Interaction between angiotensin II and Smad proteins in fibroblasts in failing heart and in vitro

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Hao, Jianming, Baiqui Wang, Stephen C. Jones, Davinder S. Jassal, and Ian M. C. Dixon. Interaction between angiotensin II and Smad proteins in fibroblasts in failing heart and in vitro. Am J Physiol Heart Circ Physiol 279: H3020–H3030, 2000.—Angiotensin II (angiotensin) and transforming growth factor (TGF)-β1 play an important role in cardiac fibrosis. We examined Smad proteins in 8-wk post-myocardial infarction (MI) rat hearts. AT1, blockade (losartan) attenuated the activation of TGF-β1, in target tissues. Losartan administration (8 wk, 15 mg·kg⁻¹·day⁻¹) normalized total Smad 2 overexpression in infarct scar and remnant heart tissue and normalized Smad 4 in infarct scar. Phosphorylated Smad 2 (P-Smad 2) staining decreased in cytosol from failing heart vs. the control, which was normalized by losartan, suggesting augmented P-Smad 2 movement into the nuclei in untreated failing hearts. Using adult primary rat fibroblasts treated with angiotensin (10⁻⁶ M), we noted rapid translocation (15 min) of P-Smad 2 into the nuclei from the cytosol. Nuclear P-Smad 2 protein level increased with angiotensin treatment, which was blocked by losartan. We conclude that angiotensin may influence total Smad 2 and 4 expression in post-MI heart failure and that angiotensin treatment is associated with rapid P-Smad 2 nuclear translocation in isolated fibroblasts. This study suggests that cross talk between angiotensin and Smad signaling is associated with fibrotic events in post-MI hearts.

AFTER MYOCARDIAL INFARCTION (MI), the myocardium undergoes a repair process involving scar formation at the site of infarction that includes fibroblast and myofibroblast proliferation and concomitant deposition of extracellular matrix proteins (38). During the early phase of MI, activation of these processes is critical for normal wound healing in the infarcted region. However, eventual interstitial fibrosis also occurs in remnant tissue and acts to increase myocardial stiffness. Further expansion of the extracellular matrix impairs diastolic stiffness and compromises systolic mechanics, contributing to subsequent cardiac hypertrophy and heart failure (17, 25). Thus the investigation of mechanism(s) underlying post-MI cardiac fibrosis has attracted considerable attention in recent years.

Mounting evidence supports the suggestion that both angiotensin II (angiotensin) and transforming growth factor-β1 (TGF-β1) stimulate the progression of cardiac fibrosis during cardiac hypertrophy and heart failure (16, 36). In this regard, TGF-β1 is a powerful initiator for the synthesis of collagen and other major extracellular matrix (ECM) components in a variety of cell types (21). The expression of TGF-β1 is increased in the myocardium during pressure overload-induced hypertrophy (19) and early after MI (35). Recently, a major advance in understanding TGF-β1 postreceptor signaling was the identification of Smad proteins as effector proteins. We observed activation of TGF-β1 and the increased expression of novel downstream Smad 2 and Smad 4 signaling proteins in infarct scar and remnant myocardium during the chronic phase of MI (12). These events were positively correlated to ongoing cardiac fibrosis in remnant tissues as well as scar remodeling in post-MI heart, which is modulated exclusively by cardiac fibroblasts and myofibroblasts (12, 24). Receptor-activated Smad 2 dimerizes with Smad 4 upon phosphorylation of tyrosine residues on the Smad 2 COOH-terminal region (22, 43). The phosphorylated Smad 2-Smad 4 dimer then translocates to the nucleus and initiates gene transcription (22, 40) by association with eukaryotic nuclear transcription factors via their specific binding to Smad 2 (22, 40). Thus the phosphorylation of Smad 2 and its subsequent translocation to the nucleus may be the critical steps in modulation of signaling by this pathway in cardiac (myo)fibroblasts.

A significant body of literature indicates that elevated angiotensin signaling is associated with the onset of cardiac fibrosis in different models of heart failure, including MI (8, 16). In the infarcted rat heart, local angiotensin generation is activated in the remnant myocardium and scar (8). The predominant collagen-synthesizing cells in heart have been identified as myofibroblasts (32), and AT1 receptor antagonism
nificantly attenuates fibrosis in both infarcted and non-infarcted rat myocardium (11, 16). Angiotensin-mediated modulation of the expression of TGF-β, ligand occurs in vitro (2, 10) and in vivo (34) in various cell types including cardiac fibroblasts. However, information about cross talk between angiotensin and TGF-β in post-MI heart at the postreceptor level (Smad proteins) is lacking. Furthermore, the role of putative angiotensin/TGF-β cross talk in the development of cardiac fibrosis and heart failure is unclear. This study addressed whether chronic AT1 receptor blockade, a known antifibrotic strategy, was associated with modulation of cardiac Smad expression and activation in failing rat heart post-MI.

MATERIALS AND METHODS

Experimental model. All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following guidelines established by the Medical Research Council of Canada. MI was produced in male Sprague-Dawley rats (weighing 200–250 g) by ligation of the left coronary artery, as described previously (6). The mortality of the animals operated on in this fashion was ~40% within 48 h. Surviving rats from sham-operated and MI groups were divided into three groups: group 1, sham-operated rats; group 2, MI rats; and group 3, MI rats treated with losartan (15 mg·kg⁻¹·day⁻¹) (4, 16, 30). All losartan treatment regimens were randomly assigned and initiated immediately after coronary occlusion by implantation of Alzet osmotic mini-pumps consecutively (models 2002 and 2ML4 in sequence; Alza, La Jolla, CA) to achieve the 8-wk treatment. For comparative purposes, sham-operated controls (group 1) and MI animals were administered vehicle (0.9% saline) in the same fashion. The experimental rats were killed after 8 wk, and cardiac tissue was isolated from (0.9% saline) in the same fashion. The experimental rats previously (16). Animals with an infarct size at 8 wk after induction of MI, as described previously (17). Briefly, a micromanometer-tipped catheter (2-0, Millar SPR-249) was inserted into the right carotid artery, advanced into the aorta to determine MAP, and then further advanced to the LV chamber to record LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), the maximum rate of isovolumic pressure development (+dP/dt max), and the maximum rate of isovolumic pressure decay (−dP/dt max).

Determination of infarct size in experimental animals. After 8 wk, the rats were killed and the hearts were excised. The LV was fixed by immersion in 10% Formalin and embedded in paraffin. Six transverse slices were cut from the apex to the base, and serial sections (5 μm) were cut and mounted. The percentage of infarcted LV was estimated at 8 wk after coronary ligation by planimetric techniques, as described previously (16). Animals with an infarct size <40% of the LV free wall were excluded.

Adult cardiac fibroblast isolation and culture. Adult cardiac fibroblasts were isolated from male Sprague-Dawley rats according to the methods of Brilla et al. (1) with minor modifications (15). The adult rat heart was subjected to Langendorff perfusion at a flow of 5 ml/min at 37°C with recirculatory Joklik’s medium containing 0.1% collagenase and 2% bovine serum albumin (BSA) for 25–35 min. Liberated cells were collected by centrifugation at 2,000 rpm for 10 min. The suspension of DMEM/F-12 was then placed on a 100-mm noncoated culture flask at 37°C with 5% CO₂ for 2 h. Cardiac fibroblasts attached to the bottom of the culture flask during 2-h incubation while nonadherent myocytes were removed by changing the culture medium. The cells were maintained in DMEM/F-12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells used for the study were from the second passage (P2), and the purity of fibroblasts used in these experiments was found to be ≥95%, using routine phenotyping methods described previously (15, 24). Briefly, endothelial cells were labeled with the use of a monoclonal antibody against factor VIII, and we found that less than ~1% of cultured cells were positive for this protein. Less than 1% of cells were positive for desmin, which is specific for smooth muscle cells (SMC), and more than ~1% of cultured cells stained positively for α-smooth muscle actin, which is produced in smooth muscle cells and myofibroblasts. On the other hand, >95% of cells in our P2 cultures stained positively for procollagen type 1, which is a major protein product of fibroblasts. For stimulation with angiotensin, fibroblasts were maintained in serum-free media for 24 h before administration of angiotensin (10⁻⁷ M) for 15 min. Equimolar losartan was added to cultured cells 1 h before angiotensin treatment to achieve AT₁ blockade.

Immunofluorescent localization of TGF-β, and Smad proteins in post-MI heart. A total of 12 rats (4 rats/group) was used in these studies. LV tissue from sham-operated rats and viable LV remote to the infarct as well as border and scar tissues from MI rats were immersed in optimum cutting temperature compound (OCT, Miles, Elkhart, IN). Serial cryostat sections (7 μm) of ventricular tissue were mounted on gelatin-coated slides. A minimum of six sections from different regions of each group was processed. Indirect immunofluorescence was performed as described in detail previously (15, 24). Tissue sections were fixed in 4% paraformaldehyde for 15 min. Polyclonal antibodies against TGF-β, Smad 2, Smad 4, and phosphorylated Smad 2 (P-Smad 2) were diluted 1:20–1:40 with 1% BSA in PBS and applied as indicated above. The anti-TGF-β₁ antibody recognizes both latent and active forms of TGF-β₁, and the Smad 2 antibody detects both phosphorylated and nonphosphorylated Smad 2. For double staining with vimentin, monoclonal mouse anti-vimentin clone no. V9 (1:100 with 1% BSA in PBS) was added to the slides at the same time. After incubation overnight at 4°C, the sections were washed with PBS and incubated with biotinylated anti-goat (or rabbit) IgG secondary antibody and subsequently incubated with FITC-labeled streptavidin for 90 min. To distinguish anti-vimentin antibody from other primary antibodies, an anti-mouse-linked Texas Red conjugate (1:20 with 1% BSA in PBS) was added with streptavidin-FITC. Thus vimentin was labeled with Texas Red, and the other primary antibodies were labeled with FITC. Slides were mounted and coverslipped, and tissue sections were examined under a Nikon Labophot microscope equipped with epifluorescence optics and appropriate filters. The results were photographed on Provia Fuji chrome 400 color film.

Immunofluorescence assay in isolated fibroblasts. Adult cardiac fibroblasts were plated on coverslips and allowed to grow for 24 h. Cells were fixed with 1% paraformaldehyde after 15-min treatment with 10⁻⁶ M angiotensin. Immun-
fluorescent staining was performed by the indirect immunofluorescence technique (26) to detect either total Smad 2 or phosphorylated Smad 2. Cells were incubated with either of these antibodies overnight at 4°C. The primary antibodies were diluted (1:20–1:140) with PBS containing 1% BSA. After being washed with PBS, cells were incubated with biotinylated anti-goat or anti-rabbit IgG secondary antibody, followed by incubation with FITC-labeled streptavidin. After being washed (3 times for 5 min) with cold PBS, slides were immersed for 30 s in 10 μg/ml of Hoechst dye 33342 to stain cellular nuclei and then were subjected to an additional wash (3 times for 5 min) in cold PBS. The slides were examined under a microscope equipped with epifluorescence optics and photographed on Provia Fujichrome 400 color film.

**Protein extraction and assay.** Cardiac tissues from sham-operated LV, viable LV, border area, and scar regions were homogenized in 100 mM Tris (pH 7.4) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin. Samples were sonicated three times for 5 s. Cytosolic fractions were isolated by centrifugation at 12,000 rpm at 4°C. Briefly, after homogenization, samples were centrifuged at 3,000 g for 10 min at 4°C to remove unbroken cells and nuclei. The resulting supernatant was further subjected to centrifugation at 48,000 g for 20 min at 4°C. The supernatant fraction was used for the protein determination of TGF-β1 and phosphorylated Smad 2. For total cardiac Smad 2 and Smad 4 protein detection, tissues were homogenized with the above buffer containing 0.1% Triton X-100 and phosphatase inhibitors (10 mM NaF, 1 mM sodium orthovanadate, and 20 mM β-glycerophosphate). This homogenate was sonicated for 5 s (repeated 5 times) to disrupt nuclear membranes. The samples were allowed to lyse for 15 min on ice. After centrifugation at 10,000 g for 20 min at 4°C, the supernatant was used for the cytosolic Smad protein assay. Total protein concentration of all samples was measured using the bicinchoninic acid (BCA) method as described previously (29).

**Nuclear isolation from cardiac fibroblasts.** Nuclei of cardiac fibroblasts were isolated using the Nuclei EZ Prep Nuclear Isolation Kit (Sigma-Aldrich, Oakville, ON, Canada) according to the manufacturer’s instructions. The purity and integrity of isolated nuclei were confirmed by flow cytometry and light microscopy following trypan blue staining (data not shown). Isolated nuclei were resuspended in 100 mM Tris (pH 7.4) containing 1 mM EDTA, 1 mM PMSF, 4 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin. Phosphatase inhibitors (10 mM NaF, 1 mM sodium orthovanadate, and 20 mM β-glycerophosphate) were also added to the solution. Samples were subjected to sonication three times for 10 s to further disrupt the nuclei, and the nuclear protein concentration analysis was performed using the BCA method (30).

**Western blot analysis of TGF-β1, Smad 2, and Smad 4.** Prestained low-molecular-weight marker (Bio-Rad, Hercules, CA) and 30 μg of protein from samples were separated on 10% or 12% SDS gels by SDS-PAGE. Separated proteins were transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membrane that was blocked at room temperature for 1 h or overnight at 4°C in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 8% skim milk and probed with primary antibodies. Primary antibodies against TGF-β1 (detects both latent and active TGF-β1) and Smad 4 were diluted 1:250 in TBS-T. Anti-Smad 2 antibody, which recognizes both phosphorylated and nonphosphorylated Smad 2, was diluted 1:250 in TBS-T. To specifically detect P-Smad 2, a polyclonal antibody against P-Smad 2 was used (1:500). Secondary antibodies included horseradish peroxidase (HRP)-labeled anti-rabbit IgG for detection of Smad 4 and TGF-β1 proteins and HRP-labeled anti-goat IgG for detection of Smad 2 proteins. All secondary antibodies were diluted 1:10,000 with TBS-T. Bands on Western blots were visualized by enhanced chemiluminescence (ECL) or by ECL-Plus (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions. Afterward, blocking peptides of TGF-β1 and Smad 2 were used to identify the band specific to each protein, and even protein loading was confirmed by staining membranes with Coomassie blue. Autoradiographs from Western blots were quantified using a charge-coupled device camera imaging densitometer (model GS 670; Bio-Rad) (12).

**Reagents.** Primary antibodies against Smad 2, Smad 4, and TGF-β1, control peptides for TGF-β1, and Smad 2, and HRP-labeled anti-goat secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). P-Smad 2 primary antibody was obtained from Upstate Biotechnology (Lake Placid, NY). For cell phenotyping, monoclonal antibody against procollagen type 1 (SP1.D8) was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), monoclonal antibody against desmin was from Calbiochem (Cambridge, MA), and antibodies against smooth muscle myosin, α-smooth muscle actin, and factor VIII (von Willebrand factor) were from Sigma-Aldrich, (Oakville, ON, Canada). Monoclonal mouse anti-vimentin antibody (clone no. V9) was obtained from Sigma. Biotinylated anti-rabbit and anti-goat secondary antibodies, anti-mouse-linked Texas Red conjugate, FITC-labeled streptavidin, and HRP-labeled anti-rabbit secondary antibody were purchased from Amersham (Arlington Heights, IL). Angiotensin II was purchased from Sigma. Losartan was a kind gift from Merck (Rahway, NJ).

**Statistical analysis.** All values are expressed as means ± SE. One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls method was used for comparing the differences among multiple groups (SigmaStat). Significant differences among groups were defined by a probability <0.05.

**RESULTS**

**General observations: cardiac hypertrophy, total cardiac collagen concentration, and heart failure.** Hearts of experimental animals were characterized by significant

<table>
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<tr>
<th>Parameters</th>
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<th>Losartan-Treated Post-MI</th>
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<td>BW, g</td>
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<td>481 ± 9</td>
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<td>4,481 ± 212†</td>
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<td>-dP/dtmax, mmHg/s</td>
<td>5,478 ± 229</td>
<td>3,894 ± 218†</td>
<td>4,652 ± 231†</td>
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</tbody>
</table>

Experimental animals were characterized by large left ventricular (LV) myocardial infarction (MI; 43 ± 4% of total LV circumference); sham-operated animals were noninfarcted age-matched controls. BW, body weight; LVW, LV weight; LVEDP, LV end-diastolic pressure; LVSP, LV systolic pressure; +dP/dtmax, maximum rate of isovolumic pressure development; -dP/dtmax, maximum rate of isovolumic pressure decay. Data are presented as means ± SE of 8–10 experiments. *P < 0.05 vs. sham-operated animals; †P < 0.05 vs. untreated post-MI animals.
Fig. 1. Immunoreactive total Smad 2 in the left ventricular myocardium from sham-operated rat heart (A), viable (remnant) tissue (B), and infarct scar (C). Experimental animals were harvested 8 wk after surgery. The same fields shown in A, B, and C were double stained for vimentin (D, E, and F, respectively) and for nuclei (G, H, and I, respectively). In all sections, relatively bright staining of total Smad 2 protein was localized to nonmyocytes, while myocytes stained less intensely. Vimentin is a common marker of nonmyocyte cells.
significant cardiac hypertrophy as reflected by an increase in the mass of the viable LV tissue and also by the increased LV-to-body mass (BW) ratio in experimental animals compared with control values (Table 1). The incidence and magnitude of LV hypertrophy noted was comparable to our previous findings (6, 24). Cardiac collagen concentrations in surviving myocardium remote to infarct (i.e., remnant heart: 58.2 ± 5.1 µg/mg dry wt) and in border + scar tissues (126.3 ± 10.8 µg/mg dry wt) were both significantly higher than control value (20.3 ± 3.2 µg/mg dry wt). Furthermore, cardiac collagen concentration in remnant heart treated with losartan (37.4 ± 3.4 µg/mg dry wt) was significantly reduced vs. values in nontreated tissues.

Heart failure, reflected by an increase in LVEDP and a decrease in $\frac{dP}{dt_{\text{max}}}$ relative to their controls, along with congested lung, has been characterized in this model from our previous studies (17). Losartan treatment was associated with normalization of indexes of cardiac hypertrophy and cardiac function (Table 1), in agreement with our previous findings (16).

**Localization and quantification of cardiac Smads in post-MI heart.** Immunofluorescent staining revealed that total Smad 2 protein was localized to the extracellular space proximal to nuclei as shown in Fig. 1. Double staining with vimentin showed that Smad 2 was mainly localized to nonmyocytes proximal to the nuclei. We observed enhanced accumulation of Smad 2 proteins in the nuclei of cells from scar tissue. Western

![Image](http://ajpheart.physiology.org/)

**Fig. 2.** A: representative Western blots for Smad 2 and Smad 4 from sham-operated control hearts (lane 1), viable remnant tissue (lane 2), viable remnant tissue with 8 wk of losartan treatment (lane 3), infarct scar tissue (lane 4), and infarct scar tissue with 8 wk of losartan treatment (lane 5) from 8-wk post-MI rat heart left ventricular samples. B: membrane from experiment shown in A was stained with Coomassie blue to verify relatively even protein loading. C: histographic representation of quantified data from multiple samples from groups shown in A. Data are means ± SE of 4–6 experiments. *P ≤ 0.05 vs. sham; †P ≤ 0.05 vs. viable; ‡P ≤ 0.05 vs. scar.

![Image](http://ajpheart.physiology.org/)

**Fig. 3.** A: representative Western blot showing latent (40 kDa) and active transforming growth factor (TGF)-β protein (25 kDa) in sham-operated control hearts (lane 1) as well as viable remnant tissue (lane 2), viable remnant tissue with 8 wk of losartan treatment (lane 3), infarct scar tissue (lane 4), and infarct scar tissue with 8 wk of losartan treatment (lane 5) from 8-wk post-MI rat heart left ventricular samples. B: membrane from experiment shown in A was stained with Coomassie blue to verify relatively even protein loading. C: histographic representation of quantified data from multiple samples from groups shown in A. Data are means ± SE of 4–6 experiments. *P ≤ 0.05 vs. sham; †P ≤ 0.05 vs. viable; ‡P ≤ 0.05 vs. scar.
blot analysis was used to determine the protein concentration of cardiac Smad 2 and Smad 4 from different groups. Cardiac Smad 2 (62 kDa) protein concentration was significantly increased in remnant and scar tissues compared with control values, while cardiac Smad 4 (62 kDa) protein concentration was only significantly elevated in scar tissue vs. control. Losartan treatment was associated with a significant inhibitory effect on Smad 2 accumulation in viable tissue and infarct scar tissue and Smad 4 accumulation in infarct scar tissue (Fig. 2).

Effect of losartan on the expression of cardiac TGF-β1. Using Western blot analysis, we quantified cardiac TGF-β1 protein concentration in control and viable LV tissues as well as in border and scar tissues of 8-wk post-MI rats. The TGF-β1 polyclonal antibody recog-
nized both the latency-associated peptide (LAP) and active forms of TGF-β1 at ~40 and 25 kDa, respectively. Although the LAP dimer of ~80 kDa binds TGF-β1 per se, we observed the monomeric LAP band due to reducing gel conditions. The active form of TGF-β1 was increased in both remnant and scar tissues from post-MI heart, which was significantly attenuated by the administration of losartan. Conversely, the latent form of TGF-β1 was decreased in both remnant and scar tissues, and this decrease was partially prevented by losartan treatment (Fig. 3). Previous studies have shown that TGF-β1 can be released from latent complexes and can be activated by cleaving an inactive high-molecular-weight precursor complex (13). We observed that the conversion of TGF-β1 from its latent to its active form was augmented in remnant myocardium and infarct scar. Losartan treatment was associated with an inhibition of this conversion. Immunofluorescent staining revealed that total TGF-β1 localized to the extracellular space in normal tissue and remnant myocardium. Furthermore, the infarct scar stained brightly for total TGF-β1 as did myocytes bordering the infarct scar region. Cardiac myocytes remote to the infarct scar expressed comparatively moderate levels of TGF-β1 (Fig. 4).

**Total and phosphorylated Smad 2 distribution in post-MI heart and cultured cardiac fibroblasts.** Immunofluorescence data indicated relatively moderate staining of P-Smad 2 in myocytes of sham-operated, remnant, and losartan-treated remnant tissues from post-MI rat heart (Fig. 5, A, C, and E). Compared with control and viable tissues, the scar and treated scar sections (Fig. 5, G and I, respectively) were characterized by brightly stained regions, and areas of punctate nuclear accumulation of P-Smad 2 were observed (Fig. 6). This pattern was associated with cellular nuclei in scar (Fig. 5, H and J). Western blot analysis of cytosolic P-Smad 2 revealed a significant decrease in band intensity from cytosolic viable and scar tissue compared with sham-operated control (Fig. 6). These trends were normalized by losartan treatment. In studies of quiescent and unstimulated cultured cardiac fibroblasts, total Smad 2 localized to cellular nuclei and cytosol (Fig. 7A), as did P-Smad 2 (Fig. 7E). Total Smad 2 staining was elevated in intensity after stimulation with angiotensin (10⁻⁶ M) for 15 min vs. unstimulated cells (Fig. 7C). Furthermore, 15-min angiotensin (10⁻⁶ M) stimulation was associated with marked translocation of P-Smad 2 from the cytosol to the nuclei (Fig. 7G). Western blot analysis of nuclei isolated from cultured cardiac fibroblasts from normal rat heart revealed that angiotensin stimulation (10⁻⁶ M) for 15 min was associated with a significant increase of P-Smad 2 protein, and this change was inhibited by AT₁ receptor blockade (Fig. 8).

**DISCUSSION**

Animals with a relatively large infarct 8 wk post-MI were considered to be in moderate heart failure as based on current data and previous observations (6, 17). Using this model, we have previously observed significant elevation in the deposition of cardiac collagen, in addition to the persistence of myofibroblasts in the remnant myocardium and scar tissue (17, 31). These findings, in addition to enhanced Smad expression in these cells, provide a strong indication that infarct scar is not quiescent in 8-wk post-MI hearts. In this regard, chronic scar remodeling has been shown to play a role in the functional preservation of the infarcted ventricle (14).

**Fibroblasts, myofibroblasts, and cardiac fibrosis.** After MI, fibroblasts arrive at the site of repair, where they undergo phenotypic transformation to myofibroblasts, a process inducible by TGF-β1 (37). Myo-
fibroblasts express α-smooth muscle actin, providing contractility and chronic mechanical tension to the remodeling scar (37). Myofibroblasts have a high synthetic capacity for fibrillar collagens and express cytokines including angiotensin and TGF-β1. These cells also express angiotensin receptors as well as TGF-β1 receptors, which potentiate fibroproliferative behavior (27). In this regard, angiotensin and TGF-β1 have been identified as contributors to cardiac fibrosis (37, 39) and angiotensin is known to influence TGF-β1 ligand expression (2); however, cross talk between the activated postreceptor mechanisms for these two systems in heart failure is unknown. We demonstrated that, in heart failure, AT1 blockade is associated with 1) altered TGF-β1 ligand processing in post-MI hearts, and 2) normalization of both increased Smad 2 expression in remnant myocardium and infarct scar and increased Smad 4 expression in infarct scar. Furthermore, these events are positively associated with normalized cardiac function and significant reduction in cardiac fibrosis in treated experimental hearts. Finally, we showed that angiotensin may elevate Smad 2 expression and nuclear accumulation in cultured adult cardiac fibroblasts, suggesting a direct mediation of this event.

Fig. 7. Immunofluorescent staining of total Smad 2 (top) and phosphorylated Smad 2 (bottom) from cultured cardiac fibroblasts stimulated by angiotensin (10^{-6} M) for 15 min. A and E represent untreated fibroblasts; C and G show angiotensin-treated fibroblasts. B, D, F, and H show nuclei (Hoechst 33342 staining) of identical sections corresponding to A, C, E, and G, respectively. Original magnification, ×400.
Angiotensin and cardiac fibrosis. Angiotensin has been shown to stimulate cardiac fibrosis in several different models of heart failure (11, 16, 28, 30). Furthermore, angiotensin stimulates collagen production in cultured cardiac fibroblasts (3), and its expression and AT1 receptor density in myofibroblasts of the infarct scar are significantly increased (33, 41). We demonstrated that AT1 blockade is associated with partial attenuation of cardiac fibrosis in post-MI rats (5, 20); however, the precise mechanism of the antifibrotic effect of this therapeutic intervention is unclear. Mounting evidence supports the existence of putative cross talk between angiotensin and TGF-β at the level of ligand expression in cultured cells including adult primary cardiac fibroblasts (2, 10). Furthermore, AT1 receptor blockade has been shown to be associated with increased steady-state abundance of TGF-β1 mRNA observed in 4-wk post-MI rat heart (34). These findings support the hypothesis that AT1 modulation of TGF-β1 ligand may occur in cardiac fibroblasts. Nevertheless, a role of angiotensin at the post-receptor levels of TGF-β1 signaling has not been identified.

AT1 activation and TGF-β ligand processing/bioavailability in failing hearts. TGF-β is secreted as an inactive precursor complex containing a signal peptide, the active TGF-β1 molecule, and the cleaved propeptide known as LAP (23). After the signal peptide is removed, the gene product undergoes proteolytic cleavage to produce mature TGF-β1 (residues 279–390) and LAP (residues 30–278) (13, 23). We found that the active form of TGF-β1 (25 kDa) is significantly elevated in remnant (viable) and scar tissues, whereas the LAP (~40 kDa in monomeric form as seen in a reducing gel) latent form of TGF-β1 is decreased vs. control in heart failure. This indicates a redistribution in expression of active TGF-β1-to-LAP ratio in the remnant myocardium and infarct scar. Because losartan treatment led to a normalization of this trend, AT1 activation may play a role in relative activation of TGF-β1 in experimental hearts and, thus, regulate the bioavailability of the active TGF-β1 molecule.

Effect of angiotensin on phosphorylation and translocation of Smad 2 in cultured cardiac fibroblasts. In recent years, Smad 2 has been well characterized as a key downstream effector of TGF-β signaling in mammalian cells, and it is clear that the phosphorylation of Smad 2 is required for nuclear translocation and subsequent regulation of transcription. Our previous data have shown that Smad 2 is upregulated in the infarct scar 8 wk after MI. However, the effect of angiotensin on the phosphorylation and nuclear translocation of Smad 2 in cardiac fibroblasts and post-MI heart has not been reported. In this study we noted increased total Smad 2 and decreased P-Smad 2 in cytosol sections from viable and scar tissues of LV 8-wk post-MI, suggesting an increased nuclear accumulation of P-Smad 2. In vivo, these trends were normalized by AT1 receptor blockade. Our in vitro study demonstrated that angiotensin (10^-6 M) stimulation of cultured adult cardiac fibroblasts is associated with an elevation of total Smad 2 protein. Furthermore, the presence of angiotensin caused an increased nuclear accumulation of P-Smad 2 in fibroblasts, as indicated by immunofluorescent staining and Western blot analysis. The protein level of P-Smad 2 in nuclei isolated from cardiac fibroblasts increased after angiotensin stimulation, an effect that was blocked by AT1 receptor blockade. Together, these results indicate a possible link between angiotensin and the phosphorylation and nuclear translocation of Smad 2. The molecular mechanism underlying this link is not yet clear, and it is currently unknown whether this action is dependent or independent of TGF-β1 ligand. It has been reported that Smad 2 activation may not be restricted to TGF-β1-stimulated cells (21, 31, 38), and our data suggest a direct role for angiotensin in this regard. Recently, Janus NH2-terminal kinase (JNK) activation has been shown to cause phosphorylation of the COOH-terminal tyrosines on receptor-activated Smads (7). Furthermore, AT1 activation causes a rapid increase (5 min) in JNK activity.
in cardiac cells in a dose-dependent manner (18). Together, these findings support a novel angiotensin-mediated pathway for phosphorylation/activation of cardiac Smad 2 proteins that is independent of TGF-β1 receptor activation. Our data indicating rapid nuclear translocation of Smad 2 in cultured fibroblasts in the presence of angiotensin support this hypothesis; however, further investigation is required in the heart to prove the existence of a direct angiotensin-Smad 2 interaction.

In conclusion, these results indicate that elevated Smad expression in experimental heart failure is normalized by long-term AT1 receptor blockade and that these changes are paralleled by modulation of fibroproliferative events in these hearts. Furthermore, AT1 activation is associated with augmented nuclear accumulation of phosphorylated Smad 2 in failing hearts and with angiotensin stimulation of cultured cardiac fibroblasts. The current results also provide a link between angiotensin receptor activation and potentiation of Smad protein function in cardiac fibroblasts.

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