Insulin Promotes the Cell Surface Recruitment of the SAT2/ATA2 System A Amino Acid Transporter from an Endosomal Compartment in Skeletal Muscle Cells*

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SAT1–3 comprise members of the recently cloned family of System A transporters that mediate the sodium-coupled uptake of short chain neutral amino acids, and their activity is regulated extensively by stimuli such as insulin, growth factors, and amino acid availability. In skeletal muscle, insulin stimulates System A activity rapidly by a presently ill-defined mechanism. Here we demonstrate that insulin induces an increase in the plasma membrane abundance of SAT2 in a phosphatidylinositol 3-kinase-dependent manner and that this increase is derived from an endosomal compartment that is required for the hormonal activation of System A. Chloroquine, an acidotropic weak base that impairs endosomal recycling of membrane proteins, induced a complete inhibition in the insulin-mediated stimulation of System A, which was associated with a loss in SAT2 recruitment to the plasma membrane. The failure to stimulate System A and recruit SAT2 to the cell surface could not be attributed to a block in insulin signaling, as chloroquine had no effect on the insulin-mediated phosphorylation of protein kinase B or glycolgen synthase kinase 3 or upon insulin-stimulated GLUT4 translocation and glucose transport. Our data indicate strongly that insulin increases System A transport in L6 cells by stimulating the exocytosis of SAT2 carriers from a chloroquine-sensitive endosomal compartment.

The plasma membranes of mammalian cells possess multiple transport systems for the cellular exchange of amino acids (1), and of the classical amino acid transport systems described the best studied has been the System A amino acid carrier, which mediates the sodium-coupled uptake of short-chain neutral amino acids (e.g. alanine). System A is expressed in many cell types. Its ability to mediate the uptake of amino acids with N-methyl substitutions has enabled the discrimination of System A from other amino acid transporters using N-methylaminoisobutyric acid (Me-AIB)\(^\dagger\) as a paradigm substrate. Functional studies using this substrate have shown that System A activity is highly pH-sensitive and that the carrier is subject to both long- and short-term modulation (1). Numerous functional studies have demonstrated, for example, that System A activity is subject to adaptive up-regulation by amino acid deprivation in multiple cell types and that this response is dependent upon gene expression, since it is inhibited by cycloheximide and actinomycin D, inhibitors of translation and transcription, respectively (recently reviewed by us (2)). Whether such modulation results from the increased expression of System A carriers or that of regulatory molecule(s) capable of effecting changes in transporter activity has remained unclear. However, the recent cloning of three isoforms of the System A transporter (SAT, also known as amino acid transporter A (ATA)), termed SAT1–3/ATA1–3 (3–6), has meant that the molecular regulation of this transport system can now be investigated in detail. The availability of molecular probes to System A has allowed us to demonstrate recently that the adaptive increase in transport activity triggered by amino acid deprivation stems from a selective up-regulation in the expression of SAT2 protein in the plasma membrane of muscle and fat cells (7). This finding is fully consistent with the observation of others that depriving human fibroblasts of amino acids induces an increase in SAT2 mRNA levels (8).

In addition to adaptive regulation, System A can also be stimulated acutely by hormones such as insulin (9–11), growth factors (12), and cell stresses (11–13). These stimuli induce a rapid increase in the \(V_{\text{max}}\) of System A transport that does not rely upon synthesis of new carriers, but which is thought to involve the acute modulation of carrier function effected by molecules participating in early insulin and growth factor signaling. We have shown previously that the stimulatory effects of insulin and IGF-1 on System A can be blocked by inhibitors of phosphatidylinositol 3-kinase (PI3K) (10–11) and that expression of a constitutively active form of protein kinase B (PKB), which lies downstream of PI3K in the insulin signaling cascade, mimics the effect of insulin on this amino acid transporter (14). Precisely how the hormonal activation of PI3K and PKB are linked to an increase in functional System A activity is not yet understood, but it is plausible that signaling from these molecules either stimulates the activity of SAT proteins resident in the plasma membrane or that additional SAT carriers are recruited to the cell surface from an intracellular compartment. The concept of carrier recruitment from intracellular compartments in response to insulin is well established for the GLUT4 glucose transporter (15). However, it remains...
In vitro. Wortmannin, insulin, chloroquine, and cytochalasin D were purchased from Sigma-Aldrich. Complete protease inhibitor tablets were obtained from Roche Molecular Biochemicals. Phospho-specific antibodies to PKB and GSK3 were obtained from New England Biolabs (Hertfordshire, UK). Horseradish peroxidase-conjugated anti-rabbit IgG were obtained from Scottish Antibody Production Unit (Carluke, Lanarkshire, UK). Hybond nitrocellulose membrane was obtained from Amersham Biosciences, and reagents for ECL were purchased from Pierce & Warriner (Chester, UK).

Cell Culture—Monolayers of L6 muscle cells were cultured to the stage of myotubes as described previously (14–20) in α-MEM containing 2% (v/v) fetal calf serum and antimycotic/antibiotic solution (100 units/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml amphotericin B) at 37 °C in an atmosphere of 5% CO₂, 95% air. Upon formation of myotubes, cells were deprived of serum by incubating muscle cells in serum-free α-MEM for 4 h followed by a 1 h incubation in amino acid-free HEPES-buffered saline (HBS, 20 mM HEPES-Na (pH 7.4), 140 mM NaCl, 2.5 mM MgSO₄, 5 mM KCl, 1 mM CaCl₂). Additions (e.g., insulin, CQ, and cytochalasin D) to the cells were made at times and at concentrations indicated in the figure legends.

Subcellular Fractionation of L6 Myotubes—Subcellular membranes from L6 myotubes were isolated as described previously (21–24). Briefly, 2 h prior to fractionation, cells were washed, and growth medium (containing 5 mM glucose) was supplemented with chloroquine (100 μM). In some dishes, insulin (100 nM) was added 30 min prior to cell harvesting; control dishes received vehicle alone. At the end of the incubation period, cells from five (15 cm) dishes were scraped off the plates with a rubber policeman, pooled, and gently pelleted. The cell pellet was resuspended in ice-cold buffer (250 mM sucrose, 20 mM Hepes, 5 mM Na₂SO₄, 2 mM EGTA, pH 7.4 plus 1 protease inhibitor tablet/50 ml) and homogenized. The cellular homogenate was subjected to a series of differential centrifugation steps that isolate crude cell membranes that were subsequently fractionated on a discontinous sucrose gradient (32, 40, and 50% sucrose by mass) at 210,000 × g for 2.5 h. Membranes from on top of the 32% sucrose cushion and those at the 32/40% and 40/50% sucrose interfaces were recovered and their protein content determined using the Bradford assay with bovine serum albumin as standard (25).

Subcellular Fractionation of Rat Skeletal Muscle—Rat skeletal muscle was excised from the hind limbs of male Sprague-Dawley rats (250–300 g) and fractionated based on a procedure established by Klip and co-workers (26). The procedure involves skeletal muscle homogenization and a series of differential centrifugation steps that allow isolation of crude muscle membranes. These were subsequently fractionated on a discontinuous sucrose density gradient (25, 30, and 35%) as described previously (26, 27). This procedure resulted in the separation of three distinct membrane bands; one band was located above the 25% sucrose cushion representing membranes enriched with plasma membranes (denoted as F25), and a second band separated on top of the...
30% sucrose layer (F30) contained membranes largely of endosomal origin (27). A third band separated out on top of the 35% sucrose layer (F35) which consisted of membranes largely of intracellular origin that contain the insulin “recruitable” pool of GLUT4 glucose transporters (28). Protein content was determined using the Bradford method (25).

Effects of CD and CQ on System A Amino Acid Transport—System A activity was assayed by measuring the uptake of Me-AIB as described previously (20). Briefly, L6 myotubes were incubated with 10 μM [3C]Me-AIB (1 μCi/ml) for 10 min. Nonspecific tracer binding was determined using either [3H]mannitol as an extracellular marker or by determining cell-associated radioactivity in the presence of an excess saturating dose of unlabeled Me-AIB (10 nm). In some experiments glucose transport was measured simultaneously in the same population of muscle cells used to assay System A activity by a dual label approach (12). Uptake of 10 μM 2-deoxy-[3H]glucose (2DG; 1 μCi/ml, 26.2 Ci/mmol) was measured for 10 min. Carrier-mediated glucose transport was determined by quantifying cell-associated radioactivity in the presence of 10 μM cytochalasin B, an inhibitor of facilitative glucose transporters (14). Uptake of both Me-AIB and 2DG was determined by aspirating the radioactive medium, followed by three successive washes in ice-cold saline (pH 7.4), 2 mg/ml bovine serum albumin) at 37 °C to deplete endogenous transferrin in the culture media. Cells were subsequently incubated in MHB buffer containing 5 μg/ml 125I-Tfn (5 × 10^4 cpm/μg) for 60 min at 4 °C followed by three successive washes in ice-cold PBS and lysis in 1% Triton X-100 prior to quantitating total cell-associated radioactivity using a Beckman LS6000IC counter. To quantitate the internalized radioactivity, the above protocol was repeated on a duplicate set of cells, but this set was washed three times with PBS/25 mM glacial acetic acid (pH 4.2) following the initial three washes with ice-cold PBS to strip surface-associated 125I-Tfn. The difference between the two samples gives an indication of surface Tfn binding. Nonspecific surface binding was corrected for by determining radioactivity associated with cells incubated at 4 °C with MHB containing 3 μg/ml 125I-Tfn with a 100-fold excess of unlabeled Tfn.

**RESULTS AND DISCUSSION**

To investigate the effects of CQ on System A activity in L6 myotubes we first performed dose and time response studies with this acidotropic agent. Muscle cells were preincubated with 100 μM CQ for up to 3 h, after which period basal and insulin-stimulated Me-AIB uptake was assayed. In the absence of any pretreatment with CQ, insulin increased Me-AIB uptake by ~65%, but this stimulation was progressively lost upon pretreatment of cells with CQ (Fig. 1A). Under these conditions CQ also induced a modest reduction (~25%) in basal System A transport activity (Fig. 1A). Insulin-stimulated System A activity was virtually undetectable in cells pre-exposed to 100 μM CQ and was maximally suppressed following preincubation with CQ for 2 h (Fig. 1, A–C). These findings indicate that System A transporters may recycle constitutively at the cell surface and that insulin stimulates System A by enhancing its exocytosis from a CQ-sensitive endosomal compartment. If this supposition is correct, then System A activity in the plasma membrane will depend on the relative rates with which the carrier protein is exocytosed and endocytosed.

Studies in yeast and mammalian cells indicate strongly that carrier free Na125I (PerkinElmer Life Sciences) for 10 min. Inodinated Tfn was isolated by passage through a Dowex-1 ion exchange column.

Muscle cells were incubated with CQ and CD for times and at concentrations indicated in the figure legend (Fig. 2) prior to incubation for 30 min in MHB buffer (serum-free or MEM medium, 20 mM HEPES (pH 7.4), 2 mg/ml bovine serum albumin) at 37 °C to deplete endogenous transferrin in the culture media. Cells were subsequently incubated in MHB buffer containing 5 μg/ml 125I-Tfn (5 × 10^4 cpm/μg) for 60 min at 4 °C followed by three successive washes in ice-cold PBS and lysis in 1% Triton X-100 prior to quantitating total cell-associated radioactivity using a Beckman LS6000IC counter. To quantitate the internalized radioactivity, the above protocol was repeated on a duplicate set of cells, but this set was washed three times with PBS/25 mM glacial acetic acid (pH 4.2) following the initial three washes with ice-cold PBS to strip surface-associated 125I-Tfn. The difference between the two samples gives an indication of surface Tfn binding. Nonspecific surface binding was corrected for by determining radioactivity associated with cells incubated at 4 °C with MHB containing 3 μg/ml 125I-Tfn with a 100-fold excess of unlabeled Tfn.

**Statistical Analysis**—Statistical analysis for multiple comparisons was performed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. Data analysis was performed using GraphPad Prism software and considered statistically significant at p values <0.05.

![Effect of CQ on System A activity](http://www.jbc.org/cgi/content/fig/273/21/13630/F5a)

**Fig. 3.** Effects of CD and CQ on insulin-stimulated System A amino acid transport in L6 myotubes. Muscle cells were incubated in the absence or presence of CD (2 μM, 2 h) and/or CQ (100 nm, 2 h). During the last 30 min of preincubation with CD or CQ, cells were incubated in the absence or presence of insulin (100 nm). Incubation of cells with wortmannin (100 nm) was performed by exposing myotubes to the inhibitor 15 min prior to treatment with CD and for the remaining incubation period with CD and uptake of Me-AIB (10 μM) assayed as described. Results represent the mean ± S.E. for 3–8 experiments, each performed in triplicate. The asterisks represent a statistically significant change (p < 0.05) from the appropriate untreated control.

![Effect of CQ on System A activity](http://www.jbc.org/cgi/content/fig/273/21/13630/F5b)

**Fig. 4.** The CQ-induced inhibition of insulin-stimulated System A amino acid transport is reversible. L6 myotubes were incubated in the absence or presence of CQ (100 μM, 2 h) and/or insulin (100 nm, 30 min) prior to assaying Me-AIB uptake. In some experiments, muscle cells were washed following pretreatment with CQ and allowed subsequently to recover. Cells were exposed subsequently to insulin (100 nm, 30 min) at times indicated during the post-wash period and insulin-stimulated Me-AIB uptake assayed. Results represent the mean ± S.E. for 3–8 experiments, each performed in triplicate. The asterisks represent a statistically significant change (p < 0.05) from the appropriate untreated control.
Actin filaments and actin-based motor proteins play a key role in receptor-mediated endocytosis (18–30). Thus, to examine further the importance of exo- and endocytic events in the regulation of System A activity, we investigated the effects of treating muscle cells with cytochalasin D, a reagent that induces depolymerization of actin filaments, in addition to CQ. To assess how each drug affects the recycling of membrane proteins we monitored surface binding of [125I]-transferrin as an index for plasma membrane transferrin receptor (TfR) abundance, a paradigm marker protein that recycles constitutively at the cell surface. Fig. 2 shows that surface TfR binding was increased significantly (by 73%) following incubation of cells with CD, whereas it fell by ~40% in cells that had been exposed to CQ (Fig. 2). These findings are consistent with the view that CD and CQ inhibit endocytosis and exocytosis of TfR, respectively. Previous work using rat adipocytes has shown that the CQ-induced disruption in IGF-II receptor recycling is reversible, suggesting that endosomal function can be restored upon withdrawal of CQ from the incubation medium (31). To test for whether this was also the case in L6 myotubes, muscle cells were washed free of CQ, and TfR binding was assayed during the post-wash recovery period. Withdrawal of CQ from the incubation medium for periods of up to 2 h led to a restoration in TfR binding to a level that was not significantly different from cells that had not been exposed to CQ (Fig. 2).

Having established that CD inhibits TfR internalization, we assessed the effects of this drug on System A transport activity. Preincubation of L6 myotubes with 2 μM CD alone induced a near 30% stimulation in Me-AIB uptake (Fig. 3). This increase most likely arises through a modest up-regulation in cell surface System A carrier number that is associated with inhibition of their internalization, whereas carrier exocytosis from the endosomal compartment remains unaffected. This proposition is supported by the finding that the CD-induced increase in System A activity did not take place when muscle cells were treated with CQ just prior to incubation with CD (Fig. 3), under which conditions both endo- and exocytosis are inhibited. Treatment of cells with insulin following incubation with CD results in a further increase in System A activity over and above that elicited by CD but to a level no greater than that observed in the presence of insulin alone (Fig. 3) This finding provides further support for the idea that exocytosis of carriers from an endosomal pool is the mechanism by which insulin stimulates System A transport.

PI3K has been implicated strongly in the control of both basal and insulin-stimulated exocytosis of recycling proteins such as TfR (32, 33). We hypothesized that if the stimulation of System A elicited by both CD and insulin relies upon carrier recruitment from an endosomal compartment, then it ought to be blocked by the PI3K inhibitor, wortmannin. Consistent with this idea, wortmannin prevented stimulation of Me-AIB uptake in response to CD and insulin (Fig. 3), and use of the structurally unrelated PI3K inhibitor, LY 294002, yielded similar results (data not shown).

The data presented in Fig. 2 indicate that endosomal function can be restored slowly when muscle cells are washed and allowed to recover for 2 h following pretreatment with CQ. We postulated that if the hormonal activation of System A was dependent on a functional endosomal compartment, then the ability of insulin to stimulate System A activity should also be recoverable during the post-wash period. Fig. 4 shows that CQ suppressed completely the hormonal activation of System A but that this stimulation was restored progressively in cells that had been washed “free” of the drug and allowed to recover subsequently in media lacking CQ for up to 2 h.

It is plausible that the inhibitory effects exerted by CQ on the hormonal activation of System A might involve direct effects of the drug on membrane transporters or through a blockade in insulin signaling. To examine these possibilities we investigated the effects of CQ on insulin-stimulated glucose transport and upon the phosphorylation of two important insulin signaling intermediates, PKB and glycogen synthase kinase-3 (GSK3) (34). Although CQ inhibited the hormonal stimulation of System A transport, the drug had no apparent effect on insulin-stimulated glucose transport when assayed in the same cell population (Fig. 5A). This observation is in agreement with previous work using adipocytes showing that although CQ disrupts the recycling of IGF-II receptors, it has little impact on insulin-stimulated glucose transport or upon transporter recycling to the cell surface (31–35). These findings imply, first, that although a proportion of the insulin-regulated glucose transporter, GLUT4, localizes to recycling endosomes (36), the effects of CQ on GLUT4 recycling are likely to be minimal. Second, because CQ inhibits insulin-stimulated System A transport and influences the recycling dynamics of TfR and the IGF-II receptor (31), this indicates that such proteins are present in a distinct population of endosomal vesicles from which GLUT4 is excluded. To assess the possibility that CQ may have impaired insulin signaling, we monitored the insulin-induced
Phosphorylation of PKB, an insulin signaling intermediate implicated in the regulation of diverse end point responses to the hormone such as glycogen synthesis (37), glucose transport, and System A transport (14). Fig. 5B shows that insulin promoted the phosphorylation of PKB and also that of its downstream physiological target, GSK3. This finding is fully compatible with the observed stimulation in glucose transport elicited by the hormone (Fig. 5A) and indicates strongly that CQ is unlikely to have inhibited activation of System A through suppression of early insulin signaling events.

Three SAT isoforms (SAT1–3, also known as ATA1–3) have recently been cloned (3–6). Of these, SAT2 appears to be the most abundantly expressed isoform in skeletal muscle, whereas SAT3 expression occurs at a much lower level, and SAT1 is not detectable (6–7). Moreover, the observation that SAT3 is a relatively poor mediator of the Me-AIB uptake compared with the other isoforms (38) implies that SAT2 is the most likely isoform to mediate insulin-stimulated Me-AIB uptake in muscle. To test this proposition, we investigated the effects of insulin and chloroquine treatment on the subcellular distribution of SAT2 in L6 myotubes. Pretreatment of cells with 100 nM CQ for 2 h followed by insulin stimulation for 30 min was associated with a 2-fold increase in SAT2 (this was associated with an increase in the Vmax of System A transport from 388 ± 16 pmol/min/mg to 584 ± 16 pmol/min/mg (p < 0.05), with no significant change in Km). The increase in cell surface SAT2 was associated with a concomitant reduction in SAT2 abundance in membranes recovered from the 40/50% sucrose interface (data not shown). However, data from three separate experiments revealed that the hormone induced a greater than 2-fold increase in SAT2 in the PM-enriched fraction, indicating that this fraction was enriched with endosomal membranes. Consistent with our recent work (7), SAT2 was detected in all three membrane fractions but was enriched in membranes recovered from the 32/40% sucrose interface (Fig. 6A).

When SAT2 abundance was investigated in the three fractions following isolation of cells treated with insulin, we did not observe any changes in SAT2 content in membranes recovered from the 40/50% sucrose interface (data not shown). However, data from three separate experiments revealed that the hormone induced a greater than 2-fold increase in SAT2 in the PM-enriched fraction, indicating that this fraction was enriched with endosomal membranes. Pretreatment of L6 cells with CQ for 2 h prior to subcellular fractionation reduced the amount of SAT2 recovered in the PM fraction, a finding consistent with the idea that this compound prevents the delivery of proteins to the cell surface from the endosomal compartment without affecting the internalization of PM proteins that are constitutively recycling at the cell surface. The loss in PM SAT2 was not recovered in any of the other membrane fractions isolated from the sucrose gradient, raising the possibility that, once internalized, SAT2 may have been routed for lysosomal degradation. Unlike SAT2, pretreatment of L6 cells with CQ had no detectable effect on SAT3 expression occur at a much lower level, and SAT1 is not detectable (6–7). Moreover, the observation that SAT3 is a relatively poor mediator of the Me-AIB uptake compared with the other isoforms (38) implies that SAT2 is the most likely isoform to mediate insulin-stimulated Me-AIB uptake in muscle. To test this proposition, we investigated the effects of insulin and chloroquine treatment on the subcellular distribution of SAT2 in L6 myotubes. Pretreatment of cells with 100 nM CQ for 2 h followed by insulin stimulation for 30 min was associated with a 2-fold increase in SAT2 (this was associated with an increase in the Vmax of System A transport from 388 ± 16 pmol/min/mg to 584 ± 16 pmol/min/mg (p < 0.05), with no significant change in Km). The increase in cell surface SAT2 was associated with a concomitant reduction in SAT2 abundance in membranes recovered from the 32/40% sucrose interface (data not shown). However, data from three separate experiments revealed that the hormone induced a greater than 2-fold increase in SAT2 in the PM-enriched fraction, indicating that this fraction was enriched with endosomal membranes. Pretreatment of L6 cells with CQ for 2 h prior to subcellular fractionation reduced the amount of SAT2 recovered in the PM fraction, a finding consistent with the idea that this compound prevents the delivery of proteins to the cell surface from the endosomal compartment without affecting the internalization of PM proteins that are constitutively recycling at the cell surface. The loss in PM SAT2 was not recovered in any of the other membrane fractions isolated from the sucrose gradient, raising the possibility that, once internalized, SAT2 may have been routed for lysosomal degradation. Unlike SAT2, pretreatment of L6 cells with CQ had no detectable effect on SAT3.
muscle cells were treated with insulin (100 nM, 30 min) alone or exposed to insulin alone and subsequent membrane isolation. Isolated PM fractions were resolved on SDS-gels and immunoblotted with antibodies to SAT2 or the α1-subunit of the Na,K-ATPase (used as loading control). The data shown in B are from two separate experiments.

![Scheme illustrating our current model for the insulin-mediated activation of System A in skeletal muscle.](image)

In summary, we have shown that treatment of skeletal muscle cells with insulin increases System A amino acid transport due to the translocation of SAT2 protein from an intracellular pool (which contains a high abundance of the endosomal marker annexin II) to the plasma membrane. CQ, a substance that disrupts the trafficking of endosomal transferrin receptors to the plasma membrane, prevents both the recruitment of SAT2 from this internal pool and the increase in System A activity following insulin treatment. Furthermore, disrupting actin microfilaments with CD moderately increases both System A activity and transferrin binding, consistent with a role for the actin network in endocytosis from the plasma membrane. Collectively these results imply that insulin stimulates System A activity by increasing the exocytosis of the SAT2 amino acid transporter from an intracellular endosomal location to the plasma membrane. Our present working model for the hormonal regulation of skeletal muscle System A transport is shown in Fig. 8. Future experiments will seek to determine the nature of the endosomal SAT2 vesicles in greater detail and how the endosomal and cytoskeletal systems interact with insulin signaling networks to regulate System A activity.

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