c-Src and Hydrogen Peroxide Mediate Transforming Growth Factor-β1–Induced Smooth Muscle Cell–Gene Expression in 10T1/2 Cells

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Objective—Transforming growth factor-β1 (TGF-β1) controls the expression of numerous genes, including smooth muscle cell (SMC)–specific genes and extracellular matrix protein genes. Here we investigated whether c-Src plays a role in TGF-β1 signaling in mouse embryonic fibroblast C3H10T1/2 cells.

Methods and Results—TGF-β1 induction of the SMC contractile protein SM22α gene expression was inhibited by PP1 (an inhibitor of Src family kinases) or by C-terminal Src kinase (a negative regulator of c-Src). Induction of SM22α by TGF-β1 was markedly attenuated in SYF cells (c-Src−, Yes−, and Fyn−) compared with Src+ cells (c-Src+, Yes+, and Fyn+). PP1 also inhibited the TGF-β1–induced expression of serum response factor (SRF), a transcription factor regulating the SMC marker gene expression. Confocal immunofluorescence analysis showed that TGF-β1 stimulates production of hydrogen peroxide. Antioxidants such as catalase or NAD(P)H oxidase inhibitors such as apocynin inhibited the TGF-β1–induced expression of SM22α. Furthermore, we demonstrate that TGF-β1 induction of the plasminogen activator inhibitor-1 (PAI-1) gene, which is known to be dependent on Smad but not on SRF, is inhibited by PP1 and apocynin.

Conclusion—Our results suggest that TGF-β1 activates c-Src and generates hydrogen peroxide through NAD(P)H oxidase, and these signaling pathways lead to the activation of specific sets of genes, including SM22α and PAI-1.


Key Words: growth factors ■ cytokines ■ oxidant stress ■ gene expression ■ smooth muscle cells
pression of which is proven to be Smad dependent but SRF independent. Thus, H₂O₂ and c-Src play a critical role in TGF-β1 signaling.

Materials and Methods

Materials
Catalase, N-acetyl-L-cysteine (NAC), diethyldithio carbamic acid (DDCA), 2',7'-dichlorofluorescein diacetate (DCF-DA), and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) were purchased from Sigma. PD98059 and calphostin C were from Calbiochem; PP1 was from Biomol; and 4′hydroxy-3′-methoxycacetophenone (apocynin) was from Aldrich. A polyclonal antibody for phosphorylated c-Src (pY418) was obtained from Biosource International. Anti-phospho-tyrosine antibody (pY20), anti-SRF antibody, and anti-Smad2/3 antibody were from Santa Cruz Biotechnology. Smad3/pcDNA3, expression vector for Smad3, Smad4/pcDNA3, expression vector for Smad4, and Smad6/pcDNA3, expression vector for Smad6, were kind gifts from Dr K. Miyazono (University of Tokyo, Japan). C-terminal Src kinase (Csk)/pcDNA1, expression vector for Csk, was a kind gift from Dr T. Kadowaki (University of Tokyo, Japan). (CAGA)₆-thymidine kinase (TK)-Lac was kindly provided by Dr J.M. Gauthier (Laboratoire Glaxo Wellcome, France).

Cell Cultures
Mouse embryonic fibroblast cells (10T1/2 cells) have been described previously. SYF cells (mouse embryonic fibroblast cell line with specific inactivation of c-Src, Yes, and Fyn) and Src⁻/-cells (mouse embryonic fibroblast cell line with specific inactivation of Yes and Fyn) were purchased from American Type Culture Collection.

Northern blot and Western blot analyses
Northern blot and Western blot were performed essentially as described previously. For Northern blot analyses, cells were treated by TGF-β1 for indicated hours, and total RNA (12 μg) was prepared with or without pretreatment with PP1 (4 μmol/L), catalase (50 μg/mL), NAC (10 mM/L). DDCA (10 mM/L), AEBSF (200 μmol/L), and apocynin (1 mM/L). Hybridization was performed with SM22α, SM α-actin and PAI-1 cDNA probe. Signals were detected by autoradiography and exposed to Kodak XAR. For Western blot analyses, cells were treated by TGF-β1 for indicated hours, and whole-cell extracts were prepared with or without pretreatment with PP1 (4 μmol/L). Activation of c-Src was assayed using a phospho-c-Src polyclonal antibody at a dilution of 1:500. Secondary antibodies were diluted to 1:10 000. Tyrosine phosphorylation was assayed using a phosphotyrosine monoclonal antibody (pY20) at a dilution of 1:1000.

Transient transfection, luciferase assay, and preparation of cell lysates
Cells were seeded at 5 × 10⁵ cells per dish and were transfected with 1 μg of reporter plasmid or, if indicated, 1 μg of expression plasmid. The following day, cells were switched to fresh medium and were harvested for luciferase assays after 4-hour culture with or without TGF-β1. Cells were lysated in 120 μL of cell-cultured lysate reagent (Promega). Luciferase activity was measured immediately with a Berthold Lumat LB9501 luminometer.

Measurement of Intracellular H₂O₂ Levels
H₂O₂ levels were measured using the peroxide-sensitive fluorophore DCF-DA (5 μmol/L). Cells were seeded at low density, grown for 24 hours in DMEM, and were stimulated with 1 ng/mL TGF-β1 for 4 hours. The H₂O₂-sensitive fluorophore DCF-DA (5 μmol/L) was used to measure intracellular H₂O₂ by laser confocal scanning microscopy (MRC-1000; Bio-Rad).

Results

Src Family Kinase and Smad Regulate TGF-β1–Induced SM22α Expression in 10T1/2 Cells
To determine the role of Src family kinase in the expression of the SM22α gene by TGF-β1, we performed Northern blot analysis. As shown in Figure 1A, specific inhibition of Src family kinase by PP1 and Csk attenuated the increase in the SM22α mRNA levels by TGF-β1. To confirm these results, we used Src⁻⁻/-cells (c-Src⁻/, Yes⁻/, and Fyn⁻/) and SYF cells (c-Src⁻-, Yes⁻, and Fyn⁻) and performed Northern blot analysis. In Src⁻⁻/-cells, the SM22α and SM α-actin mRNAs were constitutively expressed, and the SM22α mRNA levels
were increased as early as 2 hours of TGF-β1 stimulation (Figure 1B). On the other hand, in SYF cells, the levels of SM22α and SM α-actin mRNAs in steady state were much lower than those in Src<sup>++</sup> cells. In addition, an inducible expression of such genes by TGF-β1 was blunted compared with that in Src<sup>++</sup> cells (Figure 1B). These results indicate that an induction of SM22α and SM α-actin gene expression is attenuated in SYF cells compared with Src<sup>++</sup> cells. Because Smad family proteins play an important role in TGF-β1 signaling, we tested the effect of Smad6, an inhibitory Smad, on the TGF-β1–induced SM22α expression in 10T1/2 cells. As shown in Figure 1C, overexpression of Smad6 clearly inhibited the TGF-β1–mediated SM22α mRNA expression. These results indicate that Src family kinase–dependent and Smad-dependent signaling pathways are required for TGF-β1–mediated SM22α mRNA expression.

**Induction of SM22α Promoter Activity by TGF-β1 Is Regulated by Smad and c-Src**

To determine whether the specific inhibition of Src family kinase–dependent or Smad-dependent pathways regulate SM22α mRNA expression at the transcriptional levels, we performed transient transfection assays. SM22α-670, which contains the promoter region −670 to +41 of the human SM22α gene in front of the luciferase gene, showed 18-fold induction of the luciferase activity by TGF-β1. Cotransfection of Smad3 and Smad4 expression vectors resulted in a further increase in TGF-β1–induced SM22α promoter activity. These results suggest that Smad3 and Smad4 play an important role in TGF-β1–mediated SM22α gene expression. In addition, cotransfection of Smad4 (the only member of Co-Smads) with Smad3 expression vector further increased SM22α-670 activity. These results are consistent with the notion that receptor-regulated Smads, or R-Smads, form heteromeric complexes with Co-Smads to positively regulate gene expression (Figure I, available online at http://atvb.ahajournals.org). TGF-β1 also increased the luciferase activity by ~80-fold of SM22α-1309, which contains promoter region between −1309 and +41. Cotransfection of Smad6 reduced TGF-β1 responsiveness. Specific inhibition of Src kinase family–dependent pathway by cotransfection of Csk attenuated TGF-β1 responsiveness (Figure 2B). These results suggest that Smad-dependent and Src family kinase–dependent pathways regulate transcription of the SM22α gene.

**Induction of TGF-β1–Mediated SRF Protein Expression via c-Src**

SRF plays a central role in SMC marker gene expression, including SM22α and SM α-actin gene. We investigated whether TGF-β1 induces the SRF protein levels through Src family kinase–dependent pathway. Western blot analyses showed that TGF-β1 significantly induced the SRF protein levels after 4-hour stimulation in 10T1/2 cells (Figure 3A). Such an induction was completely inhibited by PP1. Smad2/3 expression levels were not affected by TGF-β1 or PP1. To confirm these results, we performed Western blot analysis using Src<sup>++</sup> cells and SYF cells. In Src<sup>++</sup> cells, TGF-β1 increased the SRF protein levels as early as 2 hours, whereas in SYF cells, inducible expression of SRF protein by TGF-β1 was blunted compared with that in Src<sup>++</sup> cells (Figure 3B). These results suggest that induction of SRF protein expression by TGF-β1 is mediated at least partly through Src family kinase–dependent pathway.

**Phosphorylation of c-Src by TGF-β1**

We next investigated whether TGF-β1 induces c-Src phosphorylation. As shown in Figure 4A, TGF-β1 rapidly induced the tyrosine phosphorylation of c-Src autophosphorylation site, which peaked at 1 minute and then declined to the basal level within 5 minutes. As illustrated in Figure 4B, Western blot analysis of the whole-cell lysates from 10T1/2 cells revealed that tyrosine residues in various kinds of proteins were phosphorylated by TGF-β1. As expected, PP1 most effectively inhibited the tyrosine phosphorylation by TGF-β1 among the 3 protein kinase inhibitors, including PD98059 (a mitogen-activated protein kinase/ERK kinase-1 inhibitor) and calphostin-C (protein kinase C inhibitor).
H2O2 Production by TGF-β1
To determine the importance of reactive oxygen species (ROS) during TGF-β1 signaling, we tested whether TGF-β1 induces H2O2 production. Confocal immunofluorescence analysis indicated that TGF-β1 increased the fluorescence levels of DCF-DA in cytosol of 10T1/2 cells. Catalase, an H2O2 scavenger, markedly reduced the fluorescence levels of DCF-DA in TGF-β1–treated 10T1/2 cells (Figure II, available online at http://atvb.ahajournals.org). We then investigated whether H2O2 acts as a second messenger to stimulate the SMC marker gene expression. Representative Northern blot analysis performed on total RNA prepared from control cells and cells treated with TGF-β1 for 8 hours in the presence or absence of various ROS scavengers is illustrated in Figure 5A. Catalase, DDCA (an inhibitor of superoxide dismutase), and NAC abolished or reduced the increase in SM22α and SM α-actin mRNA by TGF-β1. These results indicate that H2O2 plays an important role in the TGF-β1–induced SMC marker gene expression. Representative Northern blot analysis performed on total RNA prepared from control cells and cells treated with TGF-β1 for 8 hours in the presence or absence of various ROS scavengers is illustrated in Figure 5A. Catalase, DDCA (an inhibitor of superoxide dismutase), and NAC abolished or reduced the increase in SM22α and SM α-actin mRNA by TGF-β1. These results indicate that H2O2 plays an important role in the TGF-β1–induced SMC marker gene expression. Furthermore, SM22α mRNA expression was significantly inhibited by NAD(P)H oxidase inhibitors such as apocynin and AEBSF (Figure 5B). Consistent with these results, apocynin inhibited TGF-β1–induced c-Src phosphorylation (Figure III, available online at http://atvb.ahajournals.org). PP1 only minimally decreased the production of H2O2 in the TGF-β1–stimulated cells (data not shown). These results suggest that NAD(P)H oxidase–mediated H2O2 production plays an important role in c-Src activation and TGF-β1–induced SMC marker gene expression.

Src Activation Is Required for TGF-β1–Induced PAI-1 Gene Expression
To determine whether c-Src activation is involved in the SRF-independent genes, we examined the effects of c-Src inhibitors on the TGF-β1–induced expression of the PAI-1 gene because transcription from the PAI-1 promoter is strongly induced by TGF-β1 and is often used as a marker for TGF-β1 responsiveness in mammalian cells.14 Treatment of 10T1/2 cells with PP1 significantly attenuated the TGF-β1 induction of PAI-1 mRNA expression (Figure 6A). Apocynin and AEBSF strongly inhibited PAI-1 mRNA induction by TGF-β1. We then tested whether CAGA box, which has been known to be a critical element for TGF-β1–induced and Smad-dependent transcription to occur, mediates such a repression by PP1. TGF-β1 activation of (CAGA)6-TK-Luc, which contains 6 copies of CAGA boxes in front of the TK promoter–luciferase chimeric gene, was strongly inhibited by PP1 (Figure IV, available online at http://atvb.ahajournals.org). These results suggest that c-Src activation and H2O2 generation are involved in TGF-β1–induced activation of Smad-dependent but SRF-independent transcription of the PAI-1 gene.
Discussion

Results of this study indicate that TGF-β1 generates H$_2$O$_2$ and phosphorylates c-Src, which in turn mediate the increase in SRF expression and subsequent activation of the SMC marker genes. Furthermore, TGF-β1 induction of PAI-1 gene expression, which has been proven to be dependent on Smads but not on SRF, was inhibited in the presence of either PP1 or NAD(P)H oxidase inhibitors. These results suggest that activation of c-Src and NAD(P)H oxidase plays an obligatory role in TGF-β1 induction of SRF-dependent and SRF-independent genes. Given that modulation of SMC marker genes and extracellular matrix (ECM) protein gene expression in the myofibroblasts has been intimately related to the atherosclerosis and restenosis after coronary intervention, our results will shed light on the understanding of the molecular mechanism of fibroproliferative vascular diseases.

**c-Src Requirement in TGF-β1-Induced SM22α Expression**

Because CArG box plays a central role in SMC marker gene transcription including SM22α, SM α-actin, and SM γ-actin, the scenario indicating that TGF-β1—signaling target at SRF-dependent gene transcription appears to be compelling. However, this model sidesteps the question of how Smads execute their effects on SMC marker gene transcription and how SRF-dependent transcription is regulated by TGF-β1. The present work addresses some of these questions. We found that c-Src activation occurs as early events in the TGF-β1 signaling, and that this event is required for the increase in SRF expression. Our study showed that TGF-β1 induces SRF expression at the transcription levels, as assessed by the transient transfection assay of the human SRF promoter luciferase construct (data not shown). Qiu et al pointed to the physical interaction between Smad3 and SRF, which synergistically activates SM22α expression. Other mechanisms could include TGF-β1—induced chromatin remodeling, as seen with P19-derived A404 cell differentiation.

**c-Src Activation and H$_2$O$_2$ Production by TGF-β1**

Although the role of ROS and c-Src has been extensively studied in the signal transduction elicited by receptor tyrosine kinases such as epidermal growth factor (EGF) receptor and PDGF receptor, no previous study has specifically examined the role of c-Src activation in TGF-β1 signaling, which involves receptor serine/threonine kinases, Smads, and mitogen-activated protein kinase kinase kinase family kinase (TAK1). We observed several lines of strong evidence indicating that c-Src mediates TGF-β1 signal transduction. First, PP1, an inhibitor for c-Src kinase, effectively blocked TGF-β1 induction of SMC marker gene expression (Figures 1A and 5A). Second, Csk, a negative regulator of Src kinases, attenuated TGF-β1—induced SM22α expression, as assessed by both Northern blot analyses and transient transfection assays (Figures 1A and 2B). Third, TGF-β1—induced expression of SMC marker was severely inhibited in SFY cells, which are deficient in Src, Fyn, and Yes (Figure 1B). Fourth, TGF-β1 rapidly phosphor-
ylates the tyrosine residue of c-Src (Figure 4A). Finally, TGF-β1-mediated tyrosine phosphorylation is prevented in the presence of PP1 (Figure 4B). Together, these data unequivocally indicate that c-Src is activated by TGF-β1, and this step is crucial for TGF-β1-evoked intracellular signaling.

Our current study shows that NAD(P)H oxidase inhibitors apocynin and AEBSF, 2 structurally unrelated NAD(P)H oxidase inhibitors, potently inhibited TGF-β1–induced expression of SM22α and PAI-1 gene expression. This suggests that H₂O₂ generation by TGF-β1 is mediated by NAD(P)H oxidase. The signaling mechanisms by which TGF-β1 activates NAD(P)H oxidase remain unclear, but recent study by Touyz et al demonstrated that c-Src regulates activation of NAD(P)H oxidase by stimulating p47phox phosphorylation and translocation, critical steps in the initiation of NAD(P)H oxidase activation in angiotensin II (Ang II)–stimulated cells.²⁰ Likewise, Seshiah et al showed that protein kinase C, Rac, phosphatidylidyinositol 3-kinase, EGF receptor kinase, as well as c-Src play important roles in the Ang II–induced NAD(P)H oxidase activation.²¹ They proposed a feed-forward mechanism involving activation of c-Src by small amounts of ROS from NAD(P)H oxidase, thereby leading to sustained oxidase activation. In contrast, we did not observe the substantial decrease in the TGF-β1–induced H₂O₂ production in the presence of PP1. Thus, it is likely that c-Src is downstream but not upstream from NAD(P)H oxidase activation in the TGF-β1–stimulated cells. It is of a great interest that c-Src differentially activates NAD(P)H oxidases depending on the ligands and cells types.

The ability of TGF-β1 to stimulate cellular production of H₂O₂ has been well established, at least in some cell lines, and Thannickal and Fanburg showed that TGF-β1 induces NAD(P)H oxidation in human lung fibroblasts.²²,²³ However, a role of Src tyrosine kinase has not been determined. Our data provide the first evidence of the involvement of NAD(P)H oxidases in the activation of c-Src tyrosine kinase in the cells stimulated with receptor serine/threonine kinases.

c-Src Is Required for TGF-β1 Activation of SRF-Independent Transcription

Besides the regulatory role of TGF-β1 in the SMC marker gene expression, TGF-β1 plays a key role in regulating expression of the genes for ECM proteins. TGF-β1 stimulates expression of some ECM protease inhibitors, including PAI-1. Recent work identified potential consensus Smad3–Smad4 DNA binding motifs in the distal region of the PAI-1 promoters AG(C/A)CAGACA and AGACAGGTTGTG, which contain the 4-bp AGAC element in common and have been shown to serve as the target sequence of Smad3/Smad4 heterodimer.⁹ In the present study, we found that PP1 inhibited the TGF-β1 induction of PAI-1 expression. In addition, we showed that pretreatment of PP1 prevented TGF-β1–induced activation of (CAGA)₆-TK-Luc. These results suggest that c-Src is required for Smads-dependent but SRF-independent transactivation of the PAI-1 gene.

c-Src Activation and NAD(P)H Oxidase Activation: Double-Edged Sword for Vascular Pathophysiology?

Activation of vascular NAD(P)H oxidases and ROS production by these enzyme systems are common in cardiovascular disease.²⁴ ROS produced after Ang II–mediated stimulation of NAD(P)H oxidase lead to events such as inflammation, hypertrophy, remodeling, and angiogenesis. c-Src regulates NAD(P)H oxidase activity by phosphorylation of p47phox subunit in vascular SMCs, suggesting the key role of c-Src in Ang II–mediated ROS production.²⁰ TGF-β1, on the other hand, is a potent inhibitor of SMC proliferation and immune cell activation.²⁵ TGF-β1 also inhibits SMC migration and promotes SMC differentiation and ECM formation, and thus TGF-β1 has been proposed as a vasoprotective cytokine. Antithrombogenic effect of TGF-β1 is highlighted in the studies showing that blockade of TGF-β1 ligand or the TGF-β1–type 2 receptor accelerates development of atherosclerotic lesion formation in apolipoprotein E⁻/⁻-atherosclerotic-prone mice.²⁶ The observation in this study that c-Src and H₂O₂ play a critical role in TGF-β1 signaling indicates that c-Src serves as a double-edged sword for vascular pathophysiology. Activation of c-Src and ROS production can lead to beneficial or detrimental impacts on vessel wall architecture, depending on the extracellular signals involved. Such a concept is valuable when considering the development of a novel class of antioxidant, anti-inflammatory compounds that exhibit antithrombogenic properties.

In conclusion, we have shown for the first time that activation of c-Src is required for TGF-β1 signaling. Production of H₂O₂ is a possible mechanism for c-Src activation by TGF-β1. Although the precise mechanism how NAD(P)H oxidase–mediated H₂O₂ production and c-Src interact in TGF-β1 signaling remains to be determined, our findings not only add another intriguing aspect of the multiple biological effects of TGF-β1 but also provide the rationale to develop effective antioxidant therapy to prevent fibroproliferative vascular disease in which ROS generation is crucially involved.

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References


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![Diagram](image)

[RelativeLuciferaseActivity]

TGF-β1

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Bar graph showing the effect of TGF-β1 on luciferase activity with and without PP1 inhibition.