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The synthesis of SNAT2 transporters is required for the hypertonic stimulation of system A transport activity

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Abstract

In cultured human fibroblasts incubated under hypertonic conditions, the stimulation of system A for neutral amino acid transport, associated to the increased expression of the mRNA for SNAT2 transporter, leads to an expanded intracellular amino acid pool and to the recovery of cell volume. A protein of nearly 60 kDa, recognized by an antiserum against SNAT2, is increased both in the pool of biotinylated membrane proteins and in the total cell lysate of hypertonically stressed cells. The increased level of SNAT2 transporters in hypertonically stressed cells is confirmed by immunocytochemistry. DRB, an inhibitor of transcription, substantially inhibits the increase of SNAT2 proteins on the plasma membrane, completely suppresses the stimulation of system A transport activity, and markedly delays the cell volume recovery observed during the hypertonic treatment. On the contrary, if the transport activity of system A is adaptively increased by amino acid starvation in the presence of DRB, the increase of SNAT2 transporters on the plasma membrane is still clearly detectable and the transport change only partially inhibited. It is concluded that the synthesis of new SNAT2 transporters is essential for the hypertonic stimulation of transport system A, but accounts only in part for the adaptive increase of the system.

Keywords: Cell volume; Amino acid starvation; Human fibroblast; Hypertonic stress; Glutamine

1. Introduction

System A is a secondary active, sodium-dependent transport mechanism for neutral amino acids widely expressed in mammalian tissues. The system was operationally identified because it characteristically tolerates *N*-alkylated substrates [1]. However, cloning of system A-related transporters has been performed only recently [2–5]. This transporter, originally named ATA2, SAT2, or SA1, is now identified as SNAT2 (*S*odium-coupled *N*eutral *A*mino acid *T*ransporters, member 2), the product of SLC38A2 gene [6].

In many cell models (see, for review, Ref. [7]) hypertonic treatment causes a marked, protein synthesis-dependent increase of the transport activity of system A that leads to the expansion of the intracellular amino acid pool and contributes to the volume recovery of shrunken cells [8,9]. Also a prolonged incubation in amino acid-free saline solutions slowly stimulates the activity of system A, a mechanism named adaptive regulation (see Ref. [10] for review). Both adaptive [11] and hypertonic [12] stimulations of system A are associated with an

Abbreviations: BGT1, betaine/GABA transporter; DMEM, Dulbecco's modified Eagle's medium; DRB, 5,6-dichlorobenzimidazole riboside; EBSS, Earle's balanced salt solution; FBS, fetal bovine serum; GAPDH, glyceraldeide-3-phosphate dehydrogenase; NFAT5, nuclear factor of activated T cells 5; PBS, phosphate-buffered saline; SMIT1, sodium *myo*-inositol cotransporter 1; SNAT2, sodium-coupled neutral amino acid transporter 2; TAUT, taurine transporter; TonEBP, tonicity-responsive enhancer binding protein

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increased abundance of SNAT2 mRNA. Moreover, results from this laboratory have shown that a significant cell shrinkage follows not only hypertonic treatment, but also amino acid starvation [13]. Under both conditions, the osmosensitive transcription factor NFAT5/TonEBP localizes in the nucleus and promotes the expression of its target genes [13], thus indicating that the incubation under amino acid-free conditions activates an osmotic response.

In spite of these common features, adaptive and hypertonic stimulations of system A exhibit distinct sensitivities to inhibitors of protein processing [10] and depend upon different transduction pathways [14]. Moreover, while a marked increase in SNAT2 protein has been described during adaptive regulation [15], it is not certain whether osmotic stimulation depends on the synthesis of either new SNAT2 transporters or, rather, SNAT2-activating proteins [16].

To address this issue directly, we have studied the behaviour of the SNAT2 transporter under hypertonic conditions and demonstrate here that the osmotic stimulation of system A transport activity is associated with a markedly increased abundance of carrier proteins.

2. Materials and methods

2.1. Materials

Serum and culture medium have been obtained from Gibco. L-[5-^{3}H]Proline (32 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from DuPont deNemours. [^{14}C]Urea (4.2 Ci/mol) was from Amersham Pharmacia Biotech Italia, Milan, Italy. Sigma was the source of the other chemicals.

2.2. Cells and incubations

Cultured human foreskin fibroblasts were grown, as described previously [8], in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The conditions of culture were as follows: pH 7.4, atmosphere 5% CO_2 in air, temperature 37 °C. Cultures were passed weekly and used at confluence between the 5th and the 10th passage. Cell volume and transport experiments were made on fibroblast subcultures resulting from 1×10^5 cells seeded onto 2-cm^2 well of disposable 24-well trays (Falcon) and incubated for 3–4 days in 1 ml of growth medium.

For the hypertonic treatment, cells were incubated for the indicated times in hypertonic DMEM, obtained by adding 100 mM sucrose to complete DMEM, supplemented with 10% FBS. The osmolalities of the solutions, routinely checked with a vapor pressure osmometer (Wescor 5500), were 310±12 mosmol/kg, for isotonic DMEM, and 398±7 mosmol/kg, for hypertonic DMEM.

The experimental protocol employed to trigger the adaptive enhancement of system A transport activity consisted in an amino acid starvation obtained through a prolonged incubation in Earle's Balanced Salt Solution (EBSS) supplemented with 10% dialyzed FBS. The composition of EBSS (in mM) was 117 NaCl, 26 $NaHCO_3$, 5 KCl, 1.8 $CaCl_2$, 1 NaH_2PO_4, 0.8 $MgSO_4$, 5.5 glucose.

2.3. Transport studies

The transport activity of system A was determined, as described previously [8], from the initial influx of [^{3}H]-L-proline in EBSS. Under the conditions adopted, L-proline behaves as a site-A specific substrate in human fibroblasts [17]. The amino acid substrate was employed at a concentration of 0.1 mM and at an activity of 2 μCi/ml in 1-min uptake assays at 37 °C. Under these conditions proline influx is linear for more than 10 min (unpublished results). Cell proteins were determined as described previously [8] and transport activity was expressed as nmol/mg protein/min.

For the kinetic analysis of L-proline influx, a range of amino acid concentrations from 0.08 to 10 mM was employed. Transport data were fitted to the equation

$$v = \frac{V_{\max}[S]}{K_m + [S]} + K_d[S] \quad (1)$$

that describes the influx of the amino acid as the result of the additive contributions of a saturable component and of a non-saturable component, formally indistinguishable from diffusion.

2.4. Intracellular content of glutamine

Cell monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) 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. Values are expressed as nmol/mg protein.

2.5. Cell volume

Cell volume, expressed as μl/mg protein, was estimated from the distribution space of urea, according to a method previously validated in cultured human fibroblasts [13]. [^{14}C]Urea (2 μCi/ml, 0.5 mM final concentration) was added during the last 10 min of incubation. The experiment was stopped with two rapid washes in 3-ml ice-cold 300 mM urea in water. Alcohol-soluble pools were extracted

with absolute ethanol and added to scintillation fluid to be counted for radioactivity. Under the experimental conditions adopted, the cell content of urea reached a steady state level by 5 min of incubation (not shown). In cultured human fibroblasts a highly significant linear relationship exists between urea distribution space and the extracellular osmolality that extrapolates to the origin [13].

2.6. RNA extraction and SNAT2 ^{32}P-labelled probe preparation

Confluent cultures of human fibroblasts were incubated for the indicated periods in hypertonic DMEM, supplemented with 10% FBS, or in EBSS, supplemented with 10% dialyzed FBS. Total RNA was extracted with Omnizol (Euroclone). One microgram of total RNA, primed by oligo-dT, was reverse-transcribed by ImProm II Reverse Transcriptase (Promega Italy srl, Milano). Following RNAse H (USB) treatment, 200 ng of cDNA was amplified by "hot start" PCR to generate a 623-bp amplicon. PCR reaction was performed in a thermal cycler (I-Cycler, Bio-Rad Laboratories srl, Italy) for 30 cycles with the primers 5′-TCAACTACTCCTACCCCACCA (sense) and 5′-CAGAAAGAACACCATACACAACAAG (antisense) using DyNAzyme EXT DNA polymerase (Finnzymes Oy, Finland). The amplicon of 623 bp was then ligated into pCRII-TOPO vector (TOPO TA cloning, Invitrogen BV), following the producer's instruction, to generate the plasmid pSNAT2-623 and used to transform $CaCl_2$ competent *E. coli*. Plasmid DNA was extracted with Jetquick-plasmid miniprep spin kit (Genomed GmbH); both sense and antisense strands of the cDNA were sequenced (MWG-Biotech AG, Germany).

To generate a ^{32}P-labelled primer, 5 ng of pSNAT2-623 was amplified by asymmetric PCR. The PCR reaction was performed in a 25-μl reaction tube containing 50 nM sense primer, 0.5 μM antisense primer, 50 μCi of α-^{32}P-dCTP, SA 3000 Ci/mmol (PerkinElmer life Sciences srl, Italy), 20 μM each dNTPs, 1× Taq DNA buffer, 0.5 U DyNAzyme DNA polymerase (Finnzymes Oy, Finland). The reaction consisted of 35 cycles of PCR with 30 s at 94 °C, 30 s at 59 °C and 60 s at 72 °C. The labelled probe was purified on a Sephadex G-50 (Pharmacia) column, denatured, and added to the hybridization solution.

2.7. Northern blot analysis

For Northern blot analysis, 15 μg of total RNA was subjected to electrophoresis under denaturating conditions on 1% agarose gel containing 2.2 M formaldehyde. After staining with ethidium bromide, to document equal sample loading and absence of degradation, the total RNA was transferred overnight onto positively charged nylon membranes (Nytran Super Charge, Schleicher and Schüll SpA, Italy) and then linked to the membrane at 80 °C. Blots were hybridized overnight at 42 °C in 50% formamide to ^{32}P-labeled PCR-generated human SNAT2 [18] and to a random primed human GAPDH probe [11]. The blots were washed according to the producer instructions, and then exposed to Kodak XAR film at −70 °C for the appropriate time.

2.8. Biotinylation

Biotinylation of cell surface proteins was performed as described by Davis et al. [19] with slight modifications. Briefly, cultures of human fibroblasts were grown until subconfluence on 50-cm^2 tissue culture plates. After the experimental treatment, cells were rinsed twice in PBS with Ca and Mg. The plates were then incubated in 2 ml of biotin solution (EZ-Link Sulfo-NHS-LC-Biotin, Pierce, 0.25 mg/ml in Ca/Mg-PBS) for 30 min at 4 °C with gentle shaking. Cultures were then washed three times with ice-cold PBS with Ca and Mg containing 100 mM glycine and incubated in the same solution for 45 min at 4 °C with gentle agitation to quench any unbound biotin. Fibroblasts were then incubated at 4 °C in 1 ml of RIPA buffer (100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA 630, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate), containing a cocktail of protease inhibitors (Complete, Mini, EDTA-free, Roche), and shaken vigorously for 1 h to achieve a complete lysis. Lysates were transferred in Eppendorf tubes, sonicated for 15 s, and centrifuged for 30 min at 4 °C and 16 000×*g*.

After quantification of the proteins (Bio-Rad protein assay), a portion of the supernatant was kept for the Western blot of total cell lysate, while another portion, corresponding to 500 μg of proteins, was mixed with 1/3 volume of immobilized NeutrAvidin. After a 1-h incubation at room temperature, the mixture was centrifuged at 2500×*g* for 5 min. While a portion of the supernatant was kept for the Western blot of the intracellular fraction, the pellet was washed once with RIPA buffer, once with a wash buffer (50 mM Tris–HCl, 500 mM NaCl, 0.1% Igepal CA 630), three times with 10 mM Tris–HCl, and then resuspended in 50 μl of Laemmli buffer (62.5 M Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.2 M DTT) for 30 min with occasional shaking to elute the biotinylated proteins.

2.9. Western blot analysis

The biotinylated proteins were boiled for 5 min and centrifuged for 5 min at 12 000×*g* before SDS-PAGE. Aliquots (10 μg) of total cell lysate and of the intracellular fraction were boiled for 5 min. The three fractions were then electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Bio-Rad). Nonspecific binding sites were blocked with an incubation for 2 h at room temperature in Tris-buffered saline (TBS; 50 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 1% casein, 0.33% gelatin, and 3% bovine serum albumin. The blots were then incubated overnight at 4 °C with anti-SNAT2 polyclonal antiserum

(diluted 1:5000 in blocking solution), obtained with a GST/SNAT2-fusion protein [4]. After washing, the blots were exposed to horseradish peroxidase-conjugated anti-rabbit IgG diluted 1:1000 in blocking solution for 1 h. For standardization of biotinylated fraction, membranes were stripped and exposed to rabbit antiserum against the EGF receptor (EGFR, Santa Cruz Biotechnology, 1:500). For standardization of the total cell lysate and the intracellular fraction, stripped membranes were exposed to polyclonal anti-rabbit antiserum against β-tubulin (Santa Cruz Biotechnology, 1:500). The membranes were washed, the immunoreactivity was visualized with enhanced chemiluminescence, and quantification was performed with a Molecular Dynamics densitometer.

2.10. Immunocytochemistry

Cells were seeded in four-well Labtech Chamber Slides (Nunc) at a concentration of 3×10^4 cells/well in DMEM supplemented with 10% FBS. After the treatment in hypertonic DMEM or in EBSS, the slides were rapidly washed three times in PBS, fixed for 15 min at room temperature in 2% paraformaldehyde, 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 washing in PBS, the slides were incubated in a blocking solution (PBS containing 3% bovine serum albumin) for 1 h at 37 °C. The incubation with 1:200 anti-SNAT2 antibody [4] was performed for 1 h at 37 °C in the same blocking solution diluted 1:2. Cells were then washed twice with PBS containing 0.5% Tween 20 (PBST) and incubated at 37 °C for 30 min in the blocking solution with biotinylated goat anti-rabbit Ig secondary antibody diluted 1:300. After two washings in PBST, the slides were incubated in 1 μg/ml fluorescein-conjugated streptavidin (Molecular Probe) in PBS. Cells were then washed four times with PBST and once with distilled water. Slides were observed with a confocal laser scanning microscope (Molecular Dynamics Multiprobe 2001) equipped with an argon laser and based on a Nikon inverted microscope. Images were acquired with a ×60/1.4 oil immersion PlanApo lens, converted in TIFF files, digitally composed, and directly printed on photographic paper.

2.11. Statistics

A two-tail *t*-test for unpaired data was employed to assess the significance of the differences observed in transport activity, cell volume, or the intracellular content of glutamine. Nonlinear regression and linear regression analyses of kinetic data and their statistical parameters were calculated with GraphPad Prism 3.0®.

3. Results

3.1. Transport of L-proline and expression of SNAT2 protein upon hypertonic treatment

Fig. 1, panel A, shows the kinetic analysis of proline transport in cultured human fibroblasts maintained in isotonic growth medium or incubated for 5 h in hypertonic DMEM (400 mosmol/kg). Under both conditions, proline influx was fitted satisfactorily with an equation (Eq. (1), see Materials and methods) that describes the transport process as the result of the additive contributions of a single saturable component, corresponding to system A [17], and of a diffusive component not saturable in the range of the adopted concentrations. The Eadie–Hofstee representation of the saturable component of proline influx (panel B) shows that hypertonic increase was due exclusively to a

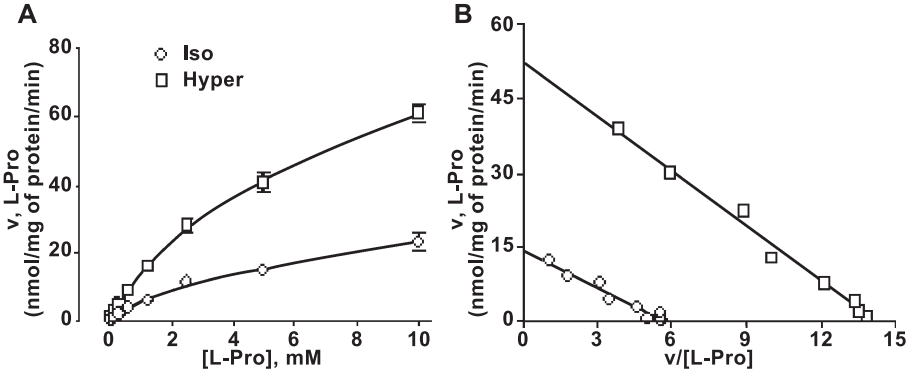

Fig. 1. Kinetic analysis of proline transport in hypertonically stressed cultured human fibroblasts. Panel A. Subconfluent cultures of human fibroblasts were incubated for 5 h in isotonic DMEM (Iso) or hypertonic DMEM at 400 mosmol/kg (Hyper). At the end of this period, cells were rapidly washed twice in Earle's Balanced Salt Solution (EBSS) and the influx of L-[^{3}H]-proline was measured in the same solution in a range of amino acid concentrations from 0.08 to 10 mM. Points are means of three independent determinations with S.D. indicated when greater than the size of the point. Lines are computer-drawn, best-fit nonlinear regressions to Eq. (1) (see Materials and methods). Goodness of fit was checked with the calculation of determination coefficients (R^2) that corresponded to 0.959, for control cells, and 0.999, for hypertonically treated cells. Panel B. The results are shown in Eadie–Hofstee graphical representation after subtraction of the diffusive component. Lines represent the saturable component of proline uptake calculated with linear regression analysis. For parameters, see text.

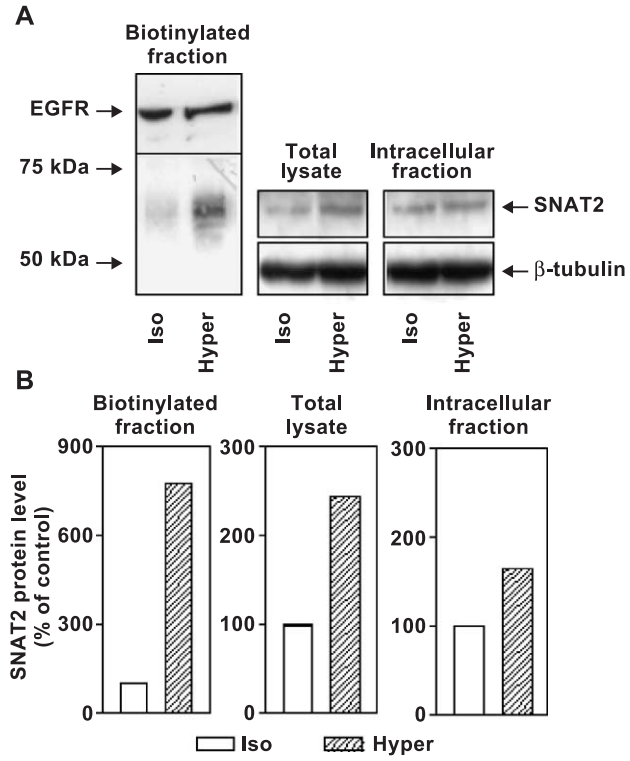

Fig. 2. Expression of SNAT2 transporters under hypertonic conditions. Subconfluent cultures of human fibroblasts were incubated for 5 h in isotonic DMEM (Iso) or hypertonic DMEM at 400 mosmol/kg (Hyper). Protein extraction and Western blot analysis were performed as described under Materials and methods. Panel A. Blots of biotinylated surface proteins were incubated with anti-SNAT2 and anti-EGFR antibodies. Blots of total cell lysate and intracellular protein fraction were incubated with anti-SNAT2 and anti-β-tubulin antibodies. Panel B. Quantification of changes in SNAT2 protein level upon hypertonic stress. Data have been obtained from the densitometric analysis of the membranes shown in Panel A, expressed as percentage of control after standardization with EGFR, for biotinylated fraction, or β-tubulin for the total lysate or the intracellular fraction. The experiment was performed three times with comparable results.

marked enhancement of the maximal velocity of transport. Linear regression analysis indicated, indeed, that V_{max} increased from 13.9±0.93 nmol/mg protein/min (r^2=0.948), in control cells, to 52.5±1.74 nmol/mg protein/min (r^2=0.989), in hypertonically treated cells. Under the same conditions, the K_m of proline influx increased from 2.6±0.43 to 3.7±0.16 mM.

Fig. 2, panel A, presents a Western blot analysis of SNAT2 protein performed on the biotinylated plasma membrane proteins, the total cell lysate, and the intracellular fraction of cultured fibroblasts maintained in isotonic medium or incubated under hypertonic conditions. In the biotinylated fraction, a broad band of the expected molecular weight for SNAT2 transporter (nearly 60,000) was barely detectable in control cells and became much more evident after hypertonic treatment. SNAT2 band, clearly detectable both in the total cell lysate and in the intracellular compartment of control cells, increased in hypertonically stressed fibroblasts. The binding of anti-SNAT2 antibody was completely suppressed by 10 μM GST/SNAT2-fusion protein and no band was detectable under these conditions in any cell fraction (not shown). Fig. 2, panel B, reports the quantification of SNAT2 protein standardized to, respectively, the receptor of EGF, for membrane SNAT2, or to β-tubulin, for SNAT2 content of the total cell lysate or the intracellular fraction. The results indicate that the relative increase of SNAT2, observed upon the hypertonic treatment, was much higher in the plasma membrane (+660%) than in the total cell lysate (+140%) or in the intracellular fraction (+60%).

The immunocytochemical analysis of SNAT2 protein abundance and distribution is shown in Fig. 3. Under control conditions (left panel), the positivity for SNAT2 protein was low and mainly intracellular. Under hypertonic conditions (5 h of incubation at 400 mosmol/kg, middle panel), the positivity for SNAT2 substantially increased with a clear reinforcement in the perinuclear zone. The specificity of the antibody binding was demonstrated by the almost complete suppression of positivity observed if the incubation with SNAT2 antiserum was performed in the presence of the GST/SNAT2 fusion protein employed for the immunization [4] (right panel).

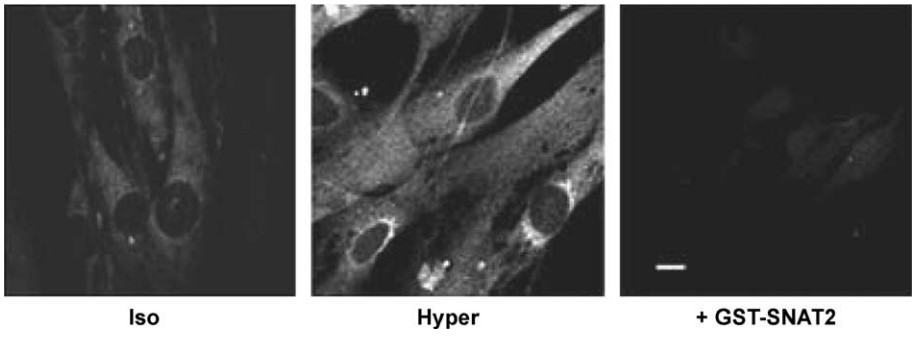

Fig. 3. SNAT2 protein expression in cultured human fibroblasts. Fibroblast cultures were maintained in isotonic DMEM (left panel, Iso) or incubated in hypertonic DMEM for 5 h (middle panel, Hyper). Cells were then fixed and exposed to anti-SNAT2 antiserum, as detailed under Materials and methods. Right panel: After the incubation in hypertonic DMEM (same treatment of middle panel) the slide was exposed to anti-SNAT2 antiserum in the presence of GST-SNAT2 fusion protein (10 μM). Confocal images of representative fields are shown with signal intensity expressed on a grey scale. Bar=10 μm. The experiment was repeated three times with comparable results.

3.2. Effect of the inhibition of transcription on transport activity, SNAT2 mRNA, and SNAT2 transporter protein

In the representative experiment described in Fig. 4, the effects of DRB, an inhibitor of transcription, on SNAT2 transporter protein (panel A), SNAT2 mRNA (panel B), and system A transport activity (panel C) were studied in cells incubated under hypertonic conditions. DRB substantially suppressed the increase of SNAT2 protein on the plasma membrane of hypertonically treated cells and, in the presence of the inhibitor, the transporter was barely detectable (panel A). Consistently, DRB suppressed the marked increase in SNAT2 mRNA abundance (panel B) and the stimulation of system A transport induced by the hypertonic treatment (panel C). The basal transport activity was unaffected by the inhibitor up to 9 h of incubation. The kinetic analysis of proline influx in fibroblasts incubated in DMEM at 400 mosmol/kg in the presence of DRB yielded a V_{max} of 21.2±1.93 nmol/mg protein/min and a K_m of 3.1±0.19 mM (r^2=0.975). These data indicated that the increase in transport V_{max}, observed under hypertonic conditions (see Fig. 1), was almost completely suppressed by the inhibitor.

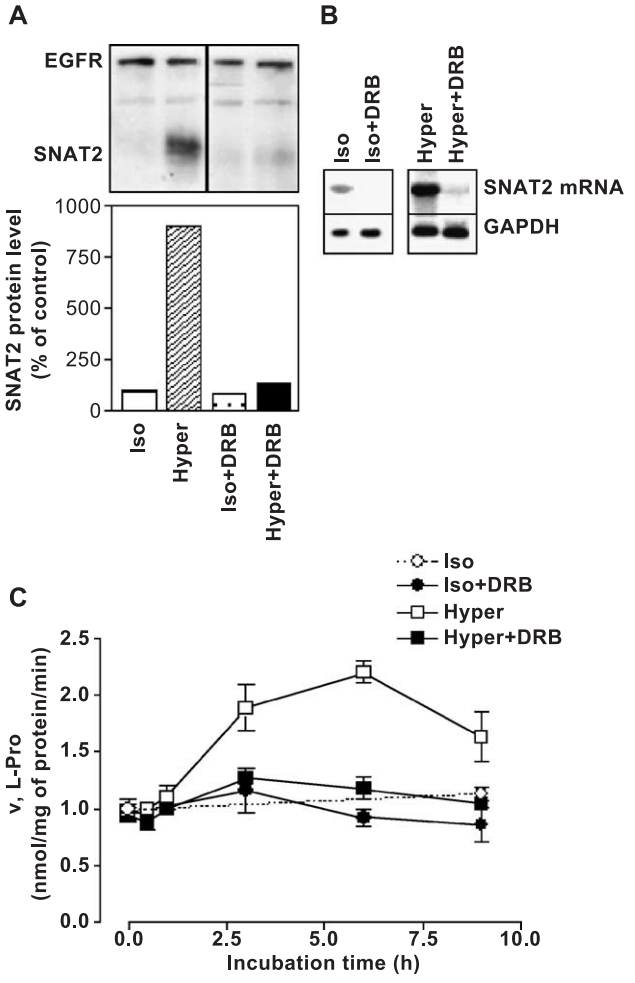

Fig. 4. Dependence on transcription of hypertonic changes in SNAT2 transporter protein, SNAT2 mRNA, and system A transport activity. Panel A, top. Western blot analysis of biotinylated membrane proteins was performed with anti-SNAT2 and anti-EGFR antisera, as described in Materials and methods, employing fibroblasts incubated for 5 h in isotonic (Iso) or hypertonic DMEM (Hyper) in the absence or in the presence of DRB. Panel A, bottom. Densitometric analysis of the membranes shown in Panel A, top, with data expressed as percentage of control after standardization with EGFR. The experiment was performed three times with comparable results. Panel B. Northern blot analysis of SNAT2 mRNA expression was performed as described in Materials and methods on total RNA extracted from cells maintained in isotonic DMEM or incubated for 3 h in hypertonic DMEM in the absence or in the presence of DRB. Panel C. One-minute influx of L-proline (0.1 mM, 2 μCi/ml) was measured, at the indicated times, as described in Materials and methods. Points are the means of three independent determinations with S.D. shown when greater than the size of the point. Where present, DRB (100 μM) was added 1 h before the treatments.

3.3. Effects of DRB on the intracellular amino acid pool and cell volume recovery of hypertonically stressed cells

In cultured human fibroblasts L-glutamine is the predominant component of the intracellular amino acid pool [8] and is a good substrate of system A [20]. The data presented in Fig. 5, panel A, indicate that a 6-h treatment with DRB had a small effect on the cell content of glutamine under isotonic conditions. As expected [8], a prolonged hypertonic incubation produced a clear-cut increase of the cell content of glutamine that was doubled compared with control. The enhanced accumulation of glutamine was substantially suppressed if the hypertonic treatment was performed in the presence of DRB. Comparable changes were observed for the total intracellular pool of amino acids (not shown).

As shown in Fig. 5, panel B, in the absence of DRB cultured human fibroblasts efficiently regulated cell volume upon hypertonic stress. Indeed, after a 6-h incubation in hypertonic medium in the absence of the inhibitor, cells had completely recovered their volume that was not significantly different from the control value. On the contrary, in the presence of DRB, cell volume recovery was significantly delayed and cells were still significantly shrunken after 6 h of hypertonic treatment.

3.4. Effect of DRB on the expression of SNAT2 transporter protein under amino acid-free conditions

Amino acid starvation causes a progressive increase in the transport activity of system A associated to enhanced abundance of SNAT2 mRNA [11]. Fig. 6 shows changes in SNAT2 protein (panel A), SLC38A2 mRNA (panel B), and system A transport activity (panel C) in DRB-treated or untreated cells incubated under amino-acid-free conditions. In the absence of DRB, amino acid starvation caused a marked increase (+680%) of the SNAT2 transporter expressed on the plasma membrane. The increase was still clearly evident, although at a lesser extent (+240%), in cells incubated under amino acid-free conditions in the presence of the inhibitor (panel A). Consistently, although the inhibitor completely suppressed the massive increase in

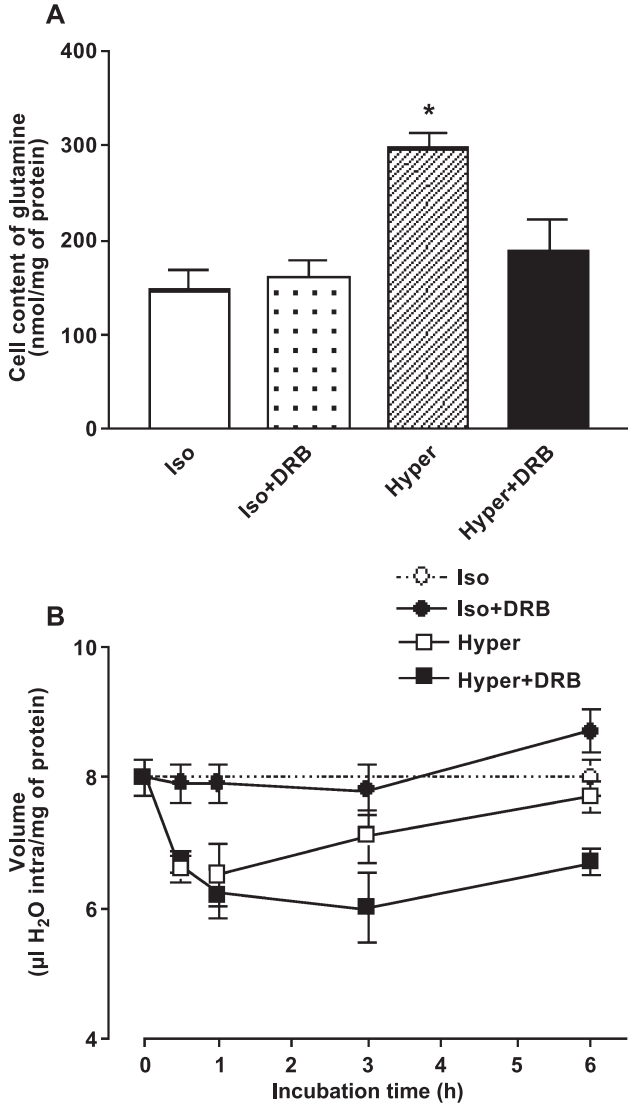

Fig. 5. Effects of DRB on the cell content of glutamine and cell volume upon hypertonic stress. The measurements of cell glutamine and of cell volume were performed in parallel cultures of fibroblasts incubated in isotonic (Iso) or hypertonic DMEM (Hyper). Where present, DRB (100 μM) was added 1 h before treatment. The cell content of L-glutamine (Panel A) was measured after 6 h of incubation under the indicated conditions. Bars are means of three independent determinations with S.D. shown. Panel B. Cell volume was determined, as described in Materials and methods, at the indicated times of incubation under the indicated conditions. Points represent the means of four independent determinations with S.D. shown. The experiment was repeated three times with comparable results. *P<0.05.

SNAT2 mRNA triggered by amino acid starvation (panel B), the adaptive increase of system A transport activity was only partially blocked by DRB (panel C). Indeed, for the first 3 h of starvation the increase in proline transport was comparable either in the presence or in the absence of the inhibitor. Moreover, after 9 h of amino acid starvation in the presence of DRB, proline transport was still more than doubled compared with control. However, a DRB-sensitive portion of transport stimulation was also evident at this time point, accounting for more than the 60% of the overall proline influx.

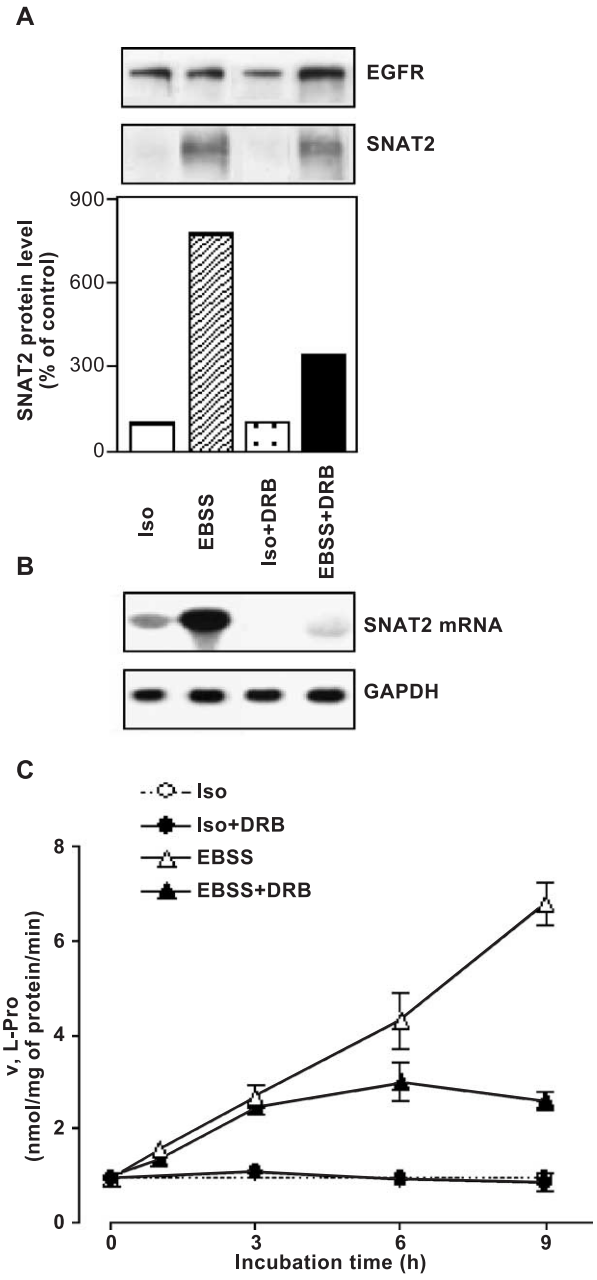

Fig. 6. Effect of DRB on SNAT2 transporter protein, SNAT2 mRNA, and system A transport activity observed upon amino acid starvation. Panel A, top. Western blot analysis of biotinylated membrane proteins was performed with anti-SNAT2 and anti-EGFR antisera, as described in Materials and methods, employing fibroblasts maintained in isotonic DMEM (Iso) or incubated for 8 h in amino acid-free EBSS (EBSS) in the absence or in the presence of DRB. Panel A, bottom. Densitometric analysis of the membranes shown in Panel A, top, with data expressed as percentage of control after standardization with EGFR. The experiment was performed three times with comparable results. Panel B. Northern blot analysis of SNAT2 mRNA expression was performed as described in Materials and methods on total RNA extracted from cells maintained in isotonic DMEM or incubated for 12 h in EBSS in the absence or in the presence of DRB. Panel C. One-minute influx of L-proline (0.1 mM, 2 μCi/ml) was measured, at the indicated times, as described in Materials and methods. Points are means of three independent determinations with S.D. shown when greater than the size of the point. Where present, DRB (100 μM) was added 1 h before the treatments.

4. Discussion

The hypertonic stimulation of system A has been referred to the increased expression of SLC38A2 gene [12], due to the activation of NFAT5/TonEBP [21], although no osmotic-response element has been yet identified in the gene. The immunoblotting data presented in this contribution indicate that SLC38A2 gene induction is followed by a clear-cut increase of the SNAT2 transporter protein in hypertonically stressed fibroblasts. In particular, employing surface biotinylation, we have demonstrated that the number of SNAT2 carriers inserted in the plasma membrane is much higher in hypertonically stressed fibroblasts than in control cells. Under the same conditions, the transport V_{max} of system A markedly increases. The discrepancy between the relative changes exhibited by membrane SNAT2 levels (7-fold increase) and transport V_{max} (4-fold increase) could be referred either to the quantitative limitations of biotinylation technique or to different transport efficiencies of SNAT2 transporters under isotonic and hypertonic conditions. The latter possibility would also be consistent with the higher K_m exhibited by system A upon hypertonic treatment (see Fig. 1).

The regulation of system A transport activity is very complex. Several aspects of biological importance, remained so far debated, have been now addressed through the assessment of the expression of SNAT2 protein. For instance, the hypertonic stimulation of system A had been attributed to the synthesis of transporter activating proteins rather than to new transporters [14,16]. Although the results presented here cannot exclude that osmotic stress triggers the synthesis of system A-activating proteins, they conclusively demonstrate that much more SNAT2 carriers are synthesized and expressed on the plasma membrane under this condition. Therefore, the increase of SNAT2 carriers constitutes at least a major mechanism for the hypertonic stimulation of system A transport activity.

Another questioned issue concerns the relationships existing between the hypertonic stimulation of system A and the adaptive enhancement of the system that follows amino acid starvation. In a previous contribution we have demonstrated that amino acid starvation actually causes an osmotic stress and activates a typical osmocompensatory response mediated by TonEBP [13]. Here we show that, like adaptive increase [15,22], also hypertonic stimulation of system A transport activity is associated with the presence of more SNAT2 proteins on the cell membrane. However, while hypertonic stimulation of transport activity is completely blocked by DRB, the adaptive increase is only partially dependent upon transcription. Consistently, in DRB-treated, starved cells membrane SNAT2 transporters are still more abundant than in fed cells, although their level is diminished if compared with fibroblasts starved in the absence of the inhibitor. These results indicate that adaptive stimulation involves both transcription-dependent and transcription-independent mechanisms, as already suggested by Ling et al. [22]. Therefore, the very high system A activity observed in starved cells should be referred both to the activation/relocalization of preformed SNAT2 transporters and to the synthesis of new carriers induced by either osmotic, TonEBP-dependent [13], or nutritional signals [23]. On the contrary, the osmotic increase of system A transport activity is substantially dependent on the synthesis of new SNAT2 transporters and, consistently, upon hypertonic treatment, the membrane abundance of SNAT2 transporters is much lower in DRB-treated cells than in untreated counterparts. Thus, a strict dependence upon newly synthesized carriers constitutes a hallmark of hypertonic increase of system A transport activity.

A considerable amount of information has been gathered about the transcriptional regulation of genes for transporters for organic osmolytes other than amino acids [24,25], such as BGT1 for betaine [26], TAUT for taurine [27] and SMIT1 for inositol [28]. On the contrary, much less is known about possible changes induced by hypertonic treatment at the level of the corresponding transporter proteins. No information is available yet for SMIT1 and TAUT transporters, while, for the hypertonic regulation of BGT1 protein, a complex picture is emerging [29–31]. Indeed, both transcriptional regulation and redistribution of preformed transporters to the plasma membrane have been observed under hypertonic conditions, at least in BGT1-transfected cells that overexpress the transporter [29–31]. At variance with those contributions, here we have studied the hypertonic stimulation of endogenous SLC38A2/SNAT2 and found evidence for only a limited redistribution of pre-existing transporters upon hypertonic stress. After 5 h of hypertonic treatment, when transport activity is maximal and volume recovery well advanced, SNAT2 positivity (see Fig. 3) still shows a clear perinuclear reinforcement, suggesting that, even under these conditions, a large subpopulation of transporters is intracellular. However, data reported in Fig. 2 indicate that a preferential targeting of SNAT2 transporters does occur upon hypertonic stress, since the increase in the membrane pool of transporters is larger than those detected in total cell lysate or in the intracellular fraction. This change is exquisitely sensitive to DRB (Fig. 4), thus suggesting that newly synthesized SNAT2 transporters, rather than the pool of pre-existing carriers, are preferentially targeted to the membrane under hypertonic conditions.

The results presented in this study contribute to define the functional role of the increased synthesis of SNAT2 transporters under hypertonic conditions. Indeed, in DRB-treated, hypertonically stressed cells, no stimulation of system A transport activity and no increase in the cell content of amino acids are observed. Under the same condition, the recovery of cell volume is significantly delayed, thus confirming that the redistribution of pre-existing carriers plays, if any, a limited role in the volume

response. It should be stressed that these effects cannot be specifically referred to the suppression of SNAT2 expression, since hypertonic stress induces several genes that would be suppressed by DRB [32]. Nevertheless, the simplest explanation for these data consists in the direct dependence of a fast volume recovery on the increased accumulation of neutral amino acids due to the synthesis of new SNAT2 transporters. However, the formal validation of this hypothesis will require additional studies with experimental devices aimed to the specific inhibition of SNAT2 expression.

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