Amino acids are compatible osmolytes for volume recovery after hypertonic shrinkage in vascular endothelial cells

Dall’Asta, Valeria, Ovidio Bussolati, Roberto Sala, Alessandro Parolari, Francesco Alamanni, Paolo Biglioli, and Gian C. Gazzola. Amino acids are compatible osmolytes for volume recovery after hypertonic shrinkage in vascular endothelial cells. Am. J. Physiol. 276 (Cell Physiol. 45): C865–C872, 1999.—The response to chronic hypertonic stress has been studied in human endothelial cells derived from saphenous veins. In complete growth medium the full recovery of cell volume requires several hours and is neither associated with an increase in cell K⁺ nor hindered by bumetanide but depends on an increased intracellular pool of amino acids. The highest increase is achieved by neutral amino acid substrates of transport system A, such as glutamine and proline, and by the anionic amino acid glutamate. Transport system A is markedly stimulated on hypertonic stress, with an increase in activity roughly proportional to the extent and the duration of the osmotic shrinkage. Cycloheximide prevents the increase in transport activity of system A and the recovery of cell volume. It is concluded that human endothelial cells counteract hypertonic stress through the stimulation of transport system A and the consequent expansion of the intracellular amino acid pool.

glutamine; system A; amino acid transport; regulatory volume increase; bumetanide

ADAPTATION TO EXTRACELLULAR hypertonicity is a property of most mammalian cells (18, 25). However, the mechanisms employed for volume restoration after hypertonic shrinkage, indicated as regulatory volume increase (RVI), are different in the various tissues. A convenient device for counteracting osmotic stress is the increased accumulation of compatible organic osmolytes through Na⁺-dependent cotransport systems. One of these mechanisms, system A, accumulates neutral amino acids and methylamines in the intracellular compartment. Stimulation of system A activity by hyperosmotic stress, known for a long time (40), is a slow, protein synthesis-dependent process that may involve the synthesis of new carriers or, alternatively, of regulatory proteins that would stimulate transport activity of preexisting transporters (29). Recent results from our group have demonstrated for a hypertonic RVI is strictly dependent on the availability of neutral amino acid substrates of system A. In these cells, volume recovery relies on the expansion of the intracellular pool of amino acids and, in particular, of glutamine (9, 15).

As far as the endothelium is concerned, it is known that many types of endothelial cells exhibit increased activities of Na⁺-K⁺-2Cl⁻ cotransport and Na⁺-H⁺ antiport after hypertonic treatment (11, 30, 32). However, it is not clear whether transport activation results in a complete volume recovery, although even the capability of endothelial cells to exert an effective RVI is uncertain (28, 32, 38). More recent data indicate that, also in endothelium, transport systems for organic osmolytes are stimulated by hypertonic stress (23, 41, 42), although no attempt has been made in those studies to demonstrate that transport stimulation is able to correct cell shrinkage efficiently.

Recent results from our group have demonstrated that, under hypertonic conditions, marked endothelial damage is detected that is significantly reduced by glutamine supplementation of the extracellular medium (35). Those data prompted us to evaluate the role of the transport of glutamine and other neutral amino acids in the endothelial response to extracellular hypertonicity. Employing an adult human model, endothelial cells derived from saphenous veins, we demonstrate that, on hypertonic stress, the human endothelium in vitro is able to complete cell volume recovery through an increase of the intracellular pool of amino acids sustained by an enhanced activity of transport system A.

METHODS AND MATERIALS

Cell Culture

Human saphenous vein endothelial cells (HSVECs) were obtained from patients undergoing coronary artery bypass grafting, as previously described (35). Cells were routinely grown in collagen-coated 10-cm-diameter dishes in medium 199 (M199), with glutamine concentration raised to 2 mM. Culture medium was supplemented with 20% fetal bovine serum (FBS), 50 µg/ml endothelial cell growth supplement, and 90 U/ml heparin. The conditions of culture were pH 7.4, atmosphere 5% CO₂ in air, and temperature 37°C. Cultures were characterized by typical cobblestone morphology and by positivity to factor VIII and CD31/PECAM-1 antigens (35). Three strains of HSVECs were isolated, characterized, and employed for the investigations presented here with qualitatively similar results.
Experimental Procedures

All the experiments were performed using the cluster-tray method for rapid measurement of solute fluxes in adherent cells (13) with appropriate modifications. The experiments were carried out on HSVEC subcultures resulting from 3.5 × 10^4 cells seeded into 2-cm^2 wells of disposable 24-well trays (Nunc, Life Technology, Milan, Italy) in 1 ml of growth medium. Cells were used after 3–5 days, when cultures were almost confluent (12 ± 2 µg protein/cm^2). Culture medium was always renewed 24 h before the experiment. Hypertonic media were obtained by additions of 50 or 100 mM sucrose to complete M199. The osmolarities of the solutions were routinely checked with a vapor pressure osmometer (model 5500, Wescor Instruments, Logan, UT). After supplementation with 20% FBS, values of 295 ± 7, 328 ± 15, and 372 ± 12 mosmol/kg were found for control M199 (hereafter indicated as isotonic M199), hypertonic M199 supplemented with 50 mM sucrose (hereafter indicated as hypertonic M199 at 330 mosmol/kg), and hypertonic M199 supplemented with 100 mM sucrose (hereafter indicated as hypertonic M199 at 370 mosmol/kg), respectively.

Determinations and Measurements

Cell volume. Cell volume was estimated as the urea distribution space, according to a method employed by our group for cultured human fibroblasts (9, 15) and validated in cultured endothelium by O'Neill and Klein (32). [14C]urea (1.5 µCi/nmol, 45 mCi/mM final concentration) was added during the last 10 min of incubation. The incubations were terminated by two rapid washings (<5 s) with an ice-cold solution of 300 mM urea in water. Cells were covered with 0.2 ml of ethanol, and the radioactivity in cell extracts was counted in a Wallac Microbeta Trilux counter after the addition of Hisafe III scintillation fluid (Wallac, Turku, Finland). Cell monolayers were dissolved with 0.5% sodium deoxycholate in 1 N NaOH for determination of protein content directly in the well with use of a modified Lowry procedure, as previously described (13). Values of cell volume are expressed as microliters per milligram of protein. The volume of control cells was the mean of the values obtained at each experimental time in cells maintained under isotonic conditions.

Intracellular ion contents and concentrations. Cell monolayers, fixed in place with 0.1 ml of ethanol, were allowed to dry. Na^+ and K^+ contents in the water-soluble pool, extracted in 2 ml of 10 mM CsCl, were determined with a Varian atomic absorption spectrophotometer, with NaCl and KCl used as standards, and expressed as micromoles per milligram of protein. Values of intracellular ion concentrations were calculated from ion content and cell volume values determined in parallel cultures under the same experimental conditions.

Intracellular amino acid contents and concentrations. Cell monolayers were washed twice with ice-cold MgCl_2 and extracted in a 5% solution of acetic acid in ethanol. The intracellular content of the single amino acid species was determined by HPLC analysis with a Biochrom 20 amino acid analyzer (Amersham Pharmacia Biotech) employing a high-resolution column (Bio 20 Peek Lithium) and the physiological fluid chemical kit (Amersham Pharmacia Biotech) for elution. The column effluent was mixed with ninhydrin reagent, passed through the high-temperature reaction coil, and read by the photometer unit. Cell contents of the single amino acid species are expressed as nanomoles per milligram of protein. The intracellular concentration of amino acids was calculated from the amino acid contents and cell volumes determined in parallel cultures under the same experimental conditions.

Amino acid transport activity. The activity of amino acid transport systems was evaluated by measuring the influx of preferential substrates under conditions described. Unless stated otherwise, the assay was performed in Earle's balanced salt solution containing (in mM) 123 NaCl, 26 NaHCO_3, 5 KCl, 1.6 CaCl_2, 1 NaH_2PO_4, 0.8 MgSO_4, and 5.5 glucose. After the transport assay the incubation was terminated by two rapid rinses with ice-cold urea (300 mM), monolayers were extracted in 0.2 ml of ethanol, and radioactivity was counted in a Wallac Microbeta Trilux counter. Amino acid uptake is expressed as nanomoles or picomoles per milligram of protein per minute.

Materials

FBS was purchased from Euroclone (Milan, Italy). Culture media (M199 and glutamine-free M199) were purchased from Life Technology. L-[2,3,4-3H]arginine monohydrochloride (45–70 Ci/mmol), [2-(1-3H)methylaminoisobutyric acid (56 mCi/mmol), L-[1-14C]glutamic acid (52 mCi/mmol), and L-[U-14C]-glutamine (249 mCi/mmol) were obtained from NEN Life Science (Boston, MA). L-[2,3-3H]proline (4.2 Ci/mmol), L-[2,3-3H]aspartic acid (33 Ci/mmol), and L-[3-3H]threonine (19 Ci/mmol) were from Amersham Pharmacia Biotech (Milan, Italy). Ethanol was obtained from Carlo Erba (Milan, Italy), 2-methylaminoisobutyric acid (MeAIB) from Aldrich-Europe (Milan, Italy), and bumetanide from Astra (Milan, Italy). Sigma-Aldrich (Milan, Italy) was the source of all other chemicals.

RESULTS

RVI of Human Endothelial Cells

The results presented in Fig. 1 demonstrate that HSVECs, incubated in complete growth medium, effectively regulate cell volume after a hypertonic stress. The substitution of isotonic medium (290 mosmol/kg) with hypertonic media provokes a decrease in cell volume close to that expected for a perfect osmometer (from 7.42 to 6.62 or 5.78 µl/mg of protein after 1 h at

![Fig. 1. Changes in cell volume on incubation of human saphenous vein endothelial cells (HSVECs) in hypertonic media. Cell monolayers were incubated in isotonic or hypertonic medium 199 (M199), and cell volumes were measured. Values are means ± SD of 6 independent experiments and within 1 representative experiment. Dashed line, control value calculated as mean of values obtained with cells maintained in isotonic medium for indicated times (7.42 ± 0.085 µl/mg of protein, n = 30). Experiment was repeated 4 times with similar results.](http://ajpcell.physiology.org/)

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330 or 370 mosmol/kg, respectively). Under either hypertonic condition an RVI restores the initial cell volume. At 330 mosmol/kg, RVI is complete after 6 h of incubation, whereas 10 h of incubation are required for a complete volume recovery in HSVECs incubated at 370 mosmol/kg.

In endothelial cells, hypertonic stress produces a marked activation of Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport and Na\(^+\)/H\(^+\) antiport (11, 30–33). Results obtained in HSVECs demonstrate, however, that endothelial RVI does not involve any increase of the cell content of inorganic cations. During the first 3 h of hypertonic incubation, cell Na\(^+\) and K\(^+\) do not change significantly, whereas a modest, albeit significant, decrease in cell K\(^+\) content is detected thereafter (Fig. 2). Because of the decrease in cell volume (Fig. 1), the intracellular concentrations of Na\(^+\) and K\(^+\) rise during the first hour of incubation but, subsequently, fall to the initial values in parallel with cell volume rescue. These data are not consistent with a significant contribution of Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport or Na\(^+\)/H\(^+\) antiport to the RVI of HSVECs. Moreover, the inhibition of Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport appears to have little effect on RVI. If hypertonic stress is carried on in the presence of bumetanide (30 µM), an inhibitor of the cotransport, neither the volume recovery (Fig. 3, top) nor the cell content of K\(^+\) (Fig. 3, bottom) is significantly modified.

Volume restoration of HSVECs is associated with dramatic changes of the intracellular amino acid pool. Table 1 presents the chromatographic analysis of free amino acids in cells incubated for 10 h in isotonic or hypertonic M199. Glutamate and glutamine are the major amino acids in cells maintained in isotonic medium representing one-half of the overall pool, with glutamate alone accounting for almost one-third of the total. After 10 h of hypertonic incubation (i.e., in cells that have successfully recovered their volume) the cell content of most amino acids is increased, with a more pronounced change in cells incubated at 370 than at 330 mosmol/kg. In hypertonically stressed cells the largest absolute change is shown by glutamine (>150 nmol/mg of protein), followed by glutamate. The highest relative change is observed for proline, the content of which exhibits a sixfold increase in cells incubated for 10 h at 370 mosmol/kg compared with cells maintained under isotonic conditions. The intracellular concentration of amino acids is calculated to be ~70 mM in isotonic M199 (290 mosmol/kg), a value substantially comparable to that obtained in other endothelial models by Manolopoulos et al. (26). The intracellular concentration of amino acids rises to 105 and 140 mM in cells incubated at 330 and 370 mosmol/kg, respectively. The expansion of the intracellular amino acid pool, detected after long-term hypertonic incubation, appears, there-
Role of Glutamine in RVI of HSVECs

The results described above demonstrate that glutamine plays an important role in endothelial RVI; in particular, the increase in the cell content of glutamine represents a major portion (~40%) of the overall expansion of the intracellular amino acid pool detected in hypertonically stressed cells. However, glutamine is the amino acid present at the highest concentration in the culture medium employed for HSVEC culture (see METHODS AND MATERIALS). Therefore, to elucidate the role of glutamine in endothelial volume recovery, the hypertonic treatment has been performed while maintaining HSVECs in a nominally glutamine-free M199. It is noteworthy that glutamine is present in this medium at a concentration of ~0.1 mM because of the contribution of serum supplement. Figure 4 shows that, under these conditions, RVI is significantly delayed and HSVECs are not able to complete volume recovery even after 10 h of hypertonic incubation.

Changes in Amino Acid Transport During Endothelial RVI

Figure 6 shows the results of an experiment aimed to ascertain the effect of hypertonic treatment on the activity of various amino acid transport systems (3). To this purpose the transport of preferential substrates has been measured under characterizing conditions. The most dramatic change (a ~6-fold increase) is exhibited by transport system A, assessed through the uptake of the specific substrate MeAIB. Moreover, increases are detected for systems y^+ for cationic amino acids (e.g., Tau and Asp), y^− for anionic amino acids (e.g., Thr and Glu), and y^0 for neutral amino acids (e.g., Ser and Gly). In contrast, transport systems B and L are not significantly affected by hypertonicity.

Table 1. Cell content and intracellular concentration of free amino acids in HSVECs incubated under isotonic or hypertonic conditions

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Isotonic M199 (290 mosmol/kg)</th>
<th>Hypertonic M199 (330 mosmol/kg)</th>
<th>Hypertonic M199 (370 mosmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg protein mM</td>
<td>nmol/mg protein mM</td>
<td>nmol/mg protein mM</td>
</tr>
<tr>
<td>Tau</td>
<td>26 3.5</td>
<td>28 3.8</td>
<td>46 6.4</td>
</tr>
<tr>
<td>Asp</td>
<td>43 5.8</td>
<td>52 7.0</td>
<td>66 9.2</td>
</tr>
<tr>
<td>Thr</td>
<td>20 2.7</td>
<td>33 4.5</td>
<td>40 5.6</td>
</tr>
<tr>
<td>Ser</td>
<td>27 3.6</td>
<td>48 6.5</td>
<td>60 8.3</td>
</tr>
<tr>
<td>Glu</td>
<td>172 23.1</td>
<td>197 26.6</td>
<td>228 31.7</td>
</tr>
<tr>
<td>Gln</td>
<td>103 13.8</td>
<td>180 24.3</td>
<td>260 36.1</td>
</tr>
<tr>
<td>Pro</td>
<td>3 0.4</td>
<td>14 1.9</td>
<td>21 2.9</td>
</tr>
<tr>
<td>Gly</td>
<td>43 5.8</td>
<td>75 10.1</td>
<td>87 12.1</td>
</tr>
<tr>
<td>Ala</td>
<td>41 5.5</td>
<td>69 9.3</td>
<td>81 11.3</td>
</tr>
<tr>
<td>Val</td>
<td>5 0.7</td>
<td>6 0.8</td>
<td>8 1.1</td>
</tr>
<tr>
<td>Met</td>
<td>3 0.4</td>
<td>5 0.7</td>
<td>9 1.3</td>
</tr>
<tr>
<td>Ile</td>
<td>8 1.1</td>
<td>10 1.4</td>
<td>11 1.5</td>
</tr>
<tr>
<td>Leu</td>
<td>10 1.3</td>
<td>15 2.0</td>
<td>21 2.9</td>
</tr>
<tr>
<td>Tyr</td>
<td>3 0.4</td>
<td>5 0.7</td>
<td>7 1.0</td>
</tr>
<tr>
<td>Phe</td>
<td>19 2.6</td>
<td>32 4.3</td>
<td>43 6.0</td>
</tr>
<tr>
<td>Lys</td>
<td>2 0.3</td>
<td>3 0.4</td>
<td>4 0.6</td>
</tr>
<tr>
<td>His</td>
<td>5 0.7</td>
<td>8 1.1</td>
<td>7 1.0</td>
</tr>
<tr>
<td>Total</td>
<td>533 71.7</td>
<td>780 105.4</td>
<td>999 139.0</td>
</tr>
</tbody>
</table>

Human saphenous vein endothelial cells (HSVECs) were incubated for 10 h under conditions indicated. At the end of this period, cells were extracted, and cell extracts were analyzed by HPLC. Contents of other amino acids were < 2 nmol/mg protein. Intracellular concentrations were calculated from values of cell volumes obtained in parallel cultures. A representative experiment is shown. Experiment was repeated twice with similar results. M199, medium 199.

Changes in cell volume on incubation of HSVECs in hypertonic media in absence of glutamine. Cell monolayers were incubated in isotonic or hypertonic M199 in presence or absence of glutamine. Values are means ± SD of 6 independent determinations within 1 representative experiment. Dashed line, control value calculated as mean of values obtained with cells maintained in isotonic medium for indicated times (7.65 ± 0.062 µl/mg of protein, n = 30). Experiment was repeated 4 times with similar results.

Changes in amino acid transport during endothelial RVI

Figure 6 shows the results of an experiment aimed to ascertain the effect of hypertonic treatment on the activity of various amino acid transport systems (3). To this purpose the transport of preferential substrates has been measured under characterizing conditions. The most dramatic change (a ~6-fold increase) is exhibited by transport system A, assessed through the uptake of the specific substrate MeAIB. Modest increases are detected for systems y^+ for cationic amino acids (e.g., Tau and Asp), y^− for anionic amino acids (e.g., Thr and Glu), and y^0 for neutral amino acids (e.g., Ser and Gly). In contrast, transport systems B and L are not significantly affected by hypertonicity.

Changes in intracellular amino acid pool. As expected, glutamine is almost completely absent, but cell glutamate is also markedly lowered. In contrast, the cell content of some other amino acids, such as proline, serine, glycine, and alanine, is higher in cells incubated under glutamine-free hypertonic medium. As a whole, the intracellular amino acid pool is 703 and 896 nmol/mg of protein in cells incubated under hypertonic conditions in the absence and in the presence of glutamine, respectively.

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acids), \(x^C\) (an \(Na^+\)-independent agency for anionic amino acids), ASC, and L (for neutral amino acids). It is noteworthy that all these transport mechanisms are transstimulated, whereas system A is transinhibited (14). No significant change is detected for system \(X_{\text{AG}}\), an \(Na^+\)-dependent route for anionic amino acid uptake.

In mesenchymal cells, glutamine entry occurs through three distinct transport systems, with major contributions from the \(Na^+\)-dependent systems A and ASC and a small contribution from the \(Na^+\)-independent system L (10). This situation is substantially similar in human umbilical vein endothelial cells, where no clear-cut evidence has been obtained that an N-type transport system is present (3). As far as HSVECs are concerned, a 10-h hypertonic incubation causes a clear-cut increase of L-proline influx; after a 6-h hypertonic treatment, when the volume recovery is substantially complete (cf. Fig. 1), system A transport activity is stimulated twofold compared with cells maintained in isotonic M199. No further increase is detectable after this period. In contrast, at 370 mosmol/kg the increase in proline transport is observed, although at a slower rate, up to 10 h, when it reaches values fourfold higher than controls maintained under isotonic conditions.

Changes in the Activity of Transport System A During the Volume Recovery Process

To follow more closely the transport activity of system A during the hypertonic incubation, the influx of L-proline, the best natural substrate of the system (3, 14), has been measured at different times during the treatment. To this purpose, a tracer amount of the labeled amino acid has been added to isotonic or hypertonic medium (Fig. 8). The switch of extracellular osmolality from 290 to 330 mosmol/kg causes a clear-cut increase of L-proline influx; after a 6-h hypertonic treatment, when the volume recovery is substantially complete (cf. Fig. 1), system A transport activity is stimulated twofold compared with cells maintained in isotonic M199. No further increase is detectable after this period. In contrast, at 370 mosmol/kg the increase in proline transport is observed, although at a slower rate, up to 10 h, when it reaches values fourfold higher than controls maintained under isotonic conditions.
Interestingly, in cells incubated under hypertonic conditions in the absence of glutamine, where cell volume is only partially recovered even after 10 h (cf. Fig. 4), system A transport activity increases massively and steadily for 10 h, when 10-fold-stimulated values are attained.

To investigate the role of protein synthesis in the upregulation of system A, the hypertonic stress has been performed in the presence of 3.5 µM cycloheximide. At this concentration, protein synthesis is inhibited by 75%, as demonstrated by determinations of leucine incorporation in the acid-insoluble cell fraction (not shown). Figure 9 shows that cycloheximide completely prevents the increase in L-proline uptake, thus demonstrating that the upregulation of system A requires an active protein synthesis (Fig. 9, top). Interestingly, volume recovery is also severely hindered in the presence of cycloheximide (Fig. 9, bottom).

**DISCUSSION**

The main conclusion reached in this report is the demonstration that amino acids are the organic osmolytes employed by endothelial cells for a successful long-term RVI on chronic hypertonic stress. The osmolyte-accumulation of amino acids is dependent on a slowly ensuing, protein synthesis-dependent stimulation of the activity of transport system A for neutral amino acids. Transport enhancement is proportional to the degree and the duration of the osmotic shrinkage. If the transport increase is suppressed with cycloheximide, volume restoration is also completely blocked. L-Glutamine is the amino acid that exhibits the highest absolute increase at the intracellular level, whereas L-proline exhibits the highest relative increase. Both these amino acids are good substrates of system A in cultured human endothelium (3, 23). However, the expansion of the intracellular pool is not restricted to these amino acids or to other substrates of system A, such as alanine, serine, or glycine. Hypertonically stressed cells, indeed, exhibit enhanced intracellular accumulation of typical substrates of systems L (e.g., phenylalanine and leucine) and ASC (e.g., threonine). Because glutamine is also a substrate of these systems, it is likely that these amino acids derive from a heteroexchange of intracellular glutamate with extracellular amino acids (8). This interpretation is consistent with the moderate stimulation of transport systems L and ASC observed in endothelial cells after RVI (cf. Fig. 6). Glutamate is also accumulated during RVI, but at variance with the results obtained in nonendothelial models (12), the activity of system X_{AG} for anionic amino acid transport does not increase on hypertonic stress in HSVECs (cf. Fig. 6). The excess intracellular content of glutamate, therefore, likely derives from glutamine hydrolysis. Consistently, in hypertonically shrunken HSVECs, glutamine is only metabolized to glutamate (unpublished results).

Most of the available literature points to a major role of Na\(^{+}\)-K\(^{-}\)-2Cl\(^{-}\) cotransport as a mechanism for endothelial volume regulation (30–33). However, these studies reach divergent conclusions as far as the response to hypertonic stress is concerned. Employing endothelial cells from bovine aorta, pulmonary artery, and cerebral microvessels, O’Donnell and co-workers (30, 31) demonstrate a rapid, although incomplete, hypertonic RVI. The adaptive response is severely hindered by bumetanide and is associated with a brisk stimulation of Na\(^{+}\)-K\(^{-}\)-2Cl\(^{-}\) cotransport. On the contrary, O’Neill and co-workers (32, 33) observe neither an increase of cell K\(^{+}\) nor cell volume recovery in hypertonically shrunken bovine aortic endothelial cells, although they also detect a clear-cut stimulation of Na\(^{+}\)-K\(^{-}\)-2Cl\(^{-}\) cotransport. These authors, however, describe an effective RVI, associated with a bumetanide-inhibitable net influx of K\(^{+}\), provided that isotonic shrinkage is caused by the replacement of extracellular Na\(^{+}\) and K\(^{+}\) for a preincubation in hypotonic medium (32).

The results presented here, obtained in human endothelial cells from saphenous veins, indicate that the role of Na\(^{+}\)-K\(^{-}\)-2Cl\(^{-}\) cotransport in long-term hypertonic RVI is, at best, marginal. This conclusion is based on the absence of significant changes in K\(^{+}\) content during the volume recovery process. Moreover, bumetanide does not affect significantly the cell volume and the cell content of K\(^{+}\) in hypertonically shrunken cells (cf. Fig. 3). Rather, as discussed above, volume recovery relies on the increased accumulation of amino...
acids due to the activity of transport system A. Preliminary data obtained in human umbilical vein endothelial cells indicate that also in this endothelial model RVI is strictly dependent on neutral amino acid accumulation (V. Dall'Asta, unpublished results). Moreover, Kempson et al. (23) reported a hypertonic upregulation of transport system A in vascular endothelial cells from calf pulmonary artery, although no assessment of the functional consequences of the transport change has been performed in that study. Osmosensitivity of system A appears, therefore, to be a consistent feature of endothelial cells as well as of many other cell models (5–7, 9, 15, 17, 19, 39, 43). It should be stressed, however, that the results presented here do not exclude the possibility that stimulation of the activity of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport does indeed occur during the first phases of the hypertonic treatment. Rather, they indicate that cotransport activation is not really important for long-term volume restoration. On the other hand, where Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport activation is associated with an effective restoration of cell volume, hypertonic treatment occurs in complete growth medium (30, 31), so that these studies cannot exclude a role for amino acid accumulation in the observed volume recovery. Interestingly, where no significant RVI is detected, amino acid-free hypertonic solutions are usually employed (32).

On the basis of the present and previous studies (23, 30–33), it is possible to compare hypertonic stimulation of transport system A and of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport. The activation of the latter mechanism is rapid (<1 min) and depends on phosphorylation of the cotransport (24, 31). As judged from K\(^{+}\) influx measurements, maximal activation is reached after 20 min of hypertonic incubation, with a slow decrease thereafter (30). Maximal sensitivity of cotransport to extracellular tonicity is detected between 280 and 320 mosmol/kg (30). In contrast, the stimulation of system A requires at least 3 h of hypertonic incubation and an active protein synthesis. Transport activity rises steadily for several hours and remains elevated until volume is completely restored. The stimulatory effect is significantly higher at 370 than at 330 mosmol/kg (23). Stimulation of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport and the increase in system A activity may represent, therefore, two complementary and, possibly, sequential aspects of the same regulatory response to hypertonic stress. Although cotransport may be employed for the rapid correction of small osmotic fluctuations, long-term restoration of cell volume in response to larger and chronic hypertonic challenges relies on a slow stimulation of neutral amino acid transport and the consequent expansion of the intracellular amino acid pool characterized here. Interestingly, the progressive and slow accumulation of amino acids parallels the fall of intracellular K\(^{+}\) concentration and the small decrease in K\(^{+}\) content. At late times of hypertonic treatment, cotransport stimulation could thus warrant a rapid efflux of K\(^{+}\) rather than a net influx of the cation.

Although Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport does not account for RVI in HSVGcs or in cultured human fibroblasts (9) under hypertonic conditions, it could work as a volume-activated mechanism in other situations of physiological relevance. For instance, a cooperation between amino acid transport system A and Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport has been described during the isotonic volume increase associated with cell cycle progression in human fibroblasts (4). It is possible that a similar mechanism works also in endothelial models, since bumetanide hinders cell cycle progression in bovine endothelial cells derived from adult aortic arch (34).

The control of cell shape and volume appears to be of pivotal importance for a variety of functions of endothelial cells (1, 2, 16, 20), particularly for their barrier activity (21, 22, 27, 36–38). Thus, if the behavior exhibited by HSVGcs in vitro reflects that of endothelium in vivo, the prolonged period required by endothelial cells to recover the original volume after hypertonic stress may have important consequences. Whether manipulations of extracellular neutral amino acid concentration affect endothelial functions deserves further investigation. However, the results presented here demonstrate that glutamine is employed as an osmolyte by endothelial cells, thus explaining the protective effect exerted by the amino acid on endothelium challenged by hypertonic stress in vitro (35). On the basis of this conclusion, potential benefits of glutamine supplementation of hypertonic solutions employed for the preservation of organs and vascular grafts should be assessed.

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RESPONSE OF ENDOTHELIAL CELLS TO HYPERTONIC STRESS


