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SNAT2 silencing prevents the osmotic induction of transport system A and hinders cell recovery from hypertonic stress

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Abstract Under hypertonic conditions the induction of SLC38A2/SNAT2 leads to the stimulation of transport system A and to the increase in the cell content of amino acids. In hypertonically stressed human fibroblasts transfection with two siRNAs for SNAT2 suppressed the increase in SNAT2 mRNA and the stimulation of system A transport activity. Under the same condition, the expansion of the intracellular amino acid pool was significantly lowered and cell volume recovery markedly delayed. It is concluded that the up-regulation of SNAT2 is essential for the rapid restoration of cell volume after hypertonic stress.

Keywords: siRNA; Amino acids; Glutamine; Cell volume

1. Introduction

To counteract hypertonic stress, cells activate a complex array of mechanisms, collectively indicated as regulatory volume increase (RVI, [1,2]). This response produces an accumulation of osmotically active compounds, called organic compatible osmolytes, that leads to the recovery of cell volume and the decrease of intracellular ionic strength.

During RVI several transduction pathways are activated and the expression of a number of genes is induced [3]. In particular, transporters for organic osmolytes, such BGT1 for betaine, TAUT for taurine, and SMIT for *myo*-inositol are up-regulated (see [4,5] for review). Several reports have also involved amino acid transport system A, a sodium dependent mechanism referable to SNAT transporters encoded by SLC38 gene family [6], in cell volume recovery after hypertonic stress (see [7] for review). Under the same conditions, also NKCC co-transporter is stimulated and accumulation of potassium chloride has been proposed as a rapid device to counteract osmotic stress [8,9].

The discrimination of the relative contributions of single mechanisms to the volume recovery process has been difficult. Many adaptations require changes in gene expression and can be blocked with inhibitors of macromolecular syntheses [10]. However, these compounds are not specific.

In this report, we employ small interfering RNAs (siRNAs) to suppress specifically the hypertonic induction of SLC38A2/SNAT2, the ubiquitous member of SLC38 gene family, so as to discriminate its role in RVI of cultured human fibroblasts.

2. Materials and methods

2.1. Cells and incubations

Cultured human fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS). The osmolality of complete growth medium (isotonic DMEM) was 306 ± 12 mosmol/kg.

For transport and volume experiments, cells were seeded in 96-well multiwell dishes (Falcon) at a density of 10 000 cells/well. For qRT-PCR and analysis of amino acid pool cells were seeded in 12-well dishes at a density of 50 000 cells/cm^2.

Hypertonic treatment consisted in the incubation in DMEM supplemented with 100 mM sucrose (hypertonic DMEM, 405 ± 9 mosmol/kg).

2.2. SNAT2 siRNAs preparation and transfection

Ambion (Austin, TX) was the source of the three siRNAs against human SLC38A2 (NM_018976): SNAT2_1 (sense GCGACUUCAA CUACUCCUAtt; antisense UAGGAGUAGUUGAAGUCGCtg; siRNA ID 43618), SNAT2_2 (sense GCAAGCUGCUCUGAAA AGCtt; antisense GCUUUUCAGAGCAGCUUGCtt; siRNA ID 43690), and SNAT2_3 (sense GAAUGAUGAAUGUGUCCAAtt; antisense UUGGACACAUUCAUCAUUCtt; siRNA ID 43761). Human fibroblasts were plated the day before transfections and grown to 50–70% confluence. Transfections were carried out according to siPORT Amine siRNA Transfection Protocol (Ambion). Negative control consisted of a scrambled siRNA with no similarity to human gene sequences (Negative Control #1 siRNA, Ambion).

2.3. Transport activity, cell volume, and intracellular amino acids

Transport activity of system A was determined from the initial influx (1 min) of the system A-specific substrate 2-methyl-aminoisobutyric acid ([^{14}C]-MeAIB, 100 μM, 1 μCi/ml). The transport activities of systems X^-_{AG} and ASC were determined from the influx of, respectively, [^{3}H]-L-aspartate (10 μM, 3 μCi/ml, uptake time 1 min, [11]) and [^{3}H]-L-threonine (50 μM, 1 μCi/ml, uptake time 30 s, [12]). Assay procedure was described previously [13].

Cell volume was estimated from the distribution space of urea, according to a method previously validated in cultured human fibroblasts [13]. The intracellular amino acid pool was determined by HPLC analysis with a Biochrom 20 Amino Acid Analyzer (Amersham Pharmacia Biotech) after cell extraction in a 5% solution of acetic acid in ethanol, as described previously [14].

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Abbreviations: BGT1, betaine/GABA transporter; NKCC, Na(+)-K(+)-2Cl(−) cotransporter; RVI, regulatory volume increase; SMIT, sodium/myo-inositol cotransporter; SNAT, sodium-coupled neutral amino acid transporter; TAUT, taurine transporter

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2.4. Reverse transcription and quantitative PCR

Total RNA, extracted with OMNIzol™ (Euroclone SpA, Pero (MI), Italy), was reverse transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen Srl, S. Giuliano Milanese (MI), Italy) according to the manufacturer's instructions. For real time PCR, aliquots of 20 ng of cDNA were amplified in Platinum® Quantitative PCR SuperMix UDG (Invitrogen Srl) with predesigned primers and specific FAM™ labelled TaqMan® MGB probes for SNAT2 or the housekeeping gene β_2-microglobulin (β2M) (Assay-on-Demand™, Applied Biosystems). Two amplifications for each transcript were performed. A no-template, no-reverse transcriptase control was included in each experiment.

Quantitative PCR was performed using a 36-well Rotor-Gene 3000™ (Corbett Research, Software Rotor-Gene 3000, version 5.0.60, Mortlake, Australia) with the following thermoprofile: initial step at 50 °C for 2 min, 95 °C for 2 min, and 40 cycles, consisting of 15 s-denaturation steps at 95 °C and an unique annealing/extension step at 60 °C for 30 s. The analysis of data was performed according to the relative standard curve method [15].

Data are expressed as SNAT2 expression normalized to the β2M expression detected in the same cDNA mixture.

2.5. Materials

Serum was obtained from Gibco. Sigma was the source of culture medium (DMEM, D5523). This formula contains 4 mM of glutamine, while aspartate, alanine, proline and asparagine are not present. Perkin–Elmer was the source of 2-[methyl-^{14}C]aminoisobutyric acid (32 mCi/mmol) and Amersham Pharmacia Biothech was the source of [2,3-^{3}H]-L-aspartate (39 Ci/mmol) and [3-^{3}H]-L-threonine (15 Ci/mmol). Unless otherwise stated, all the other chemicals were purchased from Sigma.

3. Results

3.1. SNAT2 silencing and inhibition of system A transport activity

In untransfected fibroblasts a 6 h-hypertonic treatment produced a 100% stimulation of the influx of the specific system A substrate MeAIB (Fig. 1, panel A). A 48 h-incubation with scrambled siRNA lowered MeAIB influx in both control and hypertonically treated cells, although the hypertonic stimulation was still clearly evident. On the contrary, transfection with one of the three SNAT2 siRNAs tested (each at a concentration of 100 nM) severely hindered (SNAT2_2) or completely abolished (SNAT2_1 and SNAT2_3) the hypertonic stimulation of MeAIB influx. The same treatment also lowered the basal activity of system A measured in cells maintained under isotonic conditions.

A mixture of SNAT2_1 and SNAT2_3, each at a concentration of 50 nM, completely suppressed the hypertonic increase of transport activity (Fig. 1, Panel B) after 48 h of transfection.

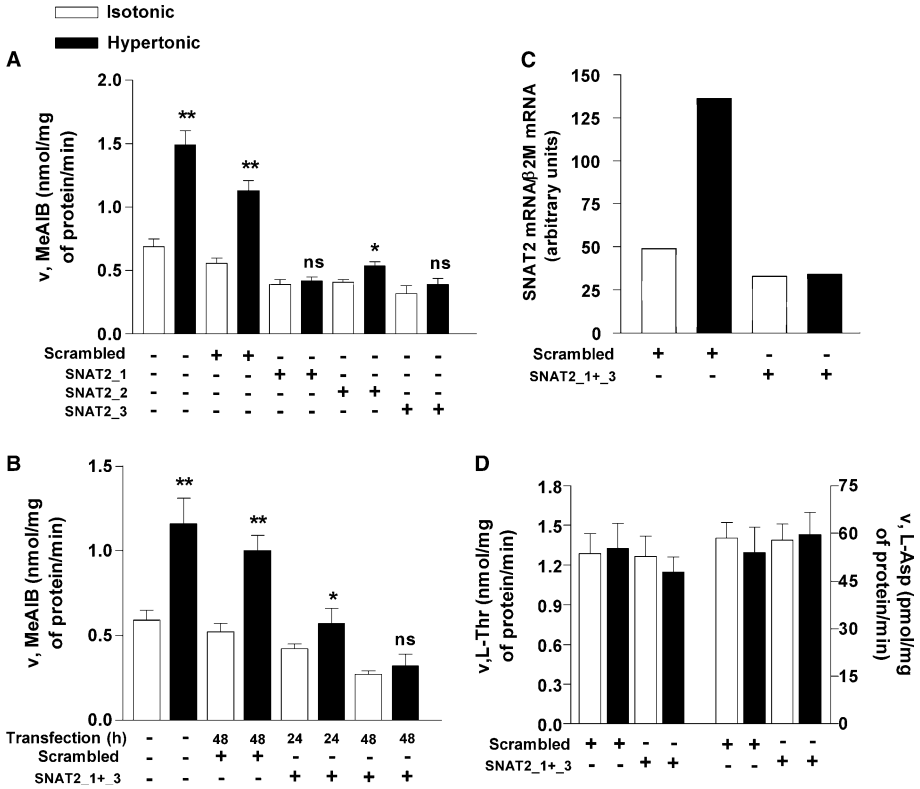

Fig. 1. Effect of SNAT2 siRNAs on amino acid transport activity and SNAT2 mRNA induction upon hypertonic stress. Human fibroblasts were transfected as detailed in the single panels. At the end of the transfection procedure, cells were incubated in isotonic or hypertonic DMEM, as indicated. (A) Cells were transfected with 100 nM of the indicated siRNAs for 48 h. After the incubation under isotonic or hypertonic conditions for 6 h, uptake of MeAIB was measured as described under Section 2. (B) Cells were transfected with 50 nM SNAT2_1 and 50 nM SNAT2_3 or 100 nM scrambled and further cultured for 24 or 48 h, as indicated. Cells were then incubated under isotonic or hypertonic conditions for 6 h and MeAIB uptake was measured. (C) Lysates of cells, transfected for 48 h as in (B) and incubated for 3 h under isotonic or hypertonic conditions, were employed for quantitative RT-PCR to assess SNAT2 mRNA levels. (D) Cells were transfected as in (B) for 48 h. Threonine or aspartate influx was measured after a 6 h incubation under isotonic or hypertonic conditions. For Panels A, B, and D, data are means ± S.D. of five independent determinations in a representative experiment. The experiment was repeated three times with comparable results. In Panels A and B, statistical analysis was performed comparing cells incubated under hypertonic conditions for 6 h vs. cells undergone the same transfection procedure, but maintained under isotonic conditions: $^{*}P < 0.05$; $^{**}P < 0.01$; ns, not significant. Panel C reports data from a representative experiment, repeated twice with comparable results.

However, a marked inhibitory effect was already detectable after a 24 h-transfection.

As expected from data in untransfected cells [13], a hypertonic incubation of 3 h caused a clear cut increase of SNAT2 mRNA abundance in cells transfected with scrambled siRNA (Fig. 1, Panel C). In contrast, no SNAT2 mRNA increase was detected in fibroblasts transfected with SNAT2_1 + SNAT2_3 siRNAs.

The specificity of system A inhibition by transfection with SNAT2_1 and SNAT2_3 was assessed by determining the activity of other sodium dependent, membrane potential sensitive, transport systems for amino acids, such as system ASC and system X^-_{AG}, both operative in cultured human fibroblasts [11,12] and encoded by genes of SLC1 family [16]. No significant inhibition of both aspartate (system X^-_{AG}) or threonine (system ASC) influx was observed in cells transfected with SNAT2_1 + SNAT2_3 siRNAs compared to cells transfected with scrambled siRNA (Fig. 1, Panel D).

3.2. SNAT2 siRNAs hinder cell volume recovery after hypertonic stress

As shown in Fig. 2, the transfection with scrambled siRNA did not prevent the hypertonic RVI expected in cultured human fibroblasts [10]. Both control cell volume and initial shrinkage, observed after the substitution of isotonic DMEM with hypertonic DMEM, were comparable in cells transfected with scrambled or antiSNAT2 siRNAs. In contrast, a compensatory swelling was progressively detectable only in cells transfected with scrambled siRNA, whose volume was already substantially restored after a 6 h-incubation. After 9 h of hypertonic treatment, cell volume was not significantly different from that of control cells, maintained in isotonic medium. Under the same conditions, cells transfected with SNAT2_1 and SNAT2_3 siRNAs exhibited a severely delayed RVI. Indeed, after 6 h of hypertonic treatment cells were still shrunken with no significant volume recovery while a little, but significant, compensatory response was only detectable after a 9 h-incubation. At this experimental time, however, cells treated with SNAT2 siRNAs were still markedly shrunken compared with control cells maintained under isotonic conditions.

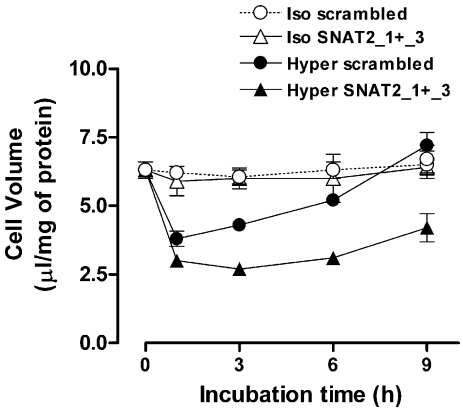

Fig. 2. Effect of SNAT2 silencing on volume recovery of human cultured fibroblasts during hypertonic stress. Cells were transfected for 48 h with scrambled (100 nM) or with SNAT2_1 + SNAT2_3 siRNA (each at 50 nM). Cells were then incubated in isotonic or hypertonic DMEM, as indicated, for 9 h and cell volume was measured at the indicated times, as described under Section 2. Data are means ± S.D. of five independent determinations in a representative experiment repeated three times with comparable results.

3.3. Changes in intracellular amino acids caused by SNAT2 siRNAs

Volume recovery of hypertonically stressed human fibroblasts is associated to the expansion of the intracellular amino acid pool [10]. After 6 h of hypertonic incubation the expected changes in intracellular amino acids were indeed evident in cells transfected with scrambled siRNA (Fig. 3, Panel A). The intracellular content of all amino acids was higher in cells incubated under hypertonic conditions than in cells maintained in isotonic medium (see also Table 1). In absolute terms, the overall expansion of amino acid pool was almost 300 nmol/mg of protein, with glutamine, a good substrate of system A in human fibroblasts [17], accounting for more than 50% of the total increase (150 nmol/mg of protein). Transfection with SNAT2-specific siRNAs (Fig. 3, Panel B) significantly lowered intracellular amino acids under isotonic conditions. Although cell content of amino acids was still increased by hyperosmotic stress (Table 1), the overall amount of amino acids accumulated upon a 6 h-hypertonic incubation was lowered from 289 to 191 nmol/mg of protein. Under these conditions cell glutamine content increased by only 87 nmol/mg of protein.

4. Discussion

Until now, RNA interference has been employed only rarely to suppress the expression of genes for amino acid transporters

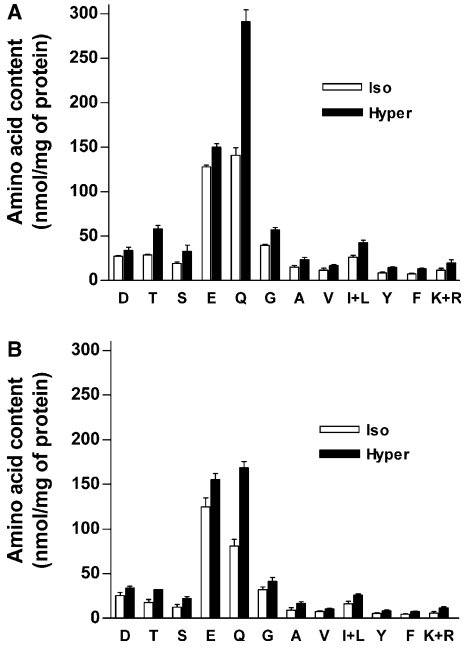

Fig. 3. Effect of SNAT2 silencing on the intracellular amino acid pool in fibroblasts incubated under isotonic or hypertonic conditions. Cells were transfected with scrambled siRNA (Panel A) or the mixture of SNAT2_1 + SNAT2_3 siRNA (Panel B) for 48 h. Cells were then incubated for 6 h in isotonic or hypertonic DMEM, as indicated, and the content of intracellular amino acids was measured as described under Section 2. The contents of P, M, N, C, H, and W were <4 nmol/mg of protein and not shown. Data are means ± S.D. of three independent experiments.

Table 1
Changes of intracellular amino acids induced by hypertonic stress in cultured human fibroblasts: effect of SNAT2 silencing

Amino acids	Scrambled siRNA		SNAT2_1 + _3 siRNA	
	Absolute increase (nmol/mg of protein)	% increase	Absolute increase (nmol/mg of protein)	% increase
Pool	289	62	191	56
D	7	20	8	33
T	20	102	14	80
S	14	71	9	75
E	23	17	30	24
Q	150	107	87	109
G	17	44	9	29
A	8	51	7	80
V	6	46	3	37
I + L	16	60	10	60
Y	6	72	3	52
F	6	70	3	65
K + R	8	71	6	109

Absolute increases have been obtained subtracting the values of the amino acid content of control cells, maintained under isotonic conditions, from values obtained in hypertonically stressed fibroblasts. Data are taken from Fig. 3.

[18,19]. Here we show that siRNA transfection can be employed to knockdown the expression of SLC38A2/SNAT2, the ubiquitous isoform of transport system A, which has escaped so far silencing attempts by either gene knockout or antisense oligos. The employment of siRNAs has yielded the effective silencing of SNAT2 not only under basal conditions but also upon hypertonic stress, an experimental situation associated with a marked induction of the gene [13,20].

Many transporters and enzymes have been involved in osmocompensatory volume increase of mammalian cells [1,2,5]. However, the absence of specific inhibitors has thus far prevented the definition of the peculiar role played by each operator in the volume recovery process. Through RNA interference we show here that SNAT2 suppression severely delays cell volume recovery, thus pointing to an essential role of SNAT2 induction in the short term restoration of cell volume after hypertonic stress. These data also demonstrate that increased SNAT2 mRNA abundance fully accounts for the stimulation of transport activity of system A detected under hypertonic conditions, thus indicating that the contribution of additive mechanisms, such as regulation at post-transcriptional or protein level, proposed for other osmotically sensitive transporters [4], is marginal.

The link between transport change and volume recovery appears now fairly clear. The increased abundance of SNAT2 mRNA [20] is followed by the enhanced membrane expression of SNAT2 protein [13]. The resulting transport stimulation is followed by the expansion of the intracellular pool of both system A substrates and other amino acids, through the operation of exchange mechanisms [7]. Amino acid accumulation sustains the recovery of cell volume, thus lowering the osmotically increased intracellular ionic strength. However, although RNA interference abolishes hypertonic SNAT2 induction, a limited, slow volume recovery is still detectable in anti-SNAT2-siRNA transfected cells incubated under hypertonic conditions. This residual compensatory response could be attributed to other osmotically sensitive transporters, characterized by delayed induction, such as BGT1 or SMIT [21]. Moreover, the increase in the intracellular amino acid pool is markedly lowered but not completely suppressed by SNAT2 silencing. Indeed, although the amount of amino acids accumulated by hypertonically stressed fibroblasts is significantly lowered by SNAT2 silencing, intracellular amino acids still significantly increase in relative terms under the same condition. This result suggests that another osmosensitive amino acid transporter may somehow substitute the silenced SNAT2. A possible candidate may be another SLC38 transporter, the neuronal isoform SNAT1, whose expression in cultured human fibroblasts is also stimulated upon hypertonic stress but not affected by anti SNAT2 siRNAs (E. Bevilacqua and R. Sala, unpublished results).

SNAT2 expression is widespread and in many cell models the activity of system A is up-regulated under hypertonic conditions. Thus, the conclusions reached in this report may point to a general clue of cell volume regulation in mammalian cells, although the consequences of SNAT2 silencing in a specific cell type will depend upon the transporter repertoire present therein. However, since failure to exert a rapid compensation of osmotic shrinkage may have severe consequences on cell proliferation and survival [22], the effects of SNAT2 silencing on cell viability and the expression of other stress-induced genes deserve further investigation. Moreover, given that system A is highly regulated under many physiological and pathological conditions [23], SNAT2 silencing may constitute a precious experimental tool for a better understanding of the functional role of the transporter.

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