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ARTICLES

Modulation of Amino Acid Uptake by TGF-β in Lung Myofibroblasts

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Abstract Hormones such as insulin, growth factors, and cell stress stimulate system A amino acid transporter. Transforming growth factor-β (TGF-β) stimulates amino acid uptake thereby inducing cell proliferation, cellular hypertrophy, and matrix synthesis. Insulin appears to activate amino acid in smooth muscle cells via a phosphatidylinositol 3-kinase (PI3-kinase)-dependent pathway. We examine the effect and interaction of TGF-β, insulin, and PI3-kinase activity on amino acid uptake in human lung myofibroblasts. TGF-β treatment induced large increases in system A activity and a small delayed increase in the phosphorylation of protein kinase B, also termed phospho-Akt. In contrast, insulin induced small increases in system A activity and large increases in phospho-Akt levels. LY294002, a PI3-kinase inhibitor, blocked the TGF-β-induced amino acid uptake only partially, but completely blocked TGF-β-induced Akt phosphorylation. Moreover, the level of phospho-Smad3 was found to be high even when LY294002 blocked TGF-β-induced phospho-Akt levels. Inhibition of PI3-kinase activity resulted in increase in K_m, consistent with a major change in transporter activity without change in transporter number. The PI3-kinase inhibitor also did not change the amino acid transporter 2 (ATA2) mRNA levels. Taken together, these results suggest that TGF-β induced Smad-3 and amino acid uptake through a PI3-kinase independent pathway. J. Cell. Biochem. 99: 71–78, 2006.

Key words: amino acid transport; transforming growth factor-β; phosphatidylinositol 3-kinase; lung; fibroblast

Activation of fibroblasts by growth factors involves a complex alteration in cellular metabolism that includes upregulation of amino acid transport. The amino acids can then be used for synthesis of new protein, as well as, for metabolic fuel. Unlike other transport systems, the sodium-dependent system A transporter for short chain amino acids is highly regulated by treatment with growth factors or nutritional deprivation (adaptive regulation) [Gazzola et al., 1981]. Insulin and insulin-like growth factor I increase amino acid transport through the activation of system A in muscle cells [Hyde et al., 2002]. The ability to transport amino acid is determined by the number of transporters and the rate of transport. Previously, we reported that exposure to prostaglandin E_2 (PGE_2) or retinoic acid, resulted in a rapid decrease in amino acid transport that is primarily mediated by decreasing the rate of transport [Goldstein et al., 1986; Krupsky et al., 1993]. In contrast, insulin increased system A activity in L6 muscle cells by stimulating the exocytosis of system A amino acid transporter 2 (ATA2) from an endosomal compartment [Hyde et al., 2002]. This process did not require protein synthesis. The cellular response to amino acid deprivation involves the production and insertion of new carriers into the membranes. This process is slower and involves induction of amino acid transporter mRNA levels [Gazzola et al., 1981].

Three amino acid transporter system A isoforms were recently cloned, ATA1, ATA2, and ATA3 [Hatanaka et al., 2000; Gazzola et al., 2001; Bode et al., 2002]. ATA1 is highly

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expressed in neuronal tissue and is an efficient glutamine transporter, whereas ATA2 is widely expressed in tissues including the lung. ATA3 is expressed almost exclusively in the liver and is comparatively less effective at mediating the transport of methyl-aminoisobutyric acid (methyl-AIB), a specific model substrate for system A [Hatanaka et al., 2001]. The signal transduction system that activates the system A transporter is not well described. In this report, we examine the activation of system A transporter by transforming growth factor-β (TGF-β). We find that stimulation of transporter activity is both dependent and independent of phosphatidylinositol 3-kinase (PI3-kinase) activity.

MATERIALS AND METHODS

Cell Culture

Human embryonic lung fibroblasts (IMR-90, Coriell Institute for Medical Research, Camden, NJ) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U penicillin/ml, 10 μg streptomycin/ml, 0.1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. After confluence was achieved, the serum content of the medium was reduced to 0.4% FBS for 6–24 h to render the cells quiescent. Experiments were conducted in 0.4% FBS. LY294002 was purchased from Calbiochem (San Diego, CA). TGF-β was purchased from R&D systems (Minneapolis, MN). RBI (SB 431542) and insulin was purchased from Sigma.

Electrophoresis and Immunoblot

Fibroblasts were seeded out to 100-cm tissue culture dishes. Confluent quiescent cells were then treated with LY294002, TGF-β, insulin, or RBI and were harvested after 24 h and 1 h. The cells were lysed in radioimmunoprecipitation buffer (RIPA) on ice for 30 min and centrifuged at 14,000*g* for 10 min at 4°C. Protein samples (100 μg) were suspended in SDS-PAGE sample buffer with LDL sample reducing agent, heated at 70°C for 10 min, and separated using 4–12% Bis–Tris gels (NuPAGE). Proteins were transferred to nitrocellulose membranes and blocked for 1 h with 5% evaporated milk in phosphate-buffered saline (PBS) with 0.1% Tween-20 (PBST). Polyclonal antibodies specific for Akt (Ser 473) and Smad3 (Ser 433/435) (Cell Signaling) were diluted 1:1,000 in the same solution and added for 24 h at 4°C. After three washes with PBST, membranes were incubated with horseradish peroxidase-coupled anti-rabbit IgG antibody (Promega) at a dilution of 1:3,000 for 1 h at room temperature. The blots were then washed thrice with PBST and developed using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Amino Acid Uptake Assay

Confluent quiescent fibroblast cultures were washed twice with Hanks' balanced salt solution (BSS) and then incubated in serum-free medium containing the radiolabeled amino acid at 37°C for the indicated time period, as previously described [Goldstein et al., 1986; Krupsky et al., 1993]. The incubations were terminated by washing the cultures three times with ice-cold Hanks' BSS and the cellular material was extracted with 1 ml of 10% trichloroacetic acid (TCA). Non-specific binding was determined by immediately removing media containing the radiolabeled amino acid. The amount of TCA-soluble radiolabeled amino acids was determined by scintillation counting. 2-amino-[1-^{14}C] isobutyric acid, 2-(methylamino)-[1-^{14}C] isobutyric acid, and L-[4, 5-^{3}H] leucine were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

Real-Time RT-PCR Analysis

The expression of ATA2 mRNA was evaluated by real-time PCR using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Reverse transcription reactions were initially carried out in the PTC-150 MiniCycler (MJ Research, Waltham, MA). Real-time amplification of the cDNA was done using TaqMan Universal Master Mix (ABI) and the TaqMan Gene Expression Assays 20× mix according to the manufacturer's protocol. Each sample was amplified in triplicate and normalized to GAPDH expression. The results were interpreted by the comparative threshold cycle value method ($\Delta\Delta C_T$) for relative quantification of gene expression.

Statistics

A *t*-test was used for means of unequal size. Probability values less than 0.05 were considered significant.

RESULTS

We examined the effect of TGF-β on system A amino acid uptake in lung myofibroblasts. The system A amino acid transporter is hormone-sensitive and sodium-dependent. The effect of TGF-β and insulin on system A amino acid transport was studied by treating human lung fibroblasts with TGF-β and insulin for 24 h and the uptake of the amino acid analog 2-amino-[1-^{14}C] isobutyric acid was determined. TGF-β induced a large increase in system A activity (129%) ($P = 0.02$), whereas, insulin induced a minimal increase in activity (18%) ($P = 0.09$). TGF-β and insulin, together, increased the system A activity by another 24% more than TGF-β alone ($P = 0.6$) (Fig. 1A). TGF-β and insulin, however, had no significant effect on the mRNA levels of ATA2 (Fig. 1B).

Previous studies suggested a role for PI3-kinase and Akt in mediating growth factor-induced increases in transport [Boerner et al., 1985; Duclos et al., 1993; Durante et al., 1996; Obata et al., 1996; Higaki and Shimokado, 1999]. We examined the effect of insulin and TGF-β on phospho-Akt levels using Western analysis. At 1 h following TGF-β stimulation, there was no change in the phosphorylation of Akt, whereas phospho-Smad3 levels were increased (Fig. 2A—lane 4). At 1 h, insulin induced a large increase in phospho-Akt levels (Fig. 2A—lane 5). At 24 h following TGF-β or insulin stimulation, Akt phosphorylation was increased approximately twofold (Fig. 2B—lanes 4 and 5). The delayed increase in phospho-Akt levels by TGF-β is more likely an indirect effect rather than through the direct action of the TGF-β signal transduction pathway. To determine the effect of endogenous TGF-β on basal phospho-Akt, we employed a TGF-β receptor antagonist, RBI (Fig. 2B—lanes 3 and 6). Basal phospho-Akt levels were decreased after treatment with a TGF-β receptor antagonist.

In order to investigate the role of PI3-kinase activity on the system A amino acid transport, we inhibited PI3-kinase with LY294002 to block Akt phosphorylation. We found that though AIB uptake was decreased by 59% in human fibroblasts ($P = 0.00005$) (Fig. 3A), there was no significant decrease in the level of ATA2 mRNA (Fig. 1B). We employed LY294002 to determine the contribution of induced phospho-Akt to amino acid uptake stimulated by TGF-β and insulin. TGF-β induced uptake by 99% ($P = 0.00003$) (Fig. 3A) even in the presence of LY294002 while insulin induced uptake by 45% ($P = 0.0005$) (Fig. 3B). Together, TGF-β and insulin induced uptake by 111% ($P = 0.0005$) (Fig. 3C). Inhibition of PI3-kinase with LY294002 partially blocked TGF-β and/or insulin-induced uptake, and completely blocked insulin and/or TGF-β-induced Akt phosphorylation (Fig. 2B—lanes 7, 8, and 9). These results suggest a PI3-kinase independent and dependent pathway, for system A amino acid transport.

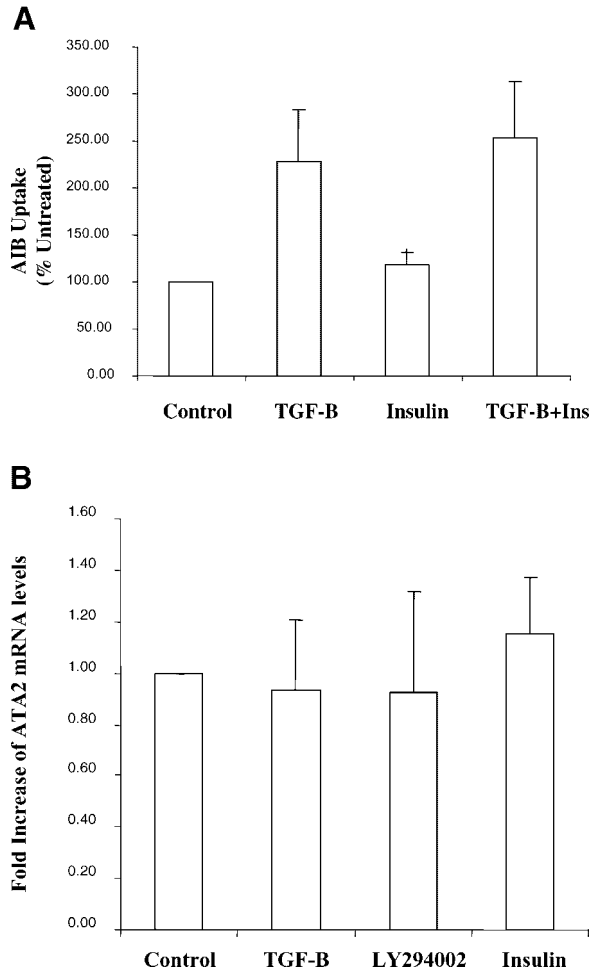

Fig. 1. **A**: Effect of TGF-β and insulin on 2-amino-[1-^{14}C] isobutyric acid (AIB) uptake. Cells were incubated in the presence of TGF-β (5 ng/ml) and/or insulin (2 μg/ml) for 24 h. AIB uptake was measured at a concentration of 0.1 mM after an incubation period of 10 min. **B**: Effect of TGF-β, LY294002, and insulin on the mRNA levels of ATA2. Cells were treated or untreated with TGF-β, LY294002, and insulin for 24 h. The ATA2 mRNA and GAPDH levels were assessed by real-time PCR. ATA2 mRNA expression was normalized to GAPDH expression. Data expressed as mean ± SD, n = 3.

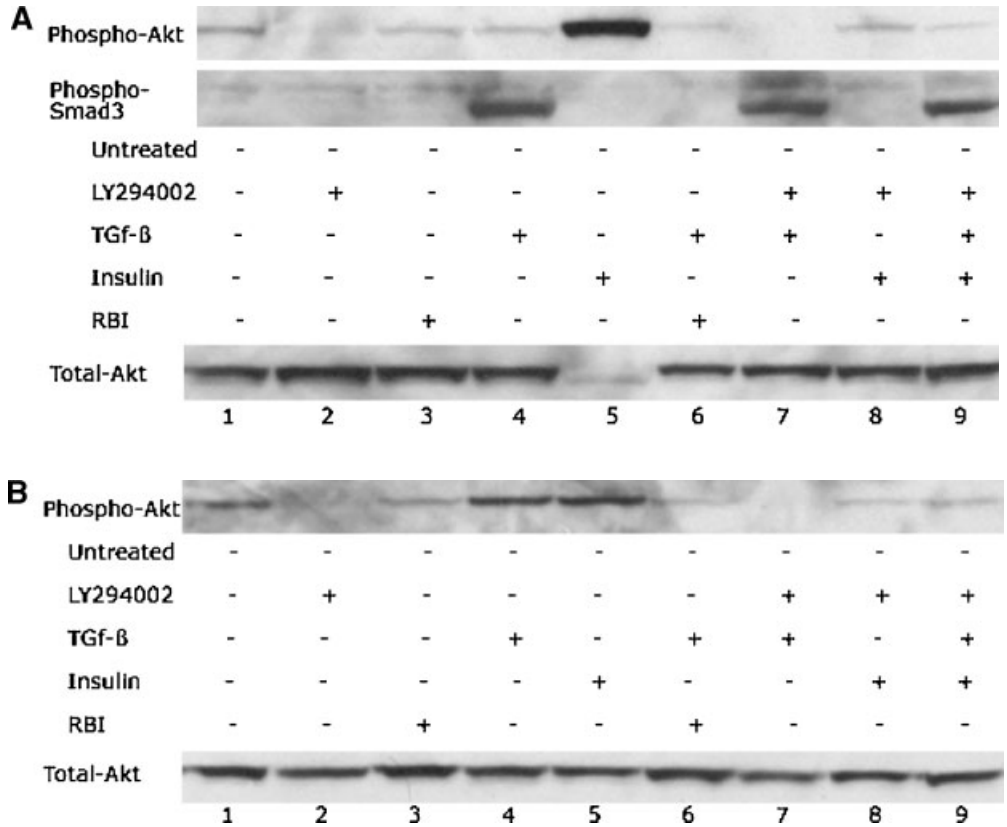

Fig. 2. **A**: Akt and Smad3 phosphorylation after 1 h treatment with TGF-β, insulin, LY294002, and/or RBI. Protein samples (100 μg) were separated by PAGE and the blots were probed with phospho-Akt and phospho-Smad3 antibodies. **B**: Akt phosphorylation after 24 h treatment with TGF-β, insulin, LY294002, and/or RBI. Aliquots of cell lysates (100 μg) were separated on SDS-PAGE and probed with anti-phospho-Akt antibody. Total Akt levels indicate the uniformity in the amounts of protein used for the analysis. Blots from a representative experiment are grouped in the figures.

These results also suggested that Akt levels regulated basal transporter activity.

We confirmed that LY294002 decreased AIB transport by inhibiting system A activity and not other transporters, such as system ASC or system L by employing 2-(methylamino)-[1-^{14}C] isobutyric acid (methyl-AIB), a substrate specific for the system A transporter [Franchi-Gazzola et al., 2001]. LY294002 decreased the uptake of methyl-AIB by 66% (untreated 1084 ± 74, LY294002 treated 365 ± 119, mean $\pm$ SD, $n = 3$, $P = 0.00005$). The effect of LY294002 on the sodium-independent system L amino acid transporter was assessed using L-[4, 5-^{3}H] leucine (Fig. 4A). Leucine uptake was not affected by LY294002 at early time points; however, uptake was decreased 29% at 6 h and 48% at 24 h.

The rate of amino acid uptake was determined at different time points following the addition of LY294002 to fibroblast cultures (Fig. 4B). Inhibition of PI3-kinase resulted in a rapid and persistent decrease in system A transport as assessed by AIB uptake. The mechanism by which LY294002 inhibited AIB uptake was defined by kinetic studies (Fig. 5). In fibroblasts treated with LY294002, the apparent K_m of the system A transporter increased 3.4-fold and the V_{max} increased slightly.

The influence of LY294002 on system A activity was dose-dependent (Fig. 6). The recovery of system A activity following LY294002 treatment was examined by washing the cells free of LY294002 and maintaining cultures in fresh media with 0.4% FBS. The cells rapidly recovered from the effect of LY294002 as system A activity increased 40% at 2 h ($P = 0.01$) (Fig. 7). After 6 h, the activity had increased by 105.7% ($P = 0.02$) and by 24 h, the cells had completely overcome the effect of LY294002 ($P = 0.0003$).

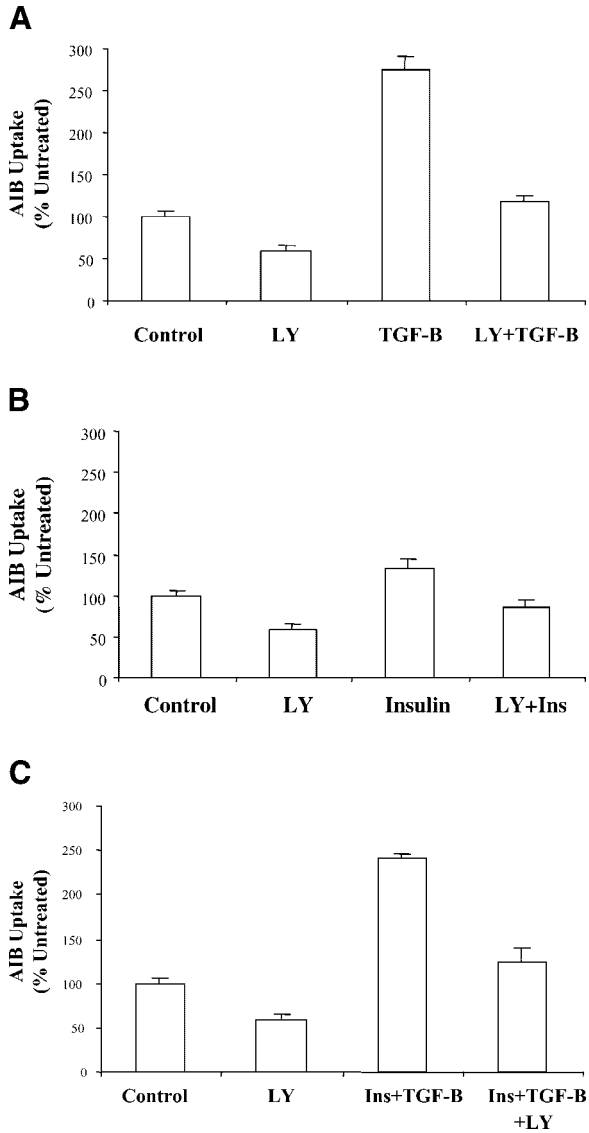

Fig. 3. **A**: Effect of TGF-β on LY294002-induced decreases in AIB uptake. Cells were incubated in the presence or absence of LY294002 (20 μM) or TGF-β (5 ng/ml) for 24 h. **B**: Effect of insulin on LY294002 induced decrease in AIB. Cells were incubated in the presence or absence of LY294002 (20 μM) or insulin (2 μg/ml) for 24 h. **C**: Combined effect of TGF-β and insulin on LY294002 induced decreases in AIB uptake. Cells were incubated in the presence or absence of LY294002 (20 μM), TGF-β (5 ng/ml) and insulin (2 μg/ml) for 24 h. AIB uptake was measured at a concentration of 0.1 mM after an incubation period of 10 min.

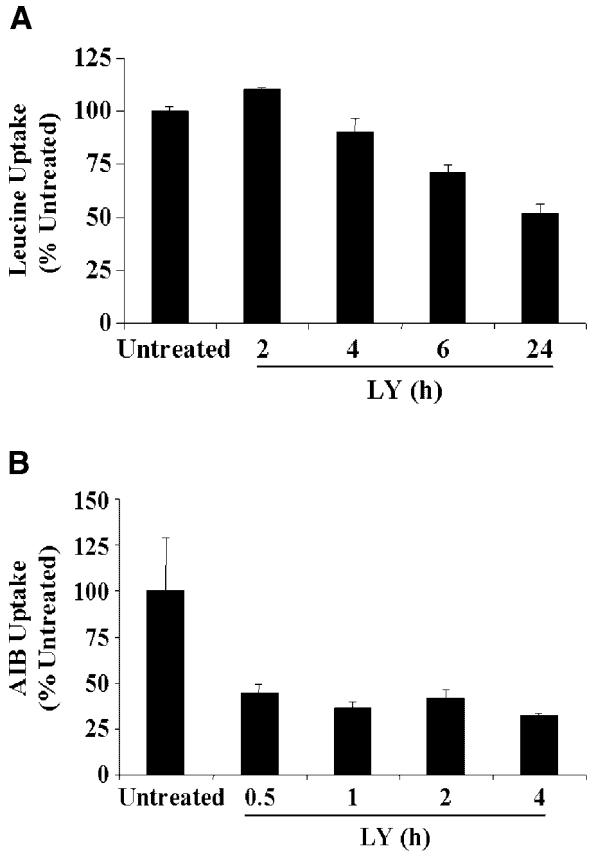

Fig. 4. Time-course analyses of the effect of LY294002 on the uptake of L-[4, 5-^{3}H] leucine and AIB in human lung fibroblasts. Fibroblast cultures were left untreated or treated with LY294002 (LY) 25 μM for varying times as indicated. The medium was replaced with medium containing (**A**) leucine or (**B**) AIB, and uptake was determined. Data are expressed as mean ± SD, n = 3.

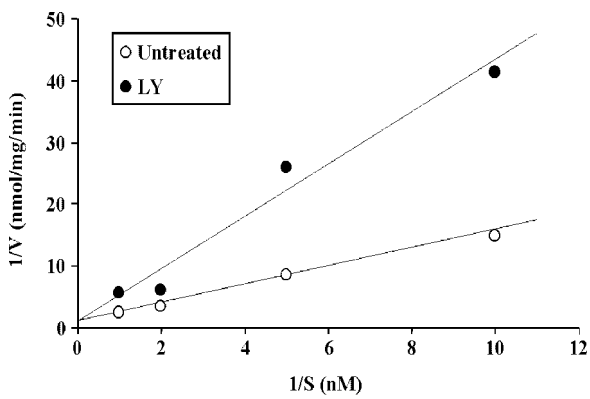

Fig. 5. Lineweaver–Burk Kinetics: Reciprocal velocity of AIB uptake plotted against reciprocal of AIB concentration. Fibroblast cultures were left untreated (open circles) or treated with LY294002 (LY) 25 μM (closed circles) for 24 h. Uptake of AIB was determined at the indicated concentration of AIB. Velocity is expressed as nmol/mg/min, n = 2. The V_{max} in untreated and LY294002-treated cultures were 0.95 and 1.08, respectively. The apparent K_m in untreated and LY294002-treated cultures were 1.32 and 4.52, respectively.

DISCUSSION

During conditions of amino acid deprivation, fibroblasts regulate the transport of neutral amino acids through the system A by adaptive mechanisms [Gazzola et al., 1981]. The abundance of ATA2/SAT2 mRNA strictly parallels the transport activity of system A during

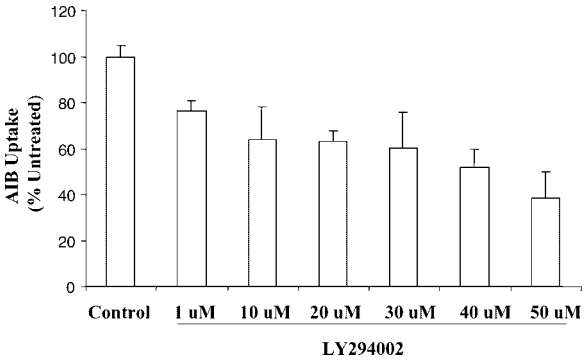

Fig. 6. Dose dependence of the influence of LY294002 on AIB uptake in IMR-90 cells. Cells were treated with increasing concentration of LY294002 for 24 h at 37°C following which AIB uptake was measured at a concentration of 0.1 mM after incubation for 10 min. Results are given as percent of corresponding control.

adaptive regulation [Gazzola et al., 2001]. In addition to adaptive regulation, growth factors, hormones such as insulin, and cell stress stimulate system A [Hyde et al., 2002]. We found that TGF-β was a strong activator of amino acid uptake in lung myofibroblasts, whereas, insulin was relatively weak. Moreover, we found that amino acid uptake was dependent on basal phospho-Akt activity but was not further enhanced by hormonal activation.

Inhibition of basal PI3-kinase activity by LY294002 induced a rapid and persistent decrease in system A amino acid transporter activity in human lung fibroblasts, but did not affect a significant change in the ATA2 mRNA levels. Insulin induces the uptake of amino acids in vascular smooth muscle cells (VSMC) but little information is available related to the effects of this hormone on uptake in fibroblasts [Obata et al., 1996; Hyde et al., 2002]. In our experiments, insulin induced a minimal increase in amino acid uptake, and large increase in Akt phosphorylation. In other systems, insulin increases system A transporter activity in the plasma membrane in a PI3-kinase dependent manner [Sanchez-Margalet et al., 1994; Le Marchand-Brustel et al., 1995; Su et al., 1998; Higaki and Shimokado, 1999]. In L6 cells, insulin stimulates the PI3-kinase-dependent exocytosis of ATA2 transporters from an endosomal compartment [Hyde et al., 2002]. In contrast, our results demonstrate that in lung fibroblasts, basal amino acid transport is dependent on PI3-kinase activity but further activation of Akt did not stimulate transport.

Kinetic studies demonstrated that inhibition of PI3-kinase resulted in large increases in the apparent K_m of the system A transporter, whereas, only small increases in the V_{max} were noted. LY294002 rapidly reduced system A transport and this effect increased with increasing doses of the inhibitor. We tested the toxicity of the dose used for this experiment (20 μM) by studying the recovery of the cells from the effect of the inhibitor. As previously reported, this

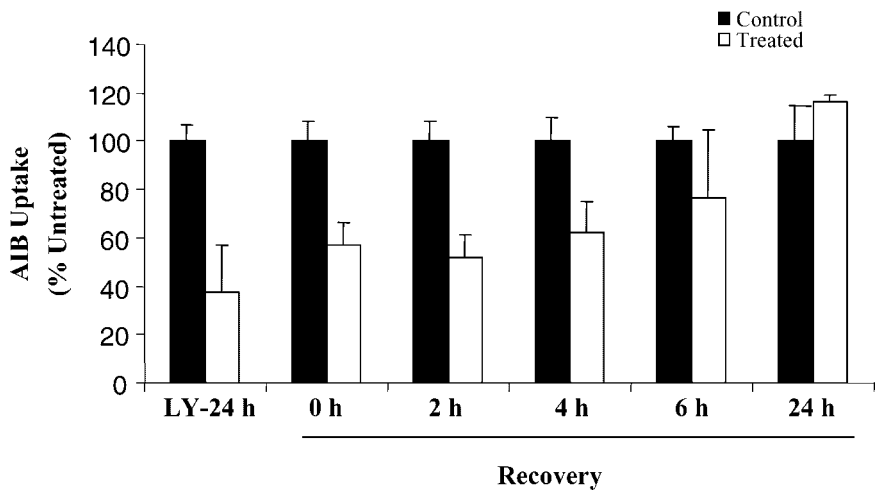

Fig. 7. Recovery effect of the LY294002-treated IMR-90 cells on AIB uptake. Cells were treated with (open bars) or without (solid bars) LY294002 (20 μM) for 24 h at 37°C. The cells were washed free of LY294002 and kept in fresh media containing 0.4% BS and the cells were allowed to recover for indicated time periods (2–24 h) at 37°C. AIB uptake was measured at a concentration of 0.1 mM after an incubation period of 10 min. Results are given as percent of corresponding control.

dosage was not toxic to fibroblasts [Ricupero et al., 2001]. The uptake of amino acids recovered rapidly in the first 2 h and by 24 h was restored to baseline, indicating that the effect of LY294002 was reversible and that the dosage used was not toxic. Moreover, LY294002 had a minimal effect on the activity of the system L transporter at early time points.

We found that TGF-β increased system A amino acid uptake by more than twofold without affecting steady state levels of ATA2. In contrast, TGF-β was shown to stimulate L-proline transport by inducing the system A amino acid transporter gene expression in VSMC [Ensenat et al., 2001]. TGF-β treatment did not affect phospho-Akt at 1 h but induced a twofold increase at 24 h. TGF-β activates PI3-kinase, thereby, initiating a signal transduction cascade, whereby, Akt is phosphorylated at serine 473 [Franke et al., 1997; Higaki and Shimokado, 1999]. In lung fibroblasts, LY294002 completely blocked TGF-β-induced phospho-Akt levels but did not inhibit TGF-β-induced amino acid uptake albeit from a lower baseline. These data indicate that TGF-β appeared to utilize a PI3-kinase independent pathway to induce amino acid uptake. This pathway likely involves the Smad family of transcriptional activators where the receptor-activated Smads, Smad2/Smad3, are phosphorylated directly by TGF-β receptor 1 kinase. We found that phospho-Smad3 levels were increased by TGF-β stimulation even in the presence of LY294002 within an hour.

Smads are believed to assist in the formation of a transcriptional complex, the activity of which is regulated by other signaling cascades. TGF-β/Smad signaling is also tightly controlled by MAP kinase signaling cascades [Attisano and Wrana, 2002; Leask and Abraham, 2004]. The induction of ERK MAPK and JNK signaling by TGF-β itself may regulate Smad activation and signaling [Derynck and Zhang, 2003]. In our studies, PI3-kinase activity was not required by TGF-β to activate Smad3 or amino acid uptake. These results demonstrate a PI3-kinase independent pathway utilized by TGF-β to induce amino acid uptake.

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