason, we investigated the regulation of AA uptake by inter-and intracellular protein kinase-mediated pathways. Significant inhibition in the presence of calmidazolium and KN-62 in HCEC and D407 cells was observed, leading to a hypothesis that the AA uptake process is under the regulation of Ca^{++} /CaM-mediated pathway. In this

study, we have assessed the effect of calmodulin modulators on AA uptake process. The effect of calmidazolium may be mediated via reduced activity and the affinity of the AA uptake process. The cellular mechanism via which the calmodulin-mediated pathway shows its effect on AA uptake is still unclear. Although, different mechanisms for calmodulin-mediated pathway have been described that include the activation/inhibition of specific protein kinases and a probable effect on the uptake transporter involved. Similar results have been reported before for carrier-mediated transport process.^{33,34} Treating both the cell lines with PMA led to a significant lowering in AA uptake, indicating the role of PKC-mediated pathway on the regulation of AA uptake. Also, addition of BIS (PKC pathway inhibitor) reverses the inhibitory effect of PMA signifying the contribution of PKC pathway in controlling this uptake process. The concentration of PMA utilized (10, 50 and 100 μ M) has the potential to modulate the uptake of ascorbic acid (AA) by affecting PKC pathway. The molecular mechanism by which activation and inhibition of PKC pathway exerts its effect on AA uptake process is not clear and requires further investigation. On the other hand, significant inhibition on AA uptake by IBMX- and forskolin-treated HCEC and D407 cells confirms that PKA-mediated pathway plays a vital role in SVCT2-mediated uptake of AA. Significant inhibition was observed in the presence of PTK pathway modulators (genistein and tyrphostin A25) showing a possible role of PTK pathway in intracellular regulation of AA (Table 1). The blockers utilized for tyrosine kinase pathways (PTK pathway) are well known. Also, we have utilized genistein as a blocker of PTK pathway (Table 1) at three different concentrations, i.e. 10, 50 and 100 μ M. The blockers utilized in this study genistein and tyrphostin A25 has been well documented by others for similar study.^{35,36} Molecular mechanism by which the protein kinase and calmodulin pathway exert their effect on AA uptake process is yet to be fully established.

Finally, RT-PCR analysis confirms the molecular evidence of a vitamin C/AA-specific carrier system in HCEC and D407 cells. The PCR product obtained at 626 bp is specific for the SVCT2 transporter system (Figure 7A). The qPCR analysis revealed that the expression of SVCT2 was significantly higher in D407 in comparison to HCEC cells (Figure 7B). We also performed the RT-PCR analysis for SVCT1 utilizing different set of primers, no band corresponding to SVCT1 was observed in both the cell lines (data not shown). Higher expression of SVCT2 in D407 provides the justification of greater catalytic transporter efficiency of SVCT2 in D407 relative to HCEC cells.

In summary, this study clearly delineates the detailed study of functional activity and molecular evidence as well as the expression, contribution and regulation of the sodium-dependent vitamin C transporter 2 (SVCT2) across HCEC and D407 cell lines. AA uptake across HCEC and D407 cells involves a carrier-mediated active process that is modulated by both protein kinases as well as Ca^{++} /CaM mediated pathways. This transporter is highly pH-dependent and requires coupling to an electrochemical Na^{+} gradient and ATP sources for cellular uptake of AA. This membrane transporter (SVCT2) can be utilized as a potential target for enhancing ocular bioavailability of AA conjugated prodrugs. This investigation demonstrates complete profiling of SVCT2-mediated AA uptake showing its dependence on calmodulin, protein kinases and intracellular ions. Also, HCEC and D407 cell line can be utilized as a valuable *in vitro* model than primary culture models to investigate ocular drug absorption of AA-conjugated therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Time course of [^{14}C] AA uptake across HCEC and D407 cells. Uptake of [^{14}C] ascorbic acid ([^{14}C] AA) was measured in DPBS buffer (pH 7.4) at 37 °C. Data are shown as mean $\pm$ S.D. $n = 4$. S.D. means standard derivation.

FIGURE 2.

(A) Temperature and (B) pH-dependent uptake study of $[^{14}\text{C}]$ AA uptake across HCEC and D407 cells in DPBS (pH 7.4). The uptake is expressed as percentage of control (37 °C).

Data are shown as mean $\pm$ S.D. $n = 4$. Asterisk (*) represents significant difference from the control (* $p < 0.05$, ** $p < 0.01$).

FIGURE 3. Uptake of [^{14}C] AA across HCEC and D407 cells, (A) in the presence of amiloride and absence of sodium and chloride ions and (B) in the presence of different concentration of sodium ions in DPBS buffer (pH 7.4) at 37 °C. * $p < 0.05$, ** $p < 0.01$.

FIGURE 4. Concentration-dependent uptake of [^{14}C] AA across (A) HCEC and (B) D407 cells. Data are shown as mean $\pm$ S.D. $n = 4$ (● represents total uptake, ▲ represents passive uptake/non-saturable component and ■ represents carrier-mediated uptake/saturable component).

FIGURE 5. Uptake of $[^{14}\text{C}]$ AA across HCEC and D407 cells in the presence of (A) metabolic inhibitors (ouabain, sodium azide and 2,4-DNP), and (B) membrane inhibitors (SITC, DIDS and probenecid). $[^{14}\text{C}]$ AA uptake was performed at 37 °C with DPBS buffer (pH 7.4) for 30 min. Data are shown as mean $\pm$ S.D. $n = 4$. * $p < 0.05$, ** $p < 0.01$.

FIGURE 6. Uptake of [14 C] AA in presence of L-ascorbic acid (L-AA), D-isoascorbic acid (D-Iso AA), dehydro ascorbic acid (DHAA), D-glucose and para-amino hippuric acid (PAHA) at three different concentrations across HCEC and D407 Cells. [14 C] AA uptake was performed at 37 $^{\circ}$ C with DPBS buffer (pH 7.4) for 30 min. Data are shown as mean $\pm$ S.D. $n = 4$. * $p < 0.05$, ** $p < 0.01$.

FIGURE 7.

(A) RT-PCR studies showing the molecular evidence of SVCT2 in HCEC and D407 cells. Lane 1 and 3 represents 100 bp molecular ladder and lane 2 and 4 represents 626 bp PCR product obtained from HCEC and D407 cells, respectively. (B) Real time-PCR comparing the expression of SVCT2 in HCEC and D407 cells. Data are shown as mean $\pm$ S.D. $n = 4$. * $p < 0.05$, ** $p < 0.01$.

TABLE 1

Uptake of [^{14}C] AA across HCEC and D407 cells in the presence of various concentrations of Ca^{++} /calmodulin pathway, PKC pathway, PKA pathway and PTK pathway modulators in DPBS (pH 7.4) at 37 °C. The uptake is expressed as percentage of control (DPBS).

Pathways	Modulators	Uptake as % of Control	
		HCEC	D407
Ca ⁺⁺ /calmodulin pathway	Control	100 ± 0.26	100 ± 1.09
	CaM (10 µM)	41.02 ± 4.03**	47.31 ± 1.43**
	CaM (50 µM)	39.35 ± 7.18**	35.43 ± 3.91**
	CaM (100 µM)	25.06 ± 0.87**	20.54 ± 2.28**
	KN-62 (0.1 µM)	51.19 ± 5.48**	40.43 ± 5.22**
	KN-62 (1 µM)	36.33 ± 3.71**	30.92 ± 4.78**
	KN-62 (10 µM)	27.75 ± 2.50**	20.42 ± 2.76**
PKC pathway	Control	100 ± 4.25	100 ± 4.91
	PMA (10 µM)	50.19 ± 5.56**	58.21 ± 4.35**
	PMA (50 µM)	46.47 ± 3.83**	50.87 ± 1.29**
	PMA (100 µM)	45.43 ± 17.43**	43.67 ± 5.48**
	PMA (100 µM) + BIS (25 µM)	91.2 ± 5.32	90.34 ± 2.95
	PMA (100 µM) + BIS (50 µM)	93.85 ± 3.49	92.69 ± 3.45
	PMA (100 µM) + BIS (100 µM)	95.34 ± 6.84	95.73 ± 5.85
PKA pathway	Control	100 ± 3.24	100 ± 2.65
	IBMX (0.1 µM)	38.52 ± 2.20**	43.67 ± 3.12**
	IBMX (1 µM)	34.42 ± 3.16**	37.77 ± 1.87**
	IBMX (5 µM)	28.12 ± 3.11**	30.43 ± 5.21**
	Forskolin (1 µM)	37.87 ± 1.15**	35.63 ± 2.35**
	Forskolin (10 µM)	31.84 ± 9.97**	33.22 ± 4.98**
	Forskolin (50 µM)	27.29 ± 2.60**	30.87 ± 3.65**
PTK pathway	Control	100 ± 3.24	100 ± 2.65
	Genistein (10 µM)	60.39 ± 2.36*	53.41 ± 2.43*
	Genistein (50 µM)	48.47 ± 4.83**	42.73 ± 1.94**
	Genistein (100 µM)	37.43 ± 7.43**	33.47 ± 3.68**
	Tyrphostin A25 (10 µM)	55.23 ± 6.59*	60.43 ± 4.59*
	Tyrphostin A25 (50 µM)	44.85 ± 1.84**	41.96 ± 7.23**
	Tyrphostin A25 (100 µM)	32.34 ± 5.39**	35.36 ± 2.58**

Data are shown as mean ± S.D. $n = 4$ (* $p < 0.05$, ** $p < 0.01$).