

Hypertrophic Stimuli Induce Transforming Growth Factor- β_1 Expression in Rat Ventricular Myocytes

Nobuyuki Takahashi, Angelino Calderone, Nicholas J. Izzo, Jr., Tiina M. Mäki, James D. Marsh, and Wilson S. Colucci
Cardiovascular Division, Departments of Medicine, Brigham and Women's Hospital and Harvard Medical School,
Boston, Massachusetts 02115

Abstract

Transforming growth factor- β_1 (TGF- β_1) is a peptide growth factor that may play a role in the myocardial response to hypertrophic stimuli. However, the cellular distribution, mechanism of induction, and source of increased TGF- β_1 in response to hypertrophic stimuli are not known. We tested the hypothesis that the cardiac myocyte responds to hypertrophic stimuli with the increased expression of TGF- β_1 . In adult rat ventricular myocardium freshly dissociated into myocyte and nonmyocyte cellular fractions, the preponderance of TGF- β_1 mRNA visualized by Northern hybridization was in the nonmyocyte fraction. Abdominal aortic constriction (7 d) and subcutaneous norepinephrine infusion (36 h) each caused ventricular hypertrophy associated with 3.1-fold and 3.8-fold increases, respectively, in TGF- β_1 mRNA in the myocyte fraction, but had no effect on the level of TGF- β_1 mRNA in the nonmyocyte fraction. In ventricular myocytes, norepinephrine likewise caused a 4.1-fold increase in TGF- β_1 mRNA associated with an increase in TGF- β bioactivity. This induction of TGF- β_1 mRNA occurred at norepinephrine concentrations as low as 1 nM and was blocked by prazosin, but not propranolol. NE did not increase the TGF- β_1 mRNA level in nonmyocytes, primarily fibroblasts, cultured from neonatal rat ventricle. Thus, the cardiac myocyte responds to two hypertrophic stimuli, pressure overload and norepinephrine, with the induction of TGF- β_1 . These data support the view that TGF- β_1 , released by myocytes and acting in an autocrine and/or paracrine manner, is involved in myocardial remodeling by hypertrophic stimuli. (*J. Clin. Invest.* 1994; 94:1470–1476.) Key words: myocardium • growth factors • hypertrophy • norepinephrine • α_1 -adrenergic receptors

Introduction

Transforming growth factor- β_1 (TGF- β_1) plays an important role in the regulation of growth, differentiation, and repair in a wide variety of cells and tissues (1–3). In the heart, TGF- β_1 is expressed at high levels during development (3, 4). There is also evidence that TGF- β_1 is induced in hypertrophied myocardium following myocardial infarction (5, 6), pressure overload

(7), or the infusion of norepinephrine (NE) (8). Moreover, in vitro observations indicate that TGF- β_1 can cause the induction of a fetal gene program in cardiac myocytes (9) and the stimulation of collagen production by fibroblasts (10), two characteristic features of myocardial hypertrophy (11). It is therefore possible that TGF- β_1 plays a role in the myocardial remodeling process induced by hypertrophic stimuli.

An AP-1 site regulates transcription of TGF- β_1 (12), and may thus couple TGF- β_1 expression to hypertrophic stimuli, such as cell stretch (13) and NE (14), that can induce *fos* and *jun*. However, a mechanism by which TGF- β_1 expression may be upregulated in response to such stimuli has not been elucidated. Furthermore, although TGF- β_1 mRNA and protein can be demonstrated in ventricular myocardium, the localization and cellular source of TGF- β_1 in the heart have been controversial (15–17), and no information is available regarding the cellular origin of TGF- β_1 in hypertrophied myocardium.

The cardiac myocyte is oriented circumferentially in the ventricular chamber, and is thus a good candidate for detecting changes in myocardial wall stress caused by hemodynamic stimuli. In addition, cardiac myocytes express α_1 -adrenergic receptors that have been shown in vitro to mediate myocyte hypertrophy and fetal gene expression in response to NE (14, 18). To test the hypothesis that hypertrophic stimuli upregulate TGF- β_1 expression in cardiac myocytes, we studied the effects of two hypertrophic stimuli, abdominal aortic constriction and NE infusion, on TGF- β_1 mRNA expression in the myocyte and nonmyocyte cell fractions from freshly dissociated adult rat ventricular myocardium. The expression of mRNA for preproatrial natriuretic factor (ANF)¹ was determined in parallel to serve as a marker for fetal gene induction by the hypertrophic stimuli (9). To elucidate further the mechanism of TGF- β_1 induction, we assessed the effect of NE on TGF- β_1 mRNA in myocytes and nonmyocytes cultured from neonatal rat ventricle.

Methods

Drug infusions. Male 200–225 g Sprague–Dawley rats (Charles River Labs., Wilmington, MA) received subcutaneous infusion of drugs using Alzet osmotic minipumps (model 1003D; Alza Corp., Palo Alto, CA) implanted subcutaneously on the back slightly posterior to the scapulae. Minipumps delivered *l*-norepinephrine bitartrate (NE) in ascorbic acid (2 mg/ml) at a mean flow rate of 1 μ l/h to deliver NE at a rate of 500 μ g/kg per h for 36 h. Sham-infused animals received ascorbic acid alone at a flow rate of 1 μ l/h for 36 h. The subcutaneous infusion of NE at a rate of 100 μ g/kg per h has been shown to increase systolic blood pressure by 30–40 mmHg (19).

Aortic constriction. Male (200–225 g) Sprague–Dawley rats (Charles River Labs.) were anesthetized with sodium pentobarbital (50

Address correspondence to Dr. Wilson S. Colucci, Cardiovascular Division, Brigham and Women's Hospital, Boston, MA 02115.

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1. Abbreviations used in this paper: ANF, atrial natriuretic factor; PKC, protein kinase C.

mg/kg). A suprarenal aortic constriction was established with a ligature, using a 21-gauge needle to establish the diameter of the constriction (20). Sham-operated animals had placement of a loosely tied ligature at the same location.

Isolation of myocyte and nonmyocyte enriched fractions from adult ventricular myocardium. Myocyte and nonmyocyte-enriched fractions of adult ventricular myocardium were isolated by a modification of the technique of Claycomb and Palazzo (21), as described in detail previously (22). Briefly, rats were anesthetized with ether and the heart was rapidly excised, transferred to a nominally Ca^{2+} -free solution, and perfused in a retrograde fashion through the aorta by the Langendorff technique. The heart was first perfused for 5 min with Krebs–Henseleit bicarbonate buffer containing 1.2 mM MgSO_4 , 1.25 mM CaCl_2 , 1.2 mM KH_2PO_4 , 25 mM NaHCO_3 , and 11 mM glucose, gassed with 95% $\text{O}_2/5\%\text{CO}_2$, and equilibrated to pH 7.4 at 37°C, with an osmolality of 287 mosm/liter. Next, the heart was perfused with Ca^{2+} -free Krebs–Henseleit buffer until spontaneous beating stopped, at which time 0.05% collagenase (Worthington Biochem. Corp., Freehold, NJ) and 0.03% hyaluronidase (Sigma Chemical Co., St. Louis, MO) were added to the Ca^{2+} -free Krebs–Henseleit buffer (enzyme buffer I) for an additional 20 min. The atria and great vessels were then removed, and the remaining ventricular tissue was minced to < 1 mm in enzyme buffer I containing 0.02 mg/ml trypsin (Sigma Chemical Co.) and deoxyribonuclease type II (Worthington Biochem. Corp.), and incubated in a shaking water bath (37°C) for 15 min. The tissue was then filtered through a 200- μm nylon mesh gauze, centrifuged at 500 rpm for 2 min, and resuspended in wash media (1:1 DME: Ca^{2+} -free Krebs–Henseleit buffer with 10% FBS). The pellet contained the myocyte-rich fraction, and the supernatant contained the myocyte-depleted fraction. The myocyte-enriched pellet was resuspended in wash media, washed three times with wash media, layered gently over a cushion of 6% bovine serum albumin and sedimented for 20 min. The myocytes in the sedimented pellet were then resuspended in denaturing solution for mRNA preparation (see below). This method has been shown to yield a myocyte-rich fraction consisting of > 95% myocytes (22). The myocyte-depleted supernatant was spun again at 1500 rpm for 5 min. The supernatant from this spin was discarded, and the pellet (nonmyocyte fraction) was resuspended in denaturing solution for mRNA preparation (see below).

Cultured neonatal rat ventricular myocytes and nonmyocytes. Neonatal rat ventricular myocytes were isolated by a modification of the technique of Kasten (23), as previously described (22). Briefly, hearts were removed from 2-d-old neonatal rats anesthetized with ether and killed by decapitation. Ventricular tissues were digested with 0.1% trypsin (Worthington Biochem. Corp.) in Hanks' balanced salt solution (Gibco Laboratories, Grand Island, NY) overnight at 4°C. Ventricular cells were then recovered by repeated digestions of the tissue in 10 ml of 0.1% collagenase in Hanks' solution. The supernatants collected from each digestion were first centrifuged at 1000 rpm for 3 min (4°C). The pellets were resuspended in ice cold Hanks' solution, pooled and centrifuged at 1000 rpm for 4 min (4°C). Cells were resuspended in DME containing 7% FBS and preplated in T75 flasks (Corning Inc., Corning, NY) for 75 min twice to enrich for myocytes and decrease contamination by nonmuscle cells. Nonadherent cells were then plated at a density of 1,000 cells/ mm^2 in 60-mm dishes. After 24 h, the culture medium was changed to serum-free medium containing insulin (5 $\mu\text{g}/\text{ml}$), transferrin (5 $\mu\text{g}/\text{ml}$), and sodium selenite (5 ng/ml), and all experiments were performed 24 h later.

Cultures of neonatal rat ventricular non-myocytes were prepared as described by Sadoshima et al. (24), except that cells were maintained in DME containing 7% FBS until they become confluent, when the medium was changed to the same serum-free medium described for myocytes. All experiments were performed after 24 h in serum-free medium. These cultures consist predominantly of fibroblasts (24).

Northern hybridization. Cells were resuspended in denaturing solution (4 M guanidium thiocyanate, 25 mM Na citrate, 0.5% *N*-laurylsarcosine, 0.1 M β -mercaptoethanol, pH 7.0). Total RNA was isolated by a modification of the technique of Chomczynski and Sacchi (25). RNA was denatured with formaldehyde and formamide, and separated by size

by electrophoresis on a 1.3% agarose/4% formaldehyde gel (15 μg total RNA/lane). RNA was transferred to nylon membranes (Genescreen Plus, DuPont, Boston, MA) by capillary transfer and crosslinked by UV Stratalinker 1800 (Stratagene, La Jolla, CA). Full-length cDNA probes for TGF- β_1 and ANF were labeled with [^{32}P]dCTP (DuPont) to a specific activity of 1–2 $\times 10^6$ cpm/ng cDNA by the random heximer priming method, and hybridized to nylon blots overnight at 42°C, as previously described (26). Hybridized blots were washed twice (15 min, room temperature) with 300 mM NaCl/30 mM trisodium citrate (pH 7.0) and 0.1% SDS, and twice (15 min, 60°C) with 30 mM NaCl/3 mM trisodium citrate (pH 7.0) and 0.1% SDS. The relative amount of mRNA per lane was determined by exposing the washed blots to Kodak XAR film with an intensifying screen at –70°C (2 h–overnight), and measuring the density of the exposed film with a laser densitometer (Ultrascan 2202; LKB, Bromway, Sweden). The sizes of the hybridized species were estimated using the 18S and 28S ribosomal RNA bands as standards. After the analysis of TGF- β_1 and ANF mRNA, blots were reprobated with a ^{32}P -labeled oligonucleotide complementary to 18S ribosomal RNA, washed and autoradiographed as described previously (26). All levels of mRNA reported in this manuscript are normalized to the level of 18S ribosomal RNA to correct for potential differences in the amount of RNA loaded and transferred.

TGF- β bioassay. The bioactivity of TGF- β was assayed by determining the inhibition of DNA synthesis in mink lung epithelial cells (27). CCL-64 mink lung epithelial cells were maintained in a high glucose formulation of DME supplemented with 10% FBS (37°C, 5% CO_2). Subconfluent cells were suspended by exposure to trypsin, resuspended in DME with 10% FBS, pelleted at 250 g for 10 min, washed once with 10 ml of assay buffer (DME supplemented with 0.2% FBS and 10 mM Hepes, pH 7.4), resuspended in assay buffer and seeded at a density of 5×10^4 cells per 0.5 ml in 24-well plastic dishes (Costar, Cambridge, MA). After 1 h, conditioned medium or TGF- β_1 (Genzyme Corp., Cambridge, MA), diluted in assay buffer, was added to each well in a volume of 0.5 ml. After 22 h, cells were pulsed with 2 μCi of [^3H]thymidine (60–80 Ci/mmol, DuPont) for 2 h. Cells were fixed with ice-cold 5% TCA and lysed with 0.5 ml of 0.4N NaOH at room temperature for 30 min.

Conditioned medium was obtained from myocyte cultures exposed to NE (1 μM) or vehicle (ascorbic acid) for 24 h. The volume of conditioned medium was adjusted to fall within the linear range of the standard curve. TGF- β is normally secreted in a latent form that is biologically inactive (28). To activate latent TGF- β , conditioned medium was adjusted to pH 1.5 with HCl, incubated for 1 h at room temperature, neutralized with NaOH, and immediately added to assay plates (29). To determine the specificity of the bioassay for TGF- β , a purified IgG fraction of mouse monoclonal TGF- β_1 , - β_2 , - β_3 antibody (Genzyme Corp.) or control mouse IgG (Sigma Chemical Co.) was preincubated with conditioned medium (1 h, 22°C) before addition of the medium to the mink lung epithelial cells (30).

Data analysis. All data are presented as the mean \pm SEM. Statistical analysis was performed by Student's *t* test or Student–Newman–Keuls test with one way analysis of variance, as appropriate. A *P* value less than 0.05 was considered significant.

Results

Effect of NE infusion on TGF- β_1 mRNA expression in adult rat ventricular myocardium. NE infusion for 36 h had no effect on body weight (sham-infused, 206 \pm 10 g; NE-infused, 198 \pm 13 g), but increased heart weight from 0.80 \pm 0.11 g to 0.94 \pm 0.08 g such that the heart weight/body weight ratio increased by \sim 27%, from 3.9 \pm 0.3 to 4.9 \pm 0.2 mg/g (*P* < 0.05, *n* = 3).

In ventricular myocardium from sham-infused rats, the TGF- β_1 cDNA hybridized to a single band at 2.4 kb. In ventricular myocardium from two NE-infused rats, the level of TGF- β_1 mRNA increased by 35 and 56% relative to sham-infused

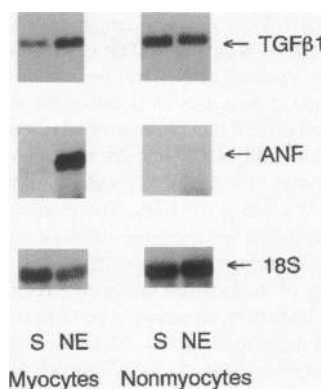


Figure 1. A representative northern hybridization depicting the effect of norepinephrine (NE; 500 $\mu\text{g}/\text{kg}/\text{h}$ in 0.2% ascorbic acid) or sham (S; 0.2% ascorbic acid) infusion for 36 h on the levels of TGF- β_1 and ANF mRNA in the myocyte and nonmyocyte fractions of freshly dissociated adult rat ventricular myocardium. The cDNAs for TGF- β_1 and ANF hybridized to bands at 2.4 and 0.9 kb, respectively. The filter was also hybridized with an oligonucleotide complementary to 18S rRNA (18S) to correct for differences in loading.

rats (data not shown). In both myocardium and cultured cells, additional transcripts of ~ 1.9 and 1.6 kb were frequently visualized (6), albeit at a much lower intensity than the 2.4-kb signal. The intensity of these smaller transcripts generally paralleled that of the 2.4-kb transcript (for example, see Fig. 5). ANF mRNA, which was faintly visualized in myocardium from sham-infused rats, was markedly induced (~ 10 -fold) in myocardium from animals infused with NE (data not shown).

To clarify the cellular source of ventricular TGF- β_1 mRNA, ventricular myocardium was dissociated into myocyte and nonmyocyte enriched fractions. In sham-infused rats, TGF- β_1 mRNA, although visualized in both fractions, was 5.9 ± 0.7 -fold more abundant in nonmyocytes vs. myocytes ($P < 0.001$; $n = 5$) (Figs. 1 and 2A). In NE-infused animals, the level of TGF- β_1 mRNA was increased 3.8 ± 0.4 -fold in the myocyte fraction ($P < 0.001$ vs. sham-infused; $n = 5$), but was unchanged in the nonmyocyte fraction (1.3 ± 0.3 -fold; $P = \text{NS}$; $n = 5$) (Figs.

1 and 2B). In cells from sham-infused rats, ANF mRNA was faintly detectable in the myocyte fraction, and was not detectable or only faintly detectable in the nonmyocyte fraction (Fig. 1). In cells from NE-infused animals, ANF mRNA was increased 17.5 ± 3.6 -fold in the myocyte fraction, and was not induced in the nonmyocyte fraction (Fig. 1).

Effect of abdominal aortic constriction on myocardial TGF- β_1 mRNA expression. Abdominal aortic constriction for 1 wk resulted in an $\sim 33\%$ increase in the heart weight/body weight ratio from 3.3 ± 0.1 to 4.4 ± 0.1 ($P < 0.001$; $n = 6$ for controls and $n = 8$ for aortic constriction). In rats with aortic constriction, the level of TGF- β_1 mRNA in the myocyte fraction was increased 3.1 ± 0.6 -fold ($P < 0.05$; $n = 3$) compared to sham-operated animals, but was unchanged in the nonmyocyte fraction (Figs. 3 and 4).

Effect of NE on TGF- β_1 mRNA in cultured neonatal rat myocytes and nonmyocytes. The experiments with freshly dissociated myocardium suggested that NE induces TGF- β_1 mRNA in cardiac myocytes, cultured neonatal rat ventricular myocytes were exposed to NE ($1 \mu\text{M}$) for 24 h. NE caused a 4.1 ± 0.8 -fold increase in the level of TGF- β_1 mRNA ($P < 0.05$; $n = 4$), associated with a 3.9 ± 1.2 -fold increase in ANF mRNA level (Figs. 5 and 6). The induction of TGF- β_1 mRNA was detectable within 2 h, sustained for at least 24 h (data not shown), and occurred with a NE threshold of 1 nM (Fig. 5). To elucidate the adrenergic receptor pathway involved, myocytes were incubated with NE in combination with prazosin ($1 \mu\text{M}$) or propranolol ($2 \mu\text{M}$). Prazosin inhibited the NE-induced increase in TGF- β_1 mRNA by $75 \pm 6\%$ ($P < 0.05$; $n = 4$), whereas propranolol had no effect (Fig. 6).

To determine whether NE can induce TGF- β_1 mRNA in cardiac nonmyocytes, neonatal rat cardiac nonmyocytes were exposed to NE ($1 \mu\text{M}$) or angiotensin II (100 nM) for 24 h. TGF- β_1 mRNA was readily visualized in control cells (Fig. 7).

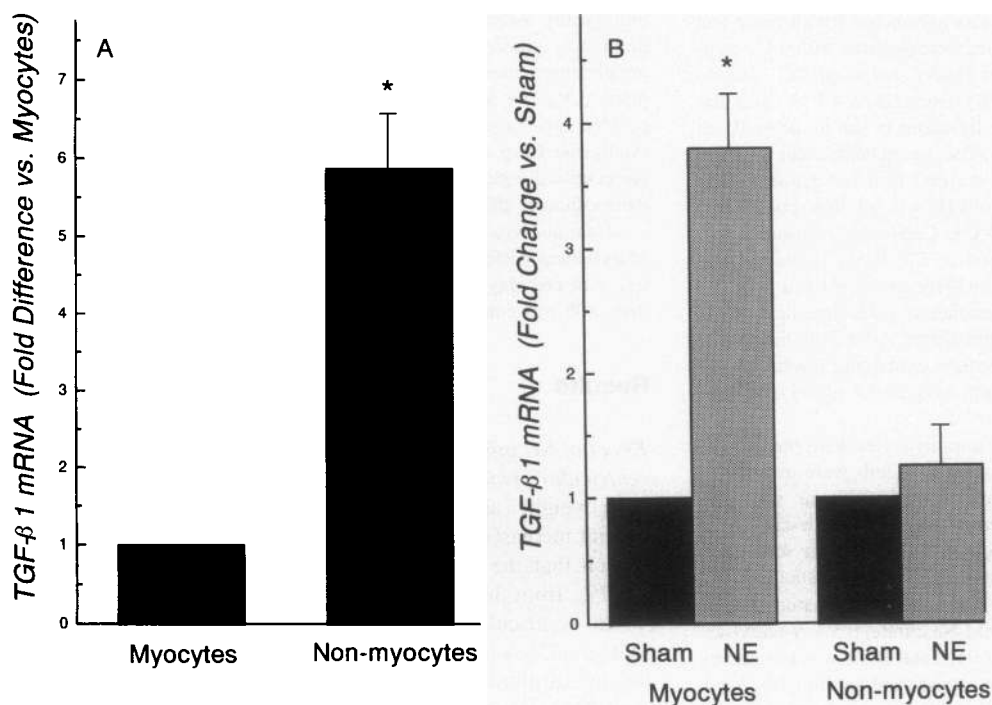


Figure 2. (A) Relative levels of TGF- β_1 mRNA in myocyte vs. nonmyocyte fractions from adult rat ventricle. The mean data from 5 experiments are expressed relative to the level of TGF- β_1 mRNA in the myocyte fraction, and are normalized to the level of 18S rRNA determined by Northern hybridization (23). $*P < 0.05$ vs. myocytes. (B) Effect of norepinephrine (NE) infusion on the level of TGF- β_1 mRNA in myocyte and nonmyocyte fractions. Data for each cell fraction are expressed relative to the levels in sham-infused (Sham) animals, and are the mean of 5 experiments. $*P < 0.05$ vs. sham-infused animals.

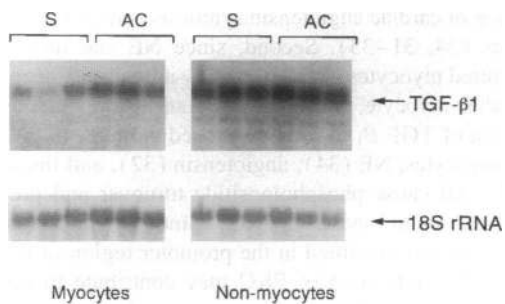


Figure 3. Northern hybridization showing the effect of a 7-d abdominal aortic constriction (AC) as compared to sham-operation (S) on the expression of TGF- β_1 mRNA in myocyte and nonmyocyte fractions of freshly dissociated adult rat ventricular myocardium.

Although angiotensin II induced TGF- β_1 mRNA by approximately twofold, NE had no effect ($+20 \pm 30\%$; $P = \text{NS}$; $n = 3$).

Effect of NE on TGF- β bioactivity in myocyte-conditioned medium. To determine whether the induction of TGF- β_1 mRNA is associated with an increase in TGF- β activity, conditioned medium from cultured neonatal myocytes treated with vehicle or NE was applied to mink lung epithelial cells on assay plates (27). Conditioned medium (diluted 1:20) from myocytes treated with vehicle or NE had no detectable TGF- β activity ($P = \text{NS}$; $n = 2$). However, in many cell types TGF- β is expressed in an inactive form which is activated by cell surface/interstitial enzymes (28). This latent TGF- β can be activated by acidification (29). After activation of latent TGF- β by acidification, conditioned medium from both vehicle- and NE-treated myocytes demonstrated marked TGF- β bioactivity, and NE treatment caused a further significant increase ($P < 0.001$; $n = 7$) in TGF- β activity compared to vehicle-treated cells (Fig. 8 A). The addition of a TGF- β -neutralizing antibody (100 $\mu\text{g}/\text{ml}$) abolished TGF- β bioactivity in acid-activated conditioned media (diluted 1:100) from NE-treated myocytes (Fig. 8 B).

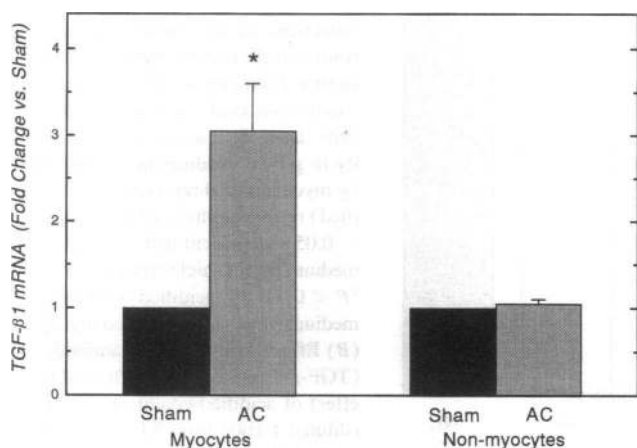


Figure 4. Effect of a 7-d aortic constriction (AC) on TGF- β_1 mRNA level in myocyte and nonmyocyte fractions depicted in Fig. 3. Data for each cell fraction are expressed relative to the levels in sham-operated animals (Sham), and are the mean of 3 experiments. * $P < 0.05$ vs. sham operated.

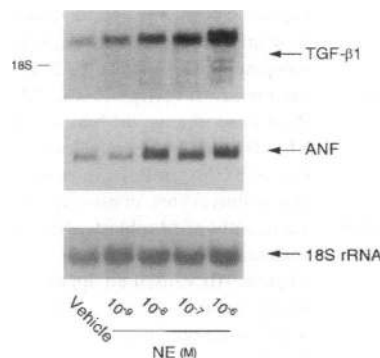


Figure 5. Northern hybridization showing the concentration-dependent induction of TGF- β_1 and ANF mRNAs in cultured neonatal rat ventricular myocytes exposed to either vehicle (ascorbic acid, 100 μM) or norepinephrine (NE) for 24 h. The cDNA for TGF- β_1 hybridized strongly to a 2.4-kb transcript. Smaller transcripts at approximately 1.6 and 1.9 kb were frequently visualized, and generally paralleled the 2.4-kb transcript in intensity.

Smaller transcripts at approximately 1.6 and 1.9 kb were frequently visualized, and generally paralleled the 2.4-kb transcript in intensity.

Discussion

The major new finding of this study is that two types of hypertrophic stimuli, pressure overload and NE, each induced the expression of TGF- β_1 mRNA in cardiac myocytes. Although the preponderance of basal TGF- β_1 mRNA in adult rat ventricular myocardium was localized in the nonmyocyte cell fraction which consists primarily of fibroblasts and endothelial cells (16), pressure overload and NE infusion induced 3.1-fold and 3.8-fold increases, respectively, in TGF- β_1 mRNA expression in the myocyte fraction, but had no effect on the level of expression in the nonmyocyte fraction.

In cultured neonatal rat ventricular myocytes, NE likewise induced a 4.1-fold increase in TGF- β_1 mRNA level that was associated with a significant increase in TGF- β bioactivity. The induction of TGF- β_1 by NE was evident at nanomolar concentrations and was abolished by the α_1 -adrenergic receptor selective antagonist prazosin. Thus, the induction of TGF- β_1 by NE can be mediated by an α_1 -adrenergic receptor located on the cardiac myocyte, and does not require a change in hemodynamic load.

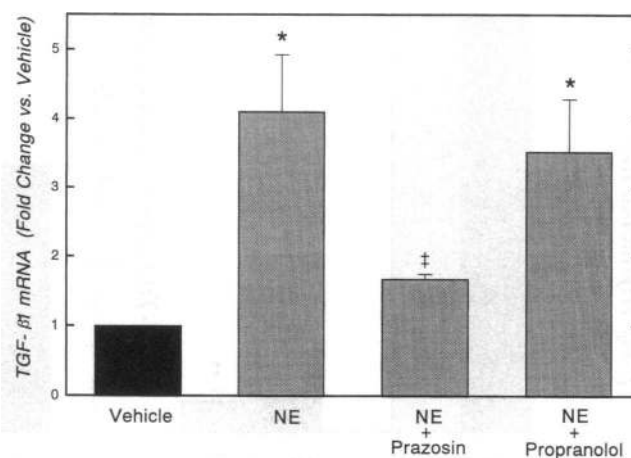


Figure 6. Effects of α_1 -adrenergic receptor selective- (prazosin, 1 μM) and β -adrenergic receptor selective- (propranolol, 2 μM) antagonists on the induction of TGF- β_1 mRNA by norepinephrine (NE, 1 μM) in cultured neonatal rat ventricular myocytes. Data are expressed relative to vehicle-treated cultures, and are the means of 4 experiments. * $P < 0.05$ vs. vehicle; † $P < 0.05$ vs. NE and NE + propranolol.

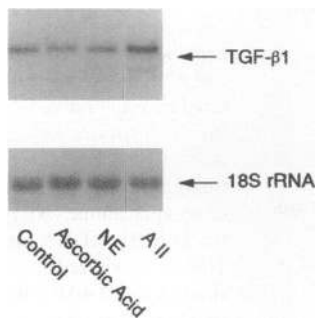


Figure 7. Northern hybridization showing the effect of exposure to norepinephrine (NE; 1 μ M, 24 h), angiotensin II (AII; 100 nM, 24 h) and ascorbic acid (vehicle for NE; 100 μ M, 24 h) on the expression of TGF- β_1 mRNA in ventricular nonmyocytes, consisting predominantly of fibroblasts. NE did not induce TGF- β_1 mRNA, whereas AII caused an approximately twofold increase. Similar results were obtained in two additional experiments.

Increased TGF- β_1 mRNA expression has been observed previously in whole ventricular myocardium following aortic constriction (7), norepinephrine infusion (8), and in the noninfarcted portion of the ventricle after myocardial infarction (5, 6, 17). Our report is, to our knowledge, the first to implicate the cardiac myocyte as the source of increased TGF- β_1 mRNA in response to hypertrophic stimuli. Since the large majority of TGF- β_1 mRNA in the myocardium is located in nonmyocytes, it is particularly striking that only myocytes responded to these hypertrophic stimuli with the induction of TGF- β_1 .

Two potential mechanisms may account for the induction of TGF- β_1 in cardiac myocytes. First, both aortic constriction and NE infusion cause an increase in myocardial wall stress, which might serve as a mechanical stimulus for TGF- β_1 induction (13). The coupling between mechanical stress and TGF- β_1 induction may involve increased levels of circulating angio-

tensin, induction of cardiac angiotensin synthesis and/or release from myocytes (24, 31–33). Second, since NE can induce TGF- β_1 in cultured myocytes, activation of α_1 -adrenergic receptors on the cardiac myocyte, per se, may be a sufficient stimulus for the induction of TGF- β_1 in the NE-infused animals. In cultured cardiac myocytes, NE (34), angiotensin (32), and linear stretch (13, 35) all cause phosphoinositide turnover and presumably activate protein kinase C (PKC). Since an AP-1 consensus region has been identified in the promoter region of the TGF- β_1 gene (12), activation of PKC may contribute to the induction of TGF- β_1 by NE and/or pressure overload.

Cardiac fibroblasts may play an important role in the myocardial remodeling to hypertrophic stimuli, both by the synthesis of interstitial matrix components such as collagen (10) and by the elaboration of growth factors (36). NE did not induce TGF- β_1 mRNA in cultured cardiac nonmyocytes, which consist predominantly of fibroblasts (24). Since angiotensin, presumably acting via an AP-1 site, induced TGF- β_1 mRNA in cultured nonmyocytes, the failure of NE to induce TGF- β_1 mRNA in these cells most likely reflects the absence of an appropriate α -adrenergic receptor subtype and/or coupling to the induction of *fos/jun*. Our findings do, however, raise the possibility that the induction of TGF- β_1 in cardiac myocytes may contribute to myocardial remodeling via a paracrine action on cardiac fibroblasts.

Interpretation of the dissociated myocardium experiments depends on the ability to separate myocytes and nonmyocytes. Previous work using this technique, or minor modifications thereof, indicates that the myocyte fraction has < 5% contamination by nonmyocytes (22). Since nonmyocytes are a major source of TGF- β mRNA both in myocardium (as shown here)

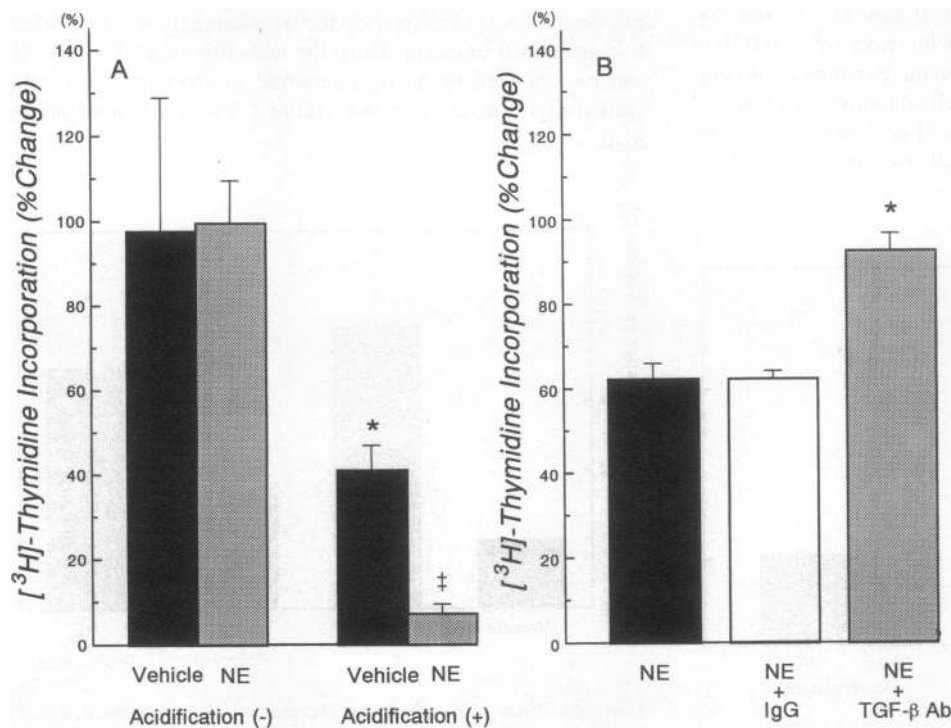


Figure 8. (A) Effect of norepinephrine (NE; 1 μ M, 24 h) on TGF- β bioactivity in the conditioned medium (diluted 1:20) from cultured neonatal rat ventricular myocytes. TGF- β bioactivity is expressed as the inhibition of [3 H]-thymidine uptake in mink lung epithelial cells (27). In the absence of acidification, no bioactivity was observed. With acidification, there was significant bioactivity in the conditioned medium from vehicle-treated myocytes, and a further significant increase in activity in conditioned medium from NE-treated cells. Data are normalized to the activity in growth medium not conditioned by myocytes, and represent 2 (nonacidified) or 7 (acidified) observations. * P < 0.05 vs. nonacidified conditioned medium from vehicle-treated myocytes; $^{\dagger}P$ < 0.001 vs. acidified conditioned medium from vehicle-treated myocytes. (B) Effect of anti-TGF- β antibody (TGF- β Ab) on the growth inhibitory effect of acidified-conditioned medium (diluted 1:100) from NE-treated myocytes. TGF- β Ab (100 μ g/ml) abolished the growth inhibitory effect of conditioned medium from NE-treated

myocytes, whereas control mouse IgG (100 μ g/ml) had no effect. Data are normalized to the activity in growth medium not conditioned by myocytes with or without an equivalent amount of IgG or TGF- β Ab. * P < 0.05 vs. conditioned medium from NE-treated myocytes with or without IgG.

and in culture (37), it is possible that even a small contamination of the myocyte fraction by nonmyocytes is important. However, it is unlikely that our observations are due to nonmyocytes contaminating the myocyte fraction, since basal TGF- β_1 mRNA expression in the nonmyocyte fraction failed to increase with either hypertrophic stimulus, and NE failed to induce TGF- β_1 in cultured fibroblasts. It is also possible that hypertrophic stimuli increase the nonmyocyte contamination of our preparation. However, based on the relative abundance of TGF- β_1 mRNA in myocytes and nonmyocytes, nonmyocyte contamination would have to increase to approximately 70% to account for the four-fold increase in TGF- β_1 mRNA that was observed in the myocyte fraction. Finally, we cannot exclude the possibility that the dissociation procedure, which has been shown to induce early response genes such as *fos* TGF- β_1 , (38), contributed to our observations. However, the induction of TGF- β_1 in cultured myocytes and the lack of induction in the nonmyocyte fraction in vitro argue against this possibility.

In summary, we have shown that the cardiac myocyte responds to hypertrophic stimuli with the induction of TGF- β_1 , both in vivo and in vitro. The functional significance of these observations remains to be established. The TGF- β_1 released from cultured myocytes in response to NE was latent. Thus, it is unlikely that TGF- β_1 contributes to the effects of NE in this in vitro system. However, proteinases capable of activating TGF- β may be present in the cardiac interstitium (39), and might activate TGF- β released from myocytes in vivo. If this were the case, TGF- β_1 might act in an autocrine and/or paracrine manner to affect the phenotype, growth and/or function of neighboring cardiac cells (40), including myocytes, fibroblasts and microvascular endothelial cells. However, it is also possible that the induction of TGF- β_1 mRNA in cardiac myocytes is merely a marker for myocyte stress, akin to the induction of certain fetal genes, and does not necessarily have functional significance. In either case, our data suggest that the cardiac myocyte plays a primary role in detecting hypertrophic stimuli.

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References

1. Sporn, M. B., and A. B. Roberts. 1992. Transforming growth factor- β : Recent progress and new challenges. *J. Cell Biol.* 119:1017-1021.
2. Barnard, J. A., R. M. Lyons, and H. L. Moses. 1990. The cell biology of transforming growth factor β . *Biochim. Biophys. Acta.* 1032:79-87.
3. MacLellan, W. R., T. Brand, and M. D. Schneider. 1993. Transforming growth factor- β in cardiac ontogeny and adaptation. *Circ. Res.* 73:783-791.

4. Pelton, R. W., B. Saxena, M. Jones, H. L. Moses, and L. I. Gold. 1991. Immunohistochemical localization of TGF β_1 , TGF β_2 , and TGF β_3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. *J. Cell Biol.* 115:1091-1105.
5. Thompson, N. L., F. Bazoberry, E. H. Speir, W. Casscells, V. J. Ferrans, K. C. Flanders, P. Kondaiah, A. G. Geiser, and M. B. Sporn. 1988. Transforming growth factor beta-1 in acute myocardial infarction in rats. *Growth Factors.* 1:91-99.
6. Qian, S. W., P. Kondaiah, W. Casscells, A. B. Roberts, and M. B. Sporn. 1991. A second messenger RNA species of transforming growth factor β_1 in infarcted rat heart. *Cell Regul.* 2:241-249.
7. Villarreal, F. J., and W. H. Dillmann. 1992. Cardiac hypertrophy-induced changes in mRNA levels for TGF- β_1 , fibronectin, and collagen. *Am. J. Physiol.* 262:H1861-H1866.
8. Bhambi, B., and M. Eghbali. 1991. Effect of norepinephrine on myocardial collagen gene expression and response of cardiac fibroblasts after norepinephrine treatment. *Am. J. Pathol.* 139:1131-1142.
9. Parker, T. G., S. E. Packer, and M. D. Schneider. 1990. Peptide growth factors can provoke "fetal" contractile protein gene expression in rat cardiac myocytes. *J. Clin. Invest.* 85:507-514.
10. Eghbali, M., R. Tomek, V. P. Sukhatme, C. Woods, and B. Bhambi. 1991. Differential effects of transforming growth factor- β_1 and phorbol myristate acetate on cardiac fibroblasts. Regulation of fibrillar collagen mRNAs and expression of early transcription factors. *Circ. Res.* 69:483-490.
11. Morgan, H. E., and K. M. Baker. 1991. Cardiac hypertrophy. Mechanical, neural, and endocrine dependence. *Circulation.* 83:13-25.
12. Kim, S.-J., P. Angel, R. Lafyatis, K. Hattori, K. Y. Kim, M. B. Sporn, M. Karin, and A. B. Roberts. 1990. Autoinduction of transforming growth factor β_1 is mediated by the AP-1 complex. *Mol. Cell. Biol.* 10:1492-1497.
13. Sadoshima, J., and S. Izumo. 1993. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1681-1692.
14. Iwaki, K., V. P. Sukhatme, H. E. Shubeita, and K. R. Chien. 1990. α - and β -Adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. *J. Biol. Chem.* 265:13809-13817.
15. Thompson, N. L., K. C. Flanders, J. M. Smith, L. R. Ellingsworth, A. B. Roberts, and M. B. Sporn. 1989. Expression of transforming growth factor- β_1 in specific cells and tissues of adult and neonatal mice. *J. Cell Biol.* 108:661-669.
16. Eghbali, M. 1989. Cellular origin and distribution of transforming growth factor- β_1 in the normal rat myocardium. *Cell Tissue Res.* 256:553-558.
17. Casscells, W., F. Bazoberry, E. Speir, N. Thompson, K. Flanders, P. Kondaiah, V. J. Ferrans, S. E. Epstein, and M. Sporn. 1990. Transforming growth factor- β_1 in normal heart and in myocardial infarction. *Ann. N.Y. Acad. Sci.* 593:148-160.
18. Simpson, P. 1985. Stimulation of hypertrophy of cultured neonatal rat heart cells through an α_1 -adrenergic receptor and induction of beating through an α_1 - and β_1 -adrenergic receptor interaction. Evidence for independent regulation of growth and beating. *Circ. Res.* 56:884-894.
19. Johnson, M. D., A. Grignolo, C. M. Kuhn, and S. M. Schanberg. 1983. Hypertension and cardiovascular hypertrophy during chronic catecholamine infusion in rats. *Life Sci.* 33:169-180.
20. Black, F. M., S. E. Packer, T. G. Parker, L. H. Michael, R. Roberts, R. J. Schwartz, and M. D. Schneider. 1991. The vascular smooth muscle α -actin gene is reactivated during cardiac hypertrophy provoked by load. *J. Clin. Invest.* 88:1581-1588.
21. Claycomb, W. C., and M. C. Palazzo. 1980. Culture of the terminally differentiated adult cardiac muscle cell: A light and scanning electron microscope study. *Dev. Biol.* 80:466-482.
22. Springhorn, J. P., Ø. Ellingsen, H.-J. Berger, R. A. Kelly, and T. W. Smith. 1992. Transcriptional regulation in cardiac muscle. Coordinate expression of Id with a neonatal phenotype during development and following a hypertrophic stimulus in adult rat ventricular myocytes in vitro. *J. Biol. Chem.* 267:14360-14365.
23. Kasten, F. H. 1973. In *Tissue Culture Methods and Applications*. P. F. Kruse, Jr. and M. K. Patterson Jr., editors. Academic Press, New York. 72-86.
24. Sadoshima, J., and S. Izumo. 1993. Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT $_1$ receptor subtype. *Circ. Res.* 73:413-423.
25. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
26. Izzo, N. J., Jr., C. E. Seidman, S. Collins, and W. S. Colucci. 1990. α_1 -Adrenergic receptor mRNA level is regulated by norepinephrine in rabbit aortic smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* 87:6268-6271.
27. Danielpour, D., L. L. Dart, K. C. Flanders, A. B. Roberts, and M. B. Sporn. 1989. Immunodetection and quantitation of the two forms of transforming growth factor-beta (TGF- β_1 and TGF- β_2) secreted by cells in culture. *J. Cell. Physiol.* 138:79-86.
28. Miyazono, K., K. Yuki, F. Takaku, C. Wernstedt, T. Kanzaki, A. Olofsson,

- U. Hellman, and C.-H. Heldin. 1990. Latent forms of TGF- β : structure and biology. *Ann. NY Acad. Sci.* 593:51–58.
29. Lyons, R. M., J. Keski-oja, and H. L. Moses. 1988. Proteolytic activation of latent transforming growth factor- β from fibroblast-conditioned medium. *J. Cell Biol.* 106:1659–1665.
30. Gibbons, G. H., R. E. Pratt, and V. J. Dzau. 1992. Vascular smooth muscle cell hypertrophy vs. hyperplasia. Autocrine transforming growth factor- β_1 expression determines growth response to angiotensin II. *J. Clin. Invest.* 90:456–461.
31. Baker, K. M., M. I. Chermn, S. K. Wixson, and J. F. Aceto. 1990. Renin-angiotensin system involvement in pressure-overload cardiac hypertrophy in rats. *Am. J. Physiol.* 259:H324–H332.
32. Schunkert, H., V. J. Dzau, S. S. Tang, A. T. Hirsch, C. S. Apstein, and B. H. Lorell. 1990. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. *J. Clin. Invest.* 86:1913–1920.
33. Sadoshima, J., Y. Xu, H. S. Slayter, and S. Izumo. 1993. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell.* 75:977–984.
34. Brown, J. H., I. L. Buxton, and L. L. Brunton. 1985. α_1 -Adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. *Circ. Res.* 57:532–537.
35. Komuro, I., Y. Katoh, T. Kaida, Y. Shibazaki, M. Kurabayashi, E. Hoh, F. Takaku, and Y. Yazaki. 1991. Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes. Possible role of protein kinase C activation. *J. Biol. Chem.* 266:1265–1268.
36. Long, C. S., C. J. Henrich, and P. C. Simpson. 1991. A growth factor for cardiac myocytes is produced by cardiac nonmyocytes. *Cell Regul.* 2:1081–1095.
37. Long, C. S. 1992. TGF β isoform expression and effect in neonatal rat cardiac myocytes and non-myocytes in culture. *Circulation.* 86:1–837. (Abstr.)
38. Claycomb, W. C., and N. A. Lanson, Jr. 1987. Proto-oncogene expression in proliferating and differentiating cardiac and skeletal muscle. *Biochem. J.* 247:701–706.
39. Lyons, R. M., K.-O., Jorma, H. L. Moses. 1989. Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. *J. Cell Biol.* 106:1659–1665.
40. Kurihara, H., M. Yoshizumi, T. Sugiyama, F. Takaku, M. Yanagisawa, T. Masaki, M. Hamaoki, H. Kato, and Y. Yazaki. 1989. Transforming growth factor- β stimulates the expression of endothelin mRNA by vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 159:1435–1440.