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# Involvement of Reactive Oxygen Species in Angiotensin II-Induced Endothelin-1 Gene Expression in Rat Cardiac Fibroblasts

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**OBJECTIVES** The aim of this study was to investigate the effects of angiotensin II (Ang II) on fibroblast proliferation and endothelin-1 (ET-1) gene induction, focusing especially on reactive oxygen species (ROS)-mediated signaling in cardiac fibroblasts.

**BACKGROUND** Angiotensin II increases ET-1 expression, which plays an important role in Ang II-induced fibroblast proliferation. Angiotensin II also stimulates ROS generation in cardiac fibroblasts. However, whether ROS are involved in Ang II-induced proliferation and ET-1 expression remains unknown.

**METHODS** Cultured neonatal rat cardiac fibroblasts were stimulated with Ang II, and then [$^3$H]thymidine incorporation and the ET-1 gene expression were examined. We also examined the effects of antioxidants on Ang II-induced proliferation and mitogen-activated protein kinase (MAPK) phosphorylation to elucidate the redox-sensitive pathway in fibroblast proliferation and ET-1 gene expression.

**RESULTS** Both $AT_1$ receptor antagonist (losartan) and $ET_A$ receptor antagonist (BQ485) inhibited Ang II-increased DNA synthesis. Endothelin-1 gene was induced with Ang II as revealed by Northern blotting and promoter activity assay. Angiotensin II increased intracellular ROS levels, which were inhibited with losartan and antioxidants. Antioxidants further suppressed Ang II-induced ET-1 gene expression, DNA synthesis, and MAPK phosphorylation. PD98059, but not SB203580, fully inhibited Ang II-induced ET-1 expression. Truncation and mutational analysis of the ET-1 gene promoter showed that AP-1 binding site was an important cis-element in Ang II-induced ET-1 gene expression.

**CONCLUSIONS** Our data suggest that ROS are involved in Ang II-induced proliferation and ET-1 gene expression. Our findings imply that the combination of $AT_I$ and $ET_A$ receptor antagonists plus antioxidants may be beneficial in preventing the formation of excessive cardiac fibrosis. (J Am Coll Cardiol 2003;42:1845–54) © 2003 by the American College of Cardiology Foundation

Fibroblasts play an important role in maintaining cardiac function by providing structural support for cardiomyocytes and serving as a source for paracrine growth factors (1–3). After myocardial infarction, reactive fibrosis results in excess scar formation as proliferating fibroblasts invade the necrotic area. This remodeling leads to the increase of the ventricular stiffness and ultimately compromises the function of the heart. Angiotensin II (Ang II), the effector peptide of the renin-angiotensin system, is now known to have growth promoting properties in various cell types and a mitogenic effect on cardiac fibroblasts (1,2,4). The growth related cellular actions of Ang II are mediated by the $AT_1$ receptor subtype (4). Recent reports have shown that Ang II stimulates membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which generates reactive oxygen species (ROS) in cardiac fibroblasts (3). Increased NADPH oxidase-derived ROS have recently been reported to be potentially linked with a defect in cardiac contractile function in a pathological setting (5); ROS may act as second messengers that regulate various intracellular signal transduction cascades and the activity of various transcription factors. Activator protein-1 (AP-1) is one of the best characterized transcription factors to be influenced by the cellular oxidation reduction (redox) state (6,7). The primary target of activation of AP-1 by ROS appears to be the activation of mitogen-activated protein kinase (MAPK) pathways, thereby leading to the phosphorylation and activation of the c-fos and c-jun family of protooncogenes. The potential role of ROS in the regulation of signal transduction and gene expression in the heart has recently been elucidated. Administration of antioxidants inhibited Ang II-induced cardiac hypertrophy (8,9).

Fujisaki et al. (1) previously reported that Ang II-induced

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**Abbreviations and Acronyms**

| | |
|---|---|
| Ang II | = angiotensin II |
| AP-1 | = activator protein-1 |
| $AT_1$ | = angiotensin II type 1 receptor |
| CAT | = chloramphenicol acetyltransferase |
| DCF-DA | = dichlorofluorescin diacetate |
| DPI | = diphenyleneiodonium |
| ECM | = extracellular matrix |
| ERK | = extracellular signal-regulated kinase |
| $ET_A$ | = ET-1 type A receptor |
| ET-1 | = endothelin-1 |
| GPCRs | = G-protein coupled receptors |
| JNK | = c-Jun N-terminal kinase |
| MAPK | = mitogen-activated protein kinase |
| MEK | = MAPK/ERK kinase |
| mRNA | = messenger RNA |
| NAC | = N-acetylcysteine |
| redox | = cellular oxidation reduction |
| ROS | = reactive oxygen species |

endothelin-1 (ET-1) gene expression in cardiac fibroblasts may serve as an autocrine/paracrine growth factor for cardiac fibroblasts. The promoter region of the ET-1 gene contains at least two essential protein binding motifs corresponding to the transcription factors GATA and AP-1 (10). We reported that mechanical stretch-induced intracellular ROS were involved in ET-1 gene induction partially via the AP-1 activation in endothelial cells (11). Our recent study further revealed that extracellular signal-regulated kinase (ERK) pathway plays a crucial role in ROS-mediated ET-1 gene expression in rat cardiac fibroblasts (12). Although the significance of ET-1 gene expression in cardiac fibroblasts has been recently determined, the regulatory mechanism of ET-1 gene induction by Ang II remains unclear. In the present study, we investigated the role of redox-sensitive mechanisms in fibroblast proliferation and the ET-1 gene induction in cardiac fibroblasts treated with Ang II. We further determined which redox-sensitive signal transduction pathways and transcription factors were involved in this process. This study shows that ROS are essential for Ang II-induced proliferation and ET-1 gene expression in cardiac fibroblasts. Our results indicate that Ang II up-regulates the ET-1 gene at least in part via Ras/Raf/ERK signaling pathways in cardiac fibroblasts.

## METHODS

**Materials.** Dulbecco's modified Eagle's medium (DMEM)/F-12, fetal calf serum, and tissue culture reagents were from Invitrogen (Carlsbad, California). A rat ET-1 complementary DNA probe (accession No. M64711) was obtained as previously described (13). A series of deletion mutants containing various lengths of the ET-1 promoter region fused to chloramphenicol acetyltransferase (CAT) reporter gene and the catalytically inactive mutant of ERK2 (mERK2), RasN17, RasL61, and Raf301 were previously described (11). The ECL detection system was from Amersham Pharmacia Biotech (Buckinghamshire, UK). PD98059 and SB203580 were from CALBIOCHEM (Calbiochem-Novabiochem Corporation, La Jolla, California). Losartan, Ang II, PMA, NAC, and all other chemicals were purchased from Sigma (St. Louis, Missouri).

**Cell culture.** Primary cultures of neonatal rat cardiac fibroblasts were prepared as previously described (12). Cells were incubated with minimum essential medium (MEM) supplemented with 10% fetal calf serum. Two to four days after seeding, confluent non-myocytes were trypsinized and subcultured. Subconfluent (~70% confluency) cardiac fibroblasts grown in either 60- or 100-mm culture dishes from the second to fourth passage were used for the experiments. Serum-containing medium from these cultured cells was replaced with serum-free medium and exposed to agents as indicated.

**DNA synthesis.** To measure synthesis of new DNA, cells ($1 \times 10^5$/well) were plated in six-well (35-mm) dishes 24 h before experiments. Cardiac fibroblasts were incubated with [$^3$H]thymidine (5 $\mu$Ci/ml) at 4°C. After addition of agent indicated, cells were then harvested with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH, and radioactivity was determined by scintillation counting. Data are presented as the mean ± SEM of 9 to 12 determinations in three to four different cell preparations and normalized to the untreated sample × 100 (i.e., percentage of control).

**Assay of intracellular ROS.** Intracellular ROS production was measured by using a fluorescent dye, 2′ 7′-dichlorofluorescin diacetate (DCF-DA) (Molecular Probes, Eugene, Oregon) with the ACAS Interactive Laser Cytometer (Meridian Instruments, Inc., Okemos, Michigan) as previously described (12). Baseline values from unstimulated cells were used as control values by comparing them with Ang II-stimulated cells. Values represent means ± SEM of DCF fluorescence from 20 randomly selected cells in each experiment for six separate investigations.

**RNA isolation and Northern blot analysis.** Total RNA was isolated from cardiac fibroblasts by the guanidine isothiocyanate/phenol chloroform method as previously described (11). The RNA (10 g/lane) was separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran, Schleicher & Schuell, Inc., Germany) by a vacuum blotting system (VacuGene XL, Pharmacia, Buckinghamshire, UK). After hybridization with the $^{32}$P-labeled ET-1 complementary DNA probes, the membrane was washed with 0.1× sodium chloride plus tris-sodium citrate containing 1% sodium dodecyl sulfate at 42°C for 30 min and then exposed to X-ray film at −70°C. Blots of specific messenger RNA (mRNA) bands were detected by autoradiography and analyzed with a densitometer (Computing Densitometer 300S, Molecular Dynamics, Sunnyvale, California). Blots were stripped and reprobed for 18S complementary DNA probe (obtained from American Type Culture Collection,

Manassas, Virginia) to control for loading. Expression of ET-1 mRNA was quantitated and was normalized to the 18S signal.

**Transfection and chloramphenicol acetyltransferase assays.** For the transient transfections, cardiac fibroblasts were transfected with different expression vectors by the calcium phosphate method (12). DNA concentration for all samples was adjusted to be equal with empty vector pSR$\alpha$ in each experiment. To correct for variability in transfection efficiency, 5 $\mu$g of pSV-$\beta'$-galactosidase plasmid DNA was co-transfected in all the experiments. The CAT and $\beta'$-galactosidase assays were performed as previously described (14). The relative CAT activity was corrected by normalizing the respective CAT value to that of $\beta$-galactosidase activity. Co-transfected $\beta$-galactosidase activity varied by <10% within a given experiment and was not affected by any of the experimental manipulations described. As positive and negative controls, pBLCAT2 (with thymidine kinase promoter) and pBLCAT3 (without promoter) were included in each assay.

**Western blot analysis.** Rabbit polyclonal anti-phospho-specific p38MAPK, anti-phospho-specific ERK1/2, and anti-phospho-specific c-Jun N-terminal kinase (JNK) antibodies were purchased from New England Biolabs (Beverly, Massachusetts). Anti-ERK1/2, anti-p38MAPK, and anti-JNK antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, California. Western blot analysis was performed as previously described (11).

**Statistical analysis.** Results are expressed as mean ± SEM of at least three experiments unless designated otherwise. Statistical analysis was performed using analysis of variance (ANOVA) and Student *t* test as appropriate. A value of $p < 0.05$ was considered to be statistically significant.

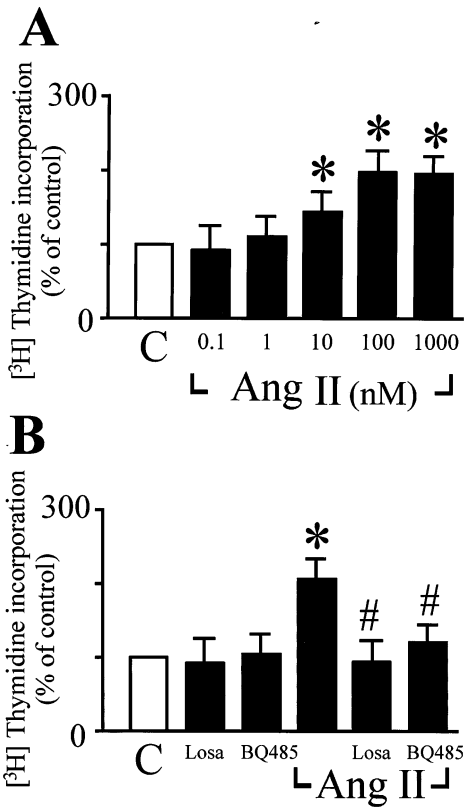


**Figure 1.** Characteristics of the activation of DNA synthesis by angiotensin II (Ang II) in cardiac fibroblasts. All experiments were performed with the incorporation of [$^3$H]thymidine into DNA. **(A)** Effect of Ang II concentration on the DNA synthesis. Cells were incubated with the indicated doses of Ang II for 24 h and then assayed for [$^3$H]thymidine incorporation. **(B)** Effect of Ang II or endothelin-1 receptor antagonists on [$^3$H]thymidine incorporation. Cells were preincubated with either losartan (Losa) (1 M) or BQ485 (1 M) for 1 h followed by an incubation with 100 nM Ang II for 24 h. Experimental details are given in the Experimental Procedures section. [$^3$H]thymidine incorporation is expressed as the percentage of increase relative to the [$^3$H] content (100%) in the control (C). All data are shown as the means ± SEM of 9 to 12 determinations in three to four different cell preparations. *$p < 0.05$ versus control (Student *t* test); #$p < 0.05$ versus Ang II alone (analysis of variance).

## RESULTS

**Ang II-induced proliferation of cardiac fibroblast is mediated via ET-$1_A$ receptor.** Angiotensin II-stimulated cardiac fibroblast proliferation was assessed by analyzing DNA synthesis with [$^3$H]thymidine incorporation. Angiotensin II increased DNA synthesis in neonatal rat cardiac fibroblasts in a dose-dependent manner (Fig. 1A). The maximum level of incorporation was 2.1-fold increase compared with the control. Angiotensin II (100 nM)–stimulated DNA synthesis in cardiac fibroblasts was inhibited by either $AT_1$ receptor antagonist losartan (1 $\mu$M) or $ET_A$ receptor antagonist BQ485 (1 $\mu$M) treatment (Fig. 1B). Both losartan and BQ485 had no effect on basal [$^3$H]thymidine uptake. These data suggest the possible role of endogenous ET-1 as an autocrine growth factor for the proliferation of cardiac fibroblasts under Ang II stimulation.

**Ang II-induced ET-1 gene expression in cardiac fibroblasts.** To confirm that Ang II increases ET-1 mRNA levels in cardiac fibroblasts, we performed Northern blot analysis (Figs. 2A and 2B). Endothelin-1 mRNA was induced by Ang II (100 nM) as early as 30 min and then returned to the basal level after 2 h (Fig. 2A). When cardiac fibroblasts were treated with Ang II for 30 min, the Ang II-induced ET-1 mRNA expression was dose-dependent with the maximum induction at 100 nM (Fig. 2B). To determine whether the Ang II-induced ET-1 expression is regulated at the transcriptional level, an ET-1 promoter construct containing the ET-1 promoter region (−4.4 kb) and the reporter gene CAT was constructed and transiently transfected into cardiac fibroblasts. Cardiac fibroblasts exposed to 24 h of Ang II (100 nM) significantly increased ET-1 promoter activity by 2.6-fold compared with untreated cells (Fig. 2C). The effect of $AT_1$ receptor blocker losartan on the Ang II-increased ET-1 promoter activity was also investigated (Fig. 2C). Cardiac fibroblasts were pretreated with losartan (1 $\mu$M) for 1 h and subsequently

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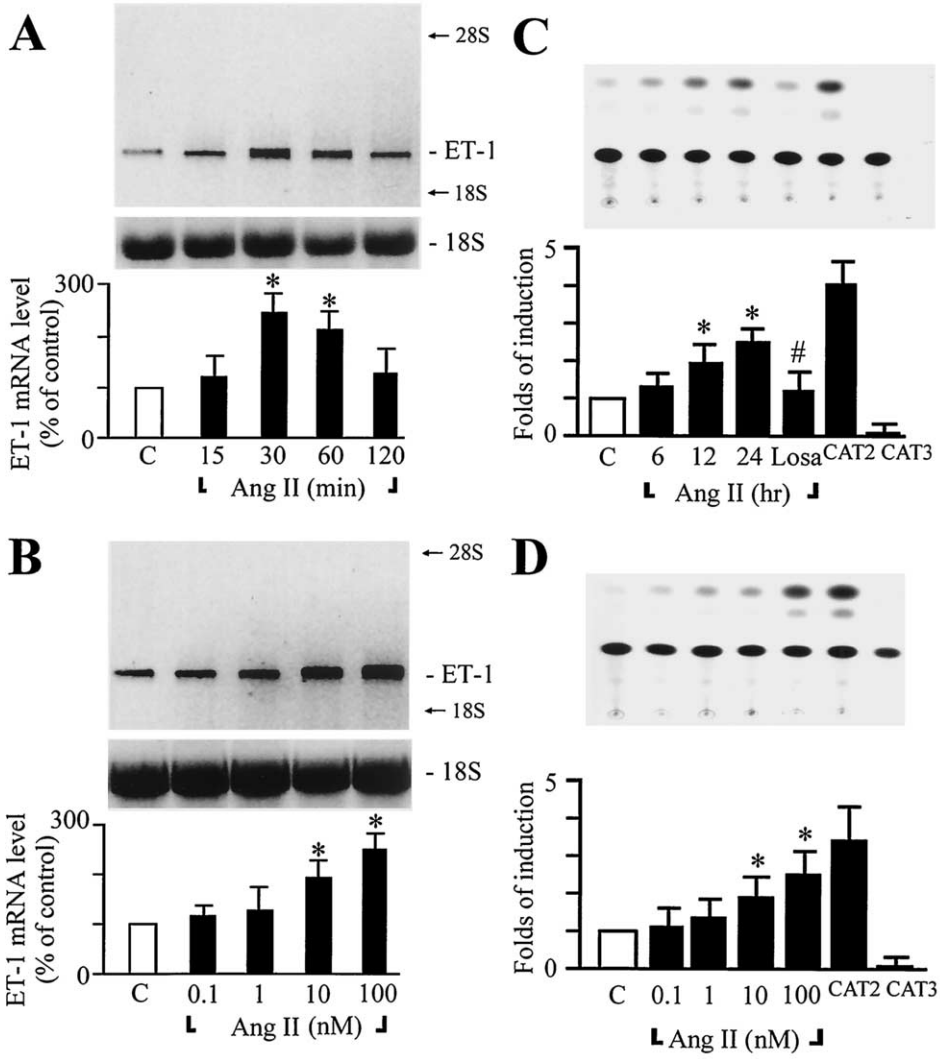


**Figure 2.** Effect of angiotensin II (Ang II) on endothelin-1 (ET-1) gene expression in neonatal rat cardiac fibroblasts. **(A)** Time course of Ang II on ET-1 messenger RNA (mRNA) expression. Cells were incubated with Ang II (100 nM) for the indicated times. **(B)** Dose-dependent effect of Ang II on ET-1 mRNA expression. Cells were incubated with various doses of Ang II for 30 min. **(C)** Time course of Ang II-increased ET-1 promoter activity. Cardiac fibroblasts were transfected with chimeric ET-1 promoter-chloramphenicol acetyltransferase (CAT) fusion genes followed by treatment with Ang II (100 nM) for the time indicated or pretreated with losartan (Losa) (1 M) for 1 h followed by Ang II stimulation. **(D)** Induction of ET-1 promoter activity by different concentration of Ang II. Cells were harvested, and CAT activities were measured as described in the Experimental Procedures section. Control (C), no drugs; CAT2 and CAT3 are shown as positive and negative control; CAT activities are shown as the percentage of incorporation after normalizing to that of $\beta$-galactosidase activities. Data are represented as difference relative to control groups. The results are shown as mean ± SEM (n = 3 per group). *$p < 0.05$ vs. control (Student $t$ test); #$p < 0.05$ vs. Ang II alone (analysis of variance). The experiment was repeated three times with reproducible results.

stimulated with Ang II for 24 h. The Ang II-induced ET-1 gene expression was inhibited by losartan. Angiotensin II dose dependently increased ET-1 promoter activity; 100 nM of Ang II also gave maximum induction (Fig. 2D). These data show that Ang II directly induces ET-1 gene expression in cardiac fibroblasts.

**Ang II-induced fibroblast proliferation and ET-1 gene expression are redox-sensitive.** We and others have demonstrated that Ang II stimulates ROS production in various cell types, including cardiomyocytes and cardiac fibroblasts (3,8,9). To confirm that Ang II induces intracellular ROS in cardiac fibroblasts, we measured intracellular ROS level by analyzing the fluorescent product DCF, a peroxidative product of DCFDA, with laser-scanning confocal microscopy. Cardiac fibroblasts treated with Ang II (100 nM) had significantly higher ROS levels than those cells treated with vehicle only (Fig. 3A). The increase of ROS was completely blocked by pretreatment of cells with either losartan or antioxidants such as catalase or N-acetylcysteine (NAC). Interestingly, cells treated with a flavoprotein containing the NADH/NADPH oxidase inhibitor, diphenyleneiodonium (DPI) also showed a significant reduction in ROS produc-

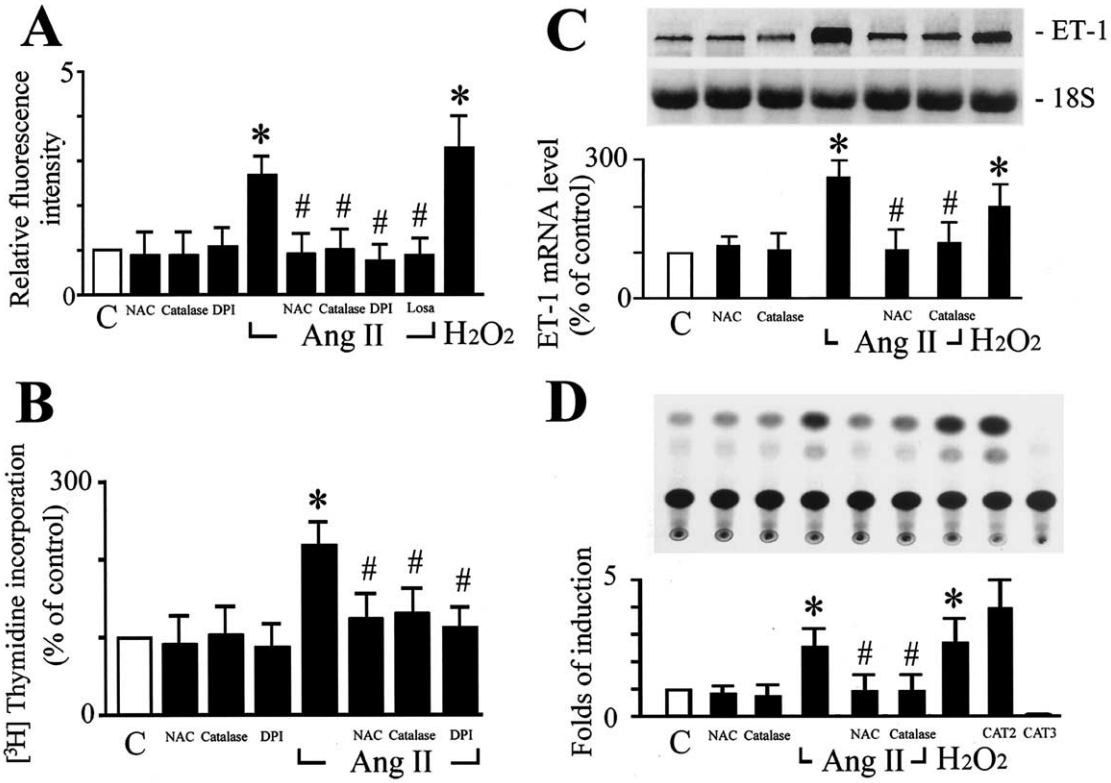


**Figure 3.** Angiotensin II (Ang II)-induced proliferation and endothelin-1 (ET-1) gene expression were mediated by reactive oxygen species (ROS) in cardiac fibroblasts. **(A)** Angiotensin II increased intracellular ROS in cardiac fibroblasts. Cardiac fibroblasts were loaded with dichlorofluorescin diacetate for 30 min and stimulated with Ang II for 30 min. Intracellular ROS levels were measured by laser-confocal microscopy. Angiotensin II (100 nM) increased ROS levels in cardiac fibroblasts, and these increases were abolished by losartan (Losa; 1 $\mu$M), the NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI) (1 $\mu$M), catalase (350 U/ml), or N-acetylcysteine (NAC) (10 mM). Cells treated with $H_2O_2$ are used as positive control. In each experiment, the densitometric analysis was performed on at least 20 cells. **(B)** Effect of antioxidants on Ang II-induced DNA synthesis in cardiac fibroblasts. Cells were preincubated with NAD(P)H oxidase inhibitor DPI (1 $\mu$M), catalase (350 U/ml), or NAC (10 mM) for 30 min followed by incubation with 100 nM Ang II for 24 h. Increases in [$^3$H]thymidine incorporation are each expressed relative to the [$^3$H] content (100%) in the respective control (C). **(C)** Effect of antioxidants on Ang II-induced ET-1 messenger RNA in cardiac fibroblasts. Cells were preincubated with either the catalase (350 U/ml) or NAC (10 mM) for 30 min followed by an incubation with 100 nM Ang II for 30 min. **(D)** Effect of antioxidants on Ang II-increased ET-1 promoter activity in cardiac fibroblasts. Cells were preincubated with either the catalase (350 U/ml) or NAC (10 mM) for 30 min followed by an incubation with 100 nM Ang II for 24 h. The results are shown as mean ± SEM (n = 3 per group). *$p < 0.05$ vs. control (Student $t$ test); #$p < 0.05$ vs. Ang II alone (analysis of variance).

tion (Fig. 3A). To elucidate the involvement of ROS in the Ang II-induced fibroblast proliferation, cardiac fibroblasts were pretreated with NAC or catalase for 30 min followed by Ang II treatment. Cardiac fibroblasts pretreated with NAC (10 mM) or catalase (350 U/ml) significantly suppressed Ang II-induced [$^3$H]thymidine uptake (Fig. 3B). To further examine the ROS involvement in the Ang II-induced ET-1 gene expression, cardiac fibroblasts were preincubated with an antioxidant NAC or catalase for 30 min and then treated with Ang II. As shown in Figure 3C, cardiac fibroblasts pretreated with NAC (10 mM) or catalase (350 U/ml) significantly suppressed Ang II-induced ET-1 mRNA level. Similarly, cells pretreated with NAC or catalase also suppressed Ang II-increased ET-1 promoter activity (Fig. 3D). These findings suggest that intracellular ROS generation plays an important role in Ang II-induced proliferation of cardiac fibroblasts and apparently mediate Ang II-induced ET-1 gene expression.

**Ang II-induced ET-1 gene expression is mediated via Ras/Raf/ERK signaling pathway.** Angiotensin II has been shown to activate ERK, JNK, and p38MAPK in cardiac fibroblasts, and the activation of these pathways is redox-sensitive (3). To study whether these pathways were involved in Ang II-induced ET-1 gene expression in cardiac fibroblasts, we examined the effect of antioxidants on each MAPK pathway and determined the effect of MAPK inhibitors on Ang II-induced ET-1 gene expression. We first confirmed that Ang II increases phosphorylation of ERK1/2, p38MAPK, and JNK in cardiac fibroblasts (Figs. 4A to 4C). Both catalase (350 U/ml) and NAC (10 mM) significantly inhibited Ang II-induced phosphorylation of ERK1/2, p38MAPK, and JNK (Figs. 4A to 4C). These

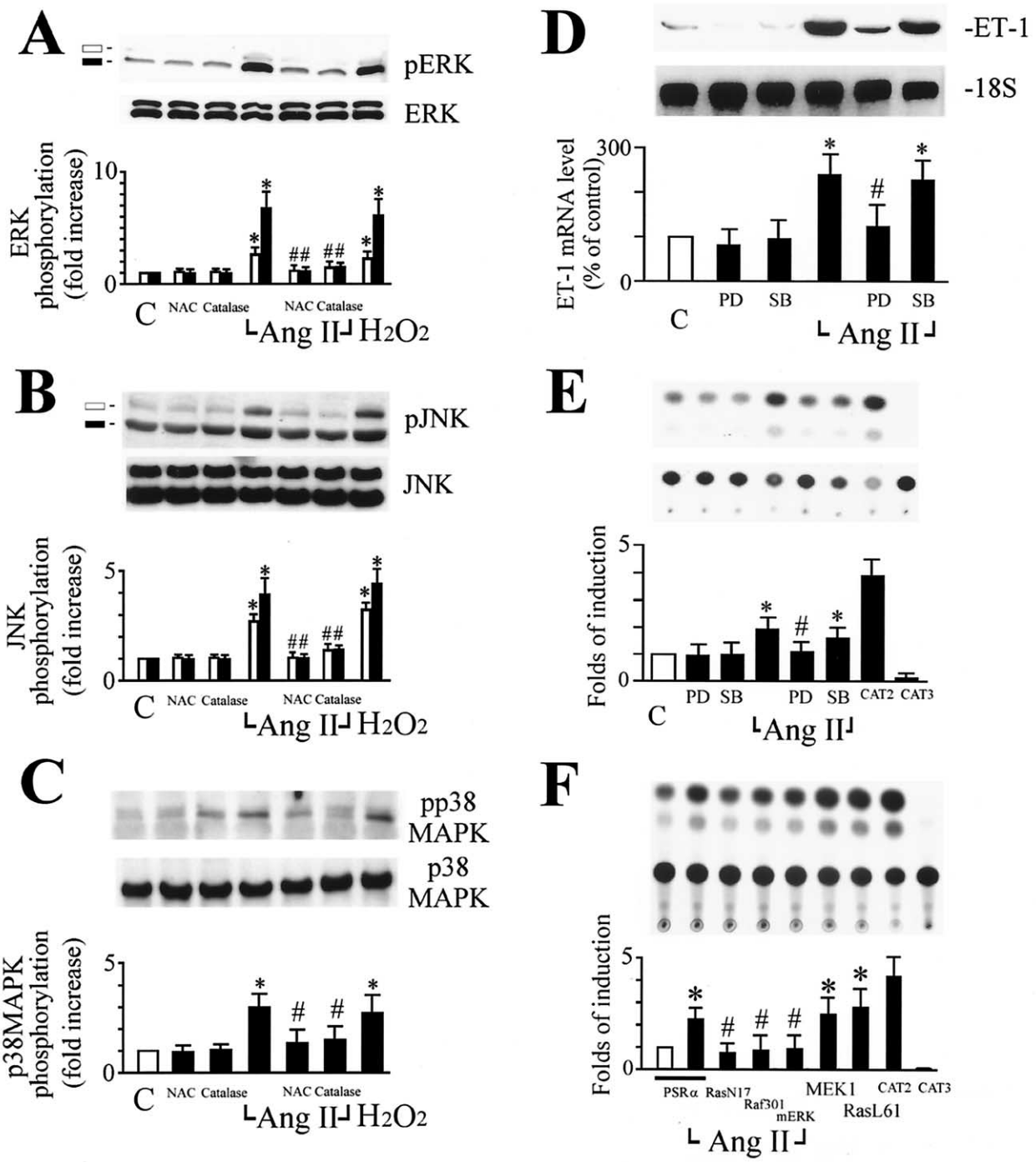


**Figure 4.** Angiotensin II (Ang II) increased ET-1 gene expression via extracellular signal-regulated kinase (ERK) in a redox-sensitive manner. **(A to C)** Angiotensin II-induced activation of ERK, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38MAPK) was mediated by reactive oxygen species-sensitive pathway. Cells were preincubated with either the catalase (350 U/ml) or N-acetylcysteine (NAC) (10 mM) for 30 min and stimulated with Ang II (100 nM) for 30 min. Phosphorylation of ERK, JNK, or p38MAPK was detected by Western blotting using anti-phospho-ERK, phospho-JNK, and phospho-p38MAPK antibodies. Both catalase and NAC inhibited Ang II-induced activation of ERK, JNK, or p38MAPK. Phosphorylations of ERK, JNK, or p38MAPK were detected, and densitometric analyses were performed. The results are shown as mean ± SEM (n = 4 per group). **(D)** Angiotensin II-induced ET-1 messenger RNA was attenuated by PD98059 in cardiac fibroblasts. Cardiac fibroblasts were stimulated with Ang II (100 nM) in the presence of PD98059 (PD; 20 M) or SB203580 (SB; 20 M), and total RNA was isolated at 30 min. **(E)** Angiotensin II-increased ET-1 promoter activity was inhibited by PD98059 in cardiac fibroblasts. Cardiac fibroblasts were stimulated with Ang II (100 nM) in the presence of PD98059 (PD; 20 M) or SB203580 (SB; 20 M), and chloramphenicol acetyltransferase (CAT) activity was assayed after 24 h. **(F)** Angiotensin II-increased ET-1 promoter activity via Ras/Raf/ERK pathway in cardiac fibroblasts. Cells, transfected with either pSRα-empty vector (5 μg), or an expression plasmid encoding the dominant negative mutant mERK, Raf301, or RasN17 (5 μg), were co-transfected with 15 μg of ET-1 promoter-CAT plasmid. Cells co-transfected with ET-1 promoter-CAT plasmid and an expression plasmid encoding MEK1 (5 μg) or RasL61 (5 μg) were used as positive controls. The results are shown as mean ± SEM (n = 3 per group). *$p < 0.05$ vs. control (Student *t* test); #$p < 0.05$ versus Ang II alone (analysis of variance).

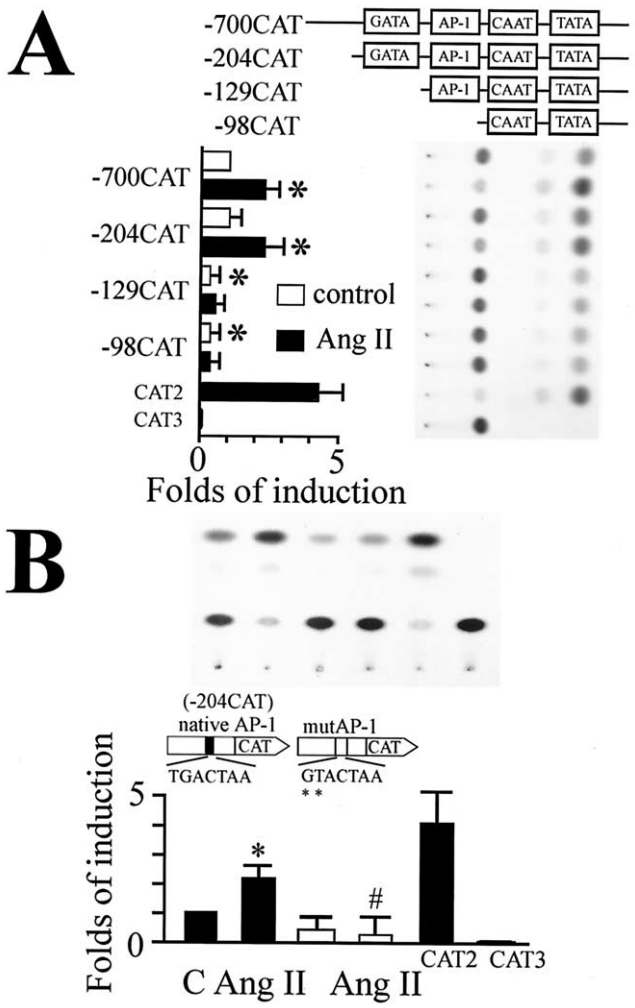


**Figure 5.** Identification of angiotensin II (Ang II)-responsive cis-elements in endothelin 1 (ET-1) promoter. **(A)** A series of deletion mutants containing various lengths of ET-1 promoter region were transfected into cardiac fibroblasts. Transfected cells were stimulated with Ang II (100 nM) for 24 h, and chloramphenicol acetyltransferase (CAT) activities were measured. Stepwise 5′-deletion constructs were depicted **(top)**. Bars represent mean (±SEM, n = 3 per group). CAT activity of each construct in the presence or absence of Ang II were assayed. CAT 2 and CAT 3 were used as positive or negative controls for CAT assay respectively **(bottom)**. **(B)** Wild type (204 bp) or AP-1 mutant of ET-1 promoter-CAT plasmid was transfected into cardiac fibroblasts. Cells were stimulated with Ang II (100 nM) for 24 h. The mutation of AP-1 strongly abolished the responsiveness to Ang II stimulation. The results are shown as mean ± SEM (n = 3 per group). *$p < 0.05$ versus control (Student $t$ test); #$p < 0.05$ versus Ang II alone (analysis of variance).

data suggest that ERK1/2, p38MAPK, and JNK are crucial components of the redox-sensitive signaling pathways activated by Ang II in cardiac fibroblasts. We next determined the role of redox-sensitive activation of MAPKs in Ang II-induced ET-1 gene expression. PD98059, a specific inhibitor of MKK-1 (ERK kinase [MEK]), inhibited augmentation of ET-1 mRNA expression stimulated with Ang II (Fig. 4D). SB203580, a specific inhibitor of p38 MAPK, failed to fully inhibit this expression (Fig. 4D). Similarly, co-incubation with PD98059 also completely abolished Ang II-increased ET-1 promoter activity, but co-incubation with SB203580 had no significant effect (Fig. 4E). These findings suggest that activation of ERK is a necessary step for ET-1 gene expression induced with Ang II.

To identify the signaling pathway involved in the Ang II-induced ET-1 expression, we also co-transfected cardiac fibroblasts with various dominant negative mutants Ras (RasN17), Raf-1 (Raf301), or a catalytically inactive mutant of ERK2 (mERK), all of which are associated with the Ras/Raf/ERK pathway. Cardiac fibroblasts co-transfected with the empty vector PSR$\alpha$, as control revealed no effect on Ang II-induced ET-1 promoter activity (Fig. 4F). However, cells co-transfected with RasN17, Raf301, or mERK resulted in a significant inhibition in Ang II-induced ET-1 promoter activity. In contrast, cardiac fibroblasts co-transfected with a dominant positive mutant of Ras (RasL61) or MEK1 greatly increased their ET-1 promoter activities. These results further suggest that the Ras/Raf/ERK signaling pathway plays an important role in Ang II-induced ET-1 gene expression in cardiac fibroblasts.

**Identification of Ang II-responsive regulatory elements in the ET-1 promoter.** The ET-1 promoter contains a number of AP-1 and GATA sites that can be regulated by multiple activation pathways (10,15). We dissected the Ang II-responsive elements of the ET-1 promoter in cardiac fibroblasts. As previously described (11), a series of deletion mutants containing various lengths of the ET-1 promoter region fused to CAT reporter gene were transfected into cardiac fibroblasts, and CAT activity was measured in response to Ang II stimulation. Angiotensin II stimulation for 24 h significantly increased CAT activity by 2.5- and 2.2-fold in −700CAT and −204CAT, respectively, both of which contain multiple transcription factor binding sites, including GATA (base pair [bp] −136 to −131) and AP-1 (bp −108 to −102) sites. However, after further truncation of the GATA and subsequent AP-1 site from the 5′-end, the increase of the Ang II-induced ET-1 promoter activity was completely abolished in both −129CAT and −98CAT. It is interesting that deletion of these two sites also resulted in a significant decrease in basal promoter activity (Fig. 5A). These findings suggest that the GATA site as well as the AP-1 site are necessary for Ang II-stimulated ET-1 gene induction. We further examined whether AP-1 site is essential for the induction of ET-1 gene by Ang II. In cells transfected with reporter construct −204CAT containing both GATA and AP-1 sites with two-bp mutation in the AP-1 site, the Ang II-induced ET-1 promoter activity was completely abolished. In addition, the basal promoter activity also decreased compared with control (Fig. 5B). These findings suggest that the AP-1 binding element is essential for the induction of ET-1 gene by Ang II. Moreover, pretreating cells with antioxidants, NAC, or catalase attenuated the Ang II-stimulated AP-1 binding activity (data not shown). These results clearly indicate that ROS mediate the transcriptional activ-

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ity of AP-1 induced by Ang II, and the AP-1 binding element is responsible for the induction of ET-1 gene expression by Ang II in cardiac fibroblasts.

## DISCUSSION

The heart is composed of not only cardiac myocytes but also nonmyocytes, particularly fibroblasts (16). Unlike cardiac myocytes, cardiac fibroblasts can proliferate and increase the deposition of extracellular matrix (ECM) proteins such as fibronectin and collagen, which leads to interstitial fibrosis (17). Therefore, fibroblasts play a crucial role in the development of cardiac fibrosis, which enhances intrinsic myocardial stiffness and results in diastolic dysfunction, accounting for 30% to 50% of congestive heart failure in clinical practice (18,19). Recently, pharmacologic intervention with angiotensin-converting enzyme inhibitors and $AT_1$ receptor antagonists has demonstrated that Ang II plays an important role in the mediation of human hypertensive cardiac fibrosis or pressure overload-induced cardiac fibrosis in rats (20–25). Angiotensin II, the central product of the renin-angiotensin system, has been shown to induce hypertrophy of cardiomyocytes and hyperplasia of cardiac fibroblasts via $AT_1$ receptor. Recent in vitro study revealed that Ang II-stimulated cardiomyocyte hypertrophy may be mediated by paracrine release of ET-1 and transforming growth factor-$\beta$1 from fibroblasts rather than by direct action (2). Animal study also revealed that the antioxidant dimethylthiourea and probucol markedly improve left ventricular remodeling in chronic heart failure, predominantly by reducing cardiac fibrosis (26,27). In the present study, we demonstrated that ROS mediate Ang II-induced fibroblast proliferation and ET-1 gene expression in cardiac fibroblasts, and antioxidants significantly inhibit these effects, consistent with findings of the above-mentioned in vivo study. We further showed that ET-1 plays an important role in Ang II-induced cardiac fibroblast proliferation in autocrine fashion, in addition to its hypertrophic effect on cardiomyocytes by paracrine action. These Ang II inductions of both cardiomyocyte hypertrophy and fibroblast proliferation, with concomitant production of ECM proteins, lead to ventricular remodeling, dilation, and heart failure. Our findings provide substantial evidence for new therapeutic options with $AT_1$ and $ET_A$ receptor antagonists combined with antioxidants in congestive heart failure.

Angiotensin II has been shown to stimulate proliferation of cardiac fibroblasts in culture (4). We characterized the proliferative response to Ang II using agents that implicated the $AT_1$ receptor (losartan) and the $ET_A$ receptor (BQ485). The Ang II-induced DNA synthesis in cardiac fibroblasts was inhibited by both losartan and BQ485. These data are compatible with Fujisaki et al. (1) in which an $ET_A$ receptor antagonist blocked DNA synthesis induced by Ang II. It is suggested that Ang II-induced proliferation of cardiac fibroblasts is mediated by endogenous ET-1 produced by cardiac fibroblasts in an autocrine/paracrine manner. Reactive oxygen species have been implicated in inflammatory processes such as fibrosis (28). It is also known that antioxidants are capable of antagonizing the actions of Ang II in many cell types (29). We previously reported that ROS could modulate Ang II-induced $\beta$-myosin heavy chain gene expression in cardiomyocytes (9). To better understand the role that ROS might play in influencing Ang II action in cardiac fibroblasts, we have characterized the induction of ET-1 gene and the activation of MAPKs in these cells by Ang II and examined the effects of antioxidants NAC and catalase on this pathway. In this study, we demonstrated that ET-1 mediates Ang II-induced fibroblast proliferation and ROS are involved in Ang II-induced activation of MAPK pathways, which leads to ET-1 gene expression. We also found that AP-1 site in the promoter region is a crucial cis-element for Ang II-mediated induction of ET-1 gene in cardiac fibroblasts.

The MAPKs are a family of serine-threonine kinases, which include ERK, JNK, and p38MAPK. Sano et al. (3) reported that Ang II activates ERK1/2, p38MAPK, and JNK partly through ROS in cardiac fibroblasts. Their observation was in agreement with our present findings that antioxidants significantly inhibit Ang II-induced activation of the three classes of MAPKs. There are multiple pathways leading to ERK activation by Ang II, which also differ between cell types. These distinctive pathways might result in different sensitivity for ROS. Several recent studies have suggested that the balance of the oxidative and reductive potentials (cellular redox state) within the cell may substantially influence this pathway. Wang et al. (30) previously reported that MEK1 was activated by Ang II and inhibited by NAC, suggesting that it could be a potential site of action for these drugs, although any site upstream of MEK1 also could be implicated. Our present findings suggest that MEK1 is not the sole site of action, because both JNK and p38MAPK activation were also inhibited by NAC and catalase and these pathways are generally believed to be independent of MEK1. We characterized the Ang II-induced ET-1 gene expression using PD98059 that implicated the requirement for MEK1 activation. However, we could not completely rule out the role of p38MAPK in Ang II-induced ET-1 gene expression, because SB203580 also slightly reduced ET-1 mRNA expression stimulated by Ang II. There is limited information regarding how activation of G-protein coupled receptors (GPCRs) by Ang II leads to activation of MEK/ERK pathway in cardiac fibroblasts. However, the molecular mechanisms by which ROS regulate upstream signaling that lead to Ang II-induced ERK phosphorylation and activation via liganded GPCRs in cardiac fibroblasts remain to be further determined.

Accumulating evidence has made it clear that GPCRs and receptor tyrosine kinases share a common pathway converging in Ras (31). The non-receptor and receptor tyrosine kinases have been implicated in Shc phosphorylation and Grb2/Sos recruitment thereby, connecting the GPCR signal to Ras and ERK activation. Co-transfection

experiments with dominant negative Ras, Raf, and ERK suggested that the Ras-Raf-ERK pathway is involved in the transcriptional activation of the ET-1 by Ang II because they all inhibited the ET-1 promoter activity induced by Ang II (Fig. 4F), whereas the empty vectors had no effect. The ET-1 promoter contains an AP-1 element that could be activated by ROS (10). Several evidences suggest that ROS serve as messengers in AP-1 activation (7). The cis-acting AP-1 element binds the protooncogene products jun and fos (32), and it is well known that the genes for jun and fos are activated by ROS (33). Using the gel mobility shift assay, we found that Ang II increased binding of jun and fos to the AP-1 site of the ET-1 gene promoter in a redox-sensitive manner (data not shown). Truncation and mutational analysis of the ET-1 gene promoter showed that the AP-1 binding sequence is an important cis-element for Ang II-induced ET-1 gene expression and that the GATA site is also necessary for ET-1 gene induction by Ang II. These results are consistent with the previous report by Kawana et al. (15), showing that both the GATA and AP-1 sites are essential for ET-1 promoter function and that cooperative interaction of GATA and AP-1 regulates the transcription of the ET-1 gene in endothelial cells.

**Conclusions.** Angiotensin II via $AT_1$ receptor increased intracellular ROS, which were at least partly involved in Ang II-induced proliferation and the Ang II-increased activation of ERK, JNK, and p38MAPK pathways in cardiac fibroblasts. Moreover, we showed that ERK activation plays a crucial role in Ang II-stimulated ET-1 gene expression and mitogenic signaling. The ROS-MAPK (ERK)–meditated AP-1-dependent transcription plays a crucial role in Ang II-induced proliferation and ET-1 gene expression in cardiac fibroblasts. The mechanism by which ROS activated various signaling pathways remains undetermined and should be clarified in the near future.

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