Norepinephrine Enhances Fibrosis Mediated by TGF-β in Cardiac Fibroblasts

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Abstract—Cardiac fibrosis results from proliferation of interstitial fibroblasts and concomitant increased biosynthesis of extracellular matrix (ECM) components and is often complicated by cardiac hypertrophy. This study was conducted to investigate whether norepinephrine (NE) potentiates transforming growth factor-β (TGF-β)–induced cardiac fibrosis. The expression of the cardiac ECM proteins, plasminogen activator inhibitor-1 (PAI-1), fibronectin, and collagen type I, was examined by Western blotting using extracts from neonatal rat primary cardiac fibroblasts. In cardiac fibroblasts, treatment with a combination of NE and TGF-β1 increased cell proliferation and ECM expression. Luciferase assays were conducted to clarify the effect of NE on TGF-β1 signaling. TGF-β1 (1 ng/mL) increased the specific signaling activity 2-fold, whereas the combination of NE (10 μmol/L) and TGF-β1 (1 ng/mL) resulted in an approximate 10-fold increase in specific signaling activity. We confirmed that treatment with NE markedly enhances TGF-β–induced phosphorylation of activating transcription factor 2 (ATF-2). These results indicated that NE has a synergistic effect on TGF-β signaling. To determine whether this activation by NE was mediated by the TGF-β1 receptor, we used a dominant negative vector of the TGF-β1 type II receptor, and the synergistic effects were inhibited. Furthermore, this synergistic effect was attenuated by a specific inhibitor of p38, SB203680. These data indicate that NE enhances cardiac fibrosis through TGF-β1 post-receptor signaling, predominantly via the p38 MAP kinase pathway. (Hypertension. 2002;40:148-154.)

Key Words: norepinephrine ■ transforming growth factors ■ fibrosis ■ extracellular matrix ■ calcium channel blockers

Cardiac hypertrophy is due not only to cardiac myocyte hypertrophy but also to proliferation of interstitial fibroblasts and increased biosynthesis of extracellular matrix (ECM) components. The development of heart failure is frequently associated with cardiac hypertrophy activated by systemic neurohumoral factors, including norepinephrine (NE) and angiotensin II (Ang II), which is characterized by impaired cardiac muscle function and increased interstitial fibrosis.1,2

Transforming growth factor-β (TGF-β) has been implicated in several fibrotic disorders, including glomerulonephritis, liver cirrhosis, lung fibrosis, and vascular restenosis.3 Increased TGF-β1 gene expression has been demonstrated in the hearts of spontaneously hypertensive rats during the transition from stable hypertrophy to failure.4 In vitro observations indicate that TGF-β1 stimulates the expression of fibronectin and collagen and their incorporation into ECM from cardiac fibroblasts.5–8 These experimental studies suggest that TGF-β1 plays a crucial role in the myocardial remodeling process, particularly in cardiac fibrosis. TGF-β1 binds to a heteromeric complex of the heterogeneous type I and type II receptors and transmits signals via mitogen-activated protein kinase (MAPK) and Smad pathways.9 Recently, activation of TGF-β1 and the increased expression of downstream Smad2 and Smad4 were reported in the infarct scar and remnant myocardium during the chronic phase of myocardial infarction.10 Elevated Smad expression was also normalized by an Ang II AT1 receptor blocker in post–myocardial infarction heart failure.11 Chronic inhibition of TGF-β1 expression by a non specific TGF-β1 inhibitor, tranilast, attenuated both left ventricular (LV) hypertrophy and perivascular fibrosis without lowering blood pressure in the hypertensive TGR (mRen2) 27 rat, a hypertrophic model characterized by increased levels of cardiac Ang II.12 Interestingly, this hypertrophic model has demonstrated only perivascular fibrosis and no interstitial fibrosis.13 These findings indicate that there is a potential signal pathway that is independent of Ang II in TGF-β–induced cardiac fibrosis. In cardiac myocytes, TGF-β1 mRNA expression increased in abdominal aortic banding–induced LV hypertrophy14 and subcutaneous NE infusion.15 α- and β-adrenergic stimulation induces distinct patterns of immediate early-response gene
expression in neonatal rat myocardial cells\textsuperscript{16} and activates the MAPK kinase (MEK)/MAPK pathway in the heart by different signaling pathways. Elevation of intracellular Ca\textsuperscript{2+} is important in the activation of MAPK by isoproterenol in the cardiac myocytes.\textsuperscript{17} Calcium antagonists are well known to have antihypertensive effects; however, their cardioprotective effects remain to be conclusively demonstrated. In the present study, we therefore focused on the role of NE in cardiac fibrosis and cardiac hypertrophy through TGF-\beta signaling. Furthermore, we determined whether Smad or MAPK were involved in TGF-\beta signaling in cardiac fibrosis.

\section*{Methods}

\subsection*{Animals}

Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories, Inc (Osaka, Japan); 2- to 4-day-old neonatal rats were used. The protocols for this experiment and all subsequent experiments were devised following the guidelines of the Animal Care and Use Committee of Nagasaki University.

\subsection*{Vectors}

A 3TP-Lux expression vector gene and the dominant negative vector of TGF-\beta-activated kinase-1 (TAKKR), an activin response element (ARE) reporter gene, and forkhead activin signal transducer-1 (FAST-1) expression vector were kindly provided by Dr Wrana, Toronto University, Canada. 3TP-Lux is a TGF-\beta–responsive luciferase reporter gene that contains 3 consecutive tetradeconyl-phorbol acetate (TPA) response elements (TREs) and a portion of the plasminogen activator inhibitor 1 (PAI-1) promoter region.\textsuperscript{18} Human FAST-1 (hFAST-1) possesses the ability to bind to human Smad2 and activates an ARE. An ARE-Lux fusion was used for the transcriptional activity determination with FAST-1, and the results indicated Smad signals. Dominant negative T\betaR-II is the kinase-defective receptor of T\betaR-II (K277R).\textsuperscript{19} pAP1-Luc and PRL-CMV vectors were kindly provided by Dr Wrana, Toronto University, Canada. A 3TP-Lux expression vector gene and the dominant negative vector of TGF-\beta-activated kinase-1 (TAKKR), an activin response element (ARE) reporter gene, and forkhead activin signal transducer-1 (FAST-1) expression vector were kindly provided by Dr Wrana, Toronto University, Canada.

\subsection*{Cell Isolation and Culture}

Neonatal cardiac myocytes and fibroblasts were prepared from Sprague-Dawley rats as described previously with slight modification.\textsuperscript{20} Briefly, cardiac myocytes were cultured in DMEM and fibroblasts in a DMEM/F12 nutrient mixture (DMEM/F12) (1:1) containing 10\% fetal bovine serum (FBS, pH 7.3). To reduce the number of nonmyocardial cells (NMCs), mainly cardiac fibroblasts, cultured rat cardiac myocytes, cardiac fibroblasts, and HepG2 cells were transduced with pRL-CMV Renilla luciferase. Twenty-four hours later, the medium was exchanged with serum-free medium containing 10 \mu g/mL insulin, 10 \mu g/mL transferrin, and 10 ng/mL sodium selenite (Sigma-Aldrich Chemie GmBH). Twenty-four hours after serum depletion, these cells were used for subsequent experiments. After treatment with various agents, cells were lysed in cell lysis buffer (Promega), and the luciferase activity was measured as the oxidation of luciferin using a luminometer (TD-20/20 Luminometer, Turner Designs). Luciferase activity was measured by comparison of its increase with that of the control. To investigate the involvement of p38 and extracellular-signal-regulated kinase (ERK) pathway in the induction of TGF-\beta, we used specific inhibitor of p38, SB203680 (10 or 20 \mu M) (Calbiochem), and MEK1 inhibitor, PD98059 (10 \mu M) (New England Biolabs. Inc).

\subsection*{Statistical Analysis}

The values of all the variables were expressed as mean±SEM. Data were analyzed using a paired t test. A value of P\textless 0.05 was considered statistically significant.

\section*{Results}

\subsection*{Cell Proliferation Assay}

To determine the effect of NE on the cell proliferation activity of cardiac fibroblasts, we performed the WST-1 assay using rat primary cardiac fibroblasts. TGF-\beta1 and NE both stimulated WST-1 activity in cardiac fibroblasts. The cells treated with the combination of NE and TGF-\beta1 exhibited substantially higher WST-1 activity than the cells treated with NE or TGF-\beta1 alone (Figure 1A). In contrast, TGF-\beta1 significantly suppressed the proliferation of HepG2 cells (Figure 1B), and NE abolished this inhibitory effect of TGF-\beta1.

\subsection*{Western Blot Analysis}

Western blot analysis was performed according to the method of Akiyama et al\textsuperscript{21} with slight modifications. Briefly, conditioned medium was obtained from cardiac fibroblast cultures exposed to NE (10 \mu M, Sigma Aldrich Chemie), TGF-\beta1 (1 ng/mL or 10 ng/mL, Austral Biologicals), or NE (10 \mu M) + TGF-\beta1 (1 or 10 ng/mL) for 24 hours. Conditioned medium (20 \mu L) was diluted 1:1.5 with SDS buffer (Prestained Protein Markers, New England Bio Labs) and then heated at 100°C for 5 minutes and cooled. The supernatant was analyzed by 7.5\% SDS-PAGE, followed by Western blotting. Detection of PAI-1, fibronectin, collagen type I, and phosphorylation of activating transcription factor 2 (ATF-2) were performed using anti-PAI-1 (American Diagnostics Inc), fibronectin (Calbiochem), collagen type I (Rockland Immunochemicals), and ATF-2 antibody (Cell Signaling Technology), respectively.
increased the activity by approximately 10-fold (Figure 3A). In contrast, 3TP-Lux activity was increased only by treatment with TGF-β1 or NE alone and significantly increased in cardiac fibroblasts after treatment with a combination of NE and TGF-β1 (A). Cell proliferation in HepG2 cells was markedly suppressed by TGF-β1; this suppression was partially reversed by treatment with NE (B). *P < 0.05 vs control; **P < 0.001 vs control; †P < 0.05 vs NE + TGF-β1, #P < 0.001 vs NE + TGF-β1. Values represent mean ± SE for WST-1 assays. NE indicates norepinephrine; TGF-β1, transforming growth factor-β1; WST, 4-[3-[4-iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio-1,3-benzene disulfonate].

Figure 1. WST-1 assay showing cell proliferation of cardiac fibroblasts and HepG2 cells. WST-1 activity increased after treatment with TGF-β1 or NE alone and significantly increased in cardiac fibroblasts after treatment with a combination of NE and TGF-β1 (A). Cell proliferation in HepG2 cells was markedly suppressed by TGF-β1; this suppression was partially reversed by treatment with NE (B). *P < 0.05 vs control; **P < 0.001 vs control; †P < 0.05 vs NE + TGF-β1, #P < 0.001 vs NE + TGF-β1. Values represent mean ± SE for WST-1 assays. NE indicates norepinephrine; TGF-β1, transforming growth factor-β1; WST, 4-[3-[4-iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio-1,3-benzene disulfonate].

cardiac fibroblasts, ARE-Lux activity was increased by only 2-fold, with no synergistic effects in the presence of FAST-1 (Figure 6A); however, in HepG2 cells an approximately 10-fold increase was observed (Figure 6B).

We next examined whether phosphorylation of ATF-2 is enhanced by combined treatment with NE and TGF-β1, because ATF-2 has been reported to be a common nuclear target of Smad and TAK1 pathways in TGF-β signaling. 24–26 NE and TGF-β1 increased the level of phospho-ATF-2 by 3.4-fold and 4.3-fold, respectively, and treatment with NE and TGF-β1 enhanced it by 8.7-fold (Figure 7A). The degree of phosphorylation of ATF-2 increased up to a maximum of 8.1-fold at 30 minutes, as shown in Figure 7B.

**Hypertrophic Effect and Ca++ Antagonist Treatment**

We evaluated the hypertrophic effect on cardiac myocytes using AP-1-Lux activity instead of 3TP-Lux, because the c-fos/AP-1 axis is an important signaling pathway in hypertrophic myocytes. NE acted with TGF-β1, resulting in an additional effect (Figure 8). The calcium antagonist nifedipine partially blocked, although not with statistical significance, the synergistic effect of NE in cardiac hypertrophy.

**Discussion**

In this report, we demonstrated that NE and TGF-β1 have a synergistic effect on cell proliferation and ECM protein expression in cultured rat primary cardiac fibroblasts. Bhambi and Eghbali 27 previously reported that NE-treated cardiac fibroblasts exhibited increased incorporation of ³H-thymidine into cell nuclei, indicating increased cardiac fibroblast proliferation. Using in vivo animal models, an increase in blood pressure was associated with increased myocardial collagen type I and TGF-β1 mRNA expression, as well as with cardiac...
hypertrophy. In aortic banding models, fibronectin and types I and III collagen mRNA expression was significantly increased. These increased levels of mRNA expression encoding ECM proteins were preceded by a marked increase in TGF-β1 mRNAs. In contrast, Nakajima et al. reported that increased TGF-β1 activity alone was insufficient to promote ventricular fibrosis in transgenic mice expressing constitutive active mutation of TGF-β1. Bhambi and Eghbali showed NE did not alter the abundance of mRNA for type I collagen in cardiac fibroblasts. These data suggest that either TGF-β1 or NE individually were insufficient to promote ventricular fibrosis. Based on our in vitro experiments using cultured cardiac fibroblasts, the treatment with a combination of NE and TGF-β1 enhanced the cell proliferation and ECM production when compared with treatment with either NE or TGF-β1 alone.

To clarify the mechanisms of their synergistic signaling, we investigated the TGF-β transacting reporter system. The 3TP-Lux activity was increased approximately 2- to 3-fold when treated individually with NE or TGF-β1; however, 3TP-Lux activity increased by approximately 10-fold after treatment with a combination of NE and TGF-β1. In the presence of the dominant negative TGF-β type II receptor, 3TP-Lux activation by a combination of NE and TGF-β1 was completely suppressed. There is substantial evidence that the synergistic effect of NE on TGF-β1 signaling might be mediated by TGF-β post-receptor pathways. Recently, several groups have reported that TGF-β1 can activate MAPKs,
extracellular-signal regulated kinase (ERK), stress-activated protein kinase 1 (SAPK)/c-jun N-terminal kinase (JNK), and p38 protein kinase.\textsuperscript{10,25,29} Because TAK1 can activate the p38 and SAPK/JNK pathways\textsuperscript{38–40}, we examined whether 3TP-Lux activity was blocked by specific inhibitors of this signaling pathway. The synergistic effect of NE and TGF-\(\beta\)1 was almost completely blocked by TAKKR and SB203580, but not by PD98059, suggesting that TAK1-p38 pathways are predominantly responsible for the regulation of induction of gene expression by TGF-\(\beta\).

On the other hand, Smads are a family of proteins that operate downstream of various members of the TGF-\(\beta\) superfamily.\textsuperscript{41,42} Smad2 and Smad3 are downstream effectors of the TGF-\(\beta\) signaling pathway. On ligand binding, they are phosphorylated by the TGF-\(\beta\)1 type I receptor kinase and translocate to the nucleus in a complex with Smad4.\textsuperscript{43} This heteromeric complex may either directly bind to the promoters of its target genes or associate with other transcription factors to induce gene transcription.\textsuperscript{44,45} Although the expression of Smad 2, 3, and 4 and TGF-\(\beta\)1 was elevated in the chronic phase of myocardial infarct scar healing,\textsuperscript{10} it is not clear whether the Smads pathway is involved in cardiac fibrosis. Recent work has identified a potential consensus Smad3-Smad4 DNA binding site, GTCTAGAC,\textsuperscript{46} which is observed within the human 3TP-Lux promoter\textsuperscript{47,48} and ARE-Lux promoter.\textsuperscript{48} This suggests that Smads pathways, as well as MAPKs pathways, are involved in the activation of 3TP-Lux. To elucidate whether the synergistic effect of NE on TGF-\(\beta\)1 signaling is mediated by MAPKs or by Smads, we examined the ARE-Lux reporter system, both in the presence and absence of FAST-1. Because FAST-1 specifically binds to human Smad2, the FAST-1--dependent activation of the ARE-Lux promoter was mediated by endogenous Smad4.\textsuperscript{31} In the present study, the ARE-Lux activity in the presence of FAST-1 increased by approximately 10-fold over the control level in HepG2 cells; however, it increased only about 3-fold in cardiac fibroblasts treated with TGF-\(\beta\)1.

Figure 6. ARE-Lux activity in cardiac fibroblasts and HepG2 cells. In the presence of FAST-1, ARE-Lux activity increased approximately 3-fold after cardiac fibroblasts were treated with TGF-\(\beta\)1; however, NE had no effect (A). In HepG2 cells, treatment with TGF-\(\beta\)1 resulted in an approximately 10-fold increase in ARE-Lux activity (B). *\(P<0.05\) vs control; **\(P<0.01\) vs control. ARE-Lux indicates activin response element (ARE)-Luciferase; FAST-1, forkhead activin signal transducer-1.

Figure 7. Western blotting of phosphorylation of ATF-2. The phosphorylated band of ATF-2 (indicated by arrow) was markedly increased in NE and TGF-\(\beta\)1 combination treatment (A). Phosphorylated ATF-2 protein levels were measured by densitometry, and the value in the control was arbitrarily expressed as 1 U. Time course of phosphorylation of ATF-2 treated by NE (10 \(\mu\)mol/L) and TGF-\(\beta\)1 (1 ng/mL) (B). Controls were prepared from total cell extracts from NIH/3T3 cells with anisomycin as positive control (p.c.) or without anisomycin as negative control (n.c.).
addition, NE alone did not enhance ARE-Lux activity in either HepG2 or cardiac fibroblast cells. Based on these results, MAPKs have significantly more influence on cardiac fibrosis than the Smads cascade.

ATF-2, a member of the ATF/cAMP response element-binding protein (CREB) family of transcription factors, can form dimers through their leucine zipper structures and bind to CRE.49,50 SAPK/JNK and p38 phosphorylate ATF-2 at Thr-69, Thr-71, and Ser-90.51 Our Western blotting analysis demonstrated that the degree of phospho-ATF-2 was markedly increased by treatment with NE and TGF-β1. Interestingly, ATF-2 has been reported to bind to Smad4 and to be phosphorylated by TGF-β signaling in C2C12 cells.25

Finally, we analyzed AP-1 Lux activation in cardiac myocytes to evaluate whether the combination effect of NE on TGF-β1 signaling is involved in myocyte hypertrophy. In cardiac myocytes, we observed only an additive effect. The Ca2+ antagonist nifedipine partially inhibited, although not with statistical significance, the synergistic effect of NE on cardiac hypertrophy. AP-1 Lux indicates activating protein-1 luciferase.

Perspectives

In conclusion, the present study demonstrates that NE and TGF-β1 have a synergistic effect on cardiac fibrosis, suggesting a pivotal role for NE in TGF-β1 signaling in advancing the process of heart failure. The mechanism of upregulation of TGF-β1 signaling was explained by both ATF-2 and Smad 3/4 directly binding to their own target sequences in the PAI-1 promoter.24 Our results also suggest that p38 MAPK-ATF-2 is predominantly involved in this process. Cross-talk between the p42/p44 MAP kinase and Smad pathways in TGF-β1–induced furin gene transactivation was reported in HepG2 cells.54 Furin is a predominant convertase of TGF-β1 in fibroblastic and synovial cells. Further studies regarding cross-talk between ERK and Smads are required to further understand these complex interactions.

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