Amino acid depletion activates TonEBP and sodium-coupled inositol transport

RENA FRANCHI-GAZZOLA, ROSSANA VISIGALLI, VALERIA DALL'ASTA,
ROBERTO SALA, SEUNG KYOON WOO, H. MOO KWON,
GIAN C. GAZZOLA, AND OVIDIO BUSSOLATI

1Dipartimento di Medicina Sperimentale, Sezione di Patologia Generale e Clinica, Università degli Studi di Parma, 43100 Parma, Italy; and 2Division of Nephrology, School of Medicine, The Johns Hopkins University, Baltimore, Maryland 21205

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Amino acid depletion activates TonEBP and sodium-coupled inositol transport. Am J Physiol Cell Physiol 280: C1465–C1474, 2001.—The expression of the osmosensitive sodium/myo-inositol cotransporter (SMIT) is regulated by multiple tonicity-responsive enhancers (TonEs) in the 5′-flanking region of the gene. In response to hypertonicity, the nuclear abundance of the transcription factor TonE-binding protein (TonEBP) is increased, and the transcription of the SMIT gene is induced.

Transport system A for neutral amino acids, another osmosensitive mechanism, is progressively stimulated if amino acid substrates are not present in the extracellular compartment. Under this condition, as in hypertonicity, cells shrink and mitogen-activated protein kinases are activated. We demonstrate here that a clear-cut nuclear redistribution of TonEBP, followed by SMIT expression increase and inositol transport activation, is observed after incubation of cultured human fibroblasts in Earle’s balanced salts (EBSS), an isotonic, amino acid-free saline. EBSS-induced SMIT stimulation is prevented by substrates of system A, although these compounds do not compete with inositol for transport through SMIT. We conclude that the incubation in isotonic, amino acid-free saline triggers an osmotic stimulus and elicits TonEBP-dependent responses like hypertonic treatment.

LONG-TERM ADAPTATION OF MAMMALIAN CELLS to hypertonic stress involves the accumulation of nonperturbing, osmoprotective solutes in the intracellular compartment (30). Increased transport of such osmolytes through secondary active, sodium-dependent mechanisms constitutes a device employed to this aim in several cell models. The expression of one of these mechanisms, the sodium/myo-inositol cotransporter (SMIT) (Ref. 28 and Dall’Asta, unpublished observations), coded by the gene SLC5A3 (2, 31), is induced by extracellular hypertonicity (29, 49). Several studies, mostly performed in renal cells (29, 49, 51), led not only to the cloning of the transporter but also to a detailed molecular characterization of its sensitivity to osmotic stress. In particular, it has been shown that SLC5A3/SMIT expression is controlled by multiple elements located at the 5′-flanking region of the gene (38), regulated by binding with an osmosensitive transcription factor named TonEBP, tonicity-responsive enhancer-binding protein (33). Upon hypertonic stress, TonEBP translocates to the nucleus and activates SLC5A3 expression, thus promoting a slow increase of inositol transport (33, 48). The osmosensitivity of myo-inositol transport is not restricted to renal models because it has also been described in endothelial (46), lens (52), and glial cells (35, 41).

Another osmosensitive mechanism is the sodium-dependent transport system A (7), a secondary active mechanism strictly coupled to the transmembrane gradient of sodium electrochemical potential (8, 18). This transport system mediates the uptake of neutral amino acids with short polar side chains and tolerates N-alkylation of the substrates. Because of this unique feature, α-(methylamino)isobutyric acid (MeAIB), a nonmetabolizable amino acid analog, is the prototypical substrate of the system (7). The osmosensitivity of the system has been investigated in a variety of cell models, such as chicken fibroblasts (44), rat thymocytes (27), bovine kidney NBL-1 cells (40), mesangial cells (50), vascular smooth muscle cells (6), and Madin-Darby canine kidney (MDCK) cells (5, 22). In cultured human fibroblasts (12) and endothelial cells (9), the stimulation of system A activity is required for volume recovery after hypertonic stress. The molecular mechanisms involved in osmotic sensitivity of system A, as well as the possible role of TonEBP therein, have not yet been investigated because cloning of system A transporter has been obtained only very recently (42). The activity of system A markedly increases also upon a prolonged incubation in an amino acid-free, isotonic saline solution. This regulatory mechanism, named...
adaptive increase, was originally described in mesenchymal cells of avian origin (19), but it has also been found in many other models (see Ref. 32 for review). The possible relationships between adaptive regulation and hypertonic enhancement of system A activity have been widely debated (10, 32). Recently, we found that incubation in isotonic, amino acid-free saline, the experimental condition that triggers adaptive upregulation of system A, is followed by a significant cell shrinkage and a persistent activation of mitogen-activated protein kinase pathways in cultured human fibroblasts (14). Both of these changes also follow hypertonic stress in either the same cell model (14) or in other cell types (23, 37, 43). On the basis of those findings, we have proposed that incubation in the absence of amino acids, although carried on under nominally isotonic conditions, triggers osmocompensatory mechanisms similar to those elicited by hypertonic treatment (14).

To validate this hypothesis, as well as to elucidate further details of the mechanisms involved in the regulation of osmolyte transport, the TonEBP-dependent signaling pathway has now been investigated in cells incubated in an amino acid-free, isotonic saline solution, employing inositol transport as an osmosensitivity reporter function. We demonstrate here that incubation in isotonic saline triggers the nuclear translocation of TonEBP, the induction of SLC5A3, and, consequently, a marked increase in inositol transport.

MATERIALS AND METHODS

Cell culture and experimental treatment. Human foreskin fibroblasts were obtained from a healthy 15-yr-old donor. Cells were routinely grown in 10-cm diameter dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The conditions of culture were as follows: pH 7.4, atmosphere 5% CO₂ in air, temperature 37°C. Cultures were passed weekly and used at confluency. The experiments were made on fibroblast subcultures resulting from 1 × 10⁵ cells seeded onto 2-cm² wells of disposable 24-well trays (Nunc) and incubated for 3–4 days in 1 ml of growth medium.

Hypertonic DMEM (398 ± 7 mosmol/kgH₂O) was obtained by adding 100 mM sucrose to complete DMEM (310 ± 12 mosmol/kgH₂O), supplemented with 10% FBS. The osmolality of the solution was checked with a vapor pressure osmometer (Wescor 5500). For incubation in saline solution, cell monolayers were washed twice in Earle’s balanced salt solution (EBSS) containing (in mM) 123 NaCl, 26 NaHCO₃, 5 KCl, 1.8 CaCl₂, 1 NaH₂PO₄, and 0.8 MgSO₄. Cells were then incubated in the same solution, supplemented with 10% dialyzed FBS for the indicated periods of time. The osmolality of the solution was 280 ± 13 mosmol/kgH₂O (n = 6). The employment of dialyzed serum was required to establish conditions of complete amino acid starvation (18).

Transport measurements. The transport of myo-inositol was evaluated according to a previously described method for the estimation of solute fluxes into adherent cells (16) with proper modifications. When not stated otherwise, inositol was employed at 40 μM. After the experimental treatment, cell monolayers were washed twice with EBSS and incubated for indicated periods of time at 37°C in 0.2 ml of the same solution containing labeled myo-inositol. The sodium-independent transport was determined in a sodium-free saline solution in which choline replaced sodium in the EBSS. Transport assay was terminated by rapidly rinsing the cell monolayer twice with 3 ml of ice-cold 300 mM urea, and cells were extracted in situ by the addition of 0.2 ml of ethanol. Extracts were added to 0.6 ml of scintillation fluid and counted for radioactivity in a Wallac Microbeta Trilux counter. Cell monolayers were then dissolved with 0.5% sodium deoxycholate in 1 N NaOH, and protein content was determined using a modified Lowry procedure (16). The influx of myo-inositol was expressed as nanomoles of polyol per milligram of protein per minute. The kinetic parameters were evaluated by nonlinear regression analysis of transport data using the following equations

\[ v = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \]  

for a single saturable system and

\[ v = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} + K_d \cdot [S] \]  

for a saturable system plus diffusion, where \( v \) is the initial velocity of inositol uptake, \( V_{\text{max}} \) is the maximal velocity, \( K_m \) is the Michaelis-Menten constant, and \( K_d \) is the diffusion constant.

Cell volume. Cell volume, expressed as microliters per milligram of protein, was estimated from the distribution space of urea according to a method previously employed in cultured human fibroblasts (12). [14C]urea (2 μCi/ml, 0.5 mM final concentration) was added during the last 10 min of incubations. The experiment was stopped with two rapid washes in 3 ml of ice-cold 300 mM unlabeled urea in water. Alcohol-soluble pools were extracted with absolute ethanol and added to scintillation fluid to be counted for radioactivity. Protein content was determined as described above. Under the experimental conditions adopted, the cell content of urea reached a steady-state level by 5 min of incubation (not shown). A highly significant linear relationship exists between urea distribution space and the extracellular osmolarity that extrapolates to the origin (Fig. 1).

Fig. 1. Measurement of cell volume with urea distribution space. Cultured human fibroblasts were incubated for 10 min at 37°C in isotonic DMEM supplemented with 0, 60, 120, or 200 mM sucrose. Urea distribution space was determined as described in MATERIALS AND METHODS. Line represents the best fit linear regression (y = 7.066 × -0.093; P < 0.01). Values are means of 4 determinations with SD shown when greater than point size.
Intracellular ion contents and concentrations. Intracellular ion contents and concentrations were determined as described previously (12) with slight modifications. Briefly, cell monolayers were fixed with ethanol (0.1 ml) that was allowed to dry. The water-soluble pool was extracted in 2 ml of 10 mM CsCl. Potassium and sodium cell contents were determined with a Varian AA-275 atomic absorption spectrophotometer, using KCl and NaCl in 10 mM CsCl as standards. Values of the intracellular concentrations of ions were calculated from values of ion contents, and cell volumes were determined in parallel cultures in the same experiment.

Immunocytochemistry. Cells were seeded in four-well Labtech chamber slides (Nunc) at a concentration of 3 × 10⁴ cells/well in DMEM supplemented with 10% FBS. After the experimental treatment, the slides were rapidly washed three times in PBS, fixed in 3.7% paraformaldehyde for 15 min at room temperature, and then rinsed twice with PBS containing 0.1% glycine. Fixed cells were permeabilized with a 1-min incubation in methanol at −20°C and immediately treated with acetone for 1 min at −20°C. After two washings in PBS, which were performed at 4°C, the slides were incubated in a blocking solution (PBS containing 3% bovine serum albumin) for 1 h at 37°C. The incubation with 1:50 polyclonal TonEBP antiserum (33) was performed for 1 h at 37°C in the same blocking solution diluted 1:2 in PBS. Cells were then washed twice with PBS containing 0.1% Tween 20 (PBST) and incubated at 37°C for 30 min in the blocking solution with biotinylated goat anti-rabbit IgG diluted 1:300 as secondary antibody. After two washings in PBST, the slides were incubated as above in 1:100 diluted fluorescein-conjugated streptavidin. Finally, the slides were washed four times with PBST and two times with water.

Preparation of nuclear extracts. Nuclear extracts were prepared using a modification of the method of Han and Brasier (20). Cells, grown in 175-cm² flasks, were washed twice with ice-cold PBS, scraped in the same solution, and collected by low-speed centrifugation. The pellet was suspended in buffer A (50 mM Tris-HCl (pH 7.4), 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Igepal CA-630 (Sigma), and protease inhibitors). After 30 min on ice, the lysates were centrifuged at 4,000 g for 5 min at 4°C, the supernatant constituting the cytoplasmic extract. For the purification of nuclei, nuclear pellets were resuspended in buffer B (50 mM Tris-HCl, pH 7.4, 10 mM KCl, 1 mM EDTA, 1.7 M sucrose, 1 mM DTT, and protease inhibitors) and centrifuged at 15,000 g for 5 min at 4°C. Pelleted nuclei, resuspended in buffer C (50 mM Tris-HCl, pH 7.4, 400 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and protease inhibitors), were kept on ice for 30 min at 4°C with frequent vortexing. The nuclear suspension was centrifuged at 15,000 g for 5 min at 4°C, and the supernatant was saved. Both cytoplasmic and nuclear extracts were normalized for protein amounts determined by the Bradford assay using bovine serum albumin as a standard (Bio-Rad).

Electrophoresis and Western blotting. Protein samples were suspended in SDS-PAGE sample buffer, separated on a 6% SDS-polyacrylamide gel, and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked in Tris-buffered saline containing 1% casein, 0.33% gelatin, and 1% bovine serum albumin for 2 h at 30°C. TonEBP antiserum (33), diluted 1:100, was added for 1 h at 30°C. After four washes, the membrane was incubated with horseradish peroxidase-coupled anti-rabbit IgG antibody (Bio-Rad), washed extensively, and developed using a colorimetric kit (Bio-Rad).

Northern blotting. Confluent fibroblasts were exposed to hypertonic medium containing 10% FBS or to isotonic EBSS supplemented with 10% dialyzed FBS for the indicated periods. Total RNA was extracted with TRIzol (Life Technologies, Italy). For Northern analysis, RNA (10–20 μg/lane) was separated under denaturing conditions on 1% agarose gel containing 2.2 M formaldehyde. After being stained with ethidium bromide to document equal sample loading and absence of degradation, total RNA was transferred for 16 h to a positively charged nylon membrane (Nytran Super Charge; Schleicher and Schüll) and then linked to the membrane at 80°C. Hybridization of the blot was carried out at 42°C overnight with a canine SMIT cDNA probe (28) that shares a homology of 94% with human SMIT (2). After being stripped, the membrane was rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase probe (gift from Dr. R. Allen, American Red Cross Laboratories). The probes were labeled with [α-32P]dCTP. The blots were washed according to the manufacturer’s instructions, with the last wash performed at 60°C for 30 min, and then exposed to Kodak XAR film at −70°C for the appropriate time. Densitometric analysis of the autoradiograms was performed with a Molecular Dynamics laser densitometer.

Statistical analysis. Whenever appropriate, statistical analysis was performed with analysis of variance, unless otherwise stated.

Materials. FBS and DMEM were purchased from Life Technologies, [14C]urea (53 mCi/mmol), [2-3H]myo-inositol (17 Ci/mmol), and [α-32P]dCTP (3,000 Ci/mmol) were from Amersham. Biotinylated goat anti-rabbit IgG and fluorescein conjugated streptavidin were obtained from Dako. Ethanol was obtained from Carlo Erba (Milan, Italy). The source of all other chemicals was Sigma.

RESULTS
TonEBP redistribution into the nucleus upon incubation of cultured human fibroblasts in isotonic saline solution. The incubation of cultured human fibroblasts in isotonic saline solution caused a marked and progressive cell shrinkage (Fig. 2A). The decrease in cell volume was already detectable after 15 min of incubation in EBSS. After 3 h of treatment, cell volume was decreased by 30% with respect to control; this value was maintained thereafter, with no apparent regulatory volume increase, for at least 12 h. Throughout this period, the intracellular concentrations of cations underwent significant alterations (Fig. 2B). However, while intracellular sodium rapidly returned to control values after a transient increase, the intracellular concentration of potassium rose steadily for the first 3 h of treatment, closely paralleling cell shrinkage. Thereafter, the intracellular potassium concentration was maintained at values higher than control by roughly 40%. These data indicate that the incubation of cultured human fibroblasts under amino acid-free conditions causes a significant cell shrinkage with an increase in cell ion strength.
In other cell models, cell shrinkage and the concurrent increase in cell ion strength, caused by a hypertonic treatment, are associated with an increase in TonEBP abundance in the nucleus (33, 47, 48). We compared TonEBP behavior in fibroblasts incubated under either hypertonic conditions or in amino acid-free solutions (Fig. 3). The immunocytochemical analysis performed with confocal microscopy indicates that TonEBP positivity was faint and widespread in control cells (Fig. 3A). After 90 min of incubation either under hypertonic conditions (Fig. 3B) or in EBSS (Fig. 3C), a clear-cut increase in nuclear localization of TonEBP in treated cells. After 3 and 6 h of incubation under either condition, an increased nuclear abundance of TonEBP was still detectable (not shown). Western blot analysis (Fig. 3D) confirms that a nuclear relocalization of TonEBP was detectable in both hypertonic medium and EBSS, starting from 4 h of incubation. Under either condition, an increase of TonEBP in the nuclear extract was detected together with a concurrent decrease in the cytoplasmic signal. These results demonstrate that both hypertonic treatment and amino acid-free incubation cause the nuclear redistribution of TonEBP.

**Characterization of inositol transport in cultured human fibroblasts.** Because SMIT carrier is a target of TonEBP (33), the evaluation of inositol transport represents a convenient device to ascertain the functional consequences of TonEBP activation induced by the incubation in EBSS. Only limited attention has been thus far devoted to the transport of the polyol in human fibroblasts (3, 15, 34). Moreover, the contradictory kinetic data reported in those studies may reflect the sensitivity of the transport process to experimental conditions such as the type and concentration of serum employed for cell culturing (1). In a series of experiments, recounted in Fig. 4, we have, therefore, performed a characterization of inositol transport in cultured human fibroblasts to define the operational features of the transporter(s) involved in the experimental conditions employed in this study.

Figure 4A reports the time course of myo-inositol accumulation either in the presence or in the absence of sodium. The polyol influx was linear up to 60 min under both conditions. It was concentration only in the presence of sodium, whereas it was exceedingly low in the absence of the cation. The kinetic analysis of inositol transport was performed in a wide range of concentrations from 0.01 to 1.28 mM (Fig. 4B). Sodium-independent influx was not saturable in the range of concentrations adopted and was thus formally undistinguishable from diffusion. Once subtracted, the sodium-independent component, sodium-dependent inositol, transport was satisfactorily fitted by an equation (Eq. 1, see MATERIALS AND METHODS) that describes the influx as the operation of a single saturable mechanism endowed with a moderately high affinity (K_m = 50 μM), a conclusion shown through Eadie-Hofstee transformation (Fig. 4C). The value of K_m is comparable to values found by other investigators for inositol transport in human fibroblasts (3) and in the basolateral membrane of MDCK cells (28). Consistent with the results obtained in MDCK cells (36) and at variance with those obtained in retinal pigment epithelial cells (26), inositol transport was markedly stimulated by protein kinase C downregulation, obtained with a prolonged incubation in the presence of phorbol esters, and significantly decreased by acute protein kinase A activation (Fig. 4D). These results indicate that the transporter expressed in cultured human fibroblasts has kinetic, operational, and regulatory features similar to those described for the SMIT transporter of MDCK cells.

Stimulation of inositol transport by amino acid-free incubation. In the experiment reported in Fig. 5, inositol transport was measured during a prolonged incubation of human fibroblasts in EBSS. The experimental treatment led to a marked, slowly ensuing stimulation of the polyol uptake. The time course of the effect, shown in Fig. 5A, indicates that the transport stimulation became detectable after 6 h of incubation in EBSS. The incubation in hypertonic DMEM also stimulated inositol uptake in human fibroblasts. The time course of the effect was comparable in the two conditions for up to 18 h of treatment, although hypertonic treatment caused a larger stimulation of inositol.

![Fig. 2. Changes in cell volume and intracellular concentrations of sodium and potassium during the incubation of cultured human fibroblasts in Earle’s balanced salt solution (EBSS).](image-url)

A: cell volume was determined at the indicated times of incubation in EBSS + 10% fetal bovine serum (FBS) as described in MATERIALS AND METHODS. Data are means of 6 independent determinations with SD. B: the intracellular concentrations of sodium (open bars) and potassium (filled bars) were calculated from the ion contents determined as described in MATERIALS AND METHODS at the indicated times of incubation in EBSS + 10% FBS in the same experiment shown in A. Data are means of 6 independent determinations with SD. The experiment was repeated 3 times with comparable results. *P < 0.05 vs. DMEM; **P < 0.01 vs. DMEM.
transport. In cells incubated in EBSS, inositol influx rose further, up to 24 h, while no further increase in polyol uptake was detected under hypertonic conditions after 18 h of treatment. In both cases, however, cycloheximide (18 μM) completely suppressed transport stimulation (not shown). Figure 5, B and C, shows the results of the kinetic analysis of inositol transport performed after 15 h of either hypertonic or EBSS incubation. The kinetic parameters, reported in Table 1, indicate that both treatments enhanced inositol transport through a marked increase of transport of \( V_{\text{max}} \), while the \( K_{\text{m}} \) was not significantly affected under either condition. At this time, the \( V_{\text{max}} \) change induced by hypertonic treatment was greater than that caused by amino acid deprivation. Northern analysis, performed with a SMIT full-length probe, consistently indicated that a markedly enhanced abundance of SMIT mRNA was detectable in both hypertonically, and, although to a lesser degree, EBSS-treated fibroblasts (Fig. 5D).

These results indicate that the nuclear translocation of TonEBP, promoted by incubation in isotonic EBSS, is followed by the induction of SMIT and by the consequent increase of inositol uptake.

Characteristics of EBSS-induced stimulation of inositol transport. Upregulation of membrane transport caused by substrate deprivation is a very well-known phenomenon, described for several kinds of solutes, such as neutral amino acids (17, 19), and, in lower organisms, inositol itself (39). These regulatory mechanisms are usually called “adaptive changes.” Because inositol is present in DMEM formula, the possibility exists that the stimulation of polyol transport observed after a prolonged incubation in EBSS is referable to an adaptive response. Figure 6A shows the results of an experiment in which the incubation of cultured human fibroblasts in EBSS was performed in the presence of different concentrations of inositol. Although transport increase was only partially blocked by 100 μM inositol, i.e., at the concentration of the polyol present in FBS-supplemented DMEM, an almost complete suppression was reached at a very high concentration of inositol (5 mM). Because, at this huge, supraphysiological concentration, inositol could have little nutritional role, but, rather, an osmoprotective effect, we hypothesized that other organic osmolytes could interfere with EBSS-induced SMIT expression.

To test this hypothesis, we performed an experiment in which EBSS incubation was carried out in a saline solution supplemented with single amino acids employed at a concentration of 0.5 mM (Fig. 6B). Amino acids that are not transported through system A in cultured human fibroblasts, such as L-glutamate (11) and L-arginine (45), did not affect EBSS-induced stimulation of inositol transport significantly. L-Leucine and L-valine, relatively poor substrates of system A (18), produced a significant repression of the induction of inositol transport. However, cells incubated in EBSS supplemented with these amino acids still exhibited inositol transport values significantly higher \( (P < 0.05) \) than control cells maintained in DMEM. On the contrary, other amino acids, such as L-proline and L-glu-
tamine, which are good substrates of system A, and, hence, actively accumulated in the intracellular compartment (13, 18), completely suppressed the stimulation of inositol transport. Interestingly, the nonmetabolizable amino acid analog MeAIB, the prototypical substrate of system A, also abolished SMIT induction.

However, the amino acids that repress SMIT induction do not interact with the transporter as substrates. Indeed, inositol uptake was not inhibited by MeAIB, proline, glutamine, valine, or leucine if these compounds were present only during the uptake of the polyol (Fig. 7). Therefore, the capability of specific amino acids to suppress SMIT induction cannot be referred to a direct competition between these compounds and myo-inositol for the transporter.

**Effect of inositol on cell volume recovery.** In cells shrunken through an amino acid-free incubation, neutral amino acids, which are substrates of system A, exert a rapid osmocompensatory effect leading to a rapid recovery of cell volume (14). The effect of inositol supplementation on the recovery of cell volume was tested under the same conditions (Fig. 8). The addition of 5 mM myo-inositol to EBSS after a 12-h incubation in the absence of organic osmolytes led to a gradual recovery of cell volume that required more than 12 h. Cell volume restoration paralleled the accumulation of the polyol. After 21 h of inositol supplementation, both cell volume and cell content of the polyol reached a steady state.

**DISCUSSION**

The volume-restoring accumulation of most compatible osmolytes is due to the stimulation of several sodium-coupled transporters, such as the sodium-chloride-betaine cotransporter BGT1 (21) and the SMIT transporter (51). The regulatory mechanism occurs at the transcription level and involves (a) tonicity-responsive element(s) that present(s) a binding site for an osmosensitive transcription factor, TonEBP. The activity of this Rel-like DNA binding protein is significantly stimulated through both an increase in its abundance and a nuclear redistribution when cells are incubated under hypertonic conditions (33). Here we show that the incubation of human fibroblasts in isotonic EBSS (280 ± 13 mosmol/kgH₂O), an amino acid-free saline solution, produces an osmotic stimulus, triggering TonEBP redistribution and activation. When these cells are transferred into the amino acid-free saline,

![Graph](http://example.com/graph.png)

![Graph](http://example.com/graph2.png)

![Graph](http://example.com/graph3.png)

![Graph](http://example.com/graph4.png)

**Fig. 4. Influx of myo-inositol in cultured human fibroblasts.** A: cultured human fibroblasts, grown onto 24-well culture multidish in DMEM, were washed with EBSS. The intracellular accumulation of 100 μM [³H]myo-inositol was then measured in EBSS ([Na⁺]₀ = 142 mM; ○, sodium present) or in a modified sodium-free EBSS (□, sodium absent). B: kinetic analysis of 10-min myo-inositol uptake in a range of polyol concentrations from 0.01 to 1.28 mM (□, sodium-dependent uptake; ○, sodium-independent uptake). The sodium-dependent uptake was obtained by subtracting the uptake measured in the absence of sodium from the total uptake of the polyol. Lines represent the linear regression (○) or the best fit nonlinear regression calculated from Eq. 1 (□). C: Eadie-Hofstee plot of the sodium-dependent component shown in B. Line represents the best fit linear regression. D: effects of chronic treatment (24 h) with 1 μM phorbol 12,13-dibutyrate (PDBu) or acute treatment (1 h) with 400 μM 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) on 10-min myo-inositol uptake. Experimental treatments were performed in complete growth medium, whereas transport assay was performed in EBSS. In all cases, data are means of 3 independent determinations with SD shown when greater than the size of the point. *P < 0.05 vs. control; **P < 0.01 vs. control.
they progressively shrink as a consequence of the depletion of the intracellular amino acid pool. Under normal conditions, the overall concentration of intracellular amino acids reaches a value of 140 mM that is nearly reduced by half after 6 h of incubation under amino acid-free conditions (14). In amino acid-starved cells, the osmotic equilibrium relies only on the inorganic ions, thus leading to a significant increase of intracellular potassium concentration after 3 h of EBSS incubation (Fig. 2A). Therefore, the availability of extracellular amino acids is required not only for the recovery of hypertonically stressed cells (9, 12, 14) but also for the maintenance of cell volume and the control of cell ionic strength under isotonic conditions.

We have also demonstrated that the TonEBP redistribution induced by isotonic amino acid-free incubation is functionally effective. For this purpose, we have employed SMIT transporter as a TonEBP-dependent reporter gene, although no data were available on the osmosensitivity of inositol transport in cultured human fibroblasts before those reported here. However, in cultured human fibroblasts, SMIT is induced not

**Table 1. Kinetic constants of inositol transport and effect of hypertonic treatment and EBSS incubation**

<table>
<thead>
<tr>
<th>Condition</th>
<th>$K_m$, mM</th>
<th>$V_{max}$, nmol-mg$^{-1}$-min$^{-1}$</th>
<th>$K_d$, min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.049 ± 0.011</td>
<td>0.102 ± 0.009</td>
<td>0.051 ± 0.008</td>
</tr>
<tr>
<td>Hypertonic DMEM</td>
<td>0.064 ± 0.007</td>
<td>1.096 ± 0.053</td>
<td>0.017 ± 0.047</td>
</tr>
<tr>
<td>EBSS</td>
<td>0.056 ± 0.009</td>
<td>0.369 ± 0.023</td>
<td>0.054 ± 0.022</td>
</tr>
</tbody>
</table>

Cultured human fibroblasts were incubated for 15 h in isotonic (control) or hypertonic DMEM or in Earle’s balanced salt solution (EBSS). The parameters are the results of the analysis shown in Fig. 5B, with SE indicated. $V_{max}$ (maximal velocity) values determined after incubation either in EBSS or in hypertonic DMEM were both significantly different from control values, as determined with a two-tailed t-test ($P < 0.001$). No significant difference was detected for $K_m$ (Michaelis-Menten constant) or $K_d$ (diffusion constant) values.

Fig. 5. Changes of inositol transport and sodium/myo-inositol cotransporter (SMIT) expression induced by hypertonic treatment or incubation in EBSS. A: cultured human fibroblasts were transferred from DMEM (●) into EBSS (■) or hypertonic DMEM (●). At the indicated times, cells were washed twice in EBSS, and 10-min inositol uptake was assayed as described in MATERIALS AND METHODS. B: kinetic analysis of 10-min myo-inositol uptake measured in a range of concentrations from 0.01 to 1.28 mM in cells maintained in DMEM (●), incubated for 15 h in hypertonic DMEM (●), or incubated for 15 h in EBSS (●). Data are means of 3 independent determinations with SD shown when greater than the size of the point. Lines represent the best fit of data to Eq. 2 (see MATERIALS AND METHODS). C: Eadie-Hofstee transformation of the data presented in B after subtraction of the sodium-independent component. Lines represent best fit linear regressions. D: analysis of SMIT mRNA levels by Northern blot. Total RNA (15 μg) was isolated from human fibroblasts maintained in DMEM (control, C) or exposed for 9 h to hypertonic DMEM (H) or to isotonic EBSS (E). The membrane was sequentially hybridized with SMIT or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. The increase of expression of SMIT, normalized for GAPDH signal, was +980% in hypertonically treated cells and +250% in cells incubated in EBSS. The experiment was repeated 3 times with comparable results.
only after hypertonic stress, as expected from the results obtained in other models (29, 35, 41, 46, 49, 51, 52) but also after an isotonic, amino acid-free incubation. As a result of SMIT induction, the transport of inositol in EBSS-incubated human fibroblasts undergoes a marked increase. The osmoprotective role of the transport stimulation appears evident from the results reported in Fig. 8. Inositol works as an effective osmoprotective agent, provided that it is added to the extracellular medium at a high, supraphysiological concentration for at least 21 h. It should be recalled that in cells shrunken through an incubation in EBSS, volume is rapidly restored to control values when amino acid substrates of transport system A are added to the extracellular medium (14). However, the transport capacity of transport system A, measured in human fibroblasts after a 6-h incubation in EBSS (>100 nmol·mg protein⁻¹·min⁻¹), is impressively higher than that exhibited by SMIT transporter after a 15-h incubation in the same saline solution (369 pmol·mg protein⁻¹·min⁻¹, this report). As expected, system A substrates induce a faster volume recovery than inositol.

The incubation in isotonic, osmolyte-free saline stimulates both SMIT and transport system A for neutral amino acids. The activation of TonEBP, detected under the same condition, suggests that hypertonic stress is
fully mimicked and strengthens the hypothesis that the adaptive regulation of system A (i.e., the increase in the activity of the system observed after amino acid starvation) (19) is actually a consequence of the well-known osmotic sensitivity of this transport system. These considerations would also suggest that TonEBP may be involved in the upregulation of transport system A, an issue that should now be directly addressed, given that the system has been recently cloned (42). However, it should be recalled that amino acid deprivation can activate several signaling pathways, some of which appear to be specific for single amino acids (see Ref. 25 for review).

Although the substrates of transport system A exert no inhibition of inositol transport (Fig. 7), they effectively suppress the EBSS-induced stimulation of SMIT if added to the saline solution (Fig. 6B). Under this condition, cell shrinkage and the upregulation of system A transport activity are also prevented (14). Amino acids that are not transported by system A, such as glutamic acid or arginine (11, 45), do not interfere with the stimulation of inositol transport caused by incubation in EBSS. These data suggest that only amino acids that can be effectively accumulated into the intracellular compartment through system A can block SMIT induction. This result points, therefore, to a regulatory interaction of SMIT and system A transporters and suggests that both transport systems respond to a common stimulus, i.e., cell shrinkage and/or the increase in intracellular ionic strength. The transport activity of system A (14) exhibits markedly faster changes than inositol uptake (this contribution) after either hypertonic stress or amino acid-free incubation. Consistently, the hypertonic activation of betaine (5) and myo-inositol carriers (21) is slower than the stimulation of system A in MDCK cells. System A stimulation may thus be the most rapid transport mechanism employed to counteract the increase in intracellular ionic strength, immediately following the transient protection yielded by the induction of heat shock proteins. Interestingly, in mutant Chinese hamster ovary cells that overexpress system A, changes of heat shock protein levels have also been detected (24). These considerations indicate that coordinated regulation of organic osmolytes (4) may be not restricted to renal cells. Moreover, they suggest that an ordered sequence of regulatory phenomena and changes counteracts the increase in intracellular ionic strength induced by hypertonic stress or deprivation of organic osmolytes. However, the components of this compensatory sequence, as well as the underlying mechanisms and the possible pathophysiological implications, are far from being fully elucidated.

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