Chapter 5

TGF-β inhibits Ang II-induced MAPK p44/42 signaling in vascular smooth muscle cells by Ang II type 1 receptor downregulation

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Abstract
Vascular changes in diabetes are characterized by reduced vasoconstriction and vascular remodeling. Previously, we demonstrated that TGF-ß1 impairs AngII-induced contraction through reduced calcium mobilization. However, the effect of TGF-ß1 on AngII-induced vascular remodeling is unknown. Therefore, we investigated the effect of TGF-ß1 on AngII-induced activation of the MAPK-p44/42 pathway in cultured rat aortic smooth muscle cells (RASMC).

Activation of MAPK p44/42 was determined with a phospho-specific antibody. Angiotensin type 1 receptor (AT₁) and AT₁ mRNA levels were measured by [³H]-candesartan-binding and real-time PCR, respectively. AT₁-gene transcription activity was assessed using AT₁-promoter-reporter constructs and by a nuclear runoff assay.

In TGF-ß1 pretreated cells, AngII-induced phosphorylation of MAPK-p44/42 was inhibited by 29% and 46% for p42 and p44, respectively, and AT₁ density was reduced by 31%. Furthermore, pretreatment with TGF-ß1 resulted in a 64% reduction in AT₁ mRNA levels and decreased AT₁ mRNA transcription rate by 42%. Pretreatment with TGF-ß1 blocked AngII induced proliferation of RASMC, while stimulating AngII induced upregulation of PAI-1.

In conclusion, TGF-ß1 attenuates AngII-mediated MAPK-p44/42 kinase signaling in RASMC through downregulation of AT₁ levels, which is mainly caused by the inhibition of transcription of the AT₁ gene.

Introduction
Vascular alterations are a common feature in patients with diabetes mellitus and consist of abnormal growth, proliferation and migration of cells. In previous studies, we demonstrated that in early diabetes, transforming growth factor ß1 (TGF-ß1) plays an important role in mediating vascular dysfunction in both renal microvascular smooth muscle cells and in aortic smooth muscle cells¹.

One of the prominent vascular changes induced by diabetes and mediated by TGF-ß is decreased sensitivity of vascular smooth muscle cells (VSMC) to angiotensin II (Ang II). We previously demonstrated that aortic ring contraction in response to Ang II was impaired in diabetic rats, but completely restored to normal in diabetic rats treated with anti-TGF-ß1 antibodies¹. Further, VSMC isolated from diabetic rat aorta exhibited impaired Ang II-induced cytosolic calcium signaling, but calcium signaling was normal in VSMC from diabetic rats treated with anti-TGF-ß1 antibodies in vivo. Further, the effect of diabetes on Ang II-induced calcium signaling was replicated in vitro by administration of TGF-ß1 and TGF-ß2 to aortic VSMC in culture. As a possible mechanism, a downregulation of inositol 1,4,5-trisphosphate receptors may be involved in TGF-ß-mediated impairment of Ang II-induced calcium signaling in VSMC¹.

Ang II signaling is mediated through two types of receptors, the angiotensin II type 1 (AT₁) and the angiotensin II type 2 (AT₂) receptor. In rodents two distinct isoforms or subtypes of AT₁ exist, the AT₁a and AT₁b, with the AT₁a being the predominant isoform in vascular...
TGF-β inhibits Ang II-induced MAPK p44/42 signaling

smooth muscle cells. Ang II-mediated vascular contraction and vascular hypertrophy is mediated mainly through the AT₁ receptor that signals through several signaling pathways. Ang II-induced vascular contraction is mediated mainly through the PLC/IP₃ calcium route, while Ang II-induced vascular remodeling in diabetes is mediated mainly through the MAPK p44/42 kinase signaling pathway. The involvement of the MAPK p44/42 pathway in vascular remodeling has been demonstrated in several in vitro experiments. Inhibition of the MAPK p44/42 pathway with the MAP kinase (MEK) inhibitor, PD98059, completely blocked the growth stimulatory effects of Ang II on VSMC. In addition, the migration of vascular smooth muscle cells by Ang II is mediated by the MAPK p44/42 pathway, although the signaling through MAPK p38 appears to be predominant.

Besides these effects on proliferation and migration, Ang II mediated activation of the MAPK p44/42 pathway is involved in the accumulation of extracellular matrix (ECM) in VSMC. A key mediator in this process is plasminogen activator inhibitor-1 (PAI-1), an inhibitor of the conversion of inactive plasminogen to active plasmin. Plasmin is able to degrade fibrin and ECM components and therefore PAI-1 contributes to an increased risk of thrombosis and atherosclerotic plaque formation. The expression of PAI-1 can be induced by both Ang II and TGF-β1, a process that can be inhibited by the MEK/MAPK p44/42 inhibitor PD98059, although other members of the MAPK signaling may be involved.

Although we have reported an inhibitory effect of TGF-β1 on the Ang II induced activation of the PLC/IP₃ calcium route, the effect of TGF-β1 on Ang II-induced activation of the MAPK p44/42 signaling pathway in VSMC is still unclear. Therefore, the aim of this study is to investigate the effect of TGF-β1 on Ang II-induced activation of the MAPK p44/42 pathway in cultured rat aortic smooth muscle cells.

Methods

Cell culture

Primary rat aortic smooth muscle cells (RASMC) were isolated from rat aorta as described earlier. Cells were cultured in DMEM supplemented with 1000 mg/l glucose, L-glutamine, 25 mM HEPES, pyruvate (Gibco BRL, The Netherlands), 10% of Bovine Calf Serum (PAA laboratories, Germany), 100 units/ml of penicillin and 100 μg/ml streptomycin (Gibco BRL, The Netherlands) in a humidified incubator at 37°C and 5% CO₂. When cells reached confluence, they were passaged in a 1:3 ratio with trypsin EDTA (Gibco BRL, The Netherlands). For all experiments, cells were used between passage 3 to 12.

Western blotting

Cells were washed three times using ice-cold phosphate buffered saline (PBS) and subsequently lysed in 300 μl of ice-cold RIPA buffer (1% Igepal ca-630, 1% SDS, 5 mg/ml sodium deoxycholate, 1 mM sodium orthovanadate, 10 mM β-mercapto-ethanol, 40 μg/ml PMSF, 100 μg/ml benzamidine, 500 ng/ml pepstatin A, 500 ng/ml leupeptine and 500 ng/ml...
aprotinin in PBS). Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad, The Netherlands). On a 7.5% SDS-PAGE gel 10 μg of total protein was run, transferred to nitrocellulose, and immunoblotted with the MAPK p44/42 (1:1,000 dilution) antibody (sc-7383, Santa-Cruz, Germany).

Saturation binding assay
RASMC cells were cultured on 24-well cell culture plates. Twenty-four hours before the start of the binding assay, the medium was replaced with fresh medium or fresh medium supplemented with 10 ng/ml TGF-β1. After 24 hours of incubation, cells were washed three times with HBSS at 37°C. Tritium-labeled candesartan ([3H]-candesartan) (629 Bq/mmol) was added in a concentration series of 0-10 nM in a volume of 0.5 ml HBSS. Nonspecific binding was determined by adding 1 μM cold candesartan. After 1 hour of incubation at 37°C, plates were placed on ice and the cells were washed three times with ice-cold PBS. Cells were subsequently lysed with 500 μl of 1 N NaOH and total protein content of the lysate was determined using a DC protein assay (Biorad). Total protein content did not differ between groups and the remaining lysate (490 μl) was transferred to a scintillation vial containing 3 ml of scintillation fluid. Vials were counted for three minutes in a scintillation counter. Analysis of the binding data was performed according to the method of Scatchard.

Real time PCR
The expression of AT₁ (rat vascular AT₁a), PAI-1, fibronectin, collagen I and III mRNA in rat aortic smooth cells was analyzed using real-time two-step quantitative RT-PCR. Quantification was performed with SYBR Green PCR reagents (Molecular Probes Europe, Leiden, The Netherlands) and an ABI PRISM 5700 sequence detection system (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). A 50 μl PCR mixture contained 0.5 unit Taq polymerase (Eurogentec, Belgium), 5 μl of the supplied reaction buffer, 250 nM dATP, 250 nM dCTP, 250 nM dGTP, 500 nM dUTP, 2 mM MgCl₂, 50 ng cDNA, 500 nM of each gene specific primer, 1 μl of 50× ROX reference dye (Invitrogen, Breda, The Netherlands) and 1 μl of 10× Sybr Green I (Molecular Probes Europe, Leiden, The Netherlands). The PCR profile consisted of 5 min at 95 °C, followed by 40 cycles with heating to 95°C for 15 s and cooling to 60°C for 1 min. PCR product specificity and purity was evaluated by gel-electrophoresis and by generating a dissociation curve following the manufacturer’s recommendations. Sequence-specific PCR primers were purchased from Biologio (Nijmegen, Netherlands). The PCR primers used were as follows: AT₁ sense: 5'-CACCAATATCACAGTGTGCGC-3', AT₁ antisense: 5'-AGCGTCGAATTCCGAGACT-3' (AT₁ receptor primer pair is specific for the rat vascular AT₁α receptor), PAI-1 sense: 5'-ACCACCGTGAGCAGGTTGGACT-3', PAI-1 antisense: 5'-AGCCAGGCGTTCAGCTCAT-3', fibronectin sense: 5'-CTGAGATAGTGAGACTGACT-3', fibronectin antisense: 5'-TCCCTCGTTGCGTTGCTCTC-3', collagen I sense: 5'-TGCCGTCACCTCAAGATGTG-3', collagen I antisense: 5'-CACAAGCGTGCTGAGTGA-
TGF-ß inhibits Ang II-induced MAPK p44/42 signaling

3', collagen III sense: 5' - CACAGCAGTCCAATGTAGAT - 3', collagen III antisense: 5' - TGTAGAAGGCTGTGGACATA - 3'.

**AT1 promoter assay**

An 854bp genomic DNA fragment corresponding to position -828 to +25 of the transcription initiation site of the rat AT1a receptor was cloned in pGL3-basic reporter vector (Promega, Leiden, The Netherlands). RASMC cells were cultured in a 24-well plate and infected with 2 µg of AT1 reporter vector per well. Forty-eight hours after infection, cells were treated with TGF-ß1 (10 ng/ml). Luciferase activity was determined using the Luciferase Assay System (Promega Leiden, The Netherlands).

**Real Time PCR based nuclear runoff assay**

RASMCs were cultured on 21,5 cm² Nunclon Delta dishes (Nunc, Roskilde, Denmark). When cells reached confluency, TGF-ß1 was supplemented to the medium with an end concentration of 10 ng/ml. After 4 hours, nuclei were isolated with the nuclei EZ prep isolation kit (Sigma) according to manufacturer’s protocol. Nuclei were dissolved in 250 ul of Nuclei EZ storage buffer. Directly after nuclei isolation, 200 µl of nuclei suspension were split into two aliquots. 100 µl of 20% glycerol, 30 mM Tris-HCl, pH8.0, 2.5 mM MgCl2, 150 mM KCl, 1 mM DTT and 40 U of RNasin (Promega) were added to each aliquot. 0.5 mM ribonucleotide mix (rNTPs) (New England Biolabs) were added to one aliquot. No rNTPs were added to the second aliquot. After 30 min at 30˚C, nuclei were centrifuged at 500g for 5 minutes at 4˚C and the pellet was resuspended in 350 µl RA1 lysis buffer (Macherey Nachel) with 3,5 µl of β-mercapto-ethanol. RNA was subsequently isolated with the nucleospin RNAII kit (Machery Nachel) according to manufacturer’s protocol. AT1 mRNA levels were analyzed by real time PCR.

**Proliferation assay**

Cells were seeded in 96-well plate (Nunc, Roskilde, Denmark) at 33% confluency. The next day, cells were silenced through replacement of the culture medium for culture medium without FCS for 30 hours. Subsequently, RASMCs were pre-incubated with 10ng/ml TGFβ1 for 4 hours prior to Ang II (0 M, 3 nM and 1 µM) stimulation. Twenty hours after Ang II stimulation, proliferation was measured with the CyQUANT NF cell proliferation kit (Invitrogen, The Netherlands) according to manufacturer’s instructions.

**Statistical analyses**

Results are presented as mean ± SEM unless indicated otherwise. Differences between concentration-response curves were analyzed using repetitive-measurement ANOVA (SigmaStat 1.01, Jandel Scientific) and differences between other variables were tested using a Student's t-test. Differences were considered significant at p<0.05.
Results

MAP kinase p44 and p42

Stimulation of RASMC with Ang II resulted in a dose-dependent increase in phosphorylated MAP kinase p44 and p42 (figure 1A). In control cells, maximal levels of phosphorylated p44/42 were observed at 3 nM Ang II. Pre-incubation of the cells for 24 hours with TGF-β1 (10 ng/ml) resulted in a right-shift of the dose-response curve to Ang II (figure 1A), showing a 50-fold increase in EC_{50}. Pre-incubation for 24 hours with TGF-β1 (10 ng/ml) did not affect basal phosphorylated MAPK p44/42 levels (figure 1A, see 0 M Ang II).

We next examined the dose-dependency of this Ang II-inhibiting effect of TGF-β1 on phosphorylation of p42/p44 (figure 1B). TGF-β1 impaired Ang II-induced MAPK p44/42 signaling in a dose-dependent manner with an apparent maximal effect at 10 ng/ml, although higher concentrations were not investigated.

Figure 1: TGF-β1 impairs Ang II-induced MAPK p44/42 signaling. A. Incubation of RASMC cells with TGF-β1 (10 ng/ml) for 24 hours results in a right-shift of the dose-response curve to Ang II (n= 3). ERK phosphorylation was significantly attenuated in TGF-β1 treated cells at -8.5 log[Ang II] compared to controls. B. TGF-β1 impairs Ang II-induced MAPK p44/42 signaling in a dose-dependent manner. RASMC were pretreated for 24 hours with different concentrations of TGF-β1. After 5 minutes of stimulation with Ang II (3 nM), cells were harvested and phosphorylation of MAPK p44/42 was determined by Western blot (n=3, values normalized to untreated cells).

TGF-β1 and AT₁ binding characteristics

To establish the mechanism of TGF-β1-impaired Ang II-induced MAPK p44/42 signaling, we first examined regulation of AT₁ by assessing receptor binding characteristics. Scatchard analysis of the equilibrium binding studies showed that B_{max} and equilibrium K_{d} values were 985 ± 24 dpm and 0.09 ± 0.01 nM for control cells, respectively (figure 2). Incubation of cells with TGF-β1 significantly decreased B_{max} to 680 ± 18 dpm, without an effect on K_{d} (0.07 ±
TGF-β inhibits Ang II-induced MAPK p44/42 signaling

0.01 nM). These data demonstrate pretreatment with TGF-β1 to result in a downregulation of AT₁, without changes in receptor affinity for candesartan.

AT₁ downregulation and MAPK p44/42 signaling

We next investigated whether the timing of AT₁ downregulation matched the timing of the inhibition of Ang II-mediated MAPK p44/42 signaling. RASMC cells were incubated with TGF-β1 (10 ng/ml) at different incubation times and both the levels of specific candesartan binding and phosphorylated p44/p42 were determined (figure 3). These results demonstrate that the timing of the inhibition of Ang II-mediated MAPK p44/42 signaling parallels the downregulation of AT₁.

TGF-β1 and AT₁ mRNA levels

To establish the mechanism of AT₁ downregulation by TGF-β1, we determined AT₁ mRNA levels in RASMC after incubation with TGF-β1 (10 ng/ml) with different incubations times (figure 4A). Incubation with TGF-β1 for 4 hours and longer resulted in decreased AT₁ mRNA levels. Next, we investigated whether decreased AT₁ mRNA levels by TGF-β1 were the result of decreased transcriptional activity of the AT₁ gene. Incubation of RASMC with TGF-β1 (10 ng/ml) resulted in decreased AT₁ promoter activity after 12 hours of incubation (figure 4B). These data were further supported by the real time PCR based nuclear runoff assay. Thirty minutes of in vitro transcription resulted in a 1.3 fold increase in AT₁ mRNA in control cells whereas 4h treatment of TGF-β1 totally blocked transcription of AT₁ mRNA (figure 5A).
Figure 3: Time course of TGF-ß1-mediated downregulation of AT1 and inhibition of Ang II-induced MAPK p44/42 activation in RASMC. A. Effect of TGF-ß1 on candesartan binding. Cells were incubated with 10ng/ml TGF-ß1 for 0-24 hours, and specific candesartan binding was subsequently determined. B. Effect of TGF-ß1 pretreatment on Ang II-induced MAPK p44/42 phosphorylation. Cells were pretreated with 10ng/ml TGF-ß1 for 0-24 hours, and subsequently stimulated with 3nM Ang II for 5 minutes. Cells were harvested and pERK phosphorylation was determined by Western blot. Data are expressed as % of values obtained in cells unexposed to TGF-ß1 (n=3).

Figure 4: Time course of TGF-ß1 effect on AT1 mRNA and AT1 promoter activity. A. Effect of TGF-ß1 on AT1 mRNA levels. B. Effect of TGF-ß1 on AT1 promoter activity. Data are expressed as % of values obtained in cells unexposed to TGF-ß1 (n=3, * p<0.05 vs. baseline).
TGF-ß inhibits Ang II-induced MAPK p44/42 signaling

Figure 5: Effect of TGF-ß1 on AT1 mRNA stability and AT1 gene transcription. A. Nuclear Runoff assay on AT1 gene transcription. AT1 gene activity was determined in nuclei from control and TGF-ß1 pretreated cells (n=9, *p<0.05 vs. baseline). B. AT1 mRNA levels were determined after blockage of transcription with actinomycin D in the absence (●) and presence of TGF-ß1 (○) (n=3). Data are expressed as % of values obtained in cells unexposed to TGF-ß1.

TGF-ß1 and AT1 mRNA stability
Further, we investigated whether the decrease in AT1 mRNA levels by TGF-ß1 is resulting from changes in AT1 mRNA stability. To this end, all transcription of mRNA was blocked by incubating the cells with actinomycin D prior to administration of TGF-ß1. In control cells, the half-life of AT1 mRNA levels was approximately 6 hours. Incubation with TGF-ß1, did not affect the half-life of AT1 mRNA levels (fig. 5B), resulting in identical rates of AT1 mRNA decay for both TGF-ß1-treated and control cells. Also, identical AT1 mRNA decay rates were observed in untreated and treated cells when TGF-ß1 was added to the cells 1 hour before the addition of actinomycin D (data not shown). Therefore, incubation with TGF-ß1 does not affect the stability of the AT1 mRNA.

Proliferation and fibrotic markers
To assess the functional implications of the interaction of TGF-ß and Ang II signaling, the proliferation and expression of profibrotic genes was assessed. Stimulation of RASMC with Ang II resulted in a dose dependent increase in proliferation in control cells. TGF-ß1 (10 ng/ml) itself increased proliferation slightly, however 4h of pre-incubation of the cells with TGF-ß1 (10 ng/ml) completely blocked Ang II induced proliferation (figure 6).
Figure 6: Effect of TGF-ß1 pretreatment on Ang II induced RASMC proliferation. RASMC were pretreated for 4 hours with TGF-ß1 (10 ng/ml) and subsequently exposed to 0 nM, 3 nM or 1 µM Ang II for 24 hours. TGF-ß1 completely blocked Ang II induced proliferation. (n≥9 \*p<0.05 vs untreated control). Data are expressed as % of values obtained in cells unexposed to TGF-ß1.

Finally, we examined the expression of the fibrotic markers PAI-1, fibronectin, collagen I and collagen III (figure 7). Stimulation of RASMC with Ang II resulted in increased mRNA levels of PAI-1 and fibronectin. TGF-ß1 (10 ng/ml) itself increased PAI-1 expression. Pre-incubation with TGF-ß1 (10 ng/ml) strongly augmented Ang II induced PAI-1 expression, indicating a synergistic effect of TGF-ß1 and Ang II on PAI-1 expression. The expression of collagen I was decreased after exposure to 3nM Ang II. The expression of collagen III was not affected by Ang II or TGF-ß1.
Figure 7: Effect of TGF-ß1 pretreatment and Ang II on the expression of fibrotic markers. RASMC were pretreated for 4 hours with TGF-ß1 (10ng/ml) and subsequently exposed to 0nM, 3nM or 1µM Ang II for 24 hours. A. TGF-ß1 and Ang II act synergistically on the expression of PAI-1. (*p<0.05 vs untreated control, #p<0.05 vs same concentration Ang II without TGF-ß1 pretreatment). B. Fibronectin expression was increased by Ang II. C. Collagen I expression was reduced by 3nM Ang II. D. Collagen III expression was not affected. Data are expressed as % of values obtained in cells unexposed to TGF-ß1.

Discussion
The present study demonstrates that TGF-ß1 inhibits the Ang II-induced MAPK p44/42 signaling pathway in cultured RASMC. In TGF-ß1 pretreated cells, we observed an inhibition of Ang II-induced phosphorylation of MAPK p44/42 and a reduction in AT1 density, without an effect on receptor affinity for candesartan. In addition, AT1 mRNA levels were downregulated and accompanied by a decrease in transcriptional activity of the AT1 gene, without a reduction in AT1 mRNA stability.

The kinetics of the inhibition of Ang II-mediated MAPK p44/42 signaling by TGF-ß1 closely followed the kinetics of AT1 mRNA downregulation. In addition, the downregulation of AT1 mRNA levels was accompanied by a reduction in AT1 promoter activity, although the reduction in AT1 promoter activity lagged behind the downregulation of AT1 mRNA levels, probably due to the stability of the luciferase reporter-protein. Furthermore, the nuclear runoff assay demonstrated that pretreatment with TGF-ß1 for 4 hours resulted in decreased
AT$_1$ mRNA levels and that AT$_1$ gene transcription had halted. As the downregulation of AT$_1$ mRNA levels by TGF-ß1 did not involve changes in the stability of the AT$_1$ mRNA, we conclude that the downregulation of AT$_1$ mRNA by TGF-ß1 is the result of decreased transcription of the AT$_1$ gene.

Downregulation of AT$_1$ levels has been described previously in TGF-ß1 treated primary renal proximal tubule cells$^{11}$. Moreover, decreased transcriptional activity of the AT$_1$ promoter by TGF-ß appears to be a general mechanism for downregulation of AT$_1$ levels in multiple tissues in experimental diabetes$^{11, 12}$. Indeed, decreased levels of AT$_1$ in VSMC exposed to high glucose was already reported in 1992$^{12}$, although the involvement of TGF-ß1 and its effects on MAPK p44/42 signaling has not been demonstrated before. In contrast to our findings, transcriptional upregulation of human AT$_1$ by TGF-ß1 has recently been reported in human lung fibroblasts$^{13}$. However, vascular smooth muscle cells contain a specific set of transcription factors$^{14}$ and therefore the difference in AT$_1$ regulation may arise from cell-type specific gene regulation. Furthermore, in rodents two subtypes of the AT$_1$ receptor exist, while in humans only a single AT$_1$ subtype is present. The promoters of the human AT$_1$ and rat vascular AT$_1a$ genes differ considerably and may explain the difference in TGF-ß1 mediated AT$_1$ regulation in human fibroblasts and rat vascular smooth muscle cells.

The decreased transcription of the AT$_1$ gene resulted in a reduction in the number of binding sites for the specific AT$_1$ blocker candesartan, indicating a reduction in the number of AT$_1$ receptors on the plasmamembrane of RASMC pretreated with TGF-ß1. Previously, it has been demonstrated that the specific downregulation of AT$_1$ mRNA levels using siRNA against AT$_1$, results in decreased AT$_1$ receptor levels and inhibition of MAPK p44/42 signaling$^{15}$. We therefore conclude that the inhibition of Ang II induced MAPK p44/42 signaling in TGF-ß1 pretreated RASMC is most likely caused by decreased gene transcription of the AT$_1$ gene and decreased AT$_1$ receptor levels. It is however unclear how the observed moderate decrease in AT$_1$ receptor levels, could result in an increase of the EC$_{50}$ of AngII by a factor of 50. Possibly, post-translational modifications to the AT$_1$ could alter the affinity of the AT$_1$ for AngII as has been demonstrated previously$^{16}$. However, we did not observe a change in the affinity of the AT$_1$ for the competitive antagonist candesartan after TGF-ß1 pretreatment. It is therefore unlikely that pretreatment with TGF-ß1 results in conformational changes to the AngII binding site of the AT$_1$. Alternatively, inhibition of downstream coupling of the AT$_1$ to G-proteins and second-messenger systems may be additional mechanisms for inhibiting Ang II induced MAPK p44/42 signaling in TGF-ß1 pretreated RASMC. Previously, it has been demonstrated that TGF-ß1 could inhibit the epidermal growth factor (EGF) and alpha1B-Adrenergic receptors by modifying their phosphorylation status$^{17, 18}$. It is however unclear whether TGF-ß1 could have similar effects on AT$_1$ receptors.

Several studies have demonstrated that the hypertrophic effects of Ang II on VSMC in experimental models of diabetes are mediated through the MAPK p44/42 signaling pathway$^2$. In a recent paper by Wang et al.$^{20}$, it has been demonstrated that activation of AT$_1$ by Ang II in VSMC, results in the activation of the Smad2/3 signaling pathway, a
TGF-β inhibits Ang II-induced MAPK p44/42 signaling

profibrotic pathway that is also activated by TGF-β. Our study demonstrates for the first time that TGF-β1 inhibits the Ang II-mediated MAPK p44/42 signaling pathway. Therefore the cross-talk between the Ang II and TGF-β1 signaling pathways appears to be bi-directional.

We demonstrated that Ang II mediated proliferation of RASMC could be completely inhibited by pretreatment with TGF-β1. This is in line with a previous study demonstrating that Ang II induced proliferation of human vascular smooth muscle cells was mediated through AT₁ and MEK2. Therefore the inhibition of Ang II mediated proliferation of TGF-β1 pretreated RASMC is most likely caused by the impaired MAPK p44/42 signaling. Furthermore, it has been demonstrated that TGF-β1 could also inhibit EGF mediated MAPK p44/42 signaling and proliferation of human epithelial cells through inhibition of the EGF receptor17. Therefore TGF-β1 mediated inhibition of MAPK p44/42 signaling and proliferation appears to apply to multiple receptor systems and cell-types.

In contrast to the effect of TGF-β1 pretreatment on proliferation, inhibition of MAPK p44/42 signaling by pretreatment with TGF-β1 augmented Ang II induced PAI-1 expression in RASMC. This is surprising, as several studies have demonstrated that PAI-1 expression is dependent on MEK8, 21-24. Possibly, PAI-1 expression may be induced through other MAPK routes. It has been demonstrated that both TGF-β1 and Ang II can signal through the Reactive Oxygen Species (ROS) p38MAP/JNK route8, 25, 26. Indeed, in fibroblasts, TGF-β1 induced PAI-1 expression is dependent on this pathway25. Furthermore, PAI-1 expression may be mediated by HIF-1α expression through Ang II mediated stimulation of AT2 receptors27. However, the involvement of the p38MAP/JNK or other pathways in the synergistic upregulation of PAI-1 by TGF-β1 and Ang II in RASMC remains to be established.

The pathophysiological relevance of the inhibitory effect of TGF-β1 on MAPK p44/42 is still unclear. Previous studies have suggested that the balance between vascular fibrosis and vascular proliferation may be controlled by the relative levels of Ang II and TGF-β20, 28. Indeed, in our study, pretreatment with TGF-β1 completely inhibited Ang II induced proliferation of RASMC, while Ang II and TGF-β1 acted synergistically on the increased expression of the early fibrotic marker PAI-1. In recent papers29, 30 a new model has emerged to describe the interaction between AT₁ and TGF-β1 signaling in vascular remodeling. In these studies, it has been demonstrated that Ang II can directly stimulate the intracellular mediators of TGF-β1 signaling, the Smads 2 and 3. This activation of Smads 2/3 appears to be bimodal in nature. In the initial phase at 15 minutes, Ang II directly activates Smads 2/ 3 through the AT₁ and via MAPK p44/42. In the delayed response to Ang II at 24 hours, paracrine production of TGF-β1 maintains Smad3 activation, leading to the activation of profibrotic genes. Our current findings suggest that the inhibition of Ang II mediated MAPK p44/42 signaling by TGF-β1 may be part of a negative feedback loop that attenuates the direct activation of Smads 2/3 by Ang II and inhibits excessive Ang II mediated smooth muscle cell proliferation.

In conclusion, in this study we provide the pharmacological characteristics of the TGF-β1-induced decrease in Ang II-mediated MAP kinase signaling in rat aortic smooth muscle cells.
We demonstrate that TGF-ß1 attenuates Ang II-mediated MAPK p44/42 signaling in rat aortic smooth muscle cells through downregulation of AT1 levels, which seems mainly dependent on the inhibition of transcriptional activity of the AT1 gene. Pretreatment with TGF-ß1 completely inhibited Ang II mediated proliferation of RASMC but synergistically increased Ang II induced PAI-1 expression.
TGF-ß inhibits Ang II-induced MAPK p44/42 signaling

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TGF-ß inhibits Ang II-induced MAPK p44/42 signaling


