Transforming growth factor-β1 stimulates vascular smooth muscle cell L-proline transport by inducing system A amino acid transporter 2 (SAT2) gene expression

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Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that contributes to arterial remodelling by stimulating vascular smooth muscle cell (SMC) growth and collagen synthesis at sites of vascular injury. Since L-proline is essential for the synthesis of collagen, we examined whether TGF-β1 regulates the transcellular transport of L-proline by vascular SMCs. L-Proline uptake by vascular SMCs was primarily sodium-dependent, pH-sensitive, blocked by neutral amino acids and α-(methylamino)isobutyric acid, and exhibited *trans*-inhibition. Treatment of SMCs with TGF-β1 stimulated L-proline transport in a concentration- and time-dependent manner. The TGF-β1-mediated L-proline uptake was inhibited by cycloheximide or actinomycin D. Kinetic studies indicated that TGF-β1-induced L-proline transport was mediated by an increase in transport capacity independent of any changes in the affinity for L-proline. TGF-β1 stimulated the expression of system A amino acid transporter 2 (SAT2) mRNA in a time-dependent fashion that paralleled the increase in L-proline transport. Reverse transcriptase PCR failed to detect the presence of SAT1 or amino acid transporter 3 (ATA3) in either untreated or TGF-β1-treated SMCs. These results demonstrate that L-proline transport by vascular SMCs is mediated predominantly by the SAT and that TGF-β1 stimulates SMC L-proline uptake by inducing the expression of the SAT2 gene. The ability of TGF-β1 to induce SAT2 expression may function to provide SMCs with the necessary levels of L-proline required for collagen synthesis and cell growth.

Key words: amino acid transport, cell growth, collagen, growth factor.

INTRODUCTION

L-Proline is a cyclic amino acid that is involved in a variety of physiological processes, including lipogenesis, glycogen synthesis and cell growth [1–3]. In addition, L-proline is an important neuromodulator in the central nervous system and an essential precursor for the synthesis of many structural proteins [4,5]. Indeed, the amino acid composition of collagen is distinctive for its high content of L-proline and its derivative hydroxy-L-proline [6]. Although amino acids can be obtained from endogenous synthesis, most cells rely on extracellular transport for their amino acid requirements. The system A amino acid transporter (SAT) mediates the sodium-coupled, pH-sensitive cellular uptake of short-chain neutral amino acids, including L-proline [7–9]. It is distinguished from other neutral amino acid transporters by the fact that it recognizes *N*-methylamino acids such as α-(methylamino)isobutyric acid (MeAIB) and exhibits *trans*-stimulation [7–10]. The SAT is the major amino acid system responsible for the net uptake of neutral amino acids and plays a key role in the overall flux of amino acids into cells. Two important features of the SAT are that its activity is regulated in a dynamic fashion by growth factors and that its induction is associated with cell growth [8,9].

Recently, genes encoding the proteins responsible for the activity of the system A carrier have been identified. A neuronal sodium-dependent glutamine transporter (GlnT; now renamed SAT1) was recently cloned and, based on its functional characteristics, was proposed as a member of the system A family of transporters [11]. Subsequent work from two independent laboratories led to the cloning of a second member of this family, SAT2 [also known as amino acid transporter (ATA) 2] [12–15]. Finally, recent work has identified a third member of the system A family of transporters, which has been designated as ATA3 [16]. These transport proteins show $\approx 50\,\%$ sequence similarity at the amino acid level and demonstrate different tissue distributions. Both SAT1 and SAT2 are high-affinity transporters ($K_m \approx 200$–$300\ \mu$M); however, they exhibit different tissue distributions. SAT1 is found primarily in the brain whereas SAT2 is expressed ubiquitously in mammalian tissues [11–15]. In contrast, ATA3 is a low-affinity transporter ($K_m \approx 4$–6 mM) for neutral amino acids that is expressed almost exclusively in the liver [16,17]. Presently, little is known regarding the expression and regulation of these transport proteins by vascular cells.

Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that plays an important role in development, differentiation, inflammation and tissue repair [18]. In the vessel wall, TGF-β1 plays a critical role in arterial remodelling by stimulating vascular smooth muscle cell (SMC) proliferation and the deposition of collagen and other extracellular matrix proteins [19–21]. Since L-proline is essential for collagen synthesis and cell growth, the present study examined the effect of TGF-β1 on the uptake of L-proline by vascular SMCs. Here we report that the transport of L-proline by vascular SMCs is primarily mediated via the system A carrier. In addition, we demonstrate that TGF-

Abbreviations used: MeAIB, α-(methylamino)isobutyric acid; SAT, system A amino acid transporter; ATA, amino acid transporter; TGF-β1, transforming growth factor-β1; SMC, smooth muscle cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase.

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β1 up-regulates SMC L-proline transport by inducing the expression of the SAT2 gene. The ability of TGF-β1 to induce the transcellular transport of L-proline may function to provide SMCs with the necessary levels of L-proline required for SMC collagen synthesis and growth.

MATERIALS AND METHODS

Materials

Collagenase, elastase, amino acids, SDS, Tris, Tes, Hepes, acrylamide, BSA, cycloheximide, actinomycin D and MeAIB were purchased from Sigma (St. Louis, MO, U.S.A.); penicillin, streptomycin and calf serum were from Gibco BRL (Rockville, MD, U.S.A.); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and RNA molecular-mass markers were from Ambion (Austin, TX, U.S.A.); TGF-β1 was from R & D Systems (Minneapolis, MN, U.S.A.); bicinchoninic acid protein assay was from Pierce (Rockford, IL, U.S.A.); [2,3,4,5-^{3}H]L-proline (105 Ci/mmol) and [α-^{32}P]UTP (400 Ci/mmol) were from Amersham Life Sciences (Arlington Heights, IL, U.S.A.).

SMC culture

Vascular SMCs were isolated by elastase and collagenase digestion of rat thoracic aortas and characterized by morphological and immunological criteria [22]. Cells were cultured serially in minimum essential medium containing 10 % calf serum, Earle's salts, 5.6 mM glucose, 2 mM L-glutamine, 5 mM Tes, 5 mM Hepes, 100 units/ml penicillin and 100 units/ml streptomycin. Subcultured strains were used between passages 6 and 28. When cells reached confluence, the culture medium was replaced with serum-free medium containing BSA (0.1 %) for 24 h and then exposed to the various treatment regimens.

L-Proline transport

L-Proline transport was determined by measuring the influx of radiolabelled L-proline into SMCs. Cells grown on 12-well plates were washed with Hepes buffer (140 mM NaCl, 5 mM KCl, 0.9 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM D-glucose and 25 mM Hepes, pH 7.4) and then incubated for various times in Hepes buffer containing 50 μM L-[^{3}H]proline (1 μCi). In some experiments, NaCl was replaced with an equimolar concentration of choline chloride. Transport activity was terminated by aspirating the medium and washing the cells rapidly with ice-cold Hepes buffer. Cells were allowed to dry, and the cell-associated radioactivity was extracted with 0.2 % SDS in 0.2 M NaOH and then assayed by liquid scintillation spectroscopy. Protein in the NaOH extracts was measured using the bicinchoninic acid method with BSA as the standard. To correct for non-specific uptake, cells were incubated in parallel wells with Hepes buffer containing 10 mM unlabelled L-proline. The fraction of the radioactivity in the cells was then determined and this fraction was subtracted from each data point.

Generation of SAT and ATA3 probes

SAT cDNA fragments were amplified from SMCs by reverse transcriptase (RT)-PCR [23]. Primers were designed according to the published sequences of rat SAT1, SAT2 and ATA3 [11,15,16]. The forward and reverse primers 5′-TCTCATCCTGACTG-TGCGAC-3′ and 5′-ATGTTAGCAGACGTCACCCC-3′ were used to amplify a 231-bp SAT1 transcript, and the forward and reverse primers 5′-TGCAGAACATCGGAGCTATG-3′ and 5′-CCGCTGGTATACCCCAAGTA-3′were used to amplify a 202-bp SAT2 transcript. In addition, the forward and reverse primers 5′-ACCCTGGAACGACCTCTTTT-3′ and 5′-CCTTCCTTG-GCTGTCTTCAG-3′ were used to amplify a 187-bp ATA3 fragment. The cDNA was amplified in reaction mixture containing 2.5 mM $MgCl_2$, 0.2 mM of each nucleoside triphosphate (dATP, dTTP, dGTP and dCTP), SAT or ATA3 primers (50 pmol each) and *Taq* DNA polymerase (2.5 units/ml) in standard reaction buffer. Amplification consisted of 30 cycles of PCR [1 min at 94 °C for denaturing, 40 s at 60 °C for annealing and 90 s at 75 °C for elongation followed by a final 5 min at 75 °C]. Products of PCR amplification were resolved by agarose-gel electrophoresis, stained with ethidium bromide, visualized on a UV transilluminator and photographed. Products of expected sizes were subcloned into pCRII plasmids (Invitrogen, San Diego, CA, U.S.A.) and sequenced to confirm their identities and orientations.

mRNA analysis

SAT and ATA3 mRNA levels were determined by ribonuclease protection assay using a commercially available kit (Ambion). Total RNA (15 μg) was hybridized with $\approx 1 \times 10^6$ c.p.m. of [^{32}P]UTP-labelled antisense SAT, ATA3 and GAPDH (316 bp) riboprobes. For SAT and ATA3 analysis, samples were digested in a 1:200 dilution of ribonuclease A and T1, whereas a 2-fold higher concentration of ribonuclease was used for GAPDH digestion. Protected RNA was analysed by electrophoresis using 6 % acrylamide/8 mM urea gels. Gels were exposed overnight to X-ray film at -70 °C in the presence of intensifying screens. The sizes of the protected nucleotide fragments was confirmed using a ^{32}P-labelled RNA ladder. Relative mRNA levels were quantified by scanning densitometry (LKB 2222-020 Ultrascan laser densitometer, Bromma, Sweden) and normalized with respect to GAPDH mRNA.

Statistics

Results are expressed as means $\pm$ S.E.M. Statistical analysis was performed with the use of Student's two-tailed *t* test and ANOVA when more than two treatments were compared. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Specific transport of L-[^{3}H]proline by vascular SMCs increased over time and was linear for 30 min (Figure 1A). Substitution of sodium in the uptake buffer with equimolar amounts of choline dramatically reduced L-proline transport (Figure 1B). Approx. 70 % of L-proline uptake by SMCs occurred through a sodium-dependent pathway. Given the predominance of sodium-dependent transport, all subsequent experiments measured L-proline uptake over 15 min using sodium-containing buffer.

To characterize the substrate specificity of the L-proline transporter, the effects of various amino acids (10 mM) were studied. L-Proline transport was markedly inhibited by other neutral amino acids whereas anionic and cationic amino acids had no significant effect (Figure 2). Interestingly, the model substrate for the SAT, MeAIB, inhibited L-proline uptake by $\approx$ 80 % (Figure 2). L-Proline uptake by vascular SMCs was also pH-sensitive. Low extracellular pH inhibited L-proline transport whereas optimal rates of uptake were observed between pH 8.0 and 8.5 (Figure 3). In addition, preloading vascular SMCs with L-proline (10 mM) for 2 h inhibited L-proline uptake by $\approx$ 30 % (results not shown).

Treatment of vascular SMCs with TGF-β1 stimulated the transport of L-proline in a concentration-dependent manner

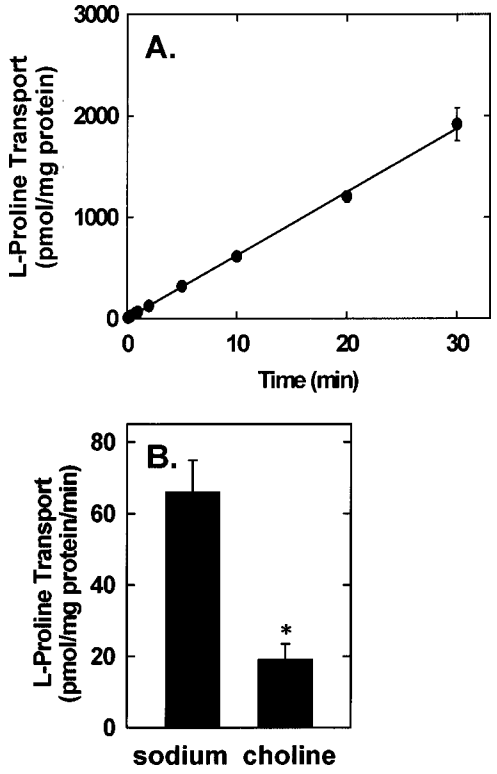

Figure 1 L-Proline transport by vascular SMCs

(**A**) Time course of L-proline uptake. Specific transport of L-[^{3}H]proline (50 μM) was measured at the indicated times in sodium-containing Hepes buffer. Results are means $\pm$ S.E.M. from four separate experiments, each performed in triplicate. (**B**) Effect of extracellular sodium on L-proline transport. Specific transport of L-[^{3}H]proline (50 μM) was measured for 15 min in sodium- or choline-containing Hepes buffer. Results are means $\pm$ S.E.M. from five separate experiments, each performed in triplicate. *, Statistically significant inhibitory effect of choline compared with sodium.

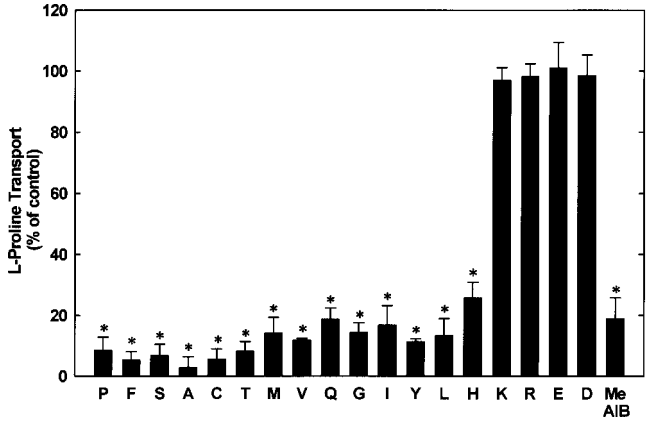

Figure 2 Inhibition of L-proline transport by amino acids in vascular SMCs

Specific transport of L-[^{3}H]proline (50 μM) was measured for 15 min in sodium-containing Hepes buffer in the presence of the indicated amino acids (10 mM). Results are means $\pm$ S.E.M. from four separate experiments, each performed in triplicate. *, Statistically significant effect of amino acid addition, as compared with a control in the absence of amino acids.

(Figure 4A). Time-course studies demonstrated that TGF-β1 significantly increased L-proline uptake by 4 h of pretreatment and this was increased further after 24 h of stimulation (Figure

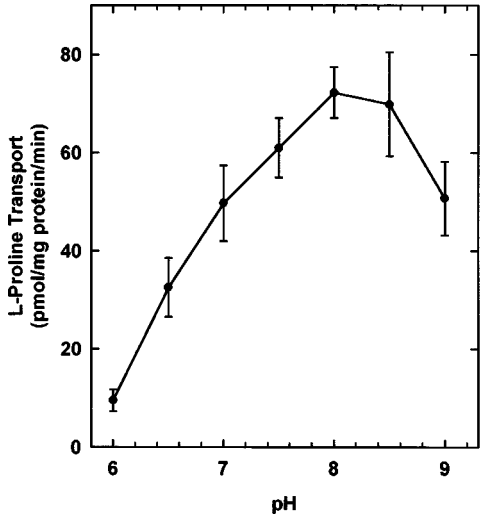

Figure 3 pH dependence of L-proline transport by vascular SMCs

Specific transport of L-[^{3}H]proline (50 μM) was measured for 15 min in sodium-containing Hepes buffers with different pH values. Results are means $\pm$ S.E.M. from five separate experiments, each performed in triplicate.

4B). The stimulation of L-proline transport by TGF-β1 was blocked by cycloheximide (5 μg/ml) or actinomycin D (1 μg/ml; results not shown). In the absence of TGF-β1, cycloheximide and actinomycin D affected L-proline transport only minimally (results not shown).

In subsequent kinetic experiments, saturable uptake of radiolabelled L-proline was measured. Figure 5 shows a representative Eadie–Hofstee plot demonstrating that saturable, high-affinity uptake of L-proline by vascular SMCs was mediated by a single carrier. Data from several experiments ($n = 5$) indicated that this transporter had a Michaelis constant (K_m) of 217 ± 29 μM and a maximum transport velocity (V_{max}) of 142 ± 19 pmol/mg of protein per min. Treatment of vascular SMCs with TGF-β1 (10 ng/ml) for 24 h had no effect on the K_m (233 ± 34 μM), but it significantly ($P < 0.05$) increased the V_{max} (276 ± 34 pmol/mg of protein per min) of L-proline transport.

RT-PCR identified a cDNA encoding SAT2 in vascular SMCs (Figure 6). However, it failed to detect cDNAs for either SAT1 or ATA3. No RT-PCR products were obtained with RNA samples in the absence of RT or when cDNA was omitted from the PCR reaction. Ribonuclease protection assays demonstrated that TGF-β1 stimulated the expression of SAT2 mRNA in a time-dependent manner (Figure 7). An increase in SAT2 message was observed following 4 h of TGF-β1 exposure and levels remained elevated after 24 h (Figure 7).

DISCUSSION

Plasma amino acid transport is a dynamic, tissue-specific process that involves discrete membrane-bound transport proteins. In the current study, we are the first to demonstrate that L-proline transport by vascular SMCs is mediated primarily by the SAT. The sodium-dependence, substrate specificity, pH-sensitivity and inhibition by MeAIB indicate that L-proline transport is mediated by the classical system A transport system [8,9]. In addition, loading SMCs with L-proline causes inhibition of L-proline

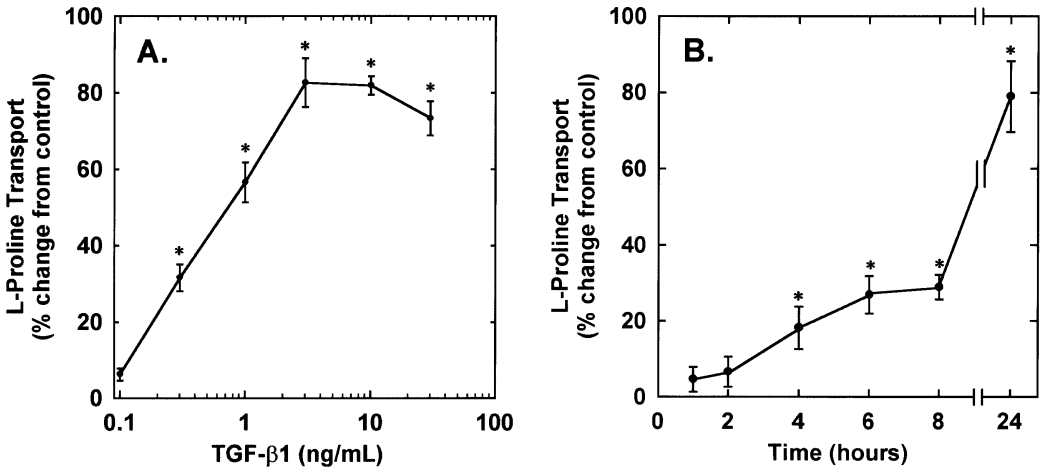

Figure 4 Effect of TGF-β1 on L-proline transport by vascular SMCs

(**A**) Concentration-dependent increase in L-proline transport by TGF-β1. SMCs were treated with TGF-β1 (0.1–30 ng/ml) for 24 h, then the specific transport of L-[^{3}H]proline (50 μM) was measured for 15 min in sodium-containing Hepes buffer. (**B**) Time course of TGF-β1-stimulated L-proline transport. Specific transport of L-[^{3}H]proline (50 μM) was measured for 15 min in sodium-containing Hepes buffer after preincubation with TGF-β1 (10 ng/ml) for the indicated times. Results are means $\pm$ S.E.M. from four or five separate experiments, each performed in triplicate. *, Statistically significant effect of TGF-β1 compared with a non-TGF-β1-treated control.

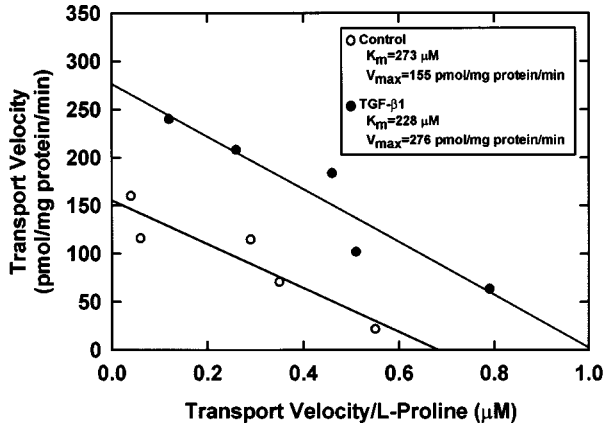

Figure 5 Representative Eadie–Hofstee plot of saturable L-proline transport in vascular SMCs

Specific transport of L-[^{3}H]proline (10–1000 μM) was measured for 15 min in sodium-containing Hepes buffer in untreated control SMCs (○) and in SMCs pretreated with TGF-β1 (10 ng/ml; ●) for 24 h. Data from one representative experiment are shown in the Figure. Cumulative data from five separate experiments indicated that this transporter had a mean K_m of 217 $\pm$ 29 μM and a mean V_{max} of 142 $\pm$ 19 pmol/mg of protein per min. Treatment of vascular SMCs with TGF-β1 for 24 h had no effect on the mean K_m (233 $\pm$ 34 μM), but it significantly ($P < 0.05$) increased the mean V_{max} (276 $\pm$ 34 pmol/mg of protein per min) of L-proline transport.

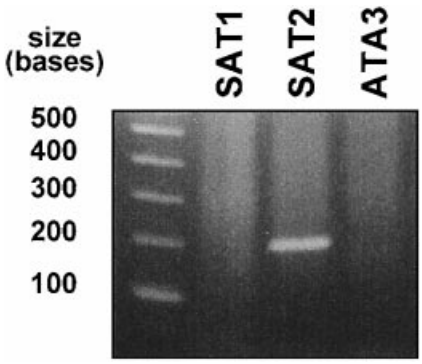

Figure 6 Ethidium-stained agarose electrophoresis gel showing PCR-amplified SAT2 cDNA from vascular SMCs

Similar findings were made in four separate experiments.

influx. This *trans*-inhibition is another characteristic feature of the SAT [10]. Minor amounts of L-proline are also transported via a sodium-independent transporter. Interestingly, MeAIB does not completely block the sodium-dependent uptake of L-proline, suggesting a small contribution by another transporter. Our finding of a major role for L-proline transport by the SAT in vascular SMCs is consistent with an earlier study examining L-proline transport in osteoblasts [24]. In the case of vascular cells, L-proline transport may play an important role in vessel wall remodelling and extracellular matrix (collagen) synthesis following vascular injury.

Both RT-PCR and ribonuclease protection assays identified SAT2 mRNA in vascular SMCs, similar to findings in other cell types [13–15]. In addition, SAT2's affinity for L-proline is similar to the affinity for L-proline transport ($K_m \approx$ 200–300 μM) by SMCs [14], suggesting that SAT2 mediates the transport of L-proline in these cells. The failure to detect SAT1 and ATA3 in SMCs is consistent with their restricted distributions. SAT1 is found exclusively in the brain whereas ATA3 is expressed primarily in the liver and skeletal muscle [11,16].

Treatment of vascular SMCs with TGF-β1 stimulates the transport of L-proline in both a time- and a concentration-dependent manner. Kinetic experiments indicate that TGF-β1 selectively increases the V_{max} without affecting the K_m of this transport system. These kinetic data suggest that the TGF-β1-induced increase in L-proline uptake probably arises from the *de novo* synthesis of additional transport proteins. Consistent with this, we found that cycloheximide blocks TGF-β1-induced

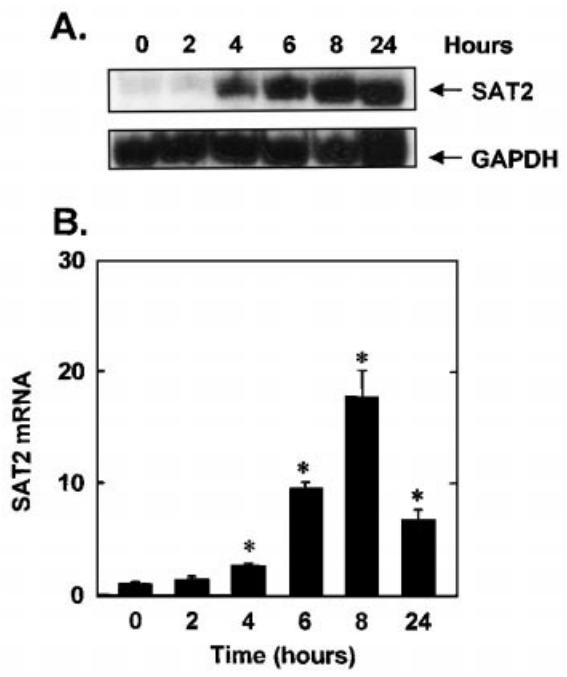

Figure 7 Effect of TGF-β1 on the expression of SAT2 mRNA by vascular SMCs

(**A**) Ribonuclease protection analysis of SAT2 and GAPDH mRNAs following treatment of SMCs with TGF-β1 (10 ng/ml) for the indicated times. (**B**) Quantification of relative SAT2 mRNA levels by laser densitometry after treatment with TGF-β1 (10 ng/ml). *, Statistically significant effect of TGF-β1 compared with a non-TGF-β1-treated control. SAT2 mRNA is expressed as arbitrary units.

transport. Moreover, we observed that TGF-β1 stimulates SAT2 mRNA expression. However, the maximum increase in SAT2 message preceded the maximum increase in L-proline transport. This may reflect the time required for the translation, post-translational modifications and insertion of the SAT2 protein into the plasma membrane to yield a functional transporter. Although the molecular mechanism by which TGF-β1 induces SAT2 gene expression is not known, it probably involves the transcriptional activation of the SAT2 gene, since the transcriptional inhibitor actinomycin D blocks this effect.

Despite several reports demonstrating that humoral agents stimulate system A activity [24–27], the molecular events associated with this activation are not known. However, results from our study indicate that SAT2 gene expression may contribute to the induction of system A activity by humoral stimuli. Interestingly, the induction of SAT2 gene expression has also been implicated in the substrate-induced adaptive up-regulation of system A amino acid transport activity, suggesting a general mechanism by which neutral amino acid transport is regulated [28,29].

The concentration of TGF-β1 (0.3–30 ng/ml) required to stimulate L-proline transport is physiologically relevant. Serum levels of TGF-β1 range from 2 to 10 ng/ml in healthy individuals [30]. However, at sites of vascular injury, where vessel wall TGF-β1 synthesis is induced and activated platelets release TGF-β1, the local concentration of this cytokine may be significantly higher.

The ability of TGF-β1 to stimulate SAT2 gene expression may be of pathophysiological significance. Considerable evidence indicates that TGF-β1 plays an important role in stimulating intimal thickening at sites of vascular injury. Arterial injury results in a marked increase in TGF-β1 expression that correlates with SMC proliferation and collagen synthesis [20]. In addition, inhibition of TGF-β1 activity by injection of neutralizing antibodies to TGF-β1 or a soluble TGF-β1 receptor significantly diminishes SMC growth and collagen formation following vascular injury [31,32]. Thus our finding that physiologically relevant concentrations of TGF-β1 stimulate SAT2 gene expression may provide a mechanism by which increased levels of precursor (L-proline) are provided to SMCs during periods of collagen formation. Moreover, we found recently [33] that TGF-β1-mediated increases in collagen production are associated with a marked rise in the intracellular synthesis of L-proline by vascular SMCs. These results suggest that TGF-β1-mediated increases in both the transcellular transport and intracellular synthesis of L-proline are co-ordinated to maximize the cellular capacity for collagen biosynthesis. Since SAT2 also mediates the uptake of other neutral amino acids, the induction of SAT2 by TGF-β1 may also serve to provide the necessary amino acids required for the synthesis of new proteins during SMC growth. In support of this proposal, system A activity correlates with cell growth in many cell types [8,9]. Moreover, induction of system A activity is recognized as a permissive step in the early phases of liver regeneration [8]. Thus the ability of TGF-β1 to stimulate SAT2 gene expression may contribute to its fibrotic and mitogenic actions at sites of vascular injury.

In summary, we have demonstrated that vascular SMC L-proline transport is mediated by the system A amino acid transporter. In addition, we found that TGF-β1 stimulates L-proline uptake by inducing expression of the SAT2 gene. The ability of TGF-β1 to up-regulate the transport of L-proline and other neutral amino acids may contribute to arterial remodelling at sites of vascular injury by providing the necessary precursors required for vascular SMC collagen deposition and growth.

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