Amino Acids (2009) 36:219–230
DOI 10.1007/s00726-008-0050-9

ORIGINAL ARTICLE

Regulatory mechanisms of SNAT2, an amino acid transporter, in L6 rat skeletal muscle cells by insulin, osmotic shock and amino acid deprivation

Hitoshi Kashiwagi · Kojiro Yamazaki · Yoh Takekuma · Vadivel Ganapathy · Mitsuru Sugawara

Received: 25 July 2007 / Accepted: 15 February 2008 / Published online: 11 March 2008

Abstract Several studies have demonstrated that the activity of system A is upregulated by insulin, osmotic shock and amino acid deprivation. However, the mechanisms are not clear. We carried out studies using L6 rat skeletal muscle cells to clarify the mechanisms of upregulation of system A activity by insulin, osmotic shock and amino acid deprivation. The upregulation was found to be due to an increase in V_{max}, not K_m. Chloroquine and wortmannin inhibited the upregulation induced by insulin stimulation and amino acid deprivation but not that induced by osmotic shock. On the other hand, cycloheximide and actinomycin D inhibited the upregulation by each stimulation. Moreover, PD98059 and SP600125 inhibited only amino acid deprivation-induced upregulation and SB202190 inhibited only insulin-induced upregulation. Our findings indicate that the mechanisms of upregulation of system A activity by insulin, osmotic shock and amino acid deprivation are different in L6 cells. Western blot and RT-PCR analysis showed an increase in system A at the protein and mRNA levels with each stimulation.

Keywords SNAT2 · Insulin stimulation · Osmotic shock · Amino acid deprivation · Transporter recruitment · de novo protein synthesis

H. Kashiwagi · K. Yamazaki · Y. Takekuma · M. Sugawara (✉)
Department of Pharmacy, Hokkaido University Hospital, Sapporo, Hokkaido, Japan
e-mail: msuga@med.hokudai.ac.jp

V. Ganapathy
Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA, USA

Abbreviations

SNAT2	Sodium-coupled neutral amino acid transporter 2
Me-AIB	α-Methylaminoisobutyric acid
MAPK	Mitogen-activated protein kinase
ERK	Extracellular regulated kinase
JNK	Jun N-terminal kinase
MDCK	Madin–Darby canine kidney
CHO	Chinese hamster ovary
PI_3K	Phosphatidylinositol 3-kinase
MEM	Minimum essential medium Eagle
FBS	Fetal bovine serum
SDS	Sodium dodecyl sulfate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

Introduction

System A is an amino acid transport system that mediates the Na^+-dependent transport of short-chain neutral amino acids such as glycine and alanine (Oxender and Christensen 1963a, b) and is widely expressed in most mammalian cells (Hatanaka et al. 2000; Sugawara et al. 2000a; Yao et al. 2000). α-Methylaminoisobutyric acid (Me-AIB) is thought to be a specific substrate for this transport system (Christensen et al. 1965). Therefore, in practice, the activity of this transport system is routinely monitored as Na^+-dependent Me-AIB uptake. However, recently, three subtypes of system A transporter have been cloned (Sugawara et al. 2000a, b; Yao et al. 2000; Varoqui et al. 2000). They were named sodium-coupled neutral amino acid transporter [SNAT, also known as amino acid transporter A (ATA)] 1, 2 and 4 (ATA 1, 2 and 3). After that, investigations about characteristics of this transporter including the study in molecular level in detail became available.

Many studies have demonstrated that system A activity is subject to upregulation by hyperosmotic shock (Horio et al. 1997; Alfieri et al. 2001; Franchi-Gazzola et al. 2004) and amino acid deprivation (Gazzola et al., 2001; Ling et al. 2001; Palii et al. 2004; Iresjo et al. 2005). It is known that osmotic shock activates mitogen-activated protein kinase (MAPK) cascades. For instance, extracellular regulated kinase (ERK), the first MAPK characterized as a transducer of mitogenic signals, was activated markedly in rat pheochromocytoma (PC12) cells by osmotic shock (Matsuda et al. 1995). Although at least three major distinct MAPK cascades (ERK, JNK and p38) are known in mammalian cells, it is believed that some stimulus can activate more than one cascade simultaneously. ERK has recently been shown to be a component of the adaptive response of system A to amino acid deprivation, and PD98059, a specific inhibitor of ERK activation, suppressed this adaptive increase of system A activity (Franchi-Gazzola et al. 1999). Furthermore, it has been shown that Jun N-terminal kinase (JNK), originally characterized by its involvement in stress responses, was necessary for the amino acid deprivation-regulated response of system A (Lopez-Fontanals et al. 2003). It is known that SP600125 is a specific inhibitor of JNK. On the other hand, p38 MAP kinase activity was essential for osmotic induction of the betaine transporter gene in Madin–Darby canine kidney (MDCK) cells (Sheikh-Hamad et al. 1998). p38 is known to be blocked by SB202190. In a previous study (Lopez-Fontanals et al. 2003), it was found that osmotic shock had no effect on SNAT2 mRNA level but that amino acid deprivation induced an increase in mRNA level in Chinese hamster ovary (CHO) cells. It was shown in that study that specific inhibition of both ERK (PD98059) and JNK (SP600125) had no effect on the response of system A to osmotic shock but that amino acid-regulated response was blocked. It was also shown that SB202190, a specific inhibitor of p38, had no effect on the relative response to amino acid deprivation but reduced the osmoregulatory response.

On the other hand, system A activity is also stimulated by hormones such as insulin (Kletzien et al. 1976; Su et al. 1998; McDowell et al. 1998) and glucagon (Cariappa and Kilberg 1990; Mailliard et al. 1994). It is thought that insulin induces an increase in the plasma membrane abundance of SNAT2, a subtype of system A that is the product of *SLC38A2* gene (Mackenzie and Erickson 2004) and that this additional SNAT2 is recruited from an intracellular compartment. Furthermore, it has been shown that phosphatidylinositol 3-kinase (PI_3K) is involved in the signaling pathway in transporter recruitment after insulin stimulation in rat skeletal muscle (L6) cells (Hyde et al. 2002). It is also thought that this mechanism, transporter recruitment, can respond quickly after stimulation. There is another mechanism involving de novo synthesis of new transporter protein associated with the stimulation of system A activity, although the response via this mechanism is much slower than that by recruitment.

Although the regulation of SNAT2 expression by insulin, osmotic shock, and amino acid deprivation and the underlying mechanisms have been examined in previous studies, most of those studies were carried out using different cell types, making it difficult to compare the signaling pathways involved in each of these manipulations. It would thus be advantageous to investigate the regulation of SNAT2 by all three manipulations (i.e., insulin, osmotic shock, and amino acid deprivation) using a single cell type. Therefore, the aim of the present study was to investigate the mechanisms of regulation of SNAT2 by comparing the effects of inhibitors of different signaling pathways on the upregulation of system A induced by insulin, osmotic shock, and amino acid deprivation using a rat skeletal muscle cell line (L6). We selected this cell line for our studies because of the well-recognized role of system A in the supply of amino acids to skeletal muscle for metabolism. Furthermore, the regulation of system A by insulin and amino acid deprivation is of physiologic relevance. Therefore, elucidation of the signaling pathways involved in the regulation of this important amino acid transport system in skeletal muscle will have profound implications on the biology of this tissue.

Materials and methods

Materials

L6 rat skeletal muscle cells (CRL-1458) were obtained from American Type Culture Collection (Manassas, VA, USA). Minimum essential medium Eagle (MEM) and penicillin–streptomycin were purchased from SIGMA (St Louis, MO, USA). Fetal bovine serum (FBS) and human insulin were obtained from MP Biochemicals (Solon, OH, USA) and Eli Lilly Japan (Kobe, Japan), respectively. Me-AIB, chloroquine and actinomycin D were obtained from SIGMA. Cycloheximide and wortmannin were obtained from Wako Pure Chemical Industries (Osaka, Japan). PD98059, SB202190 and SP600125 were obtained from EMD Biosciences (San Diego, CA, USA). Anti-peptide antibodies specific for rat SNAT2 (Ling et al. 2001) and mouse α-tubulin (used to detect α-tubulin from many organisms) obtained from SIGMA were used for Western blot analysis of SNAT2 and α-tubulin expression, respectively. [^{14}C] Me-AIB (50.50 mCi/mmol) was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA). Reverse transcriptase (ReverTra Ace) and Taq polymerase were purchased from TOYOBO (Osaka, Japan).

Cell culture

L6 cells were cultured in MEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 1 mM sodium pyruvate at 37°C in an atmosphere of 5% CO_2 and 95% air. The cells were cultured for 3 days and used at confluence between the 38th and the 67th passages. For the transport experiments, cells were seeded in 2-cm^2 wells of disposable 24-well trays (Greiner Japan, Tokyo, Japan) and incubated for 4 days in 1 mL of growth medium.

For insulin stimulation, cells were incubated in serum-free MEM containing 100 U/mL penicillin, 100 μg/mL streptomycin and 1 mM sodium pyruvate for 24 h, and then 20 mU/mL (172 nM) insulin was added to the medium. To generate osmotic shock, cells were incubated in hyperosmotic MEM, obtained by adding 200 mM sucrose to the growth medium. For the study of amino acid deprivation, cells were incubated in an amino acid-free medium that basically consisted of inorganic salts of regular MEM plus 5.6 mM glucose and 0.03 mM phenol red.

Control values were obtained from experiments without each stimulation, that is, in the experiment on insulin stimulation, cells were incubated in serum-free MEM for 24 h and then incubated for 8 h after changing the medium to the same serum-free medium without insulin. In the experiment on osmotic shock, cells were incubated in growth medium (without sucrose) for 8 h. In the study on amino acid deprivation, cells were incubated in a medium that basically consisted of amino acid and inorganic salts of regular MEM plus 5.6 mM glucose and 0.03 mM phenol red.

Cells were stimulated by insulin, osmotic shock and amino acid deprivation before determining the transport activity of system A and the changes in expression levels of SNAT2 protein and mRNA, as indicated below. Inhibitors of transporter recruitment (chloroquine, 10, 20, 40 μM) (Sibille et al. 1989; Puertollano and Alonso 1999), translation (cycloheximide, 1, 2, 4 μM), transcription (actinomycin D, 1, 2, 4 μM), PI_3K (wortmannin, 50, 100, 200 nM), ERK (PD98059, 2.5, 5, 10 μM), p38 (SB202190, 0.25, 0.5, 1 μM) and JNK (SP600125, 2.5, 5, 10 μM) were added 2 h before starting each challenge with insulin stimulation, osmotic shock and amino acid deprivation, which were then maintained for 8 h.

Measurement of system A activity

Transport activity of system A was determined by measuring the initial (15 min) uptake of [^{14}C]Me-AIB in a transport buffer (25 mM Tris-HEPES, pH 7.5, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose) at room temperature. The substrate concentration was 15.8 μM containing an activity of 0.2 μCi/250 μL. Under these conditions, Me-AIB uptake is linear for more than 20 min (data not shown). Uptake of Me-AIB was terminated by aspirating the radioactive buffer, followed by two successive washes with ice-cold transport buffer. Cells were lysed in 0.2 N NaOH containing 1% sodium dodecyl sulfate (SDS), and the associated radioactivity was determined by liquid scintillation counting.

For kinetic analysis of Me-AIB uptake, a range of Me-AIB concentration from 0.05–2 mM was used. Transport data were fitted to the following equation:

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

Non-saturable components were determined from the uptake of [^{14}C]Me-AIB in the presence of 10 mM alanine, a good substrate for system A, to inhibit system A-mediated uptake of [^{14}C]Me-AIB.

Western blot analysis

Cells stimulated by insulin, osmotic shock and amino acid deprivation were homogenized in a buffer (1 mM Na_2HPO_4, 1 mM KH_2PO_4) containing protease inhibitor cocktail tablets (complete, Mini) (Roche, Penzberg, Germany) using a supersonic homogenizer, and a portion of total cell lysate was kept for Western blot analysis and the rest was centrifuged at 500×g for 10 min at 4°C. The supernatant was transferred to a fresh tube and centrifuged at 14,000×g for 20 min at 4°C. The pellet was resuspended in the same buffer as that used for Western blot analysis as crude membrane.

For Western blot analysis, 20 μg protein of total cell lysate and of crude membrane were used for SDS-PAGE. The two fractions were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred on to a nitrocellulose membrane (Bio-Rad, Richmond, CA, USA). Nonspecific binding was blocked by incubation for 1 h at room temperature in blocking solution [Tris-buffered saline (TBS; 20 mM Tris–HCl, pH 7.5, 500 mM NaCl) containing 5% skim milk or 0.2% Blotting Grade Blocker (Bio-Rad), respectively]. The blot of total cell lysate was then incubated overnight at room temperature with an antibody against mouse α-tubulin at the concentration of 2 μg/mL in antibody buffer [T-TBS (0.1% Tween 20 in TBS) containing 5% skim milk]. On the other hand, the blot of crude membrane was incubated overnight at room temperature with an antibody against rat SNAT2 at the concentration of 1 μg/mL in antibody buffer[T-TBS (0.1% Tween 20 in TBS) containing 0.2% Blotting Grade Blocker]. After washing, the blots were exposed to

peroxidase-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) diluted 1:1,000 or alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, catalog number 170-5014) diluted 1:3,000 in antibody buffer for 2 h. Then the blots were washed and signals were detected using an ECL Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA, catalog number RPN2109) or Immun-Star chemiluminescent detection kit (Bio-Rad, catalog number 170-5014). The blots were then exposed to a high-performance chemiluminescence film (GE Healthcare Bio-Sciences) for 10 min at room temperature. Band intensity was quantified using Luminous Imager Ver. 2.0 (Aisin Cosmos R&D Co., Aichi, Japan) and normalized with that of α-tubulin.

RT-PCR analysis

Total RNA was extracted from cultured cells using the TRIzol reagent from Invitrogen (Carlsbad, CA, USA). RT-PCR reaction was performed in a PCR Thermal Cycler (Dice TP600, TaKaRa, Tokyo, Japan). One microgram of total RNA, primed by oligo-dT, was reverse-transcribed for 1 h at 42°C and then heated for 5 min at 99°C. The PCR program was for 2 min at 94°C, followed by 30 s at 94°C, 30 s at 57°C and 1 min at 72°C for 27 cycles (rSNAT2, forward primer: 5′-AACTACTCATACCCCACGAAG-3′; reverse primer: 5′-AAAGGTGCCATTCACCGTTTC-3′), 35 cycles (rSNAT1, forward primer: 5′-ACGACTCTA ATGACTTCACGG-3′; reverse primer: 5′-CTTACTGTT GAGTTCTGTTCC-3′ and rSNAT4, forward primer: 5′-AGCATTCAAAGCTGCTACACC-3′; reverse primer: 5′-GAGTGTAGTCCACCATGAAGT-3′) or 22 cycles (rGAPDH, forward primer: 5′-CCATCACCATCTTCCA GGAG-3′; reverse primer: 5′-CCTGCTTCACCACCTTCT TG-3′), and for 2 min at 72°C. The RT-PCR products were subjected to 0.9% agarose gel electrophoresis and stained with ethidium bromide. For a semiquantitative purpose, we measured the expression of glyceraldehide-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. Fluorescence intensity was measured by Luminous Imager Ver. 2.0 and normalized with that of GAPDH.

Data analysis

The results are expressed as means with SE ($n = 3$ or 4). Statistical differences were assessed by Student's t test or the Cochran–Cox test after an F test for equal variance. Data were considered statistically significant at P values <0.05, <0.01 or <0.001. In Western blot and RT-PCR analysis, three experiments were conducted, and representative results are shown.

Results

Upregulation of Me-AIB uptake by insulin stimulation, osmotic shock and amino acid deprivation

Figure 1 shows the time courses of the effects of insulin (Fig. 1a), osmotic shock (Fig. 1b) and amino acid deprivation (Fig. 1c) on uptake of Me-AIB into L6 cells. Me-AIB uptake was upregulated gradually by insulin stimulation (Fig. 1a) and by amino acid deprivation (Fig. 1c). On the other hand, Me-AIB uptake was upregulated by osmotic shock only after a substantial lag period (after 4 h) (Fig. 1b). It is obvious that the mechanism of upregulation of system A activity induced by insulin and amino acid deprivation are different from that induced by osmotic shock. These results suggest that there is a mechanism, such as transporter recruitment to the plasma membrane from an intracellular pool, for quick response after stimulation by insulin and amino acid deprivation. The stimulation with these short periods of treatment was not marked but was statistically significant.

Figure 2 shows the effects of insulin stimulation, osmotic shock and amino acid deprivation on the kinetic parameters of Me-AIB uptake. The kinetic parameters of Me-AIB uptake are shown in Table 1. The V_{max} value of Me-AIB uptake was increased by each stimulation compared to that without stimulation, but the K_m value was not changed. These findings suggest that upregulation of system A activity by insulin stimulation, osmotic shock and amino acid deprivation is due to an increase in the density of the transporter in the plasma membrane.

Mechanism of upregulation of system A activity

To clarify the mechanism of upregulation of system A activity, we investigated the effects of chloroquine, an inhibitor of recycling of membrane proteins, cycloheximide and actinomycin D, inhibitors of translation and transcription, respectively, wortmannin, an inhibitor of PI_3K, and PD98059, SB202190 and SP600125, inhibitors of MAP kinase ERK, p38 and JNK, respectively. Though the various inhibitors affected system A activity slightly under the condition of no stimulation (Figs. 3a, 4, 5a), we thought we could estimate only the effect of an inhibitor on upregulation of system A activity by each stimulation, by calculating the % stimulation for each column pair (stimulated divided by respective unstimulated × 100).

As shown in Fig. 3a, chloroquine, cycloheximide and actinomycin D inhibited insulin-stimulated Me-AIB uptake, suggesting that upregulation of system A activity by insulin is associated not only with a rapid recruitment of the transporter from an intracellular compartment to the plasma membrane but also with de novo synthesis of new

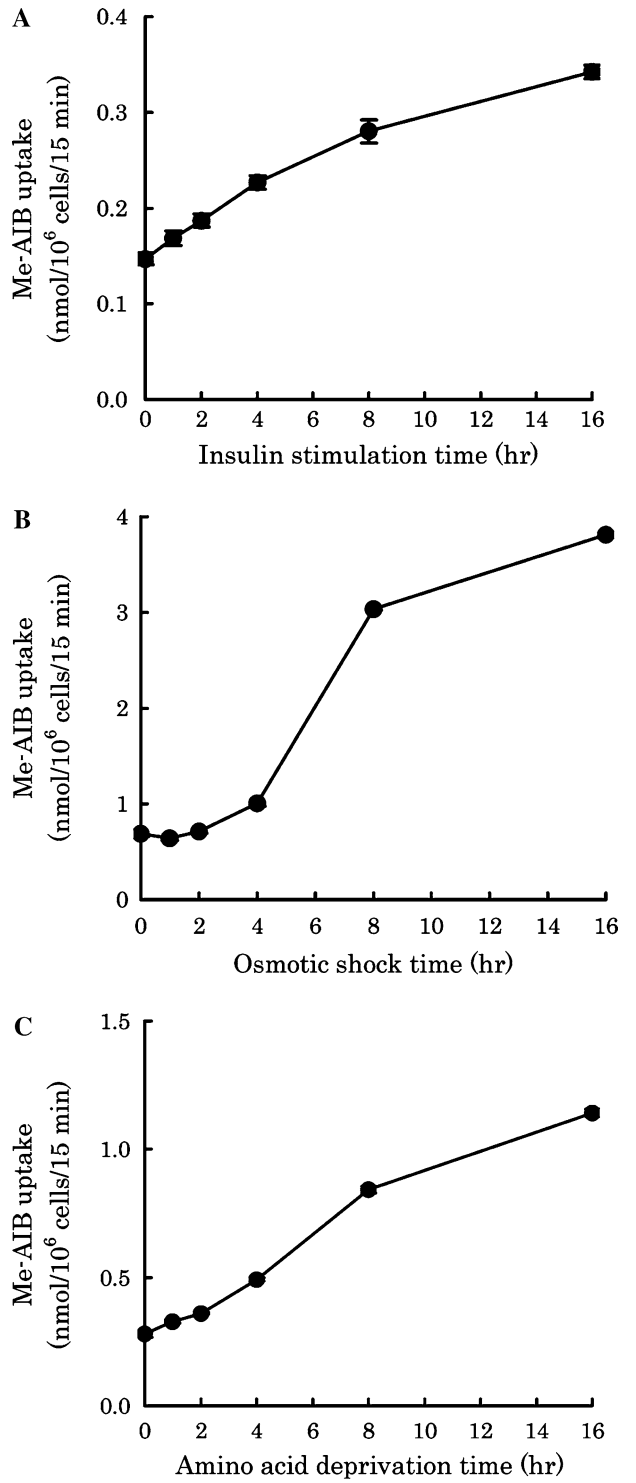

Fig. 1 Time courses of the effects of insulin (**a**), hyperosmotic shock (**b**) and amino acid deprivation (**c**) on Me-AIB uptake in L6 cells. After the respective stimulation, the cells were incubated with 15.8 μM [^{14}C]Me-AIB at room temperature for 15 min. Each value represents the mean ± SE of three or four measurements

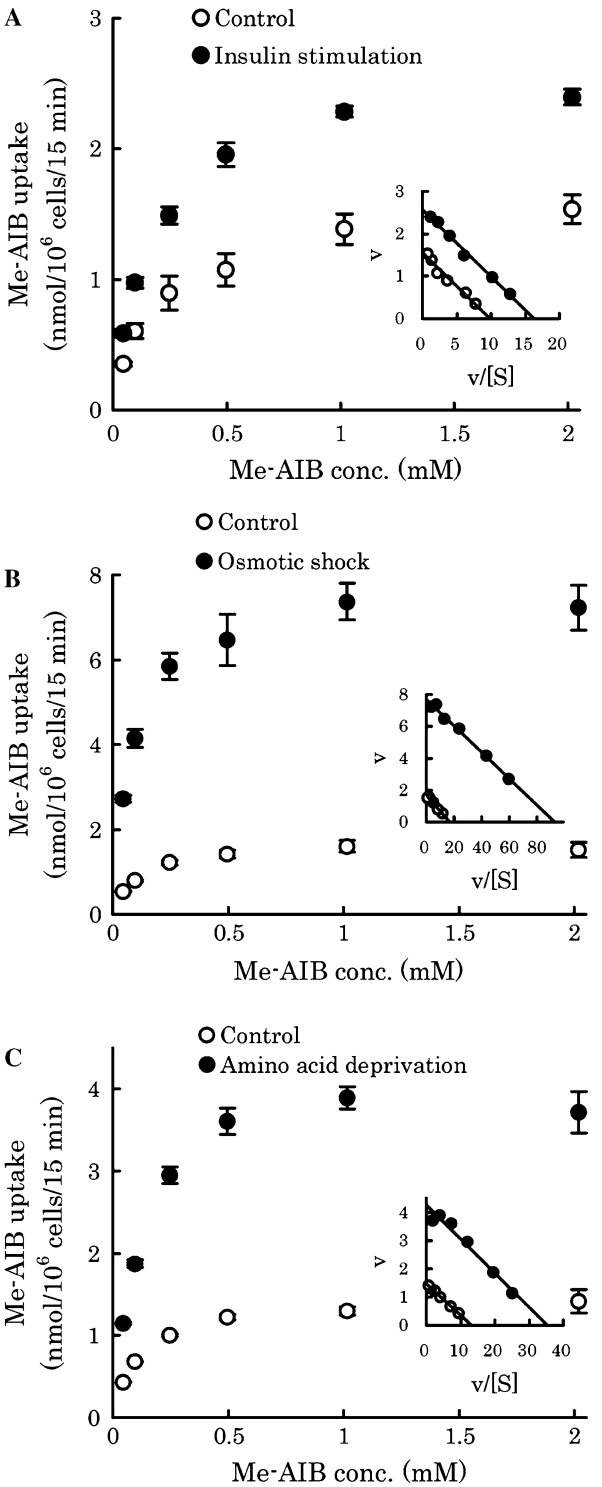

Fig. 2 Saturation kinetics of Me-AIB uptake in L6 cells. Transporter-mediated uptake was calculated by subtracting the uptake in the presence of 10 mM alanine. *Inset* Eadie-Hofstee plots of Me-AIB uptake vs. uptake rate (nmol/10^6 cells/15 min); [*S*], substrate concentration (mM). L6 cells were stimulated by insulin (**a**), hyperosmotic shock (**b**) and amino acid deprivation (**c**) at 37°C for 8 h. After the stimulation, the cells were incubated with Me-AIB (0.05–2 mM) at room temperature for 15 min. Each value represents the mean ± SE of three or four measurements

transporter mRNA and protein. Wortmannin, an inhibitor of PI_3K, which is involved in the signaling pathway in transporter recruitment, partially blocked the upregulated

Table 1 Kinetic parameters of Me-AIB uptake in L6 cells

	K_m (mM)		V_{max} (nmol/10^6cells/15 min)	
	Control	Stimulated	Control	Stimulated
Insulin stimulation	0.19 ± 0.03	0.17 ± 0.01	1.63 ± 0.08	2.62 ± 0.05
Osmotic shock	0.10 ± 0.01	0.08 ± 0.01	1.68 ± 0.05	7.69 ± 0.13
Amino acid deprivation	0.11 ± 0.01	0.11 ± 0.02	1.47 ± 0.02	4.18 ± 0.15

Each value represents the mean ± SD

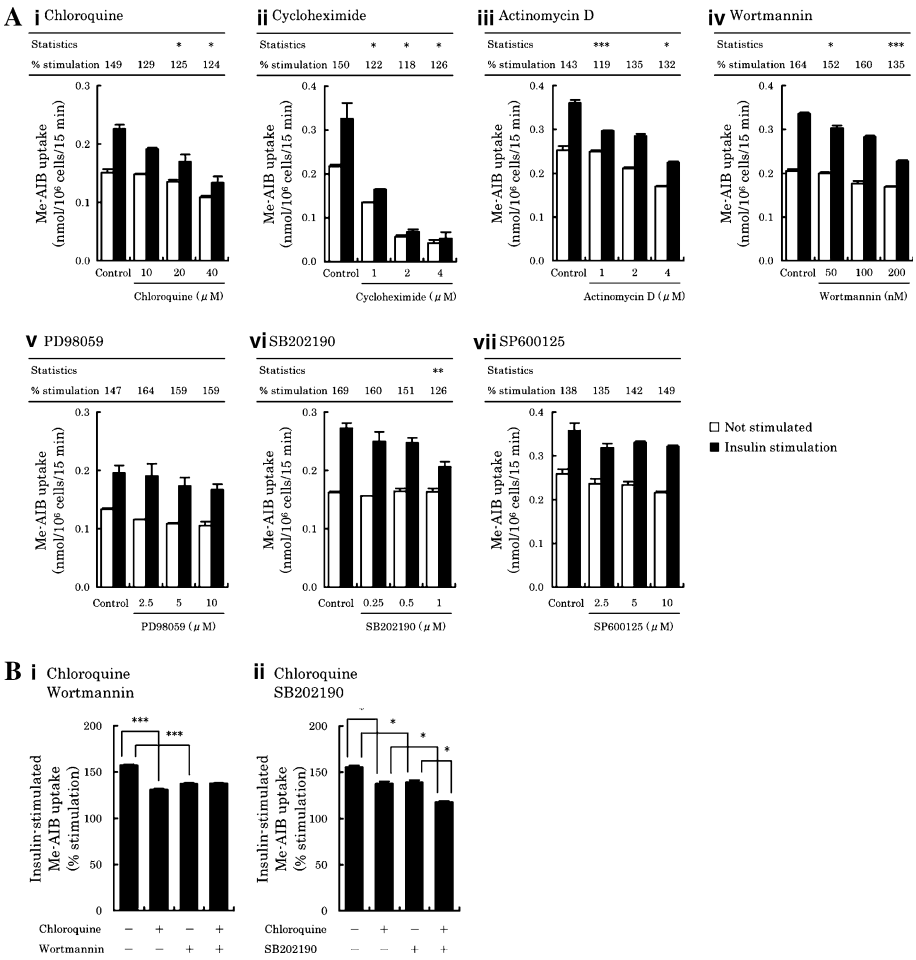

Fig. 3 Sensitivity of insulin-dependent system A stimulation to inhibitors in L6 cells. **a** Effects of inhibitors on up-regulation of system A activity. **b** Additivity of the effects of inhibitors on up-regulation of system A activity. Cells were preincubated with or without each inhibitor for 2 h at the concentrations indicated (in the experiment for which results are shown in Fig. 3b: 40 μM chloroquine, 200 nM wortmannin and 1 μM SB202190) and then stimulated by insulin at 37°C for 8 h. After the stimulation, the cells were incubated with 15.8 μM [^{14}C]Me-AIB at room temperature for 15 min. Insulin-stimulated uptake value (% stimulation) was calculated by dividing the uptake without stimulation into that with stimulation × 100. Each *column* represents the mean with SE of three measurements. Significant differences (in the case of results shown in Fig. 3a, from the control) are indicated by *asterisks*, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Me-AIB uptake induced by insulin stimulation. This indicates that PI_3K is involved in the regulation of system A by insulin stimulation. This result is similar to that obtained previously by Hyde et al. (2002). On the other hand, PD98059 and SP600125 had no effect on the response of system A activity to insulin, whereas SB202190 attenuated this response significantly. These results suggest that signaling pathways mediating the effects of insulin involve p38 but not ERK and JNK. Moreover, though the results showed that the effects of chloroquine and wortmannin on insulin stimulation were not additive, chloroquine and SB202190 in combination inhibited upregulated Me-AIB uptake by insulin stimulation more than what chloroquine or SB202190 did alone (Fig. 3b). These results suggest that PI_3K is involved in the signaling pathway in transporter recruitment but t p38 is not involved in SNAT2 trafficking after insulin stimulation; that is, upregulation of system A activity by insulin stimulation involves a mechanism of de novo protein synthesis in addition to transporter recruitment.

Figure 4 shows the effects of inhibitors on osmotic shock-stimulated Me-AIB uptake. Cycloheximide and actinomycin D caused a decrease in Me-AIB uptake, whereas chloroquine had no effect. It is therefore clear that, unlike insulin, osmotic shock stimulates system A activity only by signaling pathways involving de novo synthesis of transporter mRNA and protein without the participation of transporter recruitment from a pre-made intracellular pool. Wortmannin, PD98059, SB202190 and SP600125 showed no effect on the stimulatory effect of osmotic shock on

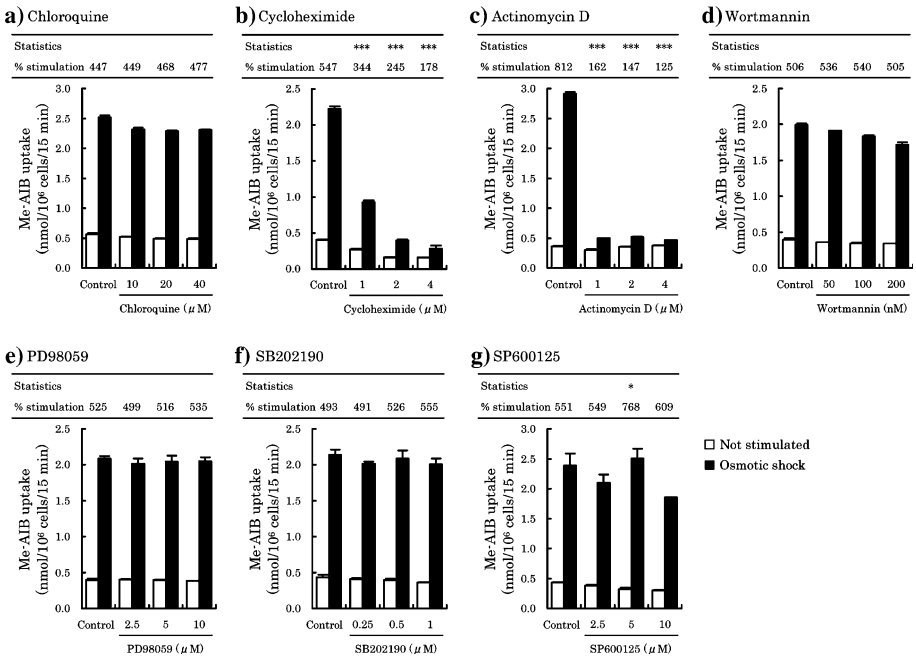

Fig. 4 Sensitivity of hyperosmotic shock-dependent system A stimulation to inhibitors in L6 cells. Cells were preincubated with or without each inhibitor for 2 h at the concentrations indicated and then stimulated by osmotic shock at 37°C for 8 h. After the stimulation, the cells were incubated with 15.8 μM [^{14}C]Me-AIB at room temperature for 15 min. Osmotic shock-stimulated uptake value (% stimulation) was calculated by dividing the uptake without stimulation into that with stimulation × 100. Each *column* represents the mean with SE of three measurements. Significant differences from the control are indicated by *asterisks*, * $P < 0.05$; *** $P < 0.001$

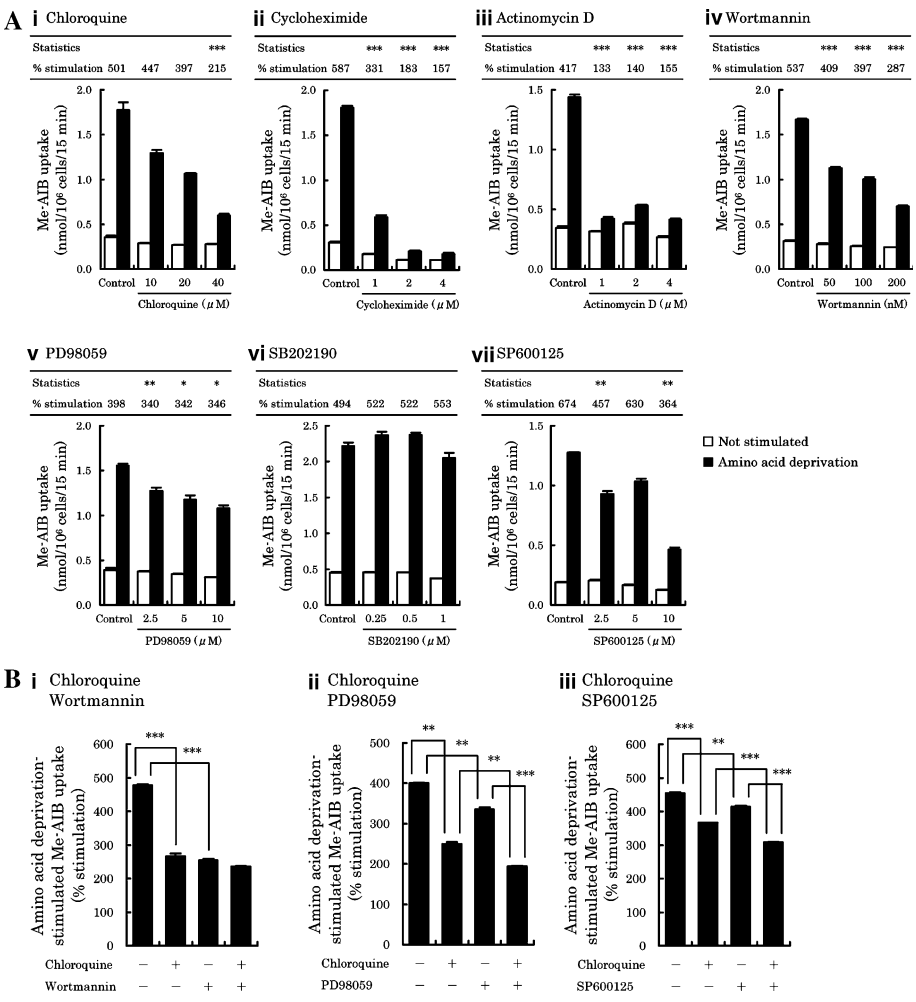

Fig. 5 Sensitivity of amino acid deprivation-dependent system A stimulation to inhibitors in L6 cells. **a** Effects of inhibitors on up-regulation of system A activity. **b** Additivity of the effects of inhibitors on up-regulation of system A activity. Cells were preincubated with or without each inhibitor for 2 h at the concentrations indicated (in the experiment for which results are shown in Fig. 5b: 40 μM chloroquine, 200 nM wortmannin, 10 μM PD98059 and 10 μM SP600125) and then stimulated by amino acid deprivation at 37°C for 8 h. After the stimulation, the cells were incubated with 15.8 μM [^{14}C]Me-AIB at room temperature for 15 min. Amino acid deprivation-stimulated uptake value (% stimulation) was calculated by dividing the uptake without stimulation into that with stimulation × 100. Each *column* represents the mean with SE of three measurements. Significant differences (in the case of results shown in Fig. 5a, from the control) are indicated by *asterisks*, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

system A activity. This indicates that the osmotic shock-induced de novo synthesis of the transporter protein does not involve PI_3K, ERK, p38 and JNK.

In the case of system A regulation by amino acid deprivation, the stimulation was blocked significantly by chloroquine, cycloheximide, and actinomycin D (Fig. 5a). This suggests the participation of recruitment of the transporter protein from a pre-made intracellular pool as well as de novo synthesis of the transporter mRNA and protein. Wortmannin inhibited amino acid deprivation-stimulated Me-AIB uptake, suggesting that PI_3K is involved in the regulation of system A by amino acid deprivation. These observations are similar to the observations with insulin. However, PD98059 and SP600125, which inhibit ERK and JNK, respectively, were able to attenuate the stimulatory effect of amino acid deprivation, whereas SB202190, an inhibitor of p38, did not have any effect. These results are different from those obtained for insulin, SB202190, but not PD98059 and SP600125, being able to block the effect. We conclude that ERK and JNK, but not p38, are involved in the regulation of system A by amino acid deprivation in L6 skeletal muscle cells. Interestingly, these results are similar to those obtained for CHO cells (Lopez-Fontanals et al. 2003). We also investigated whether the effects of two inhibitors, chloroquine and wortmannin, chloroquine and PD98059 or chloroquine and SP600125, were additive to upregulation of system A activity by amino acid deprivation. As shown in Fig. 5b, the effects of chloroquine and wortmannin on amino acid deprivation challenge were not additive. However, the inhibitors chloroquine and PD98059 in combination and the inhibitors chloroquine and SP600125 in combination blocked the upregulated Me-AIB uptake induced by amino acid deprivation more than what chloroquine, PD98059 or SP600125 did alone, similar to the results obtained for chloroquine and SB202190 in the case of insulin stimulation. These results suggest that PI_3K is involved in the signaling pathway in transporter recruitment and that ERK and JNK are involved in the signaling pathway in de novo protein synthesis.

Changes in the expression of SNAT2 protein in L6 cells

Cycloheximide, an inhibitor of protein synthesis, was able to block the stimulatory effects of insulin, osmotic shock, and amino acid deprivation on system A activity, suggesting that de novo synthesis of the transporter protein is involved, either completely or partially, in all three cases. Saturation kinetic analysis also showed that the observed stimulation in all three cases is associated with an increase in the maximal velocity with no significant change in the substrate affinity. These kinetic data also suggest an increase in the transporter density in the plasma membrane

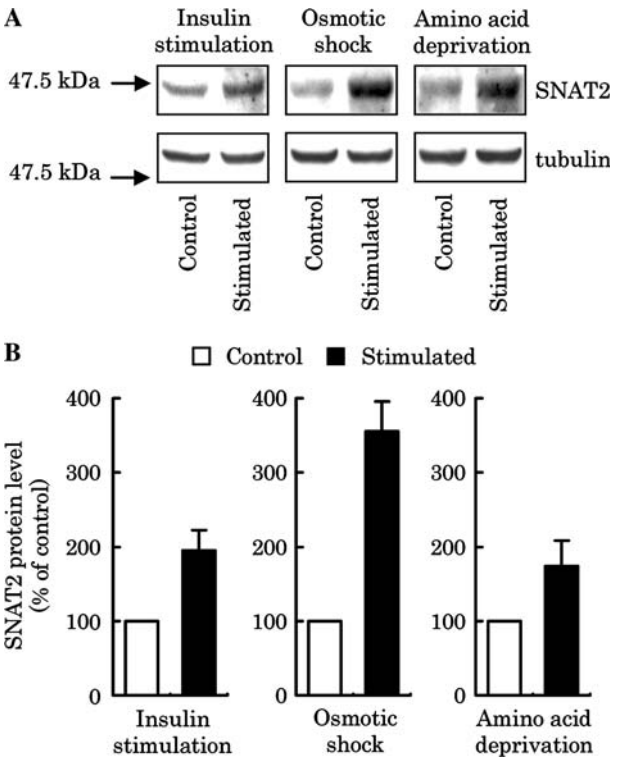

Fig. 6 Western blot analysis of expression of SNAT2 transporter in L6 cells. Cells were subjected to each stimulation for 8 h and then total cell lysate and crude membrane protein were extracted as described in "Materials and methods". **a** Western blot analysis was performed using total cell lysate with an antibody against mouse α-tubulin and using crude membrane with an antibody against rat SNAT2 as described in "Materials and methods". Similar results were obtained in three independent cell cultures. b Quantification of changes in SNAT2 protein level shown in Fig. 6a and two additional results. Each value is expressed as percentage of the control after normalization with the density of α-tubulin, and each *column* represents the mean with SE of the results from three different cell cultures

as the underlying mechanism for the stimulation of the transporter activity in all three cases. Therefore, we compared the levels of SNAT2 protein in L6 cells, by Western blot analysis using crude membrane with an antibody against rat SNAT2, under control conditions and under conditions associated with the stimulation of system A by insulin, osmotic shock, and amino acid deprivation. As shown in Fig. 6, the levels of SNAT2 protein were increased by insulin stimulation, osmotic shock, and amino acid deprivation, indicating that there is increased de novo synthesis of the transporter protein under all three conditions.

RT-PCR analysis of the expression of subtypes of system A

The results of Western blot analysis suggested that enhanced expression of SNAT2 is involved in the

upregulation of the activity of system A induced by insulin, osmotic shock, and amino acid deprivation. However, it is not clear whether the other subtypes of system A, SNAT1 and SNAT4, are also involved. Therefore, we performed RT-PCR analysis to determine the expression pattern of the three different subtypes of system A in L6 skeletal muscle cells under control conditions as well as under conditions associated with the stimulation of system A by insulin, osmotic shock, and amino acid deprivation. As shown in Fig. 7, SNAT2 was the only subtype of system A expressed in L6 cells and its expression level was increased by insulin, osmotic shock, and amino acid deprivation. SNAT1 mRNA and SNAT4 mRNA were not detected in these cells regardless of whether or not system A was stimulated by insulin, osmotic shock, or amino acid deprivation. These findings indicate that SNAT2 was exclusively responsible for system A activity in this skeletal muscle cell line not only under basal conditions but also under conditions of system A upregulation by the three different stimuli.

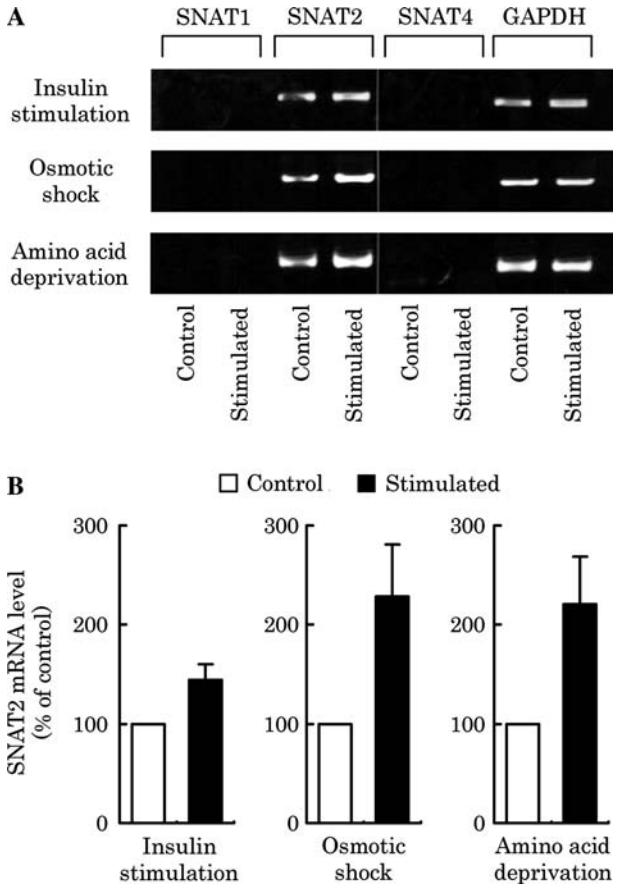

Fig. 7 Expression of each subtype of system A mRNA in L6 cells. L6 cells were subjected to each stimulation for 8 h and then total RNA was extracted. **a** RT-PCR analysis was performed as described in "Materials and methods", and PCR amplification cycle numbers of 35, 27, 35 and 22 were used for detection of SNAT1, SNAT2, SNAT4 and GAPDH, respectively. Similar results were obtained in three independent cell cultures. **b** Semiquantification of changes in SNAT2 mRNA level shown in Fig. 7a and two additional results. Each value is expressed as percentage of the control after normalization with the density of GAPDH, and each *column* represents the mean with SE of the results from three different cell cultures

Discussion

The regulation of SNAT2 expression by insulin, osmotic shock, and amino acid deprivation has already been examined. For example, it has been shown that insulin (100 nM, 30 min) stimulated system A activity by increasing the exocytosis of system A-containing vesicles from an intracellular endosomal pool to the plasma membrane and that the intracellular endosomal pool was sensitive to chloroquine in L6 rat skeletal muscle cells cultured to the stage of myotubes (Hyde et al. 2002). It has also been demonstrated that the activity of system A was increased slowly by incubation in amino acid-free EBSS (Earle's balanced salt solution) and that the adaptive regulation was associated with change in the level of SNAT2 mRNA using human fibroblasts (Gazzola et al. 2001). It has also been reported that ERK was involved in the adaptive response of system A to amino acid deprivation and that PD98059, a specific inhibitor of ERK activation, suppressed this adaptive increase of system A activity (Franchi-Gazzola et al. 1999). On the other hand, it has been reported that osmotic shock, generated by adding 200 mM sucrose to a growth medium, caused upregulation of system A activity associated with increase in the levels of SNAT2 protein and mRNA (Alfieri et al. 2001; Franchi-Gazzola et al. 2004). Lopez-Fontanals et al. (2003) demonstrated that two stimuli, osmotic shock and amino acid deprivation, did not share common signal transduction pathways when upregulating system A in CHO cells. However, as most of those studies were carried out using different cell types, it is difficult to compare the characteristics of upregulation of system A activity induced by insulin, osmotic shock and amino acid deprivation and also determine the mechanism and signaling pathways involved in each of the three manipulations.

To our knowledge, this is the first report on regulation of system A by insulin, osmotic shock, and amino acid deprivation studied under comparable experimental conditions using the same cell line. Using the L6 skeletal muscle cell line, we have demonstrated that system A is subject to upregulation by all three manipulations. However, the cellular processes and the signaling pathways involved in the upregulation process appear to differ among the three manipulations. From the time courses of the effects, it was apparent that similar cellular processes might be involved in the stimulation of system A by insulin

and amino acid deprivation. In both the cases, significant stimulation of system A was detectable even with short treatment periods and the magnitude of stimulation continued to increase with treatment time (Figs. 1a, c). Since the stimulation was detectable with short treatment periods, we speculate that recruitment of the transporter into the plasma membrane from a pre-made intracellular pool may be at least partly responsible for the stimulation of system A activity by insulin and amino acid deprivation. Inhibition of the stimulatory effect by chloroquine supports this conclusion (Figs. 3a, 5a). The fact that inhibitors of mRNA synthesis and protein synthesis were able to block the stimulatory effects of insulin and amino acid deprivation indicates that upregulation of system A activity induced by insulin and amino acid deprivation was associated with de novo synthesis of new transporter mRNA and protein. Even though the cellular processes involved in the stimulation of system A by insulin and amino acid deprivation appear similar, the signaling pathways eliciting these processes seem to be different. The results of studies using wortmannin suggested that PI_3K was involved in the regulation of system A not only by insulin but also by amino acid deprivation. However, the stimulatory effect of insulin was abrogated to a significant extent by SB202190, an inhibitor of p38, whereas it was not affected by PD98059, an inhibitor of ERK, or by SP600125, an inhibitor of JNK. The opposite was true for the stimulatory effect induced by amino acid deprivation. Thus, p38 seems to be involved in the insulin effect, whereas ERK and JNK seem to be involved in the effect induced by amino acid deprivation. These results in the case of amino acid deprivation are similar to the results of previous studies (Franchi-Gazzola et al. 1999; Lopez-Fontanals et al. 2003). Moreover, since the effects of chloroquine and wortmannin were not additive in the regulation of system A not only by insulin but also by amino acid deprivation (Figs. 3b, 5b), the two compounds seem to inhibit SNAT2 stimulation by the same mechanism; that is, PI_3K seems to be involved in the signaling pathway in transporter recruitment into the plasma membrane from a pre-made intracellular pool after insulin and amino acid deprivation. On the other hand, since the inhibitors chloroquine and SB202190, chloroquine and PD98059, and chloroquine and SP600125 blocked the stimulatory effects additively, we speculate that MAPK activation is not involved in transporter recruitment under either condition. Quantitatively, the stimulation induced by insulin was significantly smaller than the stimulation caused by amino acid deprivation. In both the cases, the increase in system A activity was due to an increase in the maximal velocity with no change in substrate affinity (Table 1). This suggests that the observed stimulatory effects are associated with an increase in the transporter density in the plasma membrane. Such a mechanism is consistent with the involvement of transporter recruitment from a pre-made intracellular pool as well as de novo synthesis of the transporter.

The stimulation caused by hyperosmotic shock was the greatest among the three experimental manipulations. Thus, the observed effects on system A activity were in the following order: osmotic shock > amino acid deprivation > insulin. The time course of stimulation caused by osmotic shock suggests that transporter recruitment from a pre-made intracellular pool makes no contribution at all to the increase in transporter activity (Fig. 1b). A lack of any detectable effect of chloroquine also supports this speculation (Fig. 4). Therefore, de novo synthesis of the transporter is likely to be the sole contributor to the effect induced by osmotic shock. Inhibitors of protein synthesis and mRNA synthesis were able to block the effect of osmotic shock. Moreover, since wortmannin, PD98059, SB202190 and SP600125 showed no effect on upregulation of system A activity by osmotic shock, we concluded that PI_3K, ERK, p38 and JNK were not involved in de novo synthesis of the transporter protein induced by osmotic shock. These results are different from those obtained by Lopez-Fontanals et al. (2003) in CHO cells. Lopez-Fontanals et al. (2003) demonstrated upregulation of system A by osmotic shock in CHO cells and showed that this upregulation could be blocked by SB202190, thus implicating p38 in the regulatory process. Therefore, it is apparent that even though osmotic shock stimulates system A in L6 skeletal muscle cells as well as in CHO cells, the specific signaling pathways involved in the process may differ between the two cell types. Furthermore, the stimulation is associated with an increase in the maximal velocity with no significant change in substrate affinity (Table 1). These findings are consistent with the notion that osmotic shock-induced increase in system A activity is due to an increase in the transporter density in the plasma membrane.

We also investigated the expression patterns of different isoforms of system A in this cell line under control conditions and under conditions associated with the stimulation of system A by all three experimental manipulations. The cell line did not express SNAT1 and SNAT4 either under control conditions or stimulated conditions (Fig. 7a). Thus, the observed system A activity in this cell line is entirely due to SNAT2. Analyses of SNAT2 protein and steady-state levels of SNAT2 mRNA showed that all the three experimental manipulations (i.e., insulin, osmotic shock, and amino acid deprivation) are associated with an increase in de novo synthesis of the transporter (Figs. 6b, 7b). In a previous study (Gaccioli et al. 2003), Gaccioli investigated the translational control of the SNAT2 during amino acid starvation. They concluded that the adaptive response of system A activity to amino acid deprivation

required internal ribosome entry site-mediated translation. In this study, from both, activity and protein (although Western blots are difficult to quantify), it seems that the major upregulation is on the protein level and not on the mRNA level. This is at least true for osmotic shock. SNAT2 expression thus seems to be regulated by translational control. The upregulation under amino acid deprivation also suggests a translational control.

The physiologic significance of these findings is obvious. Insulin plays a critical role in skeletal muscle metabolism. It is an anabolic hormone that promotes protein synthesis in muscle cells. System A is an active transporter coupled to a transmembrane electrochemical Na^+ gradient. It is also a transporter with broad substrate specificity for neutral amino acids. Stimulation of system A by insulin has biologic relevance because this might provide a mechanism to enhance the amino acid supply to skeletal muscle cells in order to support insulin-dependent protein synthesis. The involvement of transporter recruitment from a pre-made intracellular transporter pool in the insulin effect is reminiscent of insulin action on glucose uptake via GLUT4 recruitment (Walker et al. 1989; Wang et al. 1998). The stimulation of system A by amino acid deprivation is also critical for skeletal muscle biology, as this represents an adaptive mechanism to maintain amino acid supply to the cells in the presence of varying levels of amino acids in the circulation under physiologic and pathologic conditions. Regulation of system A by osmotic shock may also be of physiologic significance since amino acids, collectively, represent an important group of osmolytes. This process may enable skeletal muscle cells to maintain their cell volume under varying osmotic conditions by simply altering the intracellular concentrations of amino acids. The uphill transport nature and the broad substrate selectivity of system A are suitable for increasing the intracellular levels of amino acids in response to hyperosmotic shock. Several mechanisms operate in the maintenance of cell volume in mammalian cells (Takanaga et al. 2002). Changes in the expression of plasma membrane transporters with ability to facilitate the entry of small molecules into cells represent one such mechanism. In fact, it has been reported that transporters of taurine (Uchida et al. 1992), proline (Takenaka et al. 1994), and glutamate (Kawasaki et al. 2005) are involved in regulation of cell volume under conditions of varying osmolality of the extracellular medium. Therefore, the osmotic shock-induced upregulation of system A is likely to be an important component of cell volume regulation in skeletal muscle cells.

Acknowledgements This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Culture and Sports of Japan.

References

Alfieri RR, Petronini PG, Bonelli MA, Caccamo AE, Cavazzoni A, Borghetti AF, Wheeler KP (2001) Osmotic regulation of ATA2 mRNA expression and amino acid transport system A activity. Biochem Biophys Res Commun 283:174–178

Cariappa R, Kilberg MS (1990) Hormone-induced system A amino acid transport activity in rat liver plasma membrane and Golgi vesicles. Evidence for a differential sensitivity to inactivation by *N*-ethylmaleimide during carrier maturation. J Biol Chem 265:1470–1475

Christensen HN, Oxender DL, Liang M, Vatz KA (1965) The use of *N*-methylation to direct route of mediated transport of amino acids. J Biol Chem 240:3609–3616

Franchi-Gazzola R, Visigalli R, Bussolati O, Dall'Asta V, Gazzola GC (1999) Adaptive increase of amino acid transport system A requires ERK1/2 activation. J Biol Chem 274:28922–28928

Franchi-Gazzola R, Gaccioli F, Bevilacqua E, Visigalli R, Dall'Asta V, Sala R, Varoqui H, Erickson JD, Gazzola GC, Bussolati O (2004) The synthesis of SNAT2 transporters is required for the hypertonic stimulation of system A transport activity. Biochim Biophys Acta 1667:157–166

Gaccioli F, Huang CC, Wang C, Bevilacqua E, Franchi-Gazzola R, Gazzola GC, Bussolati O, Snider MD, Hatzoglou M (2003) Amino acid starvation induces the SNAT2 neutral amino acid transporter by a mechanism that involves eukaryotic initiation factor 2alpha phosphorylation and cap-independent translation. J Gen Physiol 122:5–16

Gazzola RF, Sala R, Bussolati O, Visigalli R, Dall'Asta V, Ganapathy V, Gazzola GC (2001) The adaptive regulation of amino acid transport system A is associated to changes in ATA2 expression. FEBS Lett 490:11–14

Hatanaka T, Huang W, Wang H, Sugawara M, Prasad PD, Leibach FH, Ganapathy V (2000) Primary structure, functional characteristics and tissue expression pattern of human ATA2, a subtype of amino acid transport system A. Biochim Biophys Acta 1467:1–6

Horio M, Yamauchi A, Moriyama T, Imai E, Orita Y (1997) Osmotic regulation of amino acids and system A transport in Madin–Darby canine kidney cells. Am J Physiol 272:C804–809

Hyde R, Peyrollier K, Hundal HS (2002) Insulin promotes the cell surface recruitment of the SAT2/ATA2 system A amino acid transporter from an endosomal compartment in skeletal muscle cells. J Biol Chem 277:13628–13634

Iresjo BM, Svanberg E, Lundholm K (2005) Reevaluation of amino acid stimulation of protein synthesis in murine- and human-derived skeletal muscle cells assessed by independent techniques. Am J Physiol Endocrinol Metab 288: E1028–E1037

Kawasaki A, Hoshi K, Kawano M, Nogami H, Yoshikawa H, Hisano S (2005) Up-regulation of VGLUT2 expression in hypothalamic-neurohypophysial neurons of the rat following osmotic challenge. Eur J Neurosci 22:672–680

Kletzien RF, Pariza MW, Becker JE, Potter VR, Butcher FR (1976) Induction of amino acid transport in primary cultures of adult rat liver parenchymal cells by insulin. J Biol Chem 251:3014–3020

Ling R, Bridges CC, Sugawara M, Fujita T, Leibach FH, Prasad PD, Ganapathy V (2001) Involvement of transporter recruitment as well as gene expression in the substrate-induced adaptive regulation of amino acid transport system A. Biochim Biophys Acta 1512:15–21

Lopez-Fontanals M, Rodriguez-Mulero S, Casado FJ, Derijard B, Pastor-Anglada M (2003) The osmoregulatory and the amino acid-regulated responses of system A are mediated by different signal transduction pathways. J Gen Physiol 122:5–16

Mackenzie B, Erickson JD (2004) Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. Pflugers Arch 447:784–795

Mailliard ME, Cariappa R, Banks RK (1994) Impairment of glucagon-induced hepatic system A activity by short-term ethanol administration in the rat. Gastroenterology 106:480–487

Matsuda S, Kawasaki H, Moriguchi T, Gotoh Y, Nishida E (1995) Activation of protein kinase cascades by osmotic shock. J Biol Chem 270:12781–12786

McDowell HE, Eyers PA, Hundal HS (1998) Regulation of System A amino acid transport in L6 rat skeletal muscle cells by insulin, chemical and hyperthermic stress. FEBS Lett 441:15–19

Oxender DL, Christensen HN (1963a) Evidence for two types of mediation of neutral and amino-acid transport in Ehrlich cells. Nature 197:765–767

Oxender DL, Christensen HN (1963b) Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. J Biol Chem 238:3686–3699

Palii SS, Chen H, Kilberg MS (2004) Transcriptional control of the human sodium-coupled neutral amino acid transporter system A gene by amino acid availability is mediated by an intronic element. J Biol Chem 279:3463–3471

Puertollano R, Alonso MA (1999) MAL, an integral element of the apical sorting machinery, is an itinerant protein that cycles between the trans-Golgi network and the plasma membrane. Mol Biol Cell 10:3435–3447

Sheikh-Hamad D, Di Mari J, Suki WN, Safirstein R, Watts BA 3rd, Rouse D (1998) p38 kinase activity is essential for osmotic induction of mRNAs for HSP70 and transporter for organic solute betaine in Madin–Darby canine kidney cells. J Biol Chem 273:1832–1837

Sibille JC, Kondo H, Aisen P (1989) Uptake of ferritin and iron bound to ferritin by rat hepatocytes: modulation by apotransferrin, iron chelators and chloroquine. Biochim Biophys Acta 1010:204–209

Sugawara M, Nakanishi T, Fei YJ, Huang W, Ganapathy ME, Leibach FH, Ganapathy V (2000a) Cloning of an amino acid transporter with functional characteristics and tissue expression pattern identical to that of system A. J Biol Chem 275:16473–16477

Sugawara M, Nakanishi T, Fei YJ, Martindale RG, Ganapathy ME, Leibach FH, Ganapathy V (2000b) Structure and function of ATA3, a new subtype of amino acid transport system A, primarily expressed in the liver and skeletal muscle. Biochim Biophys Acta 1509:7–13

Su TZ, Wang M, Syu LJ, Saltiel AR, Oxender DL (1998) Regulation of system A amino acid transport in 3T3-L1 adipocytes by insulin. J Biol Chem 273:3173–3179

Takanaga H, Tokuda N, Ohtsuki S, Hosoya K, Terasaki T (2002) ATA2 is predominantly expressed as system A at the blood-brain barrier and acts as brain-to-blood efflux transport for L-proline. Mol Pharmacol 61:1289–96

Takenaka M, Preston AS, Kwon HM, Handler JS (1994) The tonicity-sensitive element that mediates increased transcription of the betaine transporter gene in response to hypertonic stress. J Biol Chem 269:29379–29381

Uchida S, Kwon HM, Yamauchi A, Preston AS, Marumo F, Handler JS (1992) Molecular cloning of the cDNA for an MDCK cell Na(+)- and Cl(−)-dependent taurine transporter that is regulated by hypertonicity. Proc Natl Acad Sci USA 89:8230–8234

Varoqui H, Zhu H, Yao D, Ming H, Erickson JD (2000) Cloning and functional identification of a neuronal glutamine transporter. J Biol Chem 275:4049–4054

Walker PS, Ramlal T, Donovan JA, Doering TP, Sandra A, Klip A, Pessin JE (1989) Insulin and glucose-dependent regulation of the glucose transport system in the rat L6 skeletal muscle cell line. J Biol Chem 264:6587–6595

Wang Q, Khayat Z, Kishi K, Ebina Y, Klip A (1998) GLUT4 translocation by insulin in intact muscle cells: detection by a fast and quantitative assay. FEBS Lett 427:193–197

Yao D, Mackenzie B, Ming H, Varoqui H, Zhu H, Hediger MA, Erickson JD (2000) A novel system A isoform mediating Na+/neutral amino acid cotransport. J Biol Chem 275:22790–22797