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System A amino acid transporter SNAT2 shows subtype-specific affinity for betaine and hyperosmotic inducibility in placental trophoblasts

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ABSTRACT

Betaine uptake is induced by hypertonic stress in a placental trophoblast cell line, and involvement of amino acid transport system A was proposed. Here, we aimed to identify the subtype(s) of system A that mediates hypertonicity-induced betaine uptake. Measurement of [^{14}C]betaine uptake by HEK293 cells transiently transfected with human or rat sodium-coupled neutral amino acid transporters (SNATs), SNAT1, SNAT2 and SNAT4 revealed that only human and rat SNAT2 have betaine uptake activity. The Michaelis constants (K_m) of betaine uptake by human and rat SNAT2 were estimated to be 5.3 mM and 4.6 mM, respectively. Betaine exclusively inhibited the uptake activity of SNAT2 among the rat system A subtypes. We found that rat SNAT1, SNAT2 and SNAT4 were expressed at the mRNA level under isotonic conditions, while expression of SNAT2 and SNAT4 was induced by hypertonicity in TR-TBT 18d-1 cells. Western blot analyses revealed that SNAT2 expression on plasma membrane of TR-TBT 18d-1 cells was more potently induced by hypertonicity than that in total cell lysate. Immunocytochemistry confirmed the induction of SNAT2 expression in TR-TBT 18d-1 cells exposed to hypertonic conditions and indicated that SNAT2 was localized on the plasma membrane in these cells. Our results indicate that SNAT2 transports betaine, and that tonicity-sensitive SNAT2 expression may be involved in regulation of betaine concentration in placental trophoblasts.

1. Introduction

Betaine is an organic osmolyte that contributes to cytoprotection against osmotic stress. Its intracellular concentration is determined by the balance of synthesis, degradation, and net transport across the cell membrane. Betaine is highly hydrophilic and its plasma membrane permeation is mainly mediated by transporter(s). To regulate the intracellular osmolarity in response to extracellular hypertonicity, choline dehydrogenase (the first enzyme in the betaine biosynthetic pathway) and betaine uptake transporters are up-regulated, while betaine homocysteine *S*-methyltransferase, which metabolizes betaine, is down-regulated in various cells, such as hepatic cells [1,2]. Tissue distribution of betaine is not uniform, that is, its concentration in rats is quite high in liver, kidney and placenta (7.2, 6.9 and 2.6–5 μmol/g, respectively), but is less than 1.0 μmol/g in tissues such as pancreas, small intestine and heart, and is undetectable in lung and brain [3–5]. Therefore betaine is able to play more important role as an osmolyte in tissues where betaine concentration is high.

Betaine/GABA transporter (BGT-1, SLC6A12) is a well-characterized betaine transporter that is driven by inward gradients of sodium and chloride, in common with other SLC6A transport proteins. BGT-1 is osmosensitive, and plays a role in regulation of intracellular osmolarity via uptake of betaine from the extracellular fluid, particularly in renal cells [6]. Sodium/imino acid transporter 1 (SIT1, Slc6a20), which is also driven by sodium and chloride gradients, transports both betaine and proline [7]. SIT1 is also thought to be involved in osmoregulation in mammalian oocytes [8], but it is unclear whether the expression of SIT1 itself is regulated by changes of osmolarity. We have shown that betaine uptake is induced by hypertonicity in TR-TBT 18d-1 cells [9], which were used as a model for placental trophoblasts, but we concluded that amino acid transport system A, rather than BGT-1, was involved because the osmosensitive betaine uptake was inhibited by 2-(methylamino)isobutyric acid (MeAIB), a system A inhibitor and that was not inhibited by 2 mM GABA. This is consistent with observations in SV-3T3 and hepatic stellate cells [10,11]. Therefore, it appears that system A transporter(s) accepts betaine as a substrate and plays a protective role in placenta against osmotic changes in maternal or fetal plasma.

Among the sodium-coupled neutral amino acid transporters (SNATs), SNAT1, SNAT2 and SNAT4 are system A neutral amino acid

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transporter subtypes, and these three subtypes of system A have similar substrate specificities, i.e., alanine, asparagine, cysteine, serine and MeAIB are common substrates [12]. MeAIB is widely used as a specific inhibitor for system A, and it inhibits all three subtypes. The three subtypes are expressed in human placenta throughout gestation [13]. The function of system A is up-regulated by hypertonic stress in mammalian cells [14], and SNAT2 and SNAT4 show osmosensitive induction of expression within 12 h of hypertonic treatment in TR-BBB cells [15]. However, the subtype(s) of system A responsible for betaine uptake was not identified.

Here, we aimed to identify the system A subtype(s) that mediates betaine uptake and to clarify the molecular mechanism of osmosensitive upregulation of betaine transport via system A [16].

2. Materials and methods

2.1. Chemicals

[Glycine-1-^{14}C]N,N,N-trimethylglycine ([^{14}C]betaine, 55 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [1-^{14}C]Methylaminoisobutyric acid alpha ([^{14}C]MeAIB, 58.8 mCi/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). All other chemicals were commercial products of analytical grade.

2.2. Cell culture

Culture of TR-TBT 18d-1 cells as a model for placental trophoblasts was described previously [16]. Briefly, the cells were grown at 33 °C in Dulbecco's modified Eagle's medium (DMEM, 1.0 g/L glucose, Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS (fetal bovine serum, JRH Bioscience, Lenexa, KS), 2 mM L-glutamine, penicillin G (100 units/mL), and streptomycin (100 μg/mL) in a humidified incubator under an atmosphere of 5% CO_2 in air. TR-TBT 18d-1 cells were seeded at the density of 1.0×10^5 cells/mL on porcine skin collagen type-I coated 4-well plates. After 2 days of culture at 33 °C, the cells were further cultured at 37 °C for 2 days and then used for gene expression analyses and uptake studies with or without hypertonic stress.

HEK293 cells were maintained in DMEM (4.5 g/L glucose, Nacalai Tesque) supplemented with 10% FBS (JRH Bioscience), 2 mM L-glutamine, penicillin G (100 units/mL), and streptomycin (100 units/mL) at 37 °C in a humidified incubator under an atmosphere of 5% CO_2 in air.

2.3. Plasmid vectors containing a cDNA for human and rat SNAT1, SNAT2 and SNAT4

cDNA clones for hSNAT1/pCMV-SPORT6 (BC010620), hSNAT2/pCMV-SPORT6 (BC040342), hSNAT4/pCR4-TOPO (BC101827), rSNAT1/pExpressI (BC097283) and rSNAT4/pExpressI (BC097292) were commercial products. hSNAT4 cDNA was subcloned into pCMV-SPORT6 vector. rSNAT2 was cloned from TR-TBT 18d-1 cells by PCR and subcloned into pExpressI vector. The cDNA sequence of the product was confirmed to be the same as that of the reference SNAT2 sequence (NM_181090). pCMV-SPORT6 and pExpressI alone were used as negative controls in the uptake studies for human and rat SNAT subtypes, respectively.

2.4. Functional characterization of SNAT1, SNAT2 and SNAT4 in HEK293 cells

HEK293 cells were seeded at the density of 1.0×10^6 cells/mL in a 100-mm dish and 12 μg of plasmid vector containing human or rat SNAT1, SNAT2 or SNAT4 cDNA was transiently transfected using Lipofectamine (Life Technologies Inc., Rockville, MD). At 40–48 h after transfection, the cells were harvested and suspended in ECF buffer. The uptake study was performed immediately, using the silicone layer centrifugation method. The cell suspension was mixed with the solution containing a radiolabeled test compound and then the cells in 200 μL of the mixture were separated at a designated time by centrifugation at 10,000 *g* for 1 min through 100 μL of silicone layer (density of 1.03) prepared by mixing silicone oil (1.065 g/mL, SH550, Dow Corning Toray, Tokyo, Japan) and liquid paraffin (0.87 g/mL, Wako, Osaka, Japan) into 50 μL of 3 N KOH solution in a 0.4 mL tube. Radioactivity was measured with a liquid scintillation counter (LSC-6100, Hitachi-Aloka Medical, Tokyo). Cellular protein content was determined according to the Bradford's method using a BioRad protein assay kit (BioRad, Hercules, CA) with bovine serum albumin (Pierce Biotechnology, Rockford, IL) as a standard.

2.5. Kinetic analysis of betaine uptake by human and rat SNAT2

Initial uptake characteristics of betaine were kinetically analyzed by mean of the following Michaelis–Menten type equation:

$$V = \frac{V_{max} \times S}{K_m + S}$$

where V, V_{max}, K_m and S represent initial uptake velocity, maximum uptake velocity, affinity (Michaelis constant) and substrate concentration, respectively. Data were fitted to the equation by nonlinear regression analysis using GraphPad Prism4 (GraphPad Software, San Diego, CA). When the substrate concentration is much less than the K_m value, the equation can be approximated as:

$$V = \frac{V_{max} \times S}{K_m}.$$

Then, V_{max}/K_m is given by initial uptake clearance V/S.

2.6. mRNA expression analyses for rat system A subtypes

Total RNA was isolated from TR-TBT 18d-1 cells cultured in isotonic and hypertonic medium using an RNeasy mini kit (QIAGEN, Valencia, CA). Reverse transcription was performed by using a High Capacity RNA-to-cDNA kit (Life Technologies Inc.). The RT-PCR products amplified by selective PCR primers (gtggagaacggccagataaa/ggtttccttggagcagatca for rSNAT1, aaggcatacggtctggctgg/tatcggctgcggtgctgttg for rSNAT2, and accctggaacgacctctttt/aaacgccttctctcccaact for rSNAT4) were visualized after electrophoresis on 1% agarose in TAE (Tris–acetate–EDTA) buffer (Nacalai Tesque), followed by ethidium bromide staining. Quantitative RT-PCR was conducted with Power SYBR Green PCR Mater Mix (Life Technologies Inc.) on a 7300 Real-Time PCR system (Life Technologies Inc.). Relative expression was calculated by the ΔΔCt method and ß-actin amplified by the primers (tcatgaagtgtgacgttgacatccgt/cctagaagcatttgcggtgcacgatg) was used as a control.

2.7. Western blot analysis

TR-TBT 18d-1 cells exposed for 8 h to culture medium adjusted at 300 or 500 mOsm/kg with sorbitol and Cos-7 cells transfected with hSNAT2/pCMV-SPORT6 and vector alone were harvested by adding cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) supplemented with a complete protease inhibitor cocktail tablet (Roche Diagnostics, Basel, Switzerland). The samples were each homogenized by pipetting and sonication, and the supernatants obtained by centrifugation at 14,000 *g* for 10 min were stored at −80 °C until use. For plasma membrane fraction, the cells cultured in the isotonic and hypertonic conditions described above were harvested by a cell scraper and washed 3-times by phosphate-buffered saline (PBS). The cells were centrifuged at 2000 *g* for 10 min, and the pellet was re-suspended in a buffer containing 10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris (pH 7.4), and then homogenized by nitrogen cavitation at 700 psi. The

homogenate was mixed with 1 mM EDTA and centrifuged at 2000 *g* for 10 min. The supernatant was subjected to density-gradient centrifugation in 35% sucrose solution at 18,000 *g* for 90 min. The middle layer was harvested and mixed with buffer containing 150 mM mannitol, 75 mM potassium gluconate, and 10 mM HEPES (pH 7.4). This fraction was then ultracentrifuged at 100,000 *g* for 3 h and the pellet was resuspended in the last buffer. All the procedures were conducted at 4 °C. Protein concentrations were determined with a BCA protein assay kit (Pierce Biotechnology). All samples were mixed with NuPAGE LDS sample buffer and reducing agent (Invitrogen), followed by boiling at 70 °C for 10 min. Proteins (10 μg for TR-TBT 18d-1 cell lysates and 30 μg for Cos-7 cell lysates) were separated by SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide gel) and transferred onto a polyvinylidene difluoride membrane (Clear Blot Membrane-P, ATTO, Tokyo, Japan). For detection of human and rat SNAT2 protein and ß-actin, the membrane was incubated with 0.1% rabbit anti-SLC38A2 antibody (Sigma-Aldrich, St. Louis, MO, HPA035180) or 0.02% anti-ß-actin antibody (Sigma-Aldrich, Clone AC-15) in PBS containing 0.1% Tween 20 and 3% skim milk. The membranes were rinsed with PBS containing 0.1% Tween 20, and reacted with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG. Bands were visualized using an enhanced chemiluminescence detection method with ECL Western blotting detection kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

2.8. Immunocytochemistry

TR-TBT 18d-1 cells were cultured on collagen-coated cover glass at 33 °C for 2 days and at 37 °C for a further 2 days. The cells were exposed to DMEM supplemented with 200 mM sorbitol for another 8 h (hypertonic condition), and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at 4 °C for 30 min. The fixed cells were permeabilized with 0.1% Triton-X 100 in PBS and treated with protein blocking reagent (DAKO, Carpinteria, CA) at room temperature for 1 h. Next, the cells were incubated with 3% rabbit anti-SLC38A2 antibody (Sigma-Aldrich, HPA035180) at room temperature for 2 h and subsequently with Alexa Fluor 594 anti-rabbit IgG (Invitrogen) at room temperature for 1 h, followed by mounting using VECTASHIELD Hard Set Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Microscopic images were captured with a confocal laser-scanning microscope (FV1000D, Olympus, Tokyo, Japan).

2.9. Statistics

The statistical significance of differences was calculated by using Student's *t* test or analysis of variance (ANOVA) with Dunnett's post-hoc test for multiple comparisons. A p value of less than 0.05 was regarded as significant.

3. Results

3.1. Betaine uptake by amino acid transport system A subtypes

The three system A subtypes, SNAT1, 2, and 4, were each transiently expressed in HEK293 cells, and uptakes of [^{14}C]betaine and [^{14}C]MeAIB by the cells were measured. Only human and rat SNAT2 showed significant [^{14}C]betaine uptake (Fig. 1a,c), while all three subtypes showed [^{14}C]MeAIB uptake activity (Fig. 1b,d). These results indicated that SNAT2 mediates betaine uptake, while the other subtypes do not. To compare the uptake activity between substrates, the initial uptake velocities of betaine and MeAIB at 5 min were divided by their concentration (4.5 μM and 8.5 μM, respectively) and the uptake clearance (V_{max}/K_m) values were estimated to be 140 and 48 μL/mg protein/5 min, respectively. Initial uptake clearance of betaine was greater than that of MeAIB. To further characterize the betaine uptake activity of human and rat SNAT2, we analyzed concentration-dependent betaine uptake in terms of Michaelis–Menten type kinetics using the equation given in the Materials and methods (Fig. 2a,c). Saturable betaine uptake via human and rat SNAT2 gave linear Eadie–Hofstee plots (Fig. 2b,d). The values of uptake affinity (K_m) and maximum uptake velocity (V_{max}) of betaine for human and rat SNAT2 were calculated to be 5.3 ± 1.1 mM and 381 nmol/mg protein/5 min, while those for rat were 4.6 ± 0.8 mM and 773 nmol/mg protein/5 min. The inhibitory effect of betaine on SNAT-mediated uptake was examined using [^{14}C]MeAIB as a substrate. Rat SNAT1 and SNAT4-mediated [^{14}C]MeAIB uptake was hardly inhibited by betaine, but rat SNAT2-mediated [^{14}C]MeAIB uptake was almost completely inhibited by 10 mM betaine (Fig. 3).

3.2. Inducibility of system A subtypes by hypertonicity in rat placental trophoblast TR-TBT 18d-1 cells

To identify the subtype of system A responsible for hypertonicity-induced betaine uptake in TR-TBT 18d-1 cells, mRNA expression of system A subtypes was measured. All three subtypes of system A were expressed under isotonic conditions (DMEM), though the expression of SNAT4 was less than that of SNAT1 and SNAT2 (Fig. 4a). SNAT2 and SNAT4 were induced by hypertonicity (DMEM with 200 mM sorbitol), while SNAT1 showed little change (Fig. 4b). To investigate the effects of amino acids on the SNAT2 induction, TR-TBT 18d-1 cells were cultured under the hypertonic condition in the presence of taurine, betaine, MeAIB and arginine, but little effect on SNAT2 mRNA was observed (Supplementary Fig. S1).

To verify that SNAT2 expression is induced by hypertonicity in TR-TBT 18d-1 cells, protein expression of SNAT2 in cell lysate and membrane fraction was analyzed by Western blotting and immunocytochemistry. Rat SNAT2 induced by hypertonic treatment showed a band of similar size to hSNAT2 (Fig. 5a). Densitometric analysis of the 60–75 kDa region indicated that SNAT2 protein expression in cell lysate and cell membrane was increased 5.4-fold and 35-fold, respectively, under the hypertonic condition (Fig. 5b). The fraction of SNAT2 localized on cell membrane was evaluated as relative protein expression in cell membrane to that in lysate, and under the hypertonic condition this fraction was 6.3-fold higher than that under isotonic conditions (Fig. 5c). Human and rat SNAT2 bands were expressed in a slightly higher molecular weight area than would be expected from the amino acid sequence, in agreement with previous reports [17,18]. SNAT2 was localized intracellularly in TR-TBT 18d-1 cells under isotonic conditions (Fig. 6a). However, expression of SNAT2 was markedly induced by hypertonicity, and in the induced cells, SNAT2 staining was observed at the cell membrane (Fig. 6b).

4. Discussion

Betaine transport across the plasma membrane is induced in response to hypertonic stress in renal and placental cells [9,19], and BGT-1 is the best-characterized betaine transporter. Betaine uptake is also mediated by system A [9–11], but the subtype(s) involved was not established. The present results show that SNAT2 transports betaine, while the other two subtypes, SNAT1 and SNAT4, do not (Fig. 1a, c). Both human and rat SNAT2 showed betaine uptake activity and there was no marked difference in their kinetic characteristics (Fig. 2). These results suggest that betaine uptake by SNAT2 is well conserved, at least in rat and human, and SNAT2 has a physiological role in regulating betaine concentration, at least in certain types of cells.

The substrate specificity of all system A subtypes has been believed to be similar. For instance, alanine, asparagine, serine and MeAIB are common substrates [12]. This similarity has hindered characterization of the roles of individual system A subtypes in cases where plural subtypes are expressed in the same tissue. However, among system A subtypes, only SNAT4 transports cationic amino acids and therefore L-arginine-inhibitable MeAIB uptake represents SNAT4 function

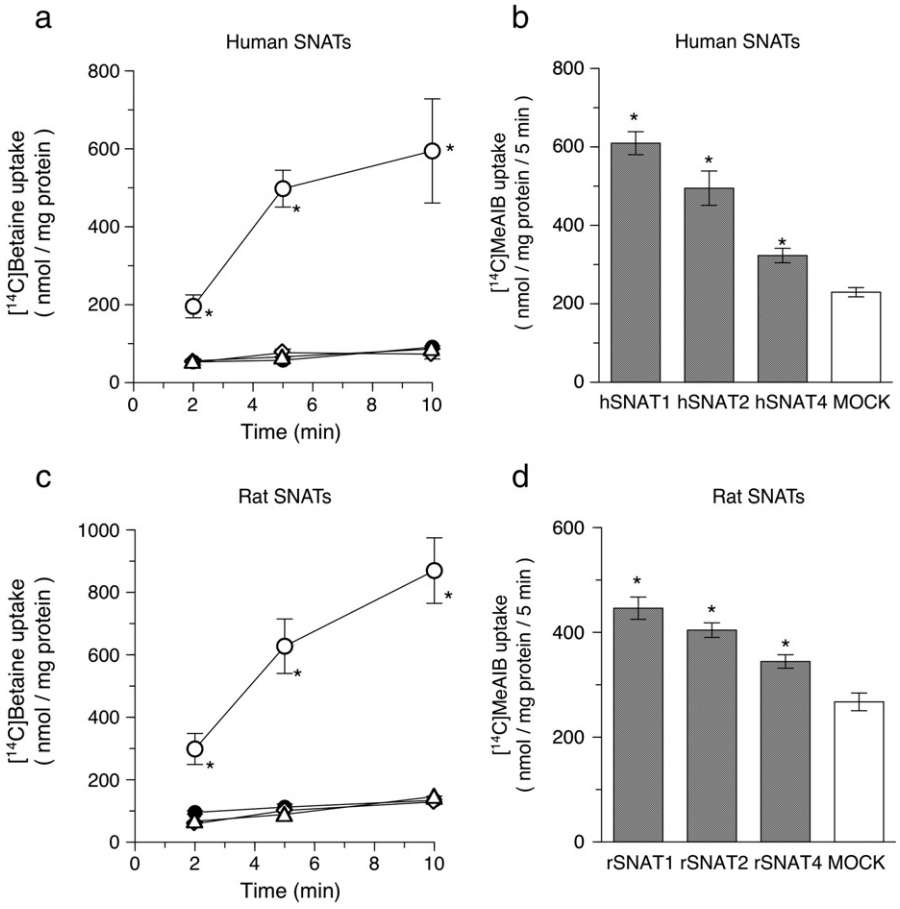

Fig. 1. Identification of system A subtype(s) involved in betaine uptake. HEK293 cells were transiently transfected with a plasmid vector containing human (a,b) or rat (c,d) SNAT1 (●), SNAT2 (○) or SNAT4 (◊) cDNA and with vector alone as a negative control (MOCK, △). [^{14}C]Betaine (4.5 μM) uptake was measured at 37 °C for 10 min (a,c). [^{14}C]MeAIB (8.5 μM) uptake was measured as a positive control (b,d). Data are presented as mean ± S.E.M. (n = 3–4). Asterisk (*) indicates statistical significance with a p value of less than 0.05 when compared to the uptake by MOCK cells.

[17,20–24]. Our present results (Fig. 3) show that betaine uptake is specific to SNAT2, and can therefore be used to distinguish this subtype from the others. Thus, the individual functions of the system A subtypes can be distinguished by using betaine and L-arginine as inhibitors.

The Michaelis constants of human and rat SNAT2 for betaine (approximately 5 mM; Fig. 2) are much higher than the reported values for BGT-1 (200–400 μM) and SIT1 (0.2 mM) [7,25]. Considering that the plasma betaine concentration is around 35–50 μM in humans and 100–200 μM in rats [26–28], the transport affinity of SNAT2 for betaine seems low. However, we consider that it would be physiologically relevant, since low affinity compared with the physiological concentration means that the transport would not easily become saturated under conditions where the betaine concentration varies substantially. Further, the capacity of SNAT2 would be partly taken up by other substrates due to its multiple substrate recognition characteristic. Initial uptake clearance of betaine by rat SNAT2 was at least greater than that of MeAIB.

Amino acid transport system A is known to be induced by amino acid depletion. Induction of SNAT2 expression by amino acid depletion is mitigated by the presence of taurine, betaine or MeAIB via ATF4 transcriptional factor [29]. To characterize the hypertonicity-induced SNAT2 expression in placental trophoblasts, we measured the effect of these amino acids on the SNAT2 induction. However, these amino acids did not inhibit SNAT2 induction in the concentration range up to 1000 μM (Supplementary Fig. S1). These results indicated that the mechanism of SNAT2 induction by the hypertonic condition is different from that of the induction by amino acid depletion.

SNAT2 was induced at both the mRNA and protein levels by hypertonicity in TR-TBT 18d-1 cells, but the induction of SNAT2 protein was much greater than would be expected from the mRNA induction (Figs. 4b, 5b). Functional upregulation of SNAT2 would be consistent with a previous report that showed enhancement of MeAIB-sensitive [^{14}C]betaine uptake in TR-TBT 18d-1 cells under hypertonic conditions [9]. Hypertonicity activates TonEBP, which enhances target gene expression by binding to TonE sites in the 5′-flanking region from the transcriptional start point. Mice lacking TonEBP showed normal growth at embryonic day 14.5, but 50% of the mice died before birth [30], indicating that osmoregulation is important for normal growth, especially in the late stage of gestation. Although BGT-1 has at least two TonE sites, which may contribute to the induction of BGT-1 by hypertonicity [31,32], there do not appear to be any TonE sites located in the upstream 1 kbp region from the transcription start site of human and rat SNAT2. Nevertheless, SNAT2 was more inducible than BGT-1 by hypertonicity in placental trophoblasts [9]. The mechanism of SNAT2 induction by hypertonicity remains uncertain. The effect of TonEBP on osmolarity-inducible SNAT2 expression should be clarified in the future.

SNAT2 was expressed even under isotonic conditions, and appeared to be localized primarily in an intracellular compartment (Fig. 6a). However, when SNAT2 was induced under hypertonic conditions, it was localized on the cell membrane, as well as intracellularly (Figs. 5, 6b). These results indicate that SNAT2 was regulated by tonicity at both the post-transcriptional and transcriptional levels. These observations are consistent at least in part with the previous report that SNAT2 is expressed in the intracellular compartment, as well as the cell membrane in fibroblasts and the microvillous membrane, in

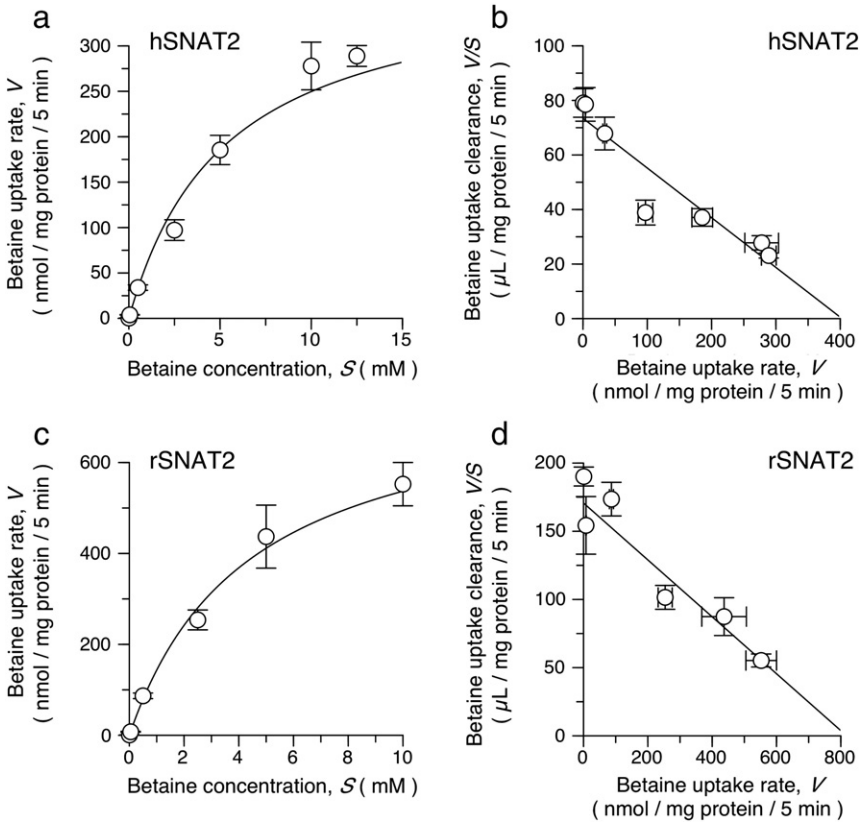

Fig. 2. Kinetics of betaine transport by human and rat SNAT2. HEK293 cells were transiently transfected with plasmid vector containing human (a,b) or rat (c,d) SNAT2 cDNA, or with vector alone. Uptake of [^{14}C]betaine (4.5 μM) was examined in the presence of unlabeled betaine at concentrations up to 12.5 mM. Betaine uptake via hSNAT2 or rSNAT2 was calculated by subtracting the uptake in vector alone-transfected HEK293 cells from that in HEK293 cells expressing human or rat SNAT2. The hSNAT2 and rSNAT2-mediated betaine uptakes were analyzed on the basis of Michaelis–Menten type kinetics (a,c) and with Eadie–Hofstee plots (b,d). Data are presented as mean ± S.E.M. (n = 4).

syncytiotrophoblasts of human placenta [17,33]. SNAT2 protein synthesis mainly contributes to the hypertonicity-induced functional expression of SNAT2 in fibroblasts [33]. In our study, protein synthesis and

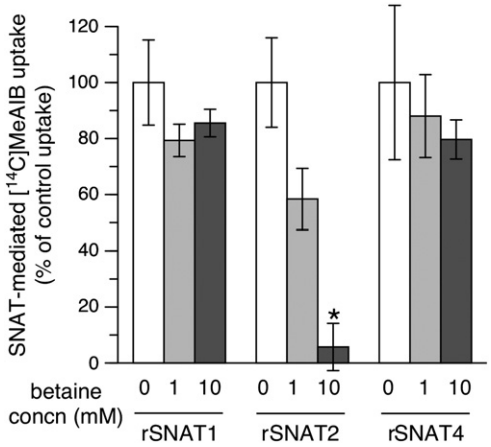

Fig. 3. Inhibitory effect of betaine on rat SNAT-mediated uptake of MeAIB. HEK293 cells were transiently transfected with plasmid vector containing rat SNAT1, SNAT2 or SNAT4 cDNA, or with vector alone. Uptake of [^{14}C]MeAIB (8.5 μM) was examined in the presence of unlabeled betaine at concentrations up to 10 mM. [^{14}C]MeAIB uptake via rSNAT2 was calculated by subtracting the uptake in HEK293 cells transfected with the vector alone from that in HEK293 cells expressing rat SNAT2. rSNAT-mediated [^{14}C]MeAIB uptake was normalized by the corresponding uptake in the absence of unlabeled betaine (control uptake). Data are presented as mean ± S.E.M. (n = 4). Asterisk (*) indicates statistical significance with a p value of less than 0.05 when compared to control uptake (open column), using ANOVA with Dunnett's post-hoc test.

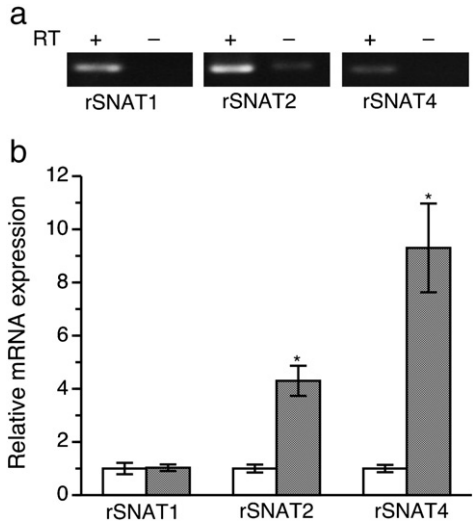

Fig. 4. Expression of system A subtypes and their response to hypertonicity in TR-TBT 18d-1 cells. Expression of rat system A subtypes (SNAT1, SNAT2 and SNAT4) was measured in TR-TBT 18d-1 cells cultured under isotonic conditions (a). PCR products with reverse transcription (RT) and without RT (negative control) were analyzed by electrophoresis. Each PCR product showed a single band, and no significant band of PCR product was observed without RT. The effect of hypertonic culture on expression of rat system A subtypes in TR-TBT 18d-1 cells was also examined (b). Quantitative RT-PCR was performed using TR-TBT 18d-1 cells incubated for 8 h under isotonic (300 mOsm/kg, open column) and hypertonic (500 mOsm/kg, closed column) conditions. Data are presented as mean ± S.E.M. (n = 3–4). Asterisk (*) indicates statistical significance with a p value of less than 0.05 when compared to the corresponding expression in isotonic culture (open column).

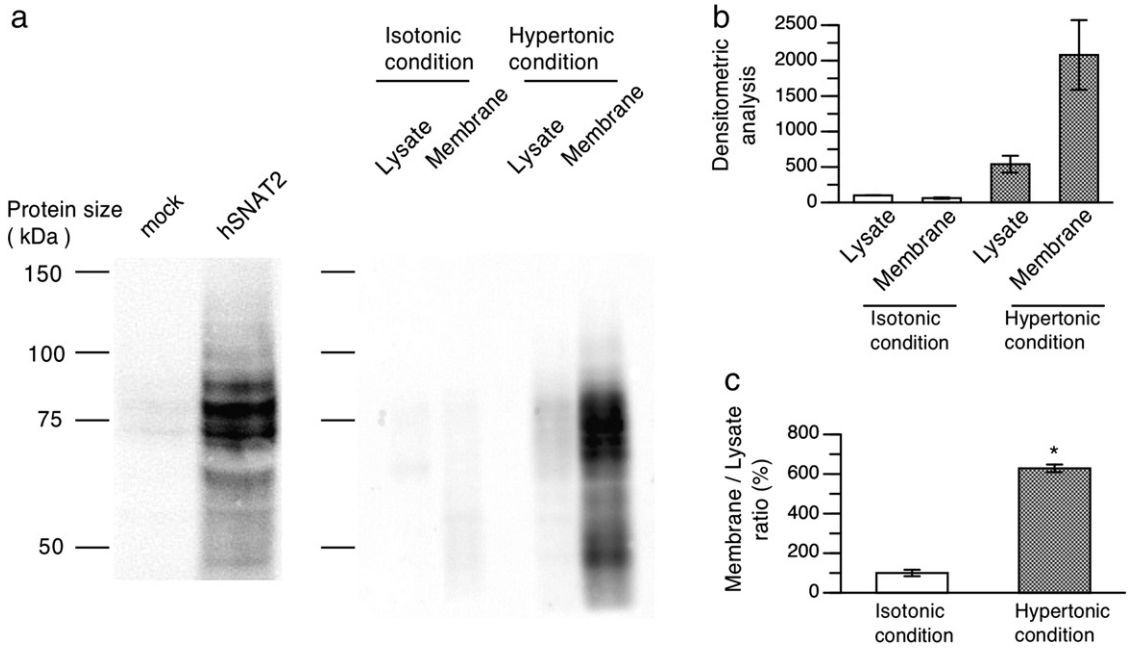

Fig. 5. Upregulation of rat SNAT2 protein by hypertonicity in TR-TBT 18d-1 cells. TR-TBT 18d-1 cells were incubated at 37 °C in isotonic or hypertonic medium for 8 h. Rat SNAT2 expression in total cell lysate and plasma membrane fraction was analyzed by Western blotting. 10 μg protein of cell lysate or membrane fraction was applied in each lane. Transient expression of human SNAT2 in Cos-7 cells and representative results of detection of rat SNAT2 in TR-TBT 18d-1 cells are shown in (a) and band intensities in 60–75 kDa of rat SNAT2 quantified by densitometry are shown in (b). Data are presented mean ± S.E.M. (n = 3). Asterisk (*) indicates statistical significance with a p value of less than 0.05.

localizational change of SNAT2 almost equally contributed to the hypertonicity-induced plasma membrane expression of SNAT2 (Fig. 5), indicating that contributions of protein synthesis and translocation to plasma membrane expression depend on cell types. Post-transcriptional regulation of SNAT2 has been investigated. SNAT2 is stored in the trans-Golgi network in 3T3-L1 adipocytes, and is released to the plasma membrane upon stimulation with insulin [34]. Inhibition of mTOR decreased microvillous membrane expression of SNAT2 [35,36]. Thus, the function of SNAT2 is dynamically regulated both post-transcriptionally and transcriptionally.

The existence of post-transcriptional regulation of SNAT2 protein localization (Fig. 6) implies that the uptake activity of SNAT2 would be rapidly increased in response to hypertonicity. As regards the transcriptional regulation, the induction of SNAT2 mRNA reached the peak within 3–6 h, and then declined almost to the original level within 24 h in endothelial cells treated with hypertonic medium adjusted to 0.5 osmol/kg [37]. Expression of TauT and BGT-1 is induced more slowly, peaking at 17 and 24 h, respectively [38,39], possibly to compensate for the decrease of system A after its transient induction. Therefore, SNAT2 may have a role in acute osmolyte regulation in response to extracellular osmotic changes.

In conclusion, our results indicate that only SNAT2 among the system A subtypes has betaine transport activity. The involvement of SNAT2 in betaine uptake by the placental trophoblasts may contribute to cytoprotection in response to osmotic stress arising from differences in osmolality between the maternal and fetal circulations. The rapid induction and high capacity of SNAT2 for betaine transport may be important in ensuring a robust response to acute osmotic changes.

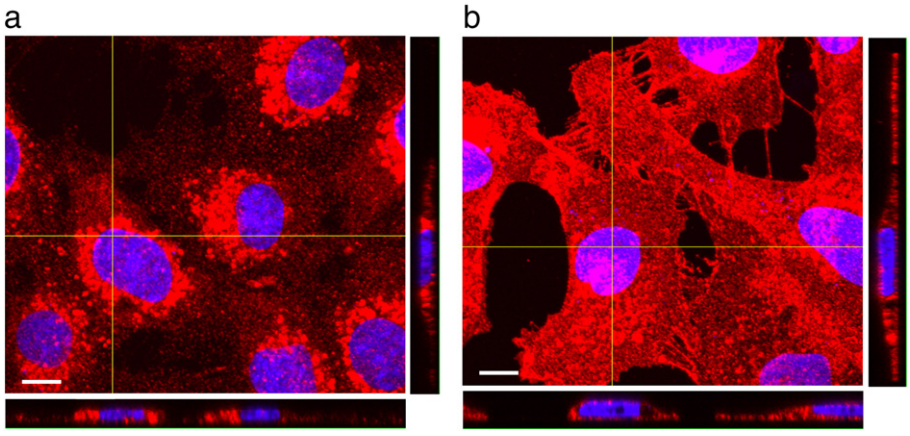

Fig. 6. Immunocytochemical analysis of upregulation of SNAT2 induced by exposure of TR-TBT 18d-1 cells to hypertonicity. Immunostaining of rat SNAT2 (red) and nuclear staining (blue) by DAPI (4′,6-diamidino-2-phenylindole, blue) in rat placental TR-TBT 18d-1 cells are shown. TR-TBT 18d-1 cells were incubated at 37 °C for 8 h in isotonic (a, 300 mOsm/kg) or hypertonic (b, 500 mOsm/kg) medium. Fixed cells were incubated with primary antibody (anti-SLC38A2 rabbit polyclonal IgG) diluted to 3% in blocking buffer for 2 h at room temperature and then incubated with Alexa594-conjugated secondary antibody for 1 h at room temperature. Images were acquired using confocal laser scanning microscopy. Cross-sectional views along the lines indicated are presented below and on the right of the confocal image. Wavelengths for excitation and emission were 559 nm and 618 nm, respectively. Magnification ×600. Bars = 10 μm.

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