Regulation of System A amino acid transport in L6 rat skeletal muscle cells by insulin, chemical and hyperthermic stress

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Abstract In this study we have investigated the effects of insulin, chemical and hyperthermic stresses upon the activity of the System A amino acid transporter in L6 rat muscle cells. Uptake of α-methyl-aminoisobutyric acid (Me-AIB), a non-metabolisable System A substrate, was increased by between 50% and 80% when muscle cells were exposed to a maximally effective concentration of insulin (100 nM), sodium arsenite (ARS, 0.5 mM) or a 42°C heat shock (HS). The observed activation in System A in response to all three stimuli was maximal within 20 min and in the case of insulin and ARS primarily involved an increase in the V_{max} of System A transport. In contrast, HS induced significant increases in both V_{max} and K_m of System A transport suggesting that hyperthermic stress may activate System A by a mechanism distinct from that mediating the effects of insulin and ARS. The hormonal stimulation of System A was blocked by the phosphoinositide 3-kinase (PI3k) inhibitor, wortmannin, but not by rapamycin or PD 98059 which respectively inhibit the mTOR and classical MAP kinase pathways. Exposure of L6 cells to ARS, but not HS, caused a 4.7-fold stimulation in MAPKAP-K2 activity that was blocked by SB 203580, a specific inhibitor of the stress activated protein kinase SAPK2/p38. However, neither SB 203580, rapamycin nor wortmannin were able to suppress the ARS- or HS-induced stimulation in System A transport. In summary, our results demonstrate that activity of the System A transporter can be rapidly upregulated in response to hormonal and stress stimuli through changes in the transport kinetics of the System A carrier. Our data show that whilst the hormonal response is PI3k dependent, the signalling mechanisms which instigate changes in System A activity in response to chemical or hyperthermic stress do not appear to involve PI3k or components of the mTOR, p42/p44 MAP kinase or SAPK2/p38 signalling pathways.

Key words: Me-AIB; PI3k; MAPKAP-K2; mTOR; $p70^{S6k}$; MAP kinase; SAPK2/p38

1. Introduction

The System A amino acid transporter is expressed in most mammalian cells and is primarily responsible for the sodium dependent uptake of short-chain neutral amino acids such as alanine and glycine [1,2]. Of the classical amino acid transport systems that have been described, System A is considered to be the most highly regulated [2] being subject to both acute and adaptive manipulation. Adaptive regulation occurs over a period of hours and is thought to involve increased synthesis of System A carriers and/or synthesis of associated regulatory proteins based on the observation that this mode of regulation is sensitive to inhibitors of both RNA and protein synthesis [2–4]. System A activity can, for example, be stimulated in numerous cell types in response to amino acid deprivation and such up-regulation can be suppressed by cycloheximide and actinomycin D which, respectively, inhibit protein synthesis and DNA transcription [5,6]. Under such circumstances the activity of other amino acid transport systems such as ASC and L remain unaffected indicating that changes in System A transport do not form part of a generalised cell response aimed at increasing amino acid uptake [7].

System A can also be acutely regulated in response to insulin [2,8] and certain growth factors such as insulin-like growth factor 1 (IGF-1) [9] and epidermal growth factor (EGF) [10] by a mechanism which does not require on-going protein synthesis. In skeletal muscle, stimulation of System A by insulin and IGF-1 is phosphoinositide 3-kinase (PI3k) dependent [11,12] and we have recently found that it can also be activated in muscle cells expressing a constitutively active form of protein kinase B (PKB), which lies downstream of PI3k [13]. Evidence is also available in the literature showing that the activity of System A can be rapidly modulated in response to changes in muscle cell volume induced by stressing cells in a hyperosmotic environment [14]. However, it remains unknown if other stress stimuli can also acutely regulate System A and, if so, whether PI3k or protein kinases activated in response stresses such as hyperthermia, osmotic shock and metabolic poisons (e.g. arsenite) [15–19] play a role in regulating the activity of this amino acid transporter.

In this study we have compared the effects of exposing L6 muscle cells to insulin, arsenite or heat shock on the activity of the System A amino acid transporter. We have evaluated what contribution, if any, PI3k and components of the mTOR, p42/p44 MAP kinase and SAPK2/p38 signalling pathways make towards changes in System A activity in response to hormonal and stress stimuli.

2. Materials and methods

2.1. Materials

Rat L6 skeletal muscle cells were kindly provided by Dr. Amira Klip (Hospital for Sick Children, Toronto, Canada). α-Minimal essential medium (α-MEM) culture medium, foetal calf serum and antimycotic/antibiotic solution for tissue culture were obtained from Gibco-BRL (Paisley, Renfrewshire, Scotland, UK) and human insulin from Novo Nordisk (Bagsvaerd, Denmark). Sterile trypsin solution, wortmannin, cycloheximide and sodium arsenite were from Sigma (Poole, Dorset, UK). All reagent grade chemicals were purchased from either Sigma or BDH (Poole, Dorset, UK). All radiochemicals were purchased from New England Nuclear (Hounslow, UK). SB 203580 and PD 98059 were obtained from Calbiochem-Novabiochem (Nottingham, UK) and protease cocktail inhibitor tablets were purchased from Boehringer Mannheim (Sussex, UK).

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2.2. Cell culture and incubations

Monolayers of L6 muscle cells were cultured to the stage of myotubes as previously described [20–22] in α-MEM containing 2% foetal calf serum and 1% antimycotic/antibiotic solution (final concentration 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B) at 37°C in an atmosphere of 5% CO_2/95% air. The cells were cultured in six-well plates and upon formation of myotubes were routinely deprived of serum for 5 h prior to analysis using serum-free α-MEM containing 25 mM glucose. During the final hour cells were depleted of amino acids by incubation in HEPES buffered saline (HBS, 20 mM HEPES-Na (pH 7.4), 140 mM NaCl, 2.5 mM $MgSO_4$, 5 mM KCl, 1 mM $CaCl_2$) containing 25 mM glucose. All subsequent additions to the cells were at the times and concentrations indicated in the figure legends.

2.3. Preparation of whole cell lysates and assay for MAPKAP-K2 activity

Monolayers of L6 myotubes were treated as described in the figure legends. HBS containing the relevant stimuli/inhibitors was rapidly aspirated and the cells washed twice in HBS, placed on ice and lysed in 0.2 ml of ice-cold lysis buffer (50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na orthovanadate, 10 mM Na-β-glycerophosphate, 50 mM NaF, 5 mM Na pyrophosphate, 0.27 M sucrose, 0.1% (v/v) β-mercaptoethanol, 2 μM microcystin-LR and one protease cocktail inhibitor tablet per 50 ml lysis buffer). Lysates were centrifuged for 10 min at $14\,000\times g$ and 50 μg of the resultant supernatant incubated with 3 μg anti-MAPKAP-K2 antibody [23] bound to 5 μl (settled volume) of protein G-Sepharose for 90 min shaking at 4°C. Immunoprecipitates were washed twice with lysis buffer containing 0.5 M NaCl and then twice with lysis buffer alone. The precipitated kinase was assayed in a 50-μl volume, containing 50 mM Na β-glycerophosphate (pH 7.4), 0.2 mM EGTA, 2.5 μM PKI (a peptide inhibitor of cyclic AMP dependent protein kinase), 30 μM of MAPKAP-K2 substrate peptide KKLNRTLSVA [23], 10 mM Mg acetate and 0.1 mM [γ-^{32}P]ATP for 10 min at 30°C. MAPKAP-K2 activity was determined by the rate of peptide phosphorylation. One unit (U) of activity was that amount of MAPKAP-K2 which catalysed the incorporation of 1 nmol of phosphate into substrate in 1 min.

2.4. System A amino acid uptake

Cells were serum and amino acid deprived as indicated previously and exposed to the appropriate stimulants or inhibitors at the times and concentrations indicated in the figure legends. System A transport was measured by incubating cells in 10 μM [^{14}C]Me-AIB (0.1 μCi/ml) for 10 min as described previously [4,12]. Non-specific tracer binding was determined by either quantitating cell associated radioactivity in the presence of a saturating dose of unlabeled Me-AIB (10 mM) or by inclusion of [^{3}H]mannitol as an extracellular marker. Uptake was determined by rapidly aspirating the radioactive incubation medium, followed by three successive washes with ice-cold isotonic saline solution (0.9% NaCl, w/v). Cell associated radioactivity was determined by cell lysis in 0.05 M NaOH followed by liquid scintillation counting. Total cell protein was determined by the Bradford method [24].

2.5. Statistical analysis

Statistical analysis was carried out using a Student's *t*-test. Data were considered statistically significant at P values ≤ 0.05.

3. Results and discussion

3.1. System A is rapidly stimulated by chemical stress and heat shock

L6 myotubes exposed to the chemical stress agent sodium arsenite (ARS) displayed a time-dependent activation in System A amino acid transport as judged by the cellular uptake of Me-AIB, a non-metabolisable substrate of the System A carrier (Fig. 1). The increase in Me-AIB uptake plateaued after 30 min and was maximally stimulated (~46%) at a concentration of 0.5 mM Na-arsenite (the induction in System A activity being assayed at ARS concentrations between 10 μM and 2 mM, data not shown). Me-AIB uptake was also acutely upregulated by over 2-fold when muscle cells were subjected to hyperthermic stress (cells were heat shocked at 42°C for maximum activation). The increase in amino acid uptake following heat shock was slightly more rapid than that seen in response to ARS, being significantly elevated within 10 min and achieving peak activation after just 20 min (Fig. 1).

The very acute nature with which both ARS and heat shock stimulate System A transport is highly reminiscent of that seen following treatment of muscle cells with either insulin or IGF-1 which increase System A activity by elevating the V_{max} of this transporter [9]. To determine whether the stress-induced increase in System A was also attributable to changes in the kinetic properties of the carrier we assayed Me-AIB uptake in L6 myotubes at different extracellular concentrations of the amino acid (5 μM–0.75 mM) following a 30-min treatment period with either 100 nM insulin, 0.5 mM ARS or heat shock (42°C). Table 1 shows that insulin and ARS significantly increased the V_{max} of Me-AIB uptake by 48% and 56%, respectively. Neither insulin nor ARS caused any significant changes in the K_m (i.e. the concentration of Me-AIB that gave half maximal activation of the System A). In contrast, both the V_{max} and K_m of the System A transporter were increased upon subjecting cells to heat shock (Table 1). This latter finding implies that whilst ARS and heat shock both acutely stimulate Me-AIB uptake, the effect is likely to be mediated by distinct cellular mechanisms. Nevertheless, neither stimulus is likely to enhance carrier activity through changes in protein synthesis based on the observation that (i) both ARS and heat shock exert their effects too rapidly for it to be accounted by increases in protein synthesis and (ii) pre-treatment of L6 cells with cycloheximide, a protein synthesis inhibitor, failed to suppress the ARS-induced increase in Me-AIB uptake (Table 2). Thus, the stress-induced activation of System A observed in this study is clearly different from that seen in response to, for example, hyperosmotic shock or cellular amino acid deprivation. These forms of cell stress enhance System A activity over a few hours and rely upon de novo synthesis of new carrier molecules and/or that of ancillary proteins that may participate in the regulation of System A [5–7].

3.2. Intracellular signalling pathways involved in the hormonal and stress-induced activation of the System A transporter in L6 myotubes

We have previously shown that in skeletal muscle insulin

Table 1
Effect of insulin, arsenite and heat shock on Me-AIB transport kinetics in L6 muscle cells

Condition	V_{max} (pmol/min/mg protein)	K_m (μM)
Control	333 ± 38	130 ± 13
Insulin	493 ± 20*	113 ± 10
ARS	519 ± 27*	141 ± 16
HS	848 ± 48*	235 ± 2*

L6 myotubes were exposed to insulin (100 nM), arsenite (0.5 mM) or heat shock (42°C) for 30 min prior to measuring System A transport. Me-AIB uptake was assayed over six different extracellular concentrations of Me-AIB (between 5 and 750 μM) and data subjected to Hanes-linear transformation to calculate the V_{max} and K_m as described in [4]. The results represent the mean ± S.E.M. from three experiments each performed in triplicate. The asterisk indicates a statistically significant change from the control value using a Student's *t*-test, $P \leq 0.05$.

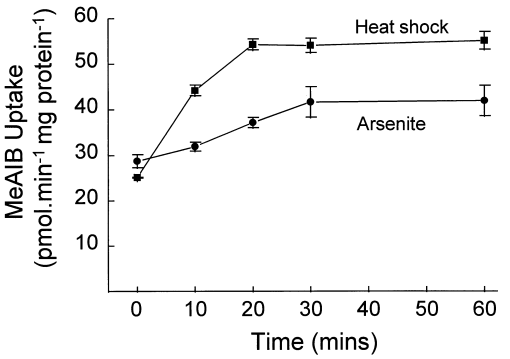

Fig. 1. Effects of heat shock and arsenite treatment on System A amino acid transport. L6 myotubes were exposed to either a 42°C hyperthermic shock or 0.5 mM sodium arsenite for times indicated in the figure prior to measuring System A (Me-AIB) uptake as indicated in Section 2.

acutely stimulates both System A and glucose transport in a phosphoinositide 3-kinase (PI3k) dependent manner [12,25]. However, whilst it is generally accepted that the hormonal stimulation of glucose transport does not involve the p42/p44 MAP kinases or $p70^{S6k}$ [26,27], the issue of whether these protein kinases participate in the hormonal and/or stress-induced activation of the System A transporter in muscle remains presently unknown. To address this we monitored the effects of wortmannin (a potent inhibitor of PI3k), PD 98059 (an inhibitor of the MAP kinase pathway) and rapamycin (an inhibitor of the mTOR pathway which also blocks the hormonal activation of $p70^{s6k}$ [28]) on basal and insulin-stimulated System A transport. The data shown in Table 2 indicates that insulin stimulated Me-AIB uptake by ~60% and that this was suppressed in the presence of wortmannin; a finding that is consistent with previous work from our group suggesting that PI3k is involved in the hormonal activation of System A [12]. In contrast, the insulin-induced increase in Me-AIB uptake was not significantly altered by PD 98059 or rapamycin indicating that although the hormone stimulates both MAP kinase and $p70^{s6k}$ in L6 myotubes [28] neither is involved in the acute upregulation of System A activity by insulin.

The magnitude with which insulin or ARS activated System A was near maximal since exposing muscle cells to both stimuli simultaneously did not result in any additive increase in Me-AIB uptake (Table 2). It is plausible that the lack of additivity signals the involvement of a common mechanism by which insulin and ARS enhance System A activity. We have recently shown that, like insulin, ARS also induces a rapid stimulation in PI3k activity based on the observation that it increases the cellular content of phosphatidyl inositol-3,4,5-P_3 in a wortmannin-sensitive fashion [25]. This finding raises the possibility that PI3k may be a common signalling element in the hormonal and stress-mediated increase in System A activity. To test this possibility we investigated whether wortmannin could block the stimulation in System A transport elicited by ARS and heat-shock. Fig. 2A shows that the inhibitor caused a small, but significant reduction in basal Me-AIB uptake in L6 myotubes, but was unable to suppress the stress-induced increase in transport activity seen in response to ARS or heat shock. Similarly, the use of rapamycin (Fig. 2B) excludes any involvement of the mTOR pathway in the acute stimulation of Me-AIB uptake by ARS and heat shock.

A number of previous studies have shown that exposing mammalian cells to ARS or heat shock results in activation of the SAPK2/p38 pathway [15,16]. MAPKAP-K2 is an in vivo substrate of the stress activated protein kinase, SAPK2/p38, whose activity is known to be enhanced by both ARS and heat shock in a number of cell lines [15,19,29]. In Fig. 3A we show that ARS stimulates MAPKAP-K2 in L6 cells by ~5-fold, but that the kinase was not activated when cells were subjected to heat shock suggesting that activation of SAPK2/p38 in L6 cells is stimulus dependent. The increase in MAPKAP-K2 activity elicited by ARS was abolished by pre-treating muscle cells with the SAPK2/p38 inhibitor, SB 203580 [16,30] (Fig. 3A). Therefore, to assess whether SAPK2/p38 was involved in the stress-induced increase in System A transport we monitored the effects of SB 203580 on Me-AIB uptake. Fig. 3B shows that the inhibitor had no detectable effect on System A activity in unstimulated muscle cells and was, moreover, unable to block the ARS- or heat shock-induced increase in Me-AIB uptake. Collectively, these findings concur with previous work from our group showing that PI3k, $p70^{s6k}$ and SAPK2/p38 are not involved in the cellular stress response that instigates an increase in muscle glucose transport [25].

The precise nature of the signalling pathway(s) that enhances amino acid and glucose uptake following exposure of muscle cells to stresses such as ARS and heat shock thus remains currently unknown. However, one potential candidate that may participate in the stress response is the serine/threonine kinase, protein kinase B (PKB, also known as Akt). PKB has been implicated in the insulin-mediated regulation of 6-phosphofructokinase-2-kinase [31] and glycogen synthase

Table 2
Effects of wortmannin, PD 98059, rapamycin and arsenite on basal and insulin-stimulated Me-AIB uptake in L6 cells

Inhibitor/compound	Me-AIB uptake (pmol/min/mg protein)	
	−Insulin (basal)	+Insulin
None	24.4 ± 1.2	39.0 ± 1.2*
Wortmannin	18.8 ± 0.9	20.7 ± 1.0
PD 98059	24.1 ± 2.2	35.1 ± 2.6*
Rapamycin	26.8 ± 1.6	40.5 ± 2.4*
Arsenite	41.7 ± 3.3**	38.4 ± 1.8**
Cycloheximide	22.7 ± 1.3	–
Cycloheximide/arsenite	39.8 ± 1.2**	–

Me-AIB uptake was assayed in L6 myotubes following treatment with insulin (100 nM, 30 min) and/or wortmannin (100 nM, 45 min), PD 98059 (50 μM, 45 min), rapamycin (100 nM, 45 min), sodium arsenite (0.5 mM, 30 min) and cycloheximide (5 μg/ml) as described in Section 2. Results represent the mean ± S.E.M. from three separate experiments each conducted in triplicate. * indicates a significant change ($P < 0.05$ by Student's *t*-test) from the respective basal uptake or ** that from the uptake observed in cells not treated with insulin or any inhibitors.

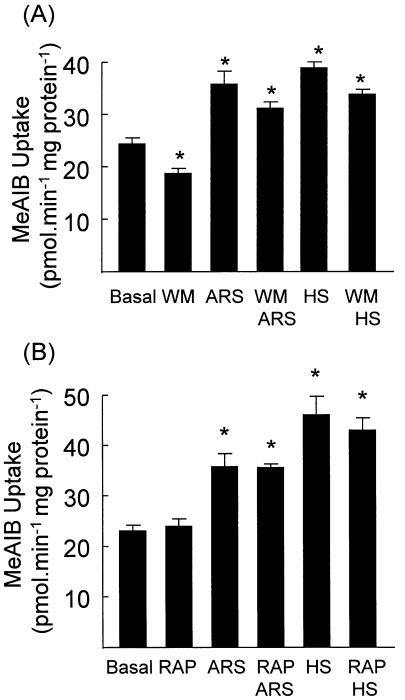

Fig. 2. Effects of wortmannin and rapamycin on the stress-induced increase in System A transport activity in L6 cells. Muscle cells were pre-treated with (A) wortmannin (WM, 100 nM) and (B) rapamycin (RAP, 100 nM) for 45 min. During the last 30 min of this incubation period cells were either exposed to sodium arsenite (ARS, 0.5 mM) or subjected to a 42°C heat shock (HS). At the end of this pre-incubation period the activity of the System A transporter was assayed as described in Section 2. Results represent the mean ± S.E.M. from three separate experiments each performed in triplicate. Asterisks indicate a significant change from the basal value (obtained from unstimulated cells) by Student's *t*-test, $P \leq 0.05$.

kinase 3 [32,33] and more recently we [13], and others [34–36], have shown that it may also be an important signalling intermediate in the hormonal activation of glucose and System A amino acid transport in muscle and fat. The activation of PKB by insulin in L6 cells takes place in PI3k dependent fashion consistent with the suggestion that it lies downstream of PI3k in the insulin signalling cascade [37]. However, it is noteworthy that PKB can also be activated by heat shock and by β-adrenergic agonists by a mechanism that does not involve the activation of PI3k [38,39]. It is conceivable, therefore, that PKB may represent a point of convergence for the insulin and stress signalling pathways. Such a scenario may, in part, help explain why the stimulation in System A activity was not additive when L6 cells were simultaneously challenged with insulin and ARS (Table 2). Unfortunately there are no specific inhibitors of PKB that would allow us to readily test its involvement in the stress-mediated increase in System A transport in skeletal muscle, but one approach that we are currently pursuing involves the over-expression of a dominant-negative form of PKB in L6 cells. This approach may not only help further define the role played by PKB in the hormonal activation of glucose and amino acid transport, but may provide important insights as to whether it also participates in the cellular stress response.

Does the stress-induced increase in amino acid uptake confer any physiological benefit to the cell? Since the 'free' intracellular amino acid pool is sufficiently large it is generally accepted that supply of amino acids from the extracellular pool is not a major determinant of the protein synthetic rate in skeletal muscle [40]. However, it is noteworthy that two recent studies have highlighted the importance of extracellular amino acid supply as a nutritional signal that initiates intracellular signalling events. For example, exposure of CHO cells, hepatocytes and L6 myotubes to high physiological concentrations of amino acids results in the activation of $p70^{s6k}$, a key participant in the initiation of protein synthesis [41,42], and PHAS-I phosphorylation [42]. Since increased synthesis of stress-related proteins (such as those belonging to the heat shock and GRP protein family) forms an integral part of cellular survival response [16,43], it is conceivable that the stress-mediated increase in amino acid uptake represents a signal transduction event that may help promote protein synthesis via activation of key regulatory molecules, such as $p70^{s6k}$.

In summary, we have shown that System A amino acid transport is rapidly stimulated by insulin, chemical and hyperthermic stress in L6 muscle cells. Whilst the hormonal stimulation of System A was mediated in a PI3k dependent fashion, the MAP kinase, mTOR and SAPK2/p38 signalling pathways do not appear to be involved in mediating the hor-

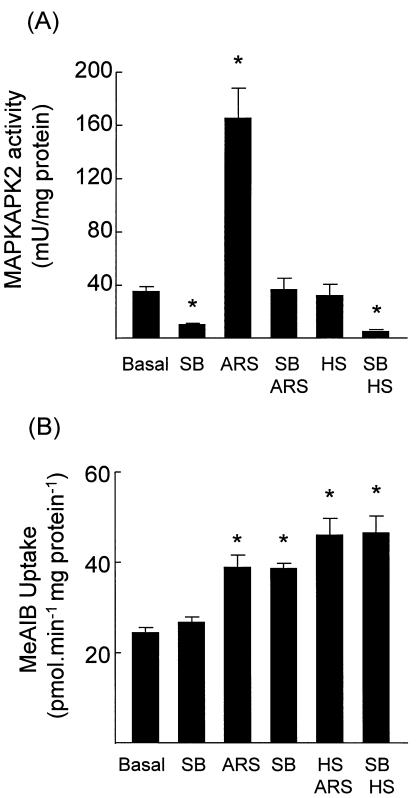

Fig. 3. MAPKAP-K2 activity and System A amino acid transport in L6 cells: the effects of arsenite, heat shock and the SAPK2/p38 inhibitor, SB 203580. L6 myotubes were exposed to either sodium arsenite (ARS, 0.5 mM), heat shock (HS, 42°C) and/or SB 203580 (SB, 10 μM) for 30 min. After this pre-treatment period cells were either (A) lysed and assayed for MAPKAP-K2 activity or (B) used for assaying of System A (Me-AIB) uptake. The results represent the mean ± S.E.M. from three experiments each performed in triplicate. Asterisks indicate a statistically significant change from the basal value (obtained from unstimulated cells) by Student's *t*-test, $P \leq 0.05$.

monal or stress-induced increases in System A amino acid transport.

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