Sodium-ascorbate cotransport controls intracellular ascorbate concentration in primary astrocyte cultures expressing the SVCT2 transporter

Jasminka Korcok, Raphael Yan, Ramin Siushansian, S. Jeffrey Dixon, John X. Wilson

Department of Physiology, Faculty of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada

Accepted 8 August 2000

Abstract

Expression of the Na+-ascorbate cotransporter, SVCT2, was detected in rat brain and in primary cultures of cerebral astrocytes by Northern blot analysis. SVCT2 expression in cultured astrocytes increased in response to the cyclic AMP analog, dibutyryl cyclic AMP. A mathematical model of ascorbic acid transport was developed to evaluate the hypothesis that Na+-ascorbate cotransport across the plasma membrane regulates the steady state intracellular concentration of ascorbic acid in these cells. The outcomes predicted by this model were compared to experimental observations obtained with primary cultures of rat cerebral astrocytes exposed to normal and pathologic conditions. Both cotransport activity and intracellular ascorbic acid concentration increased in astrocytes activated by dibutyryl cyclic AMP. Conversely transport activity and ascorbic acid concentration were decreased by hyposmotic cell swelling, low extracellular Na+ concentration, and depolarizing levels of extracellular K+. In cells incubated for up to 3 h in medium having an ascorbic acid concentration typical of brain extracellular fluid, the changes in intracellular ascorbic acid concentration actually measured were not significantly different from those predicted by modeling changes in Na+-ascorbate cotransport activity. Thus, it was not necessary to specify alterations in vitamin C metabolism or efflux pathways in order to predict the steady state intracellular ascorbic acid concentration. These results establish that SVCT2 regulates intracellular ascorbic acid concentration in primary astrocyte cultures. They further indicate that the intracellular-to-extracellular ratio of ascorbic acid concentration at steady state depends on the electrochemical gradients of Na+ and ascorbate across the plasma membrane. © 2000 Elsevier Science B.V. All rights reserved.

1. Introduction

Vitamin C occurs almost entirely in its reduced form, ascorbic acid (AA), in normal brain [4,10,16,18,19]. While the steady state concentration of AA is 200–400 μM in brain extracellular fluid, it is approximately 10-fold higher in the cellular compartment. Brain cells cannot synthesize vitamin C from glucose de novo. Instead, they obtain the vitamin through plasma membrane transporters. Intracellular AA functions as an enzyme cofactor and may contribute to antioxidant defense in brain cells. The concentration and redox state of vitamin C are crucial for these functions. On the one hand, high concentrations of AA protect brain cells from ischemic, excitotoxic and oxidative injury [9,15,16,23,35]. On the other hand, high concentrations of oxidized vitamin C (dehydroascorbic acid, DHAA) are cytotoxic [20] and especially neurotoxic [12].

Vitamin C transport systems have been characterized in an experimental model of brain cells, namely, primary cultures of rat cerebral astrocytes. Cultured astrocytes achieve intracellular AA concentrations ([AA]i) as high as 8 mM when incubated with physiologic levels of extracellular AA for 3 h [24]. Longer incubation periods to do not
Recent attempts to clone from both rat and humans have shown that under the same conditions, they can induce RNA to normalize. The blots were separated on a 0.6% formaldehyde-agarose gel, transferred to nylon membrane, and cross-linked by UV irradiation. The signal was detected as the ratio, SVCT2 mRNA/18S rRNA, which was isolated from whole brain of neonatal rats.

The amount of RNA loaded in each lane was 15 μg. The astrocytes were grown to confluence in horse serum-supplemented, minimum essential medium that did not contain detectable metabolites. Total RNA was isolated using TRIzol (GIBCO). Northern blot analysis was performed according to a published procedure [26]. All subsequent treatments were carried out at 37°C unless otherwise noted. The astrocytes were grown to confluence in horse serum-supplemented, minimum essential medium that did not contain detectable AA (<1 μM) [25]. Like astrocytes in situ, these cells are coupled by connexin43-positive gap junctions and contain glial fibrillary acidic protein [11]. They were used for transport experiments after 3–4 weeks in culture.

2. Materials and methods

2.1. Cell cultures

The experimental protocols were approved by the University of Western Ontario Council on Animal Care. Primary cultures of astrocytes were prepared from the neopallium of 1-day-old Wistar rats according to our published procedure [26]. All subsequent treatments were carried out at 37°C unless otherwise noted. The astrocytes were grown to confluence in horse serum-supplemented, minimum essential medium that did not contain detectable AA (<1 μM) [25]. Like astrocytes in situ, these cells are coupled by connexin43-positive gap junctions and contain glial fibrillary acidic protein [11]. They were used for transport experiments after 3–4 weeks in culture.

2.2. Experimental procedures

Northern blot analysis was performed according to a modification of a published procedure [22]. Total RNA was isolated from whole brain of neonatal rat and primary astrocyte cultures using TRIzol (GIBCO). RNA was separated on 0.6% formaldehyde-agarose gel, transferred to nylon membrane and cross-linked by UV irradiation. The amount of RNA loaded in each lane was 15 μg. The blot was hybridized overnight with a 1 kb rat SVCT2 cDNA 32P-labelled probe, in ULTRAhyb (Ambion) at 42°C. Following hybridization, membrane was washed 2×15 min in 0.1×SSC, 0.1% S.D.S. and 2×30 min in 0.1×SSC, 0.1% S.D.S. at 42°C. The signal was detected by exposing the membrane to X-ray film. The blots were then stripped and reprobed for rat 18S ribosomal RNA under the same conditions to normalize RNA loading. The intensities of SVCT2 and 18S rRNA autoradiographic images were quantified by scanning densitometry and were expressed as the ratio, SVCT2 mRNA/18S rRNA.

Isoosmotic normal-Na⁺ transport medium consisted of

The astrocytes also take up DHAA through facilitative hexose transporters and reduce it to AA intracellularly [26]. This recycling of DHAA to AA depends on the rate of formation of DHAA in the extracellular fluid, is inhibited by physiologic concentrations of glucose, and is not influenced by Na⁺ [26]. The only pathway by which AA has been demonstrated to exit astrocytes is through volume-sensitive organic anion channels [25]. These channels become permeant to ascorbate in response to hyposmotic cell swelling. However, this response is only transitory and the plasma membrane permeability to ascorbate returns to normal within 3 min even though regulatory volume decrease is incomplete [25].

It is possible that [AA] is influenced by several mechanisms, namely, ascorbate uptake, DHAA uptake and recycling to AA, and ascorbate efflux. However, the effects of some of these mechanisms may be transient and not determinant for [AA] at steady state. Although several transport systems and metabolic pathways have been shown to influence AA levels in cells, their relative importance has remained controversial [19]. Indeed, their respective roles may vary between tissues. For instance, the brain differs from many other organs by having an extracellular AA concentration that is several-fold higher than plasma levels. As a result, the extracellular AA concentration in brain (200–400 μM) is sufficient to saturate high affinity Na⁺-ascorbate cotransport systems [31].

Recently, the transporters SVCT1 and SVCT2 were cloned from both rat and human and shown to induce Na⁺-dependent uptake of L-ascorbate when expressed heterologously [2,14,27–29]. Northern blot analysis of mammalian tissues detected transcripts encoding SVCT1 in intestine, kidney and liver, whereas SVCT2 transcripts were ubiquitous but had relatively high expression in brain. In the present study, we report that astrocytes in primary culture express SVCT2 and that the expression level is increased by cyclic AMP. Subsequently, we develop a mathematical model to evaluate the hypothesis that Na⁺-ascorbate cotransport activity regulates [AA]. Actual values for transport rates and intracellular concentrations were obtained using primary cultures of cerebral astrocytes exposed to normal and pathologic conditions. When measured rates for Na⁺-dependent AA uptake were entered as parameters in the model, the predicted values for [AA], were found to conform closely to experimental observations. Furthermore, changes in Na⁺-ascorbate cotransport activity were sufficient to explain the sustained influences of several pathologic conditions on [AA].
146
J. Korcok et al. / Brain Research 881 (2000) 144 ± 151

(in mM): 134 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 10 glucose and 20 Hepes (pH 7.3). The final Na⁺ concentration of this medium was 138 mM and its osmolality was 300 mOsm. Other transport media were made by changing the NaCl or KCl concentrations. The specific changes were as follows: (i) Isosmotic low-Na⁺ transport media containing either 35 or 80 mM Na⁺ were prepared by partial replacement of NaCl with the chloride salt of N-methyl-D-glucamine⁺. (ii) Hyposmotic transport medium was prepared by omission of 58 mM NaCl, so that the final Na⁺ concentration was 80 mM and the osmolality was 206 mOsm. (iii) Isosmotic high-K⁺ transport medium was prepared by substitution of 50 mM KCl for an equal concentration of NaCl, thus raising the Na⁺ concentration to 55.4 mM. (iv) Hyperosmotic normal-K⁺ transport medium was prepared by addition of sufficient N-methyl-D-glucamine chloride to increase the osmolality to 400 mOsm. (iv) Hyperosmotic high-K⁺ transport medium was prepared by addition of 50 mM KCl to increase both the K⁺ concentration and the osmolality.

Stock solutions of AA and L-[1-¹⁴C]AA (8 mCi/mmol, DuPont Canada) were prepared at 4°C and contained homocysteine to prevent oxidation of the vitamin [24]. These stock solutions were diluted in prewarmed transport media immediately before addition to cell cultures.

Initial rate of AA uptake was measured by incubating astrocytes in isosmotic normal-Na⁺ transport medium containing [¹⁴C]AA (5 μM, 37°C). Uptake was linear with time for at least 90 s. To study intracellular accumulation of AA in the presence of a physiologic concentration of extracellular AA, the astrocytes were incubated for the indicated periods in transport media containing 200 μM AA (with 160 μM homocysteine to prevent AA oxidation). Washing the cultures with ice-cold Tris-sucrose solution (pH 7.3) terminated uptake. [AA], was determined by acidic extraction and HPLC-based electrochemical assay (assay sensitivity was 2 pmol AA) [25]. Total cell protein was determined by the Lowry assay.

For experiments investigating AA efflux, astrocytes were first loaded with the radiolabeled vitamin by incubation for 60 min in isosmotic normal-Na⁺ transport medium containing 200 μM [¹⁴C]AA (with 160 μM homocysteine). Next, the cells were washed and incubated in the indicated transport media containing 200 μM unlabeled AA. Aliquots of media were collected at timed intervals and then the cells were harvested. The radioactive contents of the media and cells were measured by liquid scintillation counting.

2.3. Statistics

Data are presented as mean±S.E.M. values from n number of independent experiments, with triplicate replications (i.e., 3 culture dishes per treatment) in each experiment. In the figures, error bars are omitted where the S.E.M. is less than the size of the symbol. One way ANOVA and Dunnett’s test were used to evaluate the effect of dBcAMP on SVCT2 expression levels. A randomised block factorial design was employed to evaluate the effects of treatments on observed AA concentrations by two-way ANOVA. Additionally, the paired t-test was used to compare the observed AA concentrations with the theoretical values. Finally, the effects of treatments on [¹⁴C]AA efflux rates were analysed using one-way ANOVA. P<0.05 was considered to be significant for all statistical tests.

3. Results

SVCT2 was detected by Northern blot analysis in whole rat brain and primary astrocyte cultures (Fig. 1). DBcAMP increased astrocytic expression of SVCT2 after a latent
period of 12 h (Fig. 1). The initial rates of AA uptake after 24 h of treatment were 69±2 nmol/g protein/min in vehicle control and 104±4 nmol/g protein/min in dBcAMP-treated astrocytes (n=7 experiments, P<0.05).

We next developed a mathematical model to evaluate the hypothesis that Na+-ascorbate cotransport activity regulates [AA]. The mechanisms addressed by the mathematical model are illustrated in Fig. 2 and the details are presented in the DISCUSSION below. Uptake rate through the Na+-ascorbate co-transport system is designated as $J_1$. Volume-insensitive AA efflux from astrocytes is $J_2$; this efflux may occur by reversal of the co-transport system or by other volume-insensitive pathways. The AA efflux pathway that is activated transiently by cell swelling is $J_3$. DHAA formation, uptake and intracellular reduction to AA are shown as discrete steps in Fig. 2 but are designated altogether as $J_4$ in the model. The differential equation describing the rate of change of [AA] as a function of time was solved numerically and solutions are presented as the theoretical AA concentrations plotted in Figs. 3 and 4.

The results of the HPLC-based electrochemical assay showed that the astrocytes cultured in vitamin C-free medium did not contain intracellular AA initially. However, the cells gradually accumulated the vitamin when incubated with physiologic concentrations of AA (200 μM) and Na+ (138 mM) (Fig. 3). Activation of astrocytes by dBcAMP increased the AA uptake rate and steady state [AA]. The [AA] observed at each time point were compared to values predicted by the mathematical model. For both the vehicle control and dBcAMP-activated astrocytes, observed [AA] was not significantly different from predicted values (Fig. 3). This finding demonstrates that the model is capable of predicting [AA] using values for $J_1$ calculated from previously published $V_{\text{max}}$ and $K_m$ values for Na+-ascorbate cotransport by control and dBcAMP-activated astrocytes [31]. Furthermore, the effect of dBcAMP on steady state [AA], was modeled by changing $J_1$, without altering $J_2$, $J_3$ or $J_4$ ($J_2=0.012[AA]$, $J_3=0$, and $J_4=0$).

Incubation of astrocytes in media that decreased Na+-dependent ascorbate uptake rates led to decreased steady state [AA] (Fig. 4). The mathematical model was used to calculate theoretical values for steady state [AA], using values for $J_1$ calculated from transport parameters obtained in earlier studies (as presented in the Legend of Fig. 4). When the other fluxes were kept constant ($J_2=0.012[AA]$, $J_3=0$, and $J_4=0$), the [AA] values predicted were not significantly different from the concentrations actually measured (Fig. 4).

The equations modeled the effects of treatments on [AA] by varying the AA uptake rate and leaving the efflux rate unchanged. To further test the validity of the assumption that efflux rates do not change, we measured these rates under various experimental conditions. Table 1 shows the efflux rates obtained when astrocytes were incubated in hyperosmotic normal-K+ and high-K+ media. No effects
Fig. 4. Accumulation of AA in astrocytes incubated under conditions of changed ionic gradients. Primary astrocyte cultures were incubated with 200 μM AA for 3 h in normal, low Na⁺, hyposmotic or high K⁺ medium before being harvested and assayed for intracellular AA concentration. Bars show mean ± S.E.M. values from 4 experiments. The theoretical values were generated from the equation described in the Discussion using the following parameters: $J_o = 0.39 \mu$mol/g protein/min for the isoosmotic normal-Na⁺ condition (based on transport data in [31]); $J_o = 0.22 \mu$mol/g protein/min for the isoosmotic low-Na⁺ condition (35 mM Na⁺) [31]; $J_o = 0.27 \mu$mol/g protein/min for the hyposmotic condition [25]; and $J_o = 0.26 \mu$mol/g protein/min for the hyperosmotic high-K⁺ condition [34].

Fig. 5. Efflux of [1⁴C]AA from astrocytes incubated under conditions of changed ionic gradients. Primary astrocyte cultures were loaded with [1⁴C]AA, and then were washed and incubated for 10 min in the indicated transport media containing 200 μM unlabeled AA. In the case of isoosmotic high-K⁺ medium, this efflux incubation period is long enough to reverse Na⁺-glutamate cotransport but too brief to swell astrocytes and activate volume-sensitive organic anion channels [21]. Shown are the levels of [1⁴C]AA in the transport medium (top panel) or remaining in astrocytes (bottom panel) at the end of the efflux period. * $P < 0.05$ compared to control (isoosmotic medium containing normal concentrations of Na⁺ and K⁺).

4. Discussion

The present study showed that cultured cerebral astrocytes express SVCT2. Expression of this protein in Xenopus oocytes [2,14,27] conferred vitamin C transport activity that resembles that of astrocytes [31,34], with respect to Na⁺-dependence, electrogenicity, specificity and affinity for L-ascorbate. Furthermore, dBcAMP increased astrocytic expression of SVCT2 and rate of ascorbate uptake comparably. These similarities indicate that SVCT2 mediates ascorbate uptake in these cells.

We developed a mathematical model that predicts the rise in [AA], that occurs when AA-depleted astrocytes are incubated with physiologic concentrations of AA and Na⁺ (Fig. 2). Uptake rate through the Na⁺-ascorbate cotransport system is designated as $J_u$. Volume-insensitive AA efflux from astrocytes is $J_2$; this efflux may occur by reversal of the cotransport system or by other volume-
insensitive pathways. The AA efflux pathway that is activated transiently by cell swelling is \( J_2 \). DHAA formation, uptake and intracellular reduction to AA are shown as discrete steps in Fig. 2, but are designated altogether as \( J_4 \) in the mathematical model. The rate of change of [AA] is the sum of the rates of AA accumulation, less the rates of AA disappearance. Therefore,

\[
d[\text{AA}] / dt = J_4 - J_2 - J_3 + J_4.
\]

It has been shown previously that when AA-depleted astrocytes are incubated with 200 \( \mu \text{M} \) AA, \( J_4 \) decreases exponentially to a lower bound of 67\% after 18 h [33]. Therefore,

\[
J_4 = J_4(0.67 + 0.33 \exp(-st))
\]

where \( J_4(0 \) is the Michaelian initial rate of AA uptake at time zero and \( s \) is a parameter to be determined. When the extracellular AA concentration is 200 \( \mu \text{M} \),

\[
J_4(0) = 200 V_{\text{max}} /[K_m - 200].
\]

When cells are incubated with AA in isoosmotic-Na\(^+\) medium, the relevant parameters are \( V_{\text{max}} = 0.46 \mu\text{mol/g protein/min} \) and \( K_m = 32 \mu\text{M} \) for control astrocytes and \( V_{\text{max}} = 0.69 \mu\text{mol/g protein/min} \) and \( K_m = 34 \mu\text{M} \) for dbcAMP-activated astrocytes [31]. Therefore,

\[
J_4(0) = 0.39 \mu\text{mol/g protein/min} \) for control astrocytes, and
\[
J_4(0) = 0.59 \mu\text{mol/g protein/min} \) for activated astrocytes.
\]

Because \( J_4 \) decreases to 75\% of its original (time zero) value after 3 h and to 67\% after 18 h incubation with 200 \( \mu\text{M} \) AA [33], \( s = 0.0079 \text{min}^{-1} \). The volume-insensitive component of AA efflux \( (J_2) \) was assumed to be proportional to [AA]. The best fit between predicted and observed values was found when \( J_2 = 0.012[\text{AA}] \). The volume-sensitive component of AA efflux was assumed to be negligible \( (J_3 = 0) \) during incubations in isoosmotic media. The rate of recycling of DHAA was also assumed to be negligible \( (J_3 = 0) \). The reasons for the latter assumption were that homocysteine was present in the transport medium to minimize oxidation of AA to DHAA, and glucose was present to compete with DHAA for uptake through facilitative hexose transporters. The differential equation was solved using the fourth order Runge-Kutta scheme [13].

We found that the mathematical model accurately predicted the higher [AA], in dbcAMP-activated astrocytes solely on the basis of their accelerated Na\(^+\)-ascorbate cotransport rate. Moreover, the model predicted the steady state [AA], values for astrocytes exposed to pathologic changes of medium osmolality, Na\(^+\) and K\(^+\) based on the observed inhibition of Na\(^+\)-ascorbate co-transport by these conditions.

DHAA has three adjacent carbonyl groups in a ring structure, which is not seen elsewhere in biologic systems. DHAA is absent from normal plasma [30]. It is not detectable in normal brain [18] but appears after ischemia [7,8]. DHAA can be taken up and reduced to AA within astrocytes [26]. This recycling can be expected to minimize the toxicity of the oxidized form of vitamin C. Since DHAA uptake is mediated by facilitative hexose transporters and not by the Na\(^+\)-ascorbate cotransport system [26], it is possible to model changes in the transport of each form of vitamin C independently. Incubation with high concentrations of DHAA (200 \( \mu\text{M} \) increases the [AA] in cultured astrocytes transiently when glucose is absent from the incubation medium [26]. In the present experiments however, because homocysteine was included in the incubation medium to prevent oxidation of AA to DHAA and glucose was included to block facilitative hexose transporters, the mathematical model assigned a value of zero to the rate of DHAA recycling to AA. The model also assumed that the AA efflux rate was a linear function of [AA]. Thus, the success of our model is consistent with a predominant role for AA uptake, compared to DHAA recycling or AA efflux, in determining steady state [AA], in control and activated astrocytes under physiologic conditions.

The role of the Na\(^+\)-ascorbate cotransport system was evaluated further by incubating astrocytes under conditions of altered ionic gradients. The effect of lowered extracellular Na\(^+\) on the observed [AA] was the same as that predicted mathematically on the basis of changes in the \( K_m \) and \( V_{\text{max}} \) of Na\(^+\)-ascorbate cotransport. This indicates that the intracellular-to-extracellular ratio of AA at steady state depends on the electrochemical gradients of Na\(^+\) and ascorbate across the plasma membrane. Most of the AA inside astrocytes is localized to the cytosol [24]. It is possible that higher levels of total intracellular AA can be achieved in neurons as a result of subcellular compartmentation, for example, into secretory granules that are relatively more abundant in neurons.

Extracellular K\(^+\) concentration rises to depolarizing levels after cerebral ischemia [1,3] or general anesthesia [17]. We incubated astrocytes with hyperosmotic high-K\(^+\) media to depolarize the plasma membrane during AA uptake incubations. The effect of elevated extracellular K\(^+\) on astroglial AA uptake was a diminished [AA], consistent with the decrease in the \( V_{\text{max}} \) of Na\(^+\)-ascorbate cotransport. We also incubated astrocytes with isoosmotic (Fig. 5) and hyperosmotic (Table 1) high-K\(^+\) media to depolarize the plasma membrane during AA efflux incubations. Astroglial AA efflux was not increased by K\(^+\) under either isoosmotic or hyperosmotic conditions. In the case of isoosmotic high-K\(^+\) medium, the efflux incubation period was long enough to reverse Na\(^+\)-glutamate cotransport (10 min) but too brief to swell astrocytes and activate volume-sensitive organic anion channels [21]. This finding suggests that K\(^+\), even at the high level found in the extracellular fluid of ischemic brain [1,3], cannot reverse the electrogenic Na\(^+\)-ascorbate cotransporter.

Astrogial swelling frequently is triggered by brain injury [35]. Insults that cause astrocyte swelling also elevate transiently the concentration of AA in brain
extracellular fluid [5,6,8]. This pattern is consistent with rapid release of AA from swollen astrocytes in ischemic brain regions. AA released during this astroglial swelling may contribute to scavenging of reactive oxygen species in the extracellular fluid. Hyposmotic medium stimulates AA efflux because it swells astrocytes and thereby activates volume-sensitive organic anion channels that are permeant to ascorbate [25]. However, the increase in the plasma membrane permeability is transient and terminates within 1–3 min [25]. This explains why modeling the decrease in the $V_{\text{max}}$ of Na$^+$-ascorbate cotransport was sufficient to predict the steady state [AA]$_i$ of cells incubated in hyposmotic medium. Thus, it is likely that transient changes in efflux do not explain the effects of these pathologic conditions on steady state [AA]$_i$.

Acknowledgements

We thank Dr. M.A. Hediger (Department of Medicine, Brigham and Women’s Hospital) for generously providing the rat cDNA for SVCT2 and Dr. T.M. Underhill (School of Dentistry, University of Western Ontario) for advice on Northern blot analysis. Supported by grant OGP0002200 from the Natural Sciences and Engineering Research Council of Canada.

References