Nitric Oxide Attenuates the Expression of Transforming Growth Factor-β3 mRNA in Rat Cardiac Fibroblasts via Destabilization

Nadia Abdelaziz, Federico Colombo, Isabelle Mercier, Angelino Calderone

**Abstract**—Transforming growth factor-β (TGF-β) has been implicated in the development of interstitial fibrosis in cardiac hypertrophy. NO has been regarded as a potent inhibitor of cardiac fibroblast growth, albeit the modulation of cellular events associated with interstitial fibrosis remains undefined. In this regard, the regulation of TGF-β mRNA expression by the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) was examined in neonatal rat cardiac fibroblasts. SNAP treatment for 4 hours decreased TGF-β1 mRNA levels, an effect mimicked by 8-bromo-cGMP. TGF-β3 mRNA, however, had returned to levels observed in the untreated cells after a 24-hour exposure to SNAP, whereas a decreased expression persisted with 8-bromo-cGMP. In contrast to TGF-β1, TGF-β3 mRNA levels were modestly increased in response to cGMP-generating molecules. The treatment with actinomycin D for at least 8 hours did not appreciably alter TGF-β1 mRNA levels. By contrast, SNAP treatment caused a rapid decrease of TGF-β1 mRNA with a half-life of 3.3±0.2 hours, thereby supporting a mechanism of destabilization. The pretreatment with SNAP inhibited angiotensin II–stimulated protein synthesis and the concomitant expression of TGF-β1 mRNA. These data reveal a disparate pattern of TGF-β1 and TGF-β3 mRNA regulation by NO and highlight a novel mechanism of destabilization contributing to the decreased expression of TGF-β3 mRNA. The modulation of both basal and angiotensin II–stimulated TGF-β3 mRNA expression provides a mechanism by which NO may influence the progression of interstitial fibrosis. *(Hypertension. 2001;38:261-266.)*

**Key Words:** nitric oxide ■ cyclic GMP ■ fibroblasts ■ RNA, messenger ■ transforming growth factors ■ angiotensin II

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A maladaptive consequence of cardiac hypertrophy is the development of fibrosis, characterized by uncontrolled fibroblast growth and a disproportionate accumulation of extracellular matrix proteins. The underlying mechanism(s) implicated in these processes remains equivocal, albeit the peptide growth factor transforming growth factor-β (TGF-β) may play an integral role. In various models of cardiac hypertrophy (eg, aortic-banded model, spontaneously hypertensive model, and myocardial infarct model) associated with fibrosis, ventricular mRNA levels of TGF-β1 and TGF-β3 have been found increased. This latter pattern of expression is not limited to the myocardium, because the development of lung fibrosis secondary to myocardial infarction was associated with the upregulation of TGF-β1 and TGF-β3 mRNAs. In vitro studies performed in cultured cardiac fibroblasts have demonstrated that TGF-β isoforms increased collagen expression, and in lung fibroblasts TGF-β was the most potent profibrotic TGF-β isoform. Collectively, these data support the premise that the TGF-β family may represent in part a critical local mechanism involved in the increased expression of extracellular matrix protein in various models of fibrosis.

NO is an inorganic free radical gas synthesized from L-arginine via the action of the enzyme NO synthase. The family of NO synthases consists of 3 isoforms, of which isoforms I and III are constitutively expressed in a wide variety of tissue and regulated by calcium/calmodulin. By contrast, isoform II is not constitutively expressed but is induced by various factors, including cytokines and bacterial lipopolysaccharide, and its activation occurs via a calcium/calmodulin-independent process. The immediate second messenger is cGMP, synthesized following NO activation of the enzyme soluble guanylate cyclase. In numerous cell types, including fibroblasts, the exogenous administration of NO, acting via the recruitment of cGMP-dependent pathways, has been shown to exert a potent antiproliferative action. Moreover, in vivo and in vitro studies have demonstrated that NO can negatively regulate the expression of extracellular matrix protein expression. However, the mechanism attributed to these latter effects remains unknown. Thus, because of the putative profibrotic role of the TGF-β family, the following study examined the potential modulation of these peptide growth factors by NO. Second, the...
recent observation that angiotensin (Ang) II induced TGF-β mRNA expression in cardiac fibroblasts supports the premise that this family of peptide growth factors may, via an autocrine pathway, mediate the well-documented effects of Ang II on fibroblast growth and extracellular matrix protein expression. In this regard, parallel experiments examined the effect of NO on Ang II–stimulated cardiac fibroblast growth and TGF-β mRNA expression.

Methods

Cultured Neonatal Rat Cardiac Fibroblasts

Cardiac fibroblasts were isolated from 1- to 3-day-old Sprague-Dawley rat pups (Charles River Canada, St Constant, Quebec), as previously described. The primary culture of fibroblasts was maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 7% FBS until confluent (5 to 7 days) and subsequently passaged with trypsin-EDTA (0.25%, 0.53 mmol/L; Gibco BRL). Experiments were performed on first and second passaged cells.

Protein Synthesis Experiments

Cardiac fibroblasts were plated at a density of 100 to 200 cells per square millimeter in 24-well plates for 24 to 36 hours in DMEM containing 7% FBS and subsequently washed and maintained in serum-free DMEM containing insulin (5 μg/mL), transferrin (5 μg/mL), and sodium selenite (5 ng/mL) (Collaborative Biomedical) for 48 hours before the experimental protocol. Fibroblasts were treated with either the NO donor S-nitroso-N-acyetyl-penicillamine (SNAP) (100 μmol/L; Calbiochem) or 8-bromo-cGMP (1 mmol/L; Sigma) for 15 to 30 minutes before the addition of Ang II (1 μmol/L; Calbiochem). The growth response was permitted to continue for 24 hours, and protein synthesis was determined by the addition of 2 μCi/μL of [3H]leucine (ICN Biomedicals Inc). After completion of the experimental protocol, fibroblasts were washed twice with PBS (4°C), and cold 5% trichloroacetic acid was added for 30 minutes to precipitate protein. The precipitates were washed twice with cold water and resuspended in 0.4 mol/L NaOH. Aliquots were counted in a scintillation counter.

Measurement of cGMP Levels

Cardiac fibroblasts (100 to 200 cells per square millimeter) were treated with 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX, Sigma) for 15 minutes before treatment with SNAP (100 μmol/L) and incubated for an additional 30 minutes at 37°C. The reaction was stopped by the addition of 0.1 mol/L HCl, the plates were placed on ice for 30 minutes, and the supernatant was collected and titrated to pH 7.4 with 2.5 mol/L sodium acetate (pH 5.8) and 5 mol/L NaOH. cGMP levels were quantified with a standard radioimmunoassay kit (Biomedical Technologies, Inc). NaOH (0.2 mol/L) was subsequently added to the plates for 30 minutes, the cells were scrapped, and the protein content was measured by the Bradford technique (Bio-Rad Laboratories Canada Ltd) with BSA as a standard.

Northern Hybridization

Cardiac fibroblasts were plated at a density of 200 cells per square millimeter in p100 plates for 7 days in DMEM containing 7% FBS; the medium was changed twice during this time. The cells were subsequently washed, and the medium was replaced with serum-free DMEM containing insulin (5 μg/mL), transferrin (5 μg/mL), and sodium selenite (5 ng/mL). The cells were maintained in serum-free DMEM for 48 hours before the experimental protocol. Fibroblasts were treated with either the NO donor SNAP (100 μmol/L) or 8-bromo-cGMP (1 mmol/L) for 4 and 24 hours. In parallel experiments, SNAP or 8-bromo-cGMP was added for 15 to 30 minutes before the addition of Ang II (1 μmol/L), and stimulation proceeded for an additional 4 hours. Total RNA was isolated by a modification of the technique of Chomczynski and Sacchi. A 0.985-kb fragment of rat TGF-β3 (American Type Culture Collection [ATCC]), a 1.2-kb fragment of mouse TGF-β3 (ATCC), and a 2-kb fragment of rat GAPDH (ATCC) were labeled with [32P]dCTP (NEN) to a specific activity of 1 to 2×106 cpm/μg cDNA by the random hexamer (Pharmacia) priming method and hybridized to nylon membranes (Gene screen-plus; NEN Life Sciences) for 18 to 24 hours at 42°C, as previously described. The filters exposed to the cDNA probes were washed twice (15 minutes, room temperature) with 300 mmol/L NaCl/30 mmol/L trisodium citrate and 0.1% SDS and twice (15 minutes, 45°C) with 30 mmol/L NaCl/30 mmol/L trisodium citrate and 0.1% SDS. Nylon membranes were subsequently exposed to Kodak XAR film with an intensifying screen at −70°C, and films were scanned with a laser densitometer (Chemilumager 4000 I v4.04 software; Alpha Innotech Corp). All levels of mRNA reported in this article are normalized to the level of GAPDH mRNA.

Assessment of Half-Life of SNAP-Mediated Decrease of TGF-β3

The data of the SNAP-mediated decrease of TGF-β3 mRNA were fit by a straight line defined by the relationship ln Rt/R0 = −kt, where R0 is the level of TGF-β3 mRNA before exposure to SNAP, R6 is the level of TGF-β3 mRNA at time t of SNAP treatment, and k6 is the apparent decay constant. The half-life (t1/2) of TGF-β3 mRNA was taken as 0.693/k, where k is the slope of ln R0/R6 versus time t calculated by linear regression.

Statistical Analysis

Data are presented as mean±SEM. Statistical analysis was performed by Student’s unpaired t test (2-tailed), and a value of P<0.05 was considered significant.

Results

Effect of SNAP and 8-Bromo-cGMP on TGF-β3 mRNA Expression in Cardiac Fibroblasts

A 4-hour treatment of cardiac fibroblasts with SNAP (100 μmol/L) caused a 64±6% decrease (n=6; P<0.01) of TGF-β3 mRNA levels (Figure 1). However, after a 24-hour exposure to SNAP, TGF-β3 mRNA returned to near normal levels (Figure 1). Previous studies have demonstrated that SNAP treatment of numerous cell types, including fibroblasts, increased intracellular cGMP levels. To evaluate whether the SNAP-mediated decrease of TGF-β3 mRNA occurred in part via a cGMP-dependent pathway, cardiac fibroblasts were treated with a cGMP analogue, 8-bromo-cGMP (1 mmol/L). A 4-hour treatment with 8-bromo-cGMP caused a 55±6% decrease (n=6; P<0.01), and TGF-β3 mRNA levels remained decreased after a 24-hour treatment (72±10% decrease versus basal; n=5; P<0.01) (Figure 1). In contrast to TGF-β3 mRNA, SNAP and 8-bromo-cGMP treatment caused a modest significant increase in the steady state mRNA levels of TGF-β1 after a 4-hour treatment, but by 24 hours had returned to levels observed in the untreated cells (Figure 1).

SNAP Treatment Increased cGMP Synthesis, and This Response Was Impaired After Chronic Exposure to SNAP

A 30-minute stimulation with 100 μmol/L SNAP increased intracellular cGMP levels (basal=3.9±0.9 versus SNAP=55±13 pmol/mg protein per minute; n=6; P=0.01) in cardiac fibroblasts. These latter data and the action of 8-bromo-cGMP support the role of a cGMP-dependent pathway in SNAP-mediated decrease in the steady state mRNA levels of TGF-β1. However, TGF-β3 mRNA levels returned
to untreated levels after a 24-hour treatment with SNAP, thereby suggesting a possible desensitization of soluble guanylate cyclase responsiveness. To assess this premise, cardiac fibroblasts were exposed to 100 μmol/L SNAP (in the absence of IBMX) for a period of 4 and 24 hours, and the subsequent production of cGMP was measured with an additional 30-minute treatment with SNAP. A 4-hour pretreatment with SNAP caused a significant decrease in cGMP production in response to a subsequent challenge to SNAP, thereby supporting a process of desensitization of soluble guanylate cyclase (Figure 2). After a 24-hour exposure to SNAP, the production of cGMP in response to the readministration of SNAP remained significantly impaired; this responsiveness was lower than that with the 4-hour exposure but did not reach statistical significance (P=0.07) (Figure 2).

Effect of SNAP on TGF-β3 mRNA Stability

A reduction in the steady state level of a transcript can occur either via a decrease in transcription and/or an increase in mRNA degradation (mRNA destabilization). The following experiments examined the role of destabilization as a mechanism of SNAP-mediated decrease of TGF-β3 mRNA levels. To address this issue, a standard approach involves determining the half-life of mRNA loss in the presence of the transcriptional inhibitor actinomycin D. The treatment of cardiac fibroblasts with actinomycin D (5 μg/mL) did not appreciably decrease the basal expression of TGF-β3 mRNA over a period of 8 hours (Figure 3). This concentration of actinomycin D inhibited [3H]thymidine uptake by >95% (data not shown). In contrast, the exposure to SNAP (100 μmol/L) resulted in a time-dependent decrease of TGF-β3 mRNA levels (Figure 3). The data for the SNAP-mediated decrease of TGF-β3 mRNA were fit by a straight line (r=0.9979; P<0.01) defined by the relationship ln R0/Rt = −kt (see Methods) (Figure 2). The Kt determined by this approach was 0.214±0.014 (n=4) and yielded an apparent t1/2 of 3.3±0.2 hours (n=4) according to the relationship t1/2=k/2 (see Methods). Analogous to SNAP, the treatment with 1 μmol/L 8-bromo-cGMP caused a temporal decrease in the steady state mRNA level of TGF-β3, which yielded an apparent t1/2 of 2.8 hours (n=2) (Figure 3). To determine whether the SNAP-mediated decrease of TGF-β3 mRNA required the transcription of a trans-acting factor, actinomycin D (5 μg/mL) was added 30 minutes before the administration of SNAP. The treatment (4 hours) with actinomycin D did not appreciably decrease TGF-β3 mRNA levels (Figure 3), although the pretreatment (30 minutes) with actinomycin D attenuated the subsequent SNAP-mediated decrease of TGF-β3 mRNA by 71% (SNAP=58±3% vs SNAP+actinomycin D=17±6% ↓; P<0.01 versus SNAP; n=3) (Figure 3).
pretreatment of cardiac fibroblasts with SNAP attenuated the subsequent increase in [3H]leucine uptake by Ang II (1 μmol/L) (Figure 4). Similarly, the pretreatment with 8-bromo-cGMP attenuated Ang II–stimulated [3H]leucine uptake by 66±4% (n=4; P<0.01 versus Ang II). In parallel experiments, the pretreatment with either SNAP or 8-bromo-cGMP attenuated Ang II–stimulated TGF-β3 mRNA expression by 96±8% (n=3; P<0.01) and 78±22% (n=3; P<0.05), respectively (Figure 4).

Discussion

Uncontrolled cardiac fibroblast growth and the excessive accumulation of collagen and fibronectin represent phenotypic events associated with the development of interstitial fibrosis. An underlying mechanism implicated in the development and/or maintenance of interstitial fibrosis is the induction of the TGF-β family of peptide growth factors. In mammalian cells, 3 distinct isoforms of TGF-β have been identified (1, 2, and 3), and in most cell lines examined, they exert qualitatively similar physiological effects. An important physiological feature of TGF-β includes the de novo synthesis of extracellular matrix proteins. Moreover, TGF-β has been shown to inhibit the expression of matrix metalloproteinases, a family of proteins that degrade extracellular matrix proteins and cause a concomitant increase in the expression of tissue inhibitors of matrix metalloproteinases. Lastly, an increased expression of TGF-β isoforms has been documented in the fibrotic myocardium and lungs of several rat models of cardiac hypertrophy and failure. By contrast, the inorganic molecule NO has been well documented in the fibrotic myocardium and lungs of several rat models of cardiac hypertrophy and failure.

SNAP Treatment Inhibited Ang II–Stimulated Protein Synthesis and TGF-β3 mRNA Expression in Cardiac Fibroblasts

A 24-hour treatment of cardiac fibroblasts with Ang II (1 μmol/L) caused 61±7% increase in [3H]leucine uptake (n=7; P<0.01 versus basal) (Figure 4), and this effect was blocked by the selective angiotensin type 1 (AT1) receptor antagonist irbesartan (data not shown). Consistent with previous data, a 4-hour treatment with Ang II (1 μmol/L) caused a 2.6±0.6-fold increase (n=5; P<0.01 versus basal) in the steady state mRNA levels of TGF-β3, and remained elevated at 24 hours (1.7±0.1-fold increase; n=2) (Figure 1). Moreover, a concomitant 1.7±0.4-fold increase (n=6; P<0.01 versus basal) in the steady state mRNA of TGF-β3 mRNA was also observed at 4 hours and remained elevated at 24 hours (1.9±0.3-fold; n=5; P<0.05 versus basal) (Figures 1 and 4). The 24-hour exposure of cardiac fibroblasts to SNAP (100 μmol/L) caused a modest nonsignificant reduction in basal [3H]leucine uptake (Figure 4). Likewise, a 24-hour exposure to 8-bromo-cGMP (1 mmol/L) had no effect on [3H]leucine uptake (n=4; data not shown). However, a 15- to 30-minute pretreatment of cardiac fibroblasts with SNAP attenuated the subsequent increase in [3H]leucine uptake by Ang II (1 μmol/L) (Figure 4). Similarly, the pretreatment with 8-bromo-cGMP attenuated Ang II–stimulated [3H]leucine uptake by 66±4% (n=4; P<0.01 versus Ang II). In parallel experiments, the pretreatment with either SNAP or 8-bromo-cGMP attenuated Ang II–stimulated TGF-β3 mRNA expression by 96±8% (n=3; P<0.01) and 78±22% (n=3; P<0.05), respectively (Figure 4).

Figure 3. NO decreased TGF-β3 mRNA levels in part via a mechanism of destabilization. A, Treatment with 100 μmol/L SNAP caused a temporal decrease in the steady state mRNA level of TGF-β3. A 4-hour treatment with the transcriptional inhibitor actinomycin D (Act-D, 5 μg/mL) had no significant effect on the steady state mRNA level of TGF-β3. A 30-minute treatment with actinomycin D (5 μg/mL) before the addition of SNAP (100 μmol/L) attenuated the subsequent SNAP-mediated decrease of TGF-β3 mRNA levels. B. Data for the SNAP-mediated decrease of TGF-β3 mRNA were fit by a straight line (r=0.9979; P<0.01) defined by the relationship ln R/ΔR = -kΔt (see Methods). The kΔ determined by this approach was 0.214±0.014 and yielded an apparent t1/2 of 3.3±0.2 hours (n=4) according to the relationship t1/2=0.693/kΔ (see Methods). Likewise, the treatment with 8-bromo-cGMP (1 mmol/L) decreased TGF-β3 mRNA level with an apparent t1/2 of 2.8 hours (n=2). By contrast, the basal expression of TGF-β3 mRNA did not appreciably change after the treatment with the transcriptional inhibitor actinomycin D (5 μg/mL) before the addition of SNAP (100 μmol/L) (Figure 4). Similarly, the pretreatment with 8-bromo-cGMP (1 mmol/L) attenuated the subsequent SNAP-mediated decrease of TGF-β3 mRNA (n=7) and this effect was blocked by the selective angiotensin type 1 (AT1) receptor antagonist irbesartan (data not shown). Consistent with previous data, a 4-hour treatment with Ang II (1 μmol/L) caused a 2.6±0.6-fold increase (n=5; P<0.01 versus basal) in the steady state mRNA levels of TGF-β3, and remained elevated at 24 hours (1.7±0.1-fold increase; n=2) (Figure 1). Moreover, a concomitant 1.7±0.4-fold increase (n=6; P<0.01 versus basal) in the steady state mRNA of TGF-β3 mRNA was also observed at 4 hours and remained elevated at 24 hours (1.9±0.3-fold; n=5; P<0.05 versus basal) (Figures 1 and 4). The 24-hour exposure of cardiac fibroblasts to SNAP (100 μmol/L) caused a modest nonsignificant reduction in basal [3H]leucine uptake (Figure 4). Likewise, a 24-hour exposure to 8-bromo-cGMP (1 mmol/L) had no effect on [3H]leucine uptake (n=4; data not shown). However, a 15- to 30-minute pretreatment of cardiac fibroblasts with SNAP attenuated the subsequent increase in [3H]leucine uptake by Ang II (1 μmol/L) (Figure 4). Similarly, the pretreatment with 8-bromo-cGMP attenuated Ang II–stimulated [3H]leucine uptake by 66±4% (n=4; P<0.01 versus Ang II). In parallel experiments, the pretreatment with either SNAP or 8-bromo-cGMP attenuated Ang II–stimulated TGF-β3 mRNA expression by 96±8% (n=3; P<0.01) and 78±22% (n=3; P<0.05), respectively (Figure 4).

Figure 4. NO treatment inhibits Ang II–stimulated protein synthesis and TGF-β3 mRNA expression in cardiac fibroblasts. A, 24-hour treatment with 1 μmol/L Ang II (All) caused a significant increase (P<0.01 vs basal; n=7) in [3H]leucine uptake. The 24-hour exposure to 100 μmol/L SNAP (n=7) did not appreciably affect basal [3H]leucine uptake, although a 15- to 30-minute pretreatment attenuated the subsequent increase in protein synthesis in response to Ang II (n=7). Data are presented as mean±SEM. *P<0.01 vs basal; †P<0.01 vs Ang II. B, 4-hour treatment of cardiac fibroblasts with Ang II (1 μmol/L) increased the steady state mRNA levels of TGF-β3. Pretreatment (15 to 30 minutes) with either 100 μmol/L SNAP (n=3) or 1 mmol/L 8-bromo-cGMP (cGMP; n=3) abrogated the subsequent Ang II–mediated increase in TGF-β3 mRNA.
treated as a potent antiproliferative factor in numerous cell types, including fibroblasts, and found to inhibit the expression of extracellular matrix proteins.\textsuperscript{12–14} The mechanism attributed to the latter effect of NO, however, remains unknown. On the basis of the profibrotic role of the TGF-\(\beta\) family, the present study examined whether their expression could be regulated by NO. Indeed, the treatment of cultured neonatal rat cardiac fibroblasts with the NO donor SNAP significantly decreased the steady state mRNA levels of TGF-\(\beta_1\) at 4 hours but returned to levels observed in untreated cells after 24 hours. The immediate intracellular second messenger of NO is cGMP, synthesized via NO binding and subsequent activation of the enzyme soluble guanylate cyclase.\textsuperscript{19} In neonatal rat cardiac fibroblasts, SNAP treatment significantly increased intracellular cGMP levels. Consistent with this finding, a significant decrease in the steady state mRNA levels of TGF-\(\beta_3\) was observed after a 4-hour exposure to the cGMP analogue 8-bromo-cGMP. However, in contrast to SNAP, TGF-\(\beta_3\) mRNA levels remained significantly decreased after a 24-hour treatment with 8-bromo-cGMP. Thus, these data demonstrate that SNAP-mediated decrease of TGF-\(\beta_3\) mRNA occurred at least in part via a cGMP-dependent mechanism.

The underlying mechanism implicated in the transient decrease of TGF-\(\beta_3\), mRNA by the NO donor SNAP remains to be defined. Both in vivo and in vitro studies have described a phenomenon of tolerance after the continuous administration of organic nitrates.\textsuperscript{20,21} Although the underlying mechanism responsible for nitrate tolerance is multifactorial, a desensitization and/or downregulation of soluble guanylate cyclase has been found to contribute in part to this process.\textsuperscript{22,23} On the basis of these observations, the transient regulation of TGF-\(\beta_3\) mRNA is consistent with a SNAP-mediated desensitization and/or downregulation of soluble guanylate cyclase. Indeed, the chronic exposure of cardiac fibroblasts to SNAP significantly reduced the subsequent production of cGMP after the reexposure to the NO donor, thereby supporting a process of desensitization of soluble guanylate cyclase. Lastly, consistent with these latter data, the 24-hour exposure to 8-bromo-cGMP, an actinomycin D, that acts downstream of soluble guanylate cyclase, caused a sustained decrease of TGF-\(\beta_3\), mRNA.

Destabilization represents a posttranscriptional mechanism regulating the steady state levels of various mRNAs. This phenomenon has been well characterized for a number of proto-oncogenes with short half-life periods and occurs via the binding of a trans-acting factor to the 3'-untranslated region of the transcript.\textsuperscript{24} Moreover, in addition to immediate early genes, the agonist-mediated downregulation of G protein–coupled receptor transcripts has been shown to occur via a process of destabilization.\textsuperscript{25,26} In the present study the contribution of mRNA destabilization to the SNAP-mediated decrease of TGF-\(\beta_3\) mRNA levels was examined. To investigate this premise, the kinetics of TGF-\(\beta_3\) mRNA decrease in response to SNAP were compared with the response to the transcriptional inhibitor actinomycin D.\textsuperscript{24,25} The exposure of cardiac fibroblasts to actinomycin D for a period of at least 8 hours did not appreciably alter the steady state mRNA level of TGF-\(\beta_3\), compared with untreated cells. By contrast, the treatment with SNAP caused a rapid decrease of TGF-\(\beta_3\) mRNA with a t1/2 of 3.3 hours. Likewise, the treatment with 8-bromo-cGMP decreased the steady state mRNA level of TGF-\(\beta_3\), with a t1/2 of 2.8 hours. On the basis of these observations, the SNAP-mediated decrease of TGF-\(\beta_3\) mRNA involved a mechanism of mRNA destabilization that occurred at least in part via a cGMP-dependent pathway. In parallel experiments, the pretreatment with actinomycin D before the administration of SNAP abrogated the SNAP-mediated decrease of TGF-\(\beta_3\) mRNA. Thus, these data suggest that SNAP induced the transcription of a destabilizing factor, although its identity and its cognate sequence on the TGF-\(\beta_3\) transcript remain to be defined.

The increased synthesis and secretion of Ang II in human and animal models of cardiac hypertrophy and failure has been considered an integral factor in the progression of interstitial fibrosis.\textsuperscript{1} The TGF-\(\beta\) family may in part contribute to the action of Ang II, since this peptide has been shown to increase TGF-\(\beta_1\) levels in numerous cell types, including cardiac fibroblasts.\textsuperscript{27,28} Consistent with these findings, the present study demonstrated that angiotensin II treatment of neonatal rat cardiac fibroblasts increased TGF-\(\beta_1\) mRNA levels and was associated with a concomitant increase in the steady state mRNA levels of TGF-\(\beta_1\). In this regard, the Ang II–mediated increase of at least 2 TGF-\(\beta\) isoforms provides an autocrine mechanism mediating in part extracellular matrix protein expression. The treatment of cardiac fibroblasts with either the NO donor SNAP or the cGMP analogue 8-bromo-cGMP inhibited Ang II–stimulated protein synthesis in cardiac fibroblasts, a finding consistent with previous studies demonstrating a growth-inhibiting action in this cell type.\textsuperscript{12,13} Moreover, SNAP pretreatment abrogated Ang II–mediated expression of TGF-\(\beta_1\), mRNA, an effect mimicked by 8-bromo-cGMP. Thus, these results demonstrate that Ang II–stimulated cardiac fibroblast growth and TGF-\(\beta_1\) mRNA expression are targets of NO regulation and may support in part a mechanism of NO-mediated attenuation of cardiac fibrosis.

In contrast to TGF-\(\beta_1\), SNAP and 8-bromo-cGMP caused a modest significant increase in the steady state mRNA levels of TGF-\(\beta_1\) mRNA after a 4-hour exposure but returned to levels observed in the untreated cells by 24 hours. These data demonstrate that the SNAP- and 8-bromo-cGMP–mediated decrease of TGF-\(\beta_1\) mRNA was not due to a nonspecific degradation of mRNA. Moreover, similar observations were observed in mesangial cells and an adenocarcinoma lung cell line, as NO and atrial natriuretic peptide, acting via a cGMP-dependent pathway, increased TGF-\(\beta_1\) expression.\textsuperscript{29,30} Second, the data observed in the present study further highlight a disparate pattern of TGF-\(\beta_1\) and TGF-\(\beta_3\) mRNA regulation. Indeed, in the volume-overloaded rat model of cardiac hypertrophy, a distinct pattern of ventricular TGF-\(\beta\) isoform regulation was also observed.\textsuperscript{3} Lastly, a well-documented role of TGF-\(\beta_1\) includes the suppression of NO synthase activity.\textsuperscript{31} In this regard, NO/cGMP-dependent expression of TGF-\(\beta_1\) may represent a negative feedback mechanism limiting the physiological actions of NO.

In conclusion, a major new finding of the present study highlights a NO-mediated decrease of TGF-\(\beta_1\) mRNA level
in neonatal rat cardiac fibroblasts in part via a posttranscriptional mechanism of destabilization. By contrast, SNAP treatment caused a transient increase in TGF-β expression, which may represent a negative feedback mechanism modulating the physiological actions of NO. Lastly, Ang II has been characterized as a profibrotic factor, and it has been suggested that this effect is mediated in part via the induction of the TGF-β family of peptide growth factors. In the present study NO inhibited Ang II–stimulated protein synthesis and TGF-β mRNA expression. On the basis of these observations, NO could negatively influence the progression of fibrosis via the modulation of TGF-β expression in cardiac fibroblasts.

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