0026-895X/03/6305-1002–1011$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:1002–1011, 2003

Vol. 63, No. 5
2169/1060484
Printed in U.S.A.

Crucial Role of Extracellular Signal-Regulated Kinase Pathway in Reactive Oxygen Species-Mediated Endothelin-1 Gene Expression Induced by Endothelin-1 in Rat Cardiac Fibroblasts

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Received September 23, 2002; accepted February 6, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Endothelin-1 (ET-1) has been implicated in fibroblast proliferation. However, the mechanism involving ET-1 is not clear. The present study was performed to examine the role of endogenous ET-1 in ET-1–stimulated fibroblast proliferation and to investigate the regulatory mechanism of ET-1–induced ET-1 gene expression in cardiac fibroblasts. Both ET_A receptor antagonist [(hexahydro-1H-azepinyl)carbonyl-Leu-D-Trp-D-OH (BQ485)] and endothelin-converting enzyme inhibitor (phosphoramidon) inhibited the increased DNA synthesis caused by ET-1. ET-1 gene was induced by ET-1, as revealed with Northern blotting and ET-1 promoter activity assay. ET-1 increased intracellular reactive oxygen species (ROS), which were significantly inhibited by BQ485 and antioxidants. Antioxidants suppressed ET-1 gene expression and DNA synthesis stimulated by ET-1. ET-1 activated mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase, which were significantly inhibited by antioxidants. Only ERK inhibitor U0126 could inhibit ET-1–induced transcription of the ET-1 gene. Cotransfection of dominant-negative mutant of Ras, Raf, and MEK1 decreased the ET-1–induced increase in ET-1 transcription, suggesting that the Ras-Raf-ERK pathway is required for ET-1 action. Truncation and mutational analysis of the ET-1 gene promoter showed that the activator protein-1 (AP-1) binding site was an important *cis*-element in ET-1–induced ET-1 gene expression. Antioxidants attenuated the ET-1–stimulated AP-1 binding activity. Our data suggest that ROS were involved in ET-1–induced fibroblast proliferation and mediated ET-1–induced activation of ERK pathways, which culminated in ET-1 gene expression.

Fibroblasts play an important role in maintaining cardiac function by providing structural support for the cardiomyocytes (Marsen et al., 2000) and by serving as a source for autocrine/paracrine growth factors (Ancey et al., 2002). After myocardial infarction, reactive fibrosis results in excessive scar formation as proliferating fibroblasts invade the necrotic area. This remodeling leads to an increase of the ventricular stiffness and ultimately compromises the function of the heart (Borer et al., 2002). Excessive myocardial fibrosis has been found in the progression of cardiac dysfunction, especially diastolic dysfunction, in hypertensive hearts (Mann, 1999).

Recent studies in humans (Graf et al., 1997) and animal models (Lapointe et al., 2002) have shown that the expression of myocardial endothelin-1 (ET-1) is increased during cardiac fibrosis. Some have suggested that ET-1 might contribute to cardiac fibroblast proliferation (Piacentini et al., 2000), resulting in cardiac fibrosis (Ammarguellat et al., 2001). ET-1 is a bioactive vasoconstrictor peptide (Yanagisawa and Masaki, 1989) formed through the specific conversion of its intermediate precursor, big ET-1, by an endothelin-converting enzyme (ECE). ET-1 works as a paracrine regulator as well as an autocrine regulator. An in vitro study

This study was in supported with grants from National Science Council (91-2314-B-038-255-) and Taipei Medical University (TMU 91-Y05-A151), Taiwan, R.O.C.

ABBREVIATIONS: ET, endothelin; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; DCF, dichlorofluorescein; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DPI, diphenylene iodonium; ECE, endothelin-converting enzyme; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; BQ485, (hexahydro-1H-azepinyl)carbonyl-Leu-D-Trp-D-OH; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynylthio)butadiene; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BQ788, *cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine; Bp, base pair(s).

revealed that angiotensin II-stimulated cardiomyocyte hypertrophy is mediated by the paracrine release of ET-1 from fibroblasts rather than by direct action (Gray et al., 1998). On the other hand, ET-1 mRNA expression in cardiac fibroblasts is induced by ET-1 itself, in an autocrine fashion (Fujisaki et al., 1995). Consequently, intracellular reactive oxygen species (ROS) in cardiomyocytes are increased (Cheng et al., 1999; Tanaka et al., 2001; Hirotani et al., 2002). ROS act as second messengers in receptor-mediated signaling pathways. An animal study revealed that the antioxidant dimethylthiourea and probucol markedly improve left ventricular remodeling in chronic heart failure, predominantly by reducing cardiac fibrosis (Kinugawa et al., 2000; Sia et al., 2002). ROS modulate c-*fos* gene expression induced by ET-1, and the administration of antioxidants inhibits such expression in cardiomyocytes (Cheng et al., 1999). In turn, the increase in ROS regulates various intracellular signal transduction cascades (Tanaka et al., 2001), as well as the activities of various transcription factors (Hirotani et al., 2002). Activator protein-1 (AP-1) and GATA2 have been shown to regulate transcription of the ET-1 gene in a cooperative fashion through the GATA and AP-1 sites located in the promoter region of ET-1 gene in endothelial cells (Kawana et al., 1995). In addition, mechanical stretch-induced intracellular ROS are involved in ET-1 gene induction through the AP-1 element of the ET-1 gene (Cheng et al., 2001). Redox-sensitive mitogen-activated protein kinases (MAPKs) have been shown to be important for postreceptor signaling; through these, growth factors stimulate cardiac fibroblast proliferation (Pages et al., 1993). However, whether ET-1 expression is induced by ET-1 itself and whether it acts through this pathway are not known.

Therefore, we set out to study the role of redox-sensitive mechanisms in the cardiac fibroblast proliferation and the modulation of ET-1–induced augmentation of ET-1 gene expression in cultured cardiac fibroblasts from neonatal rats. By using different kinds of MAPK kinase (MEK) inhibitors, we investigated the pathway by which ET-1 expression is regulated.

Materials and Methods

Materials. ET-1 cDNA was obtained from a human endothelial cell cDNA library as described previously (Wang et al., 1993). This ET-1 cDNA was cloned into pGEM (Promega, Madison, WI), then excised with restriction endonucleases *Eco*RI and *Bam*HI. A series of deletion mutants containing different lengths of the ET-1 promoter region were fused to the chloramphenicol acetyltransferase (CAT) reporter gene. Catalytically inactive mutants of extracellular signal-regulated kinase (ERK) 2 (mERK2), RasN17, RasL61, and Raf301 have been described previously (Cheng et al., 2001). The enhanced chemiluminescence detection system was obtained from Amersham Biosciences (Piscataway, NJ). ET-1, BQ485, phosphoramidon, U0126, SB203580, curcumin, and all other chemicals were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, fetal calf serum, and tissue culture reagents were obtained from Invitrogen (Carlsbad, CA).

Cell Culture. Primary cultures of neonatal rat cardiac fibroblasts were prepared as described previously (Cheng et al., 1999). Briefly, ventricles from 1- to 2-day-old neonatal Sprague-Dawley rats were cut into chunks of approximately 1 mm^3 by using scissors and were subjected to trypsin (0.125%; Invitrogen) digestion in phosphate-buffered saline (PBS). Dispersed cells were incubated on 100-mm culture dishes for 30 min in a 5% CO_2 incubator. Nonmyocytes attached to the bottom of the dishes were subsequently incubated with DMEM supplemented with 10% fetal calf serum for an additional 2 to 4 d. Confluent nonmyocytes were treated with trypsin and subcultured. Subconfluent (approximately 70% confluence) cardiac fibroblasts grown in 60- or 100-mm culture dishes from the second to fourth passages; these were used for the experiments. Serum-containing medium from these cultured cells was replaced with serum-free medium and exposed to different reagents, as indicated.

DNA Synthesis. To measure the synthesis of new DNA, cells (1×10^5 per well) were seeded in six-well (35-mm) dishes 24 h before the experiments were performed. Cardiac fibroblasts were incubated with [^{3}H]thymidine (5 μCi/ml) for 24 h before harvest. The cells were harvested by means of incubation at 4°C with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH. Radioactivity was determined by means of scintillation counting. Data are presented as the mean ± S.E.M. for 9 to 12 determinations in three to four cell preparations and normalized with data from the untreated sample and multiplied by 100 (i.e., as a percentage of control).

Assay of Intracellular ROS. Intracellular ROS production was measured by using the fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes, Eugene, OR) with the ACAS interactive laser cytometer (Meridian Instruments, Inc., Okemos, MI), as described previously (Cheng et al., 1999). A 10 mM stock solution of DCF-DA was prepared in ethanol on a daily basis and diluted to a final concentration of 10 μM just before the experiments were conducted. Cardiac fibroblasts were preincubated with 10 μM DCF-DA in DMEM for 30 min at 37°C before treatment. After exposure to the dye, the cells were rinsed with Tyrode's solution. The cells were maintained in Tyrode's solution and examined by using the laser cytometer at 37°C. Excitation of dichlorofluorescein (DCF) was achieved by using the 488-nm line of a 20-mW argon-ion laser. The emission above 515 nm was quantitated from two-dimensional scans generated by using a 1-μm laser beam and an X-Y scanning stage to obtain a fluorescence value from single cells. To provide a valid comparison, the same acquisition parameters were used for all observations. Quantification of the levels of DCF fluorescence was assessed on a relative scale from 0 to 4000 units. Baseline values from unstimulated cells were used as control values in the comparison with ET-1–stimulated cells. Values represent mean ± S.E.M. of DCF fluorescence from 20 randomly selected cells for each experiment in the five investigations.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from cardiac fibroblasts by using the guanidine isothiocyanate/phenochloroform method described previously (Cheng et al., 1999). The RNA (10 μg per lane) was separated by means of electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran; Schleicher and Schuell, Inc.) by using a vacuum blotting system (VacuGene XL; Amersham Biosciences). After hybridization with ^{32}P-labeled cDNA probes (Cheng et al., 2001), the membrane was washed with 1× standard saline citrate containing 1% SDS at 42°C for 30 min and then exposed to X-ray film at −70°C. Autoradiographic results were analyzed by using a densitometer (Computing Densitometer 300S; Amersham Biosciences). The blots were stripped and reprobed for the 18S cDNA probe (obtained from the American Type Culture Collection, Manassas, VA) to control for loading. Expression of ET-1 mRNA was quantitated and normalized to the 18S signal.

Transfection and Chloramphenicol Acetyltransferase Assays. For the transient transfections, cardiac fibroblasts were transfected with different expression vectors by using the calcium phosphate method (Cheng et al., 1999). In each experiment, DNA concentrations of all samples were adjusted to equal amounts by using the empty vector pSRα. Briefly, cardiac fibroblasts were maintained in culture for 48 h before transfection. The indicated expression vectors were mixed with calcium phosphate and immediately added to the cardiac fibroblast cell culture. After incubation for 5 h, the cells were washed three times with PBS and incubated with 10% serum DMEM. After 12 h, cells were washed with serum-free me-

dium and incubated in serum-free medium for an additional 48 h. The cells were then treated with different agents. To correct for variability in transfection efficiency, 5 μg of pSV-β-galactosidase plasmid DNA was cotransfected in all the experiments. The CAT and β-galactosidase assays were performed as described previously (Cheng et al., 1999). The relative CAT activity was corrected by normalizing the respective CAT value to that of the β-galactosidase activity. Cotransfected β-galactosidase activity varied less than 10% within a given experiment and was not affected by any of the experimental manipulations described. As positive and negative controls, pBLCAT2 (with a thymidine kinase promoter) and pBLCAT3 (without a promoter) were included in every assay.

Western Blot Analysis. Rabbit polyclonal anti-phosphospecific p38 MAPK, anti-phosphospecific ERK1/2, and anti-phosphospecific c-Jun N-terminal kinase (JNK) antibodies were purchased from New England Biolabs (Beverly, MA). Anti-ERK1/2, anti-p38 MAPK, and anti-JNK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western blot analysis was performed as described previously (Cheng et al., 2001).

Electrophoretic Mobility Shift Assay. The electrophoretic mobility shift assay was performed as described previously (Wung et al., 1997). To prepare nuclear protein extracts, cardiac fibroblasts were washed with cold PBS and then immediately removed by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in cold buffer A containing 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride for 15 min. The cells were lysed by adding 10% Nonidet P-40 and then centrifuged at 6000 rpm to obtain pellets of nuclei. The nuclear pellets were resuspended in cold buffer B containing 20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mol/L NaCl. They were vigorously agitated and then centrifuged. The supernatant containing the nuclear proteins was used for the assay or stored at −70°C until used. Double-stranded oligonucleotides (30 bp) containing the AP-1 binding site were synthesized and annealed. The oligonucleotides were end-labeled with [^{32}P]ATP. Extracted nuclear proteins (10 μg) were incubated with 0.1 ng of ^{32}P-labeled DNA for 15 min at room temperature in 25 μl of binding buffer containing 1 μg of poly(dI-dC). For competition with unlabeled oligonucleotide, a 100-fold molar excess of unlabeled oligonucleotide relative to the radiolabeled probe was added to the binding assay. Supershift experiments were performed to determine the composition of the complexes by using a c-fos antibody (Santa Cruz Biotechnology). In these experiments, 1 μg of antibody was added to the reaction 45 min before the addition of labeled probe, and the mixture was kept at 4°C overnight. The mixtures were electrophoresed on 5% nondenaturing polyacrylamide gels. Gels were dried and imaged by means of autoradiography.

Statistical Analysis. Results are expressed as mean ± S.E.M. of at least three experiments. The statistical significance of differences between groups was estimated by one-way analysis of variance. For the comparisons, p values of less than 0.05 were considered to indicate statistically significant differences.

Results

DNA Synthesis by ET-1 Is Mediated through ET_A Receptor and Endogenous ET-1 Synthesis. ET-1–induced increase in cardiac fibroblast proliferation was studied by monitoring DNA synthesis. As measured by assessing [^{3}H]thymidine incorporation, ET-1 increased DNA synthesis in neonatal rat cardiac fibroblasts in a dose-dependent fashion (Fig. 1A). The incorporation of [^{3}H]thymidine stimulated by ET-1 (10 nM) was inhibited by BQ485 (100 nM) but not BQ788 (100 nM) (Fig. 1B). BQ485 is known to be an ET_A receptor blocker (Cheng et al., 1999), whereas BQ485 alone has no effect on basal [^{3}H]thymidine uptake. Similar to BQ485, phosphoramidon (an ECE inhibitor) also inhibited

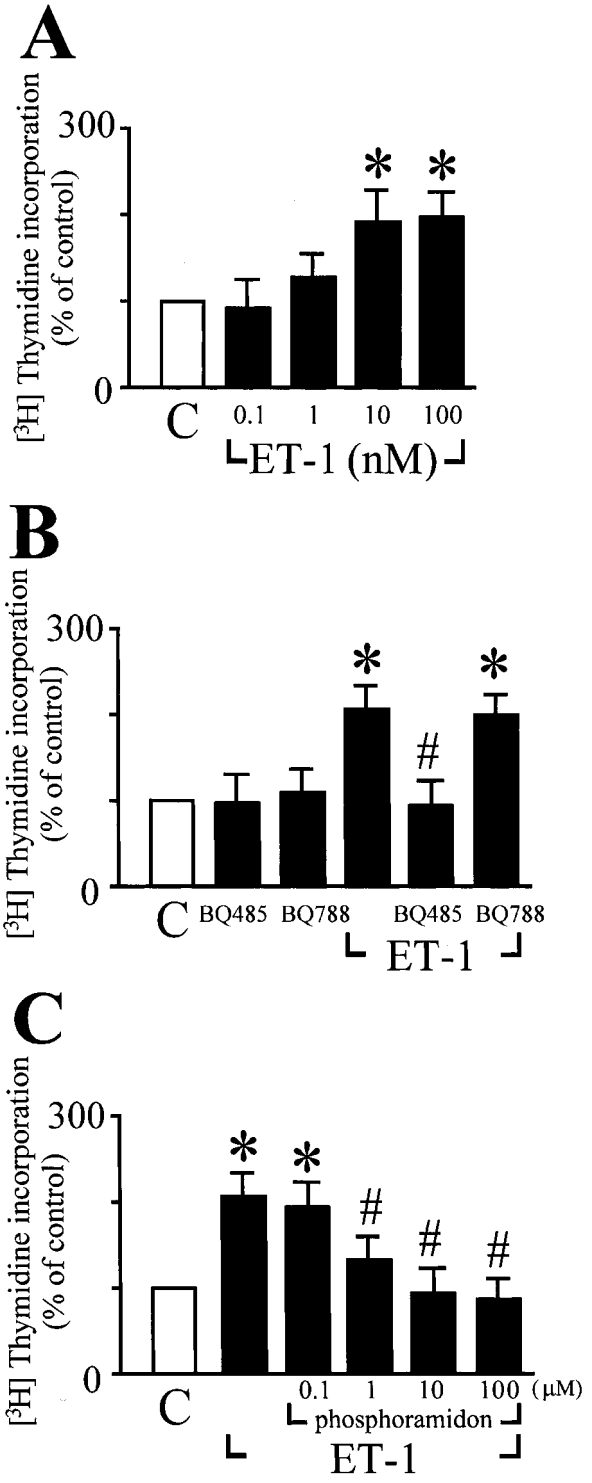

Fig. 1. Characteristics of the activation of DNA synthesis by ET-1 in cardiac fibroblasts. All experiments were performed by using the incorporation of [^{3}H]thymidine into DNA. All data are shown as the mean ± S.E.M. for 9 to 12 determinations in three to four cell preparations. *, $p < 0.05$ versus control; #, $p < 0.05$ versus ET-1 alone. A, effect of ET-1 concentration on DNA synthesis. Cells were incubated with the indicated doses of ET-1 for 24 h, and [^{3}H]thymidine incorporation was then assayed. B, effect of ET-1 receptor antagonists on [^{3}H]thymidine incorporation. Cells were preincubated with either BQ485 (100 nM) or BQ788 (100 nM) for 1 h after their incubation with 10 nM ET-1 for 24 h. C, effect of different concentration of phosphoramidon (0.1, 1, 10, and 100 μM) on ET-1–induced DNA synthesis. Increases in [^{3}H]thymidine incorporation are each expressed relative to the ^{3}H content (100%) in their respective controls.

the increased DNA synthesis caused by ET-1; it did so in a dose-dependent fashion (Fig. 1C). These data confirm that ET-1 increases DNA synthesis via ET_A receptors and suggest the role of endogenous ET-1 as an autocrine growth factor for cardiac fibroblast proliferation under ET-1 stimulation.

ET-1–Induced ET-1 Gene Expression Is Regulated Transcriptionally. Previously, investigators have shown that ET-1 increases ET-1 mRNA levels in cardiac fibroblasts (Fujisaki et al., 1995). Whether the ET-1–induced ET-1 expression is regulated at the transcriptional level is not known. ET-1 (10 nM) induced ET-1 mRNA expression as early as 30 min; this increase was sustained for additional 2 h (Fig. 2A). The ET-1–induced ET-1 mRNA expression (30 min) was dose-dependent, with the maximum induction occurring at the concentration of 10 nM (Fig. 2B). An ET-1 promoter construct containing the 4.4-kb ET-1 promoter region and the reporter gene for CAT was transiently transfected into cardiac fibroblasts. Cardiac fibroblasts exposed to ET-1 increased ET-1 promoter activity in a dose-dependent manner, with the maximal induction occurring at 10 nM (Fig. 2C). These data show that ET-1 directly induces ET-1 gene expression in cardiac fibroblasts at the transcriptional level.

ET-1–Induced Fibroblast Proliferation and ET-1 Gene Expression Is Regulated through a Redox-Sensitive Mechanism. Previously, we demonstrated that ET-1 stimulates the production of ROS by analyzing the fluorescent product DCF, a peroxidative product of DCF-DA, with laser-scanning confocal microscopy in cardiomyocytes (Cheng et al., 1999). We set out to determine whether ET-1 also induces intracellular ROS in cardiac fibroblasts. The addition of 10 nM ET-1 (Fig. 3A) significantly increased ROS, compared with the vehicle (Fig. 3B). This ROS induction depended on ET-1 concentration because the ROS level seemed to reach a plateau with 10 nM ET-1 (Fig. 3C). To specify which ET-1 receptor subtype(s) was responsible for the generation of ROS in cardiac fibroblasts, we pretreated cardiac fibroblasts with either ET_A antagonist (BQ485) or ET_B antagonist (BQ788) before the ET-1 exposure. BQ485 (100 nM) significantly inhibited the ET-1–induced generation of ROS (Fig. 3D). In contrast, BQ788 (100 nM) had no significant or obvious effect on the ET-1–induced generation of ROS. These results suggest that induction of ROS upon ET-1 treatment is mediated through the binding of ET-1 to ET_A receptors. The increase in DCF fluorescence was also inhibited by antioxidants such as catalase (350 U/ml), *N*-acetylcysteine (NAC; 10 mM), and diphenylene iodonium (DPI; 1 μM), an inhibitor of NADPH oxidase (Fig. 4A). To confirm the involvement of ROS in proliferation induced by ET-1, cardiac fibroblasts were pretreated with catalase, NAC, and DPI for 30 min, followed by ET-1 treatment. Consistent with these findings, pretreatment with antioxidants also significantly suppressed ET-1–increased [^{3}H]thymidine uptake (Fig. 4B) as well as ET-1 mRNA expression (Fig. 5A). Consistently, the pretreatment also suppressed ET-1–related increases in ET-1 promoter activity (Fig. 5B). These data suggest that the ET-1 induces ROS reaction through binding to the ET_A receptor. Intracellular ROS generation plays an important role in the ET-1–induced proliferation of cardiac fibroblasts, and ROS mediate ET-1–induced transcription of the ET-1 gene.

Effect of MAPK Pathways on ET-1–Induced Expression of the ET-1 Gene. ET-1 activates ERK, JNK, and p38MAPK in cardiomyocytes (Tanaka et al., 2001); therefore, we set out to examine whether ET-1–induced ET-1 expression is regulated through the redox-sensitive MAPK pathways. We examined the effect of antioxidants on different

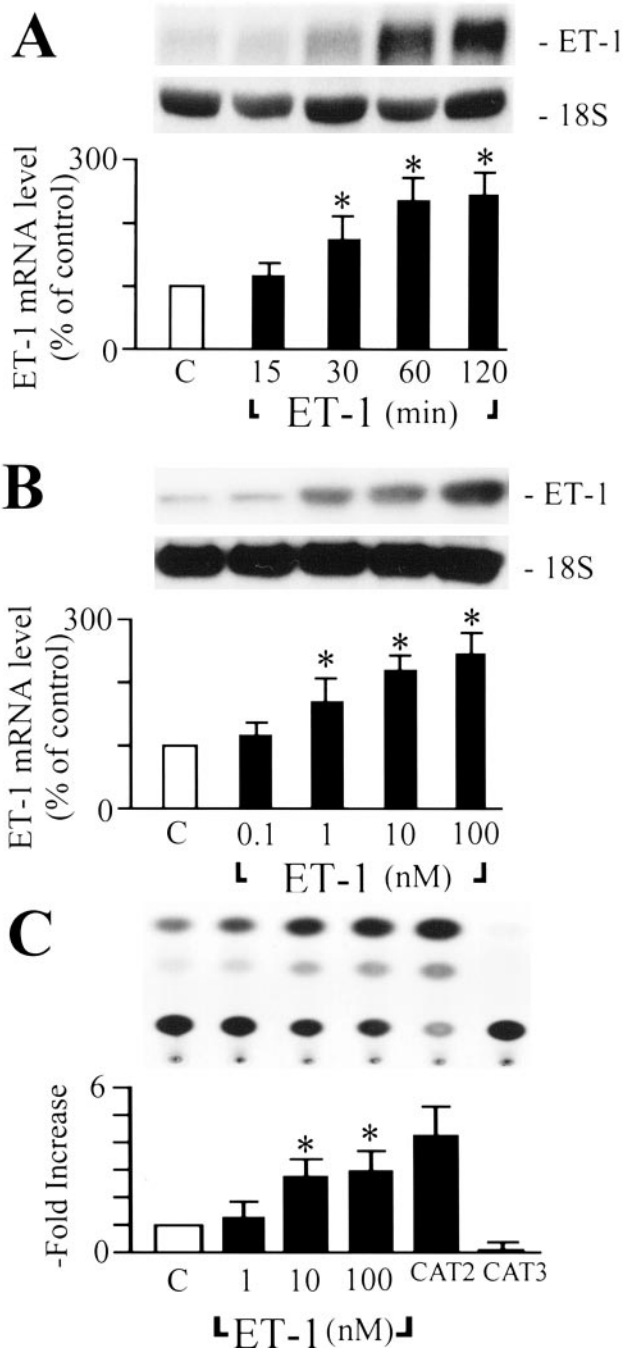

Fig. 2. Effect of ET-1 on ET-1 gene expression in neonatal rat cardiac fibroblasts. Data are represented as the difference relative to the data in the control groups. The results are shown as the mean ± S.E.M. ($n = 3$). *, $p < 0.05$ versus control. #, $p < 0.05$ versus ET-1 alone. A, time course of ET-1 on ET-1 mRNA expression. Cells were incubated with ET-1 (10 nM) for the indicated times. B, dose-response effect of ET-1 on ET-1 mRNA expression. Cells were incubated with various doses of ET-1 for 30 min. C, induction of ET-1 promoter activity by different concentrations of ET-1. Cardiac fibroblasts were transfected with chimeric CAT fusion genes and then treated with ET-1 for 24 h. Cells were harvested, and CAT activities were measured. CAT activities are shown as the percentage incorporation after the data were normalized to β-galactosidase activities. C indicates control (no drugs). CAT2 and CAT3 are positive and negative controls, respectively.

components on the MAPK pathway by using different MAPK inhibitors. As shown in Fig. 6, A-C, ET-1 increased phosphorylation of ERK1/2, p38MAPK, and JNK in cardiac fibroblasts, whereas the antioxidants catalase (350 U/ml) and NAC (10 mM) significantly inhibited ET-1–induced phosphorylation of ERK1/2, p38MAPK, and JNK. These findings suggest that ERK1/2, p38MAPK, and JNK are critical pathways of the redox-sensitive signaling pathways activated by ET-1 in cardiac fibroblasts.

To further assess the role of redox-sensitive activation of MAPK in ET-1–induced ET-1 gene expression, cells were pretreated for 1 h with the MEK inhibitors U0126 (10 μM), SB203580 (10 μM), or curcumin (10 μM). U0126, an inhibitor of ERK1/2, inhibited ET-1 mRNA expression after ET-1 stimulation. Both SB203580 and curcumin, inhibitors of p38MAPK and JNK, respectively, failed to fully inhibit this expression (Fig. 6D). Similarly, coincubation with U0126 but not SB203580 (or curcumin) also completely inhibited ET-1–induced increases in ET-1 promoter activity (Fig. 6E). These data suggest that the activation of ERK is essential for the maximal ET-1 gene expression induced by ET-1.

To further identify the signaling pathway involved, we also cotransfected cardiac fibroblasts with various dominant-negative mutants, Ras (RasN17), Raf-1 (Raf301), or a catalytically inactive mutant of mERK, all of which are associated with the Ras/Raf/ERK pathway. Cells cotransfected with RasN17, Raf301, or mERK resulted in a significant inhibition of ET-1–induced ET-1 promoter activity compared with the control (Fig. 6F). Cardiac fibroblasts cotransfected with a dominant-positive mutant of Ras (RasL61) or MEK1 consistently increased ET-1 promoter activity to a significant extent. These results further suggest that the Ras/Raf/ERK signaling pathway plays an important role in ET-1–induced ET-1 gene expression in cardiac fibroblasts.

Identification of ET-1–Responsive Elements in the ET-1 Promoter. The ET-1 promoter contained AP-1 and GATA sites. We dissected the ET-1–responsive elements of the ET-1 promoter in cardiac fibroblasts by using a series of truncation mutants (Cheng et al., 2001). As shown in Fig. 7, ET-1 stimulation for 24 h significantly increased CAT activity for −700CAT and −204CAT, both of which contained

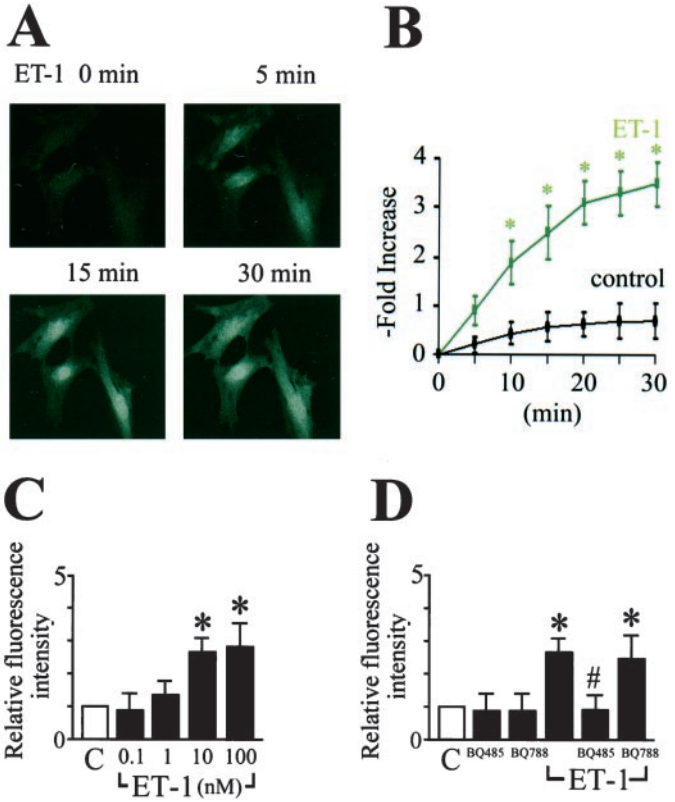

Fig. 3. ET-1–related increases in the intracellular ROS in cardiac fibroblasts. In B–D, data are presented as mean ± S.E.M. *, $p < 0.05$ versus control. #, $p < 0.05$ versus ET-1 alone. A, cardiac fibroblasts treated with ET-1 have increased intracellular ROS levels, as revealed by the fluorescent intensities of DCF. Cardiac fibroblasts were preincubated with DCF-DA for 30 min and then treated with ET-1 for the indicated times. Fluorescence intensity was analyzed with laser-confocal microscopy. B, time course of ET-1–induced ROS generation in cardiac fibroblasts, as revealed by DCF fluorescence. About 20 cells were used for the determination at each point. C, ET-1 induced ROS generation in a dose-dependent manner. D, effect of BQ485 (100 nM) or BQ788 (100 nM) on ET-1 (10 nM) increases in the ROS levels in cardiac fibroblasts. Densitometric results from five experiments are shown. Densitometric analysis was performed on at least 20 cells.

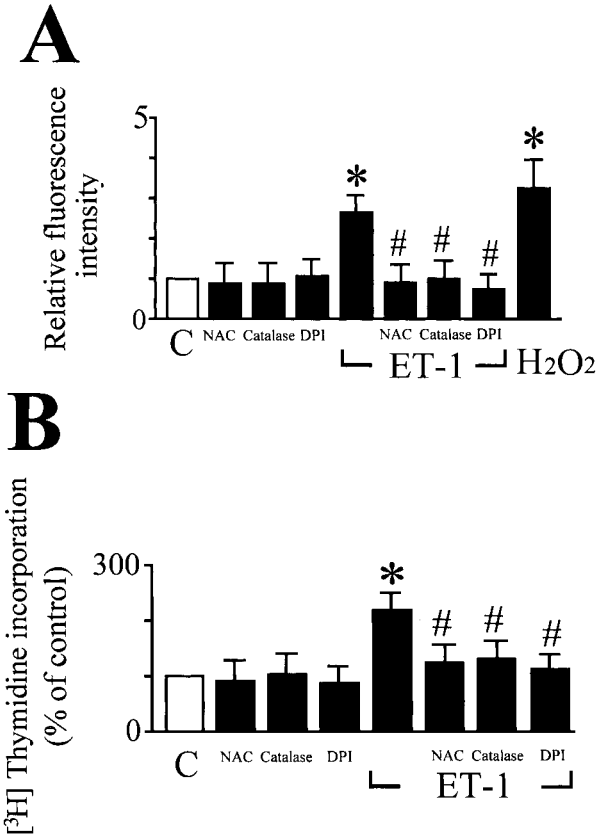

Fig. 4. ROS involvement in ET-1–induced fibroblast proliferation. All data are shown as the means ± S.E.M. of nine determinations in three cell preparations. The results are shown as the mean ± S.E.M. *, $p < 0.05$ versus control. #, $p < 0.05$ versus ET-1 alone. A, effect of antioxidants on ET-1–related increases in ROS. Cardiac fibroblasts were loaded with DCF-DA for 30 min and stimulated with ET-1. Intracellular ROS levels were measured at 30 min by using laser-confocal microscopy. ET-1 (10 nM) increased ROS levels, which were abolished by catalase (350 U/ml), NAC (10 mM), and DPI (1 μM). Cells treated with H_2O_2 (25 μM) are shown as positive controls (column 9). Densitometric results from five experiments are shown. Densitometric analysis was performed on at least 20 cells. B, effect of antioxidants on ET-1–induced DNA synthesis in cardiac fibroblasts. Cells were preincubated with catalase (350 U/ml), NAC (10 mM), or DPI (1 μM) for 30 min after incubation with 10 nM ET-1 for 24 h. Increases in [^{3}H]thymidine incorporation are expressed relative to the ^{3}H content (100%) in their respective controls (C).

multiple transcription factor binding sites, including sites for GATA (bp −136 to −131) and AP-1 (bp −108 to −102). However, further truncation of the GATA and subsequent AP-1 site from the 5′ end has resulted in the loss of the ET-1 promoter activity, as shown in −129CAT and −98CAT (Fig. 7A). In cells transfected with reporter construct −204CAT containing both GATA and AP-1 sites with a 2-bp mutation in the AP-1 site, the ET-1–induced ET-1 promoter activity was also completely abolished. The basal promoter activity also decreased, compared with the control, with the loss of either site (Fig. 7B). Therefore, the deletion of both GATA and AP-1 sites resulted in a significant decrease in basal promoter activity, suggesting that these two sites are necessary for ET-1–stimulated transcription of the ET-1 gene.

Using the electrophoretic mobility shift assay, AP-1 binding to the consensus AP-1 binding sequence was assayed in cells treated with ET-1 for 6 h (Fig. 7C). Pretreating cells with antioxidants NAC or catalase attenuated the ET-1–stimulated AP-1 binding activity. In contrast, cells pretreated with H_2O_2 had enhanced AP-1 binding activity; this result indicated that the intracellular ROS level is involved in the modulation of ET-1–stimulated AP-1 activity. This binding seems to be specific to AP-1, because it was abolished by competition experiments with excess AP-1 oligonucleotides, as well as coincubation of nuclear proteins with *c-fos* antibody. These results clearly indicate that ROS mediate the transcriptional activity of AP-1 induced by ET-1. These results indicated that the AP-1 binding element was mainly responsible for the induction of ET-1 gene expression by ET-1 in cardiac fibroblasts.

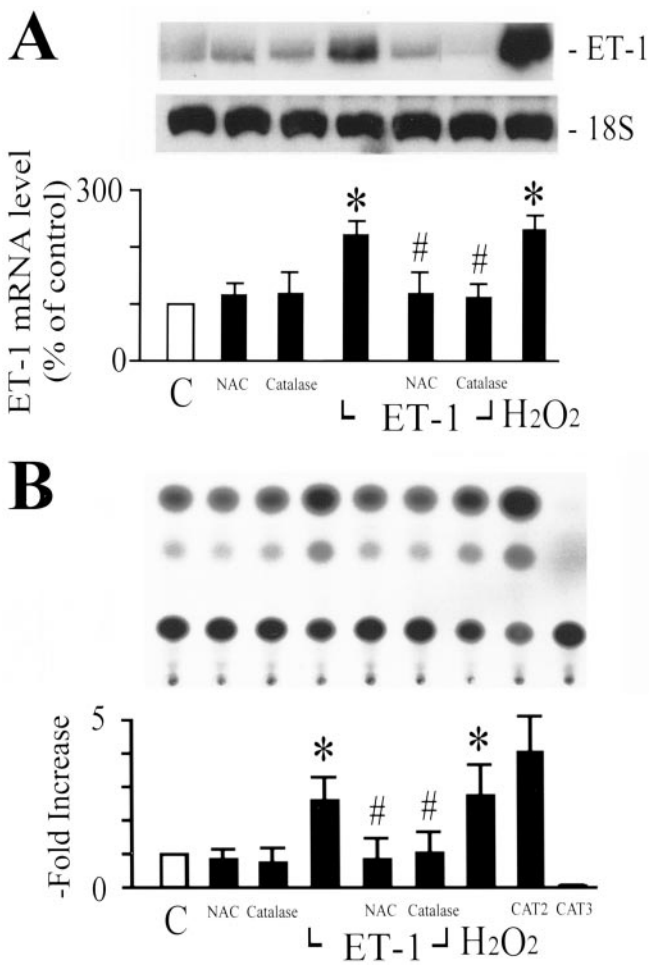

Fig. 5. ET-1–induced ET-1 gene expression mediated by ROS in cardiac fibroblasts. The results are shown as the mean ± S.E.M. ($n = 3$). *, $p < 0.05$ versus control. #, $p < 0.05$ versus ET-1 alone. A, effect of antioxidants on ET-1–induced ET-1 mRNA in cardiac fibroblasts. Cells were preincubated with catalase (350 U/ml) or NAC (10 mM) for 30 min after incubation with 10 nM ET-1 for 30 min. B, effect of antioxidants on ET-1–induced increases in ET-1 promoter activity in cardiac fibroblasts. Cells were preincubated with catalase (350 U/ml) or NAC (10 mM) for 30 min after incubation with 10 nM ET-1 for 24 h.

Discussion

Fibroblasts constitute the vast majority (>90%) of nonmyocyte cells in the heart (Eghbali, 1992). Cardiac fibroblasts increase the production of fibronectin and collagen when the heart is exposed to a variety of injuries, such as myocardial infarction, pressure overload, and myocarditis. It is believed that an increase in both the number of cardiac fibroblasts and the content of extracellular matrix proteins during cardiac remodeling are the major causes of cardiac dysfunction (Kim and Iwao, 2000). ET-1 is a potent stimulator of proliferation (Piacentini et al., 2000) and collagen synthesis (Rhaleb et al., 2001) in cultured cardiac fibroblasts. We characterized the proliferative response to ET-1 by using agents that implicated the ET_A receptor (BQ485). The ET-1–induced DNA synthesis in cardiac fibroblasts was inhibited by BQ485. Furthermore, the suppression of endogenous ET-1 synthesis by phosphoramidon (an ECE inhibitor) also inhibited the ET-1–induced DNA synthesis in cardiac fibroblasts. These findings suggested that ET-1–induced proliferation of cardiac fibroblasts is mediated by endogenous ET-1 production by cardiac fibroblasts in an autocrine/paracrine manner. We have demonstrated for the first time that phosphoramidon inhibits the stimulatory effect of ET-1 on cardiac fibroblast proliferation. ECE is an enzyme widely distributed in the lungs, kidneys, and heart, and it is known to have a role in degradation of big prepro-ET-1 (Takahashi et al., 1995). Sawamura et al. (1993) partially purified the ECE, which is sensitive to phosphoramidon and selective for the conversion of big ET-1 to ET-1 from the membrane fraction of porcine lung (Sawamura et al., 1993). Another study demonstrated that phosphoramidon blocked the vasoconstriction caused by big ET-1 in the vascular smooth muscle in vitro and in vivo (Fukuroda et al., 1990). These results suggest that phosphoramidon may inhibit hydrolysis of big ET-1. We cannot exclude the possibility that phosphoramidon modulates the degradation of not only ET-1 but also other peptides. A recent study showed that phosphoramidon decreased collagen synthesis in cardiac fibroblasts (Maki et al., 2000). We found that phosphoramidon also inhibits increased fibroblast proliferation caused by ET-1. Therefore, these results suggest that the inhibitory mechanism of ET-1–induced proliferation by phosphoramidon is mainly mediated by the decreased action of ET-1 secreted from cardiac fibroblasts.

ROS have been implicated in inflammatory processes such as fibrosis (Sorescu and Griendling, 2002). We previously reported that ROS can modulate ET-1–induced c-*fos* gene expression in cardiomyocytes (Cheng et al., 1999).

That result is consistent with our present findings and the known ability of antioxidant to antagonize the actions of ET-1 in cardiac fibroblasts. To better understand the role that ROS might have in influencing ET-1 action in cardiac fibroblasts, we characterized the induction of ET-1 gene and the activation of MAPKs by ET-1 in these cells, and we examined the effects of NAC and catalase on this pathway.

The MAPKs make up a family of serine-threonine kinases that includes ERK, JNK, and p38 MAPK. In this study, we found that ET-1 activates ERK, p38MAPK, and JNK in cardiac fibroblasts. The signaling system leading to the activation of MAPKs is subject to diverse and complex regulation. Results of several recent studies suggest that the balance of the oxidative and reductive potentials within the cell (cellular redox state) may substantially influence this pathway (Sano et al., 2001; Tanaka et al., 2001). The activation of ERK through such a signaling pathway has been implicated in other studies using cardiac fibroblasts (Sano et al., 2001), and it is consistent with

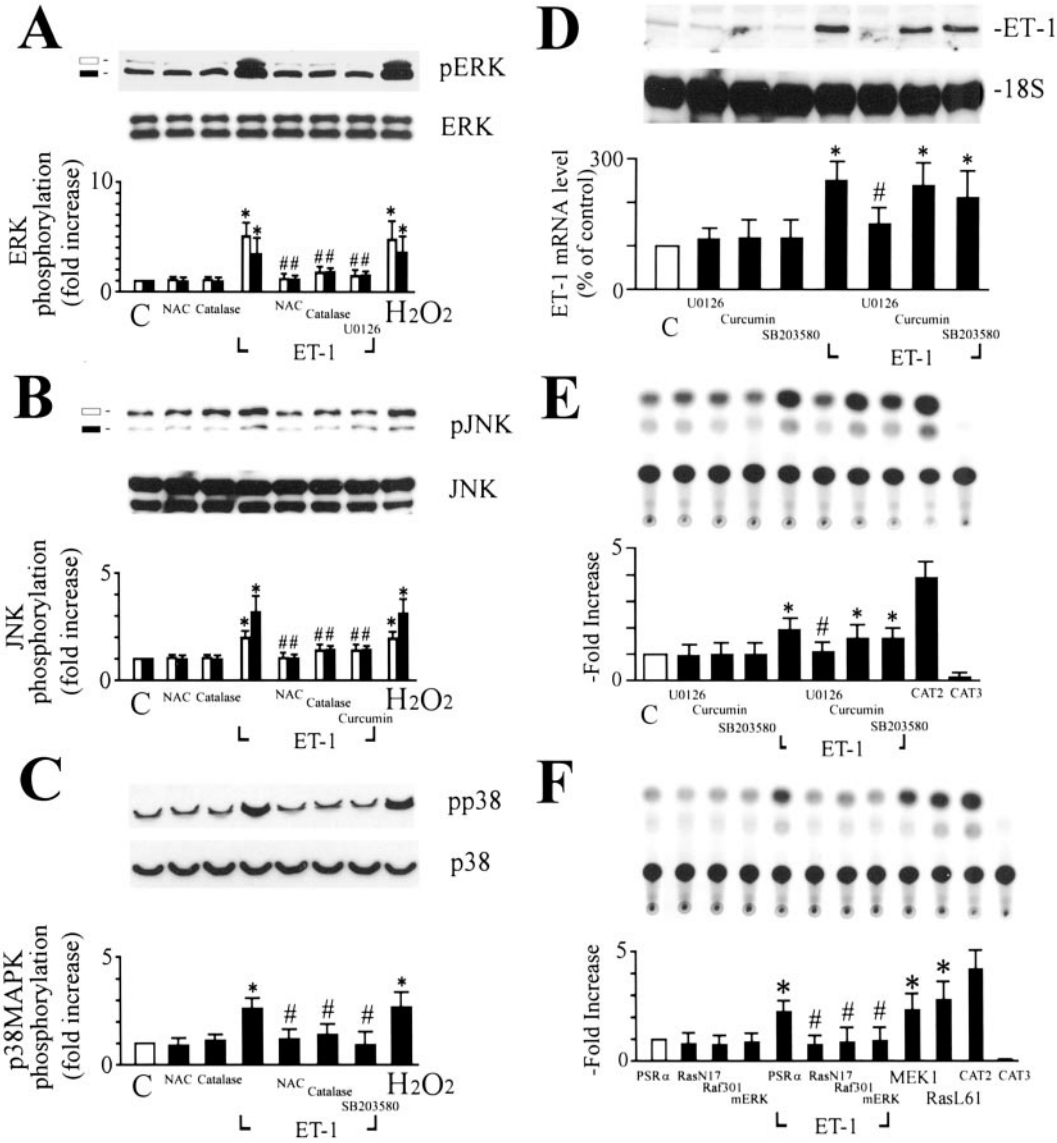

Fig. 6. ET-1–induced increases in ET-1 gene expression by means of ERK in a redox-sensitive manner. The results are shown as the mean ± S.E.M. ($n = 4–6$). *, $p < 0.05$ versus control. #, $p < 0.05$ versus ET-1 alone. A–C, ET-1–induced activation of ERK, JNK, and p38 MAPK was mediated by an ROS-sensitive pathway. Cells were preincubated with catalase (350 U/ml) or NAC (10 mM) for 30 min and stimulated with ET-1 (10 nM) for 30 min. U0126, a MEK1 inhibitor; SB203580, a p38 inhibitor; or curcumin, a JNK inhibitor, was used to block ET-1–induced MAPK phosphorylation. Phosphorylation of ERK, JNK, or p38 MAPK was detected by means of Western blotting with anti-phospho-ERK, phospho-JNK, and phospho-p38 MAPK antibodies. Both catalase and NAC inhibited ET-1–induced activation of ERK, JNK, or p38 MAPK. Phosphorylation of ERK, JNK, or p38 MAPK was detected, and densitometric analyses were performed. D, ET-1–induced ET-1 mRNA was attenuated by U0126 in cardiac fibroblasts. Cardiac fibroblasts were stimulated with ET-1 (10 nM) in the presence of U0126 (10 μM), SB203580 (10 μM), or curcumin (10 μM). Total RNA was isolated at 30 min. E, ET-1–induced increases in ET-1 promoter activity were inhibited by U0126 in cardiac fibroblasts. Cardiac fibroblasts were stimulated with ET-1 (10 nM) in the presence of U0126 (10 μM), SB203580 (10 μM) or curcumin (10 μM). CAT activity was assayed after 24 h. F, ET-1–induced increases in ET-1 promoter activity via the Ras/Raf/ERK pathway in cardiac fibroblasts. Cells were transfected with pSRα empty vector (5 μg) or an expression plasmid encoding the dominant negative mutant mERK, Raf301, or RasN17 (5 μg) was cotransfected with 15 μg of a plasmid for ET-1 and CAT. Cells cotransfected with an expression plasmid encoding MEK1 (5 μg) or RasL61 (5 μg) were used as positive controls.

the present finding that divergent pathways are involved in ET-1 action.

We characterized ET-1–induced ET-1 gene expression by using agent that implicated the requirement for ERK activation (U0126). Upstream activators of the MAPK pathways include small GTPases of the Ras family, and downstream effectors include transcription factors and other kinases (Kyriakis and Avruch, 2001). ERK activity has been extensively studied in rat cardiac fibroblasts (Bogoyevitch et al., 1994; Thorburn et al., 1994), and the stimulation of fibroblasts with angiotensin II or platelet-derived growth factor is known to stimulate MAPK activity, fibroblast proliferation, and formation of extracellular matrix proteins (Zou et al., 1998; Moriguchi et al., 1999). ET-1 has been shown to activate ERK in cardiac myocytes (Clerk and Sugden, 1999) and rat cardiac fibroblasts (Fig. 6A). The treatment of cardiac fibroblasts with ET-1 led to ERK activation, and this effect was ameliorated by pretreatment with antioxidants. This observation suggests that the ERK pathway may mediate proliferation and ET-1 gene expression in cultured fibroblasts. The inhibition of p38MAPK and JNK had no effect on ET-1–stimulated ET-1 expression. Other signaling pathways known to be activated by ET-1 include those involving protein kinase C, phosphatidylinositol-3 kinase, protein kinase B, and nonreceptor tyrosine kinase Src (Clerk and Sugden, 1999). Thus, ET-1–stimulated proliferation and ET-1 gene expression may possibly involve one or more of these other kinases.

Results of the cotransfection experiments with dominant-negative Ras, Raf, and ERK suggested that the Ras-Raf-ERK

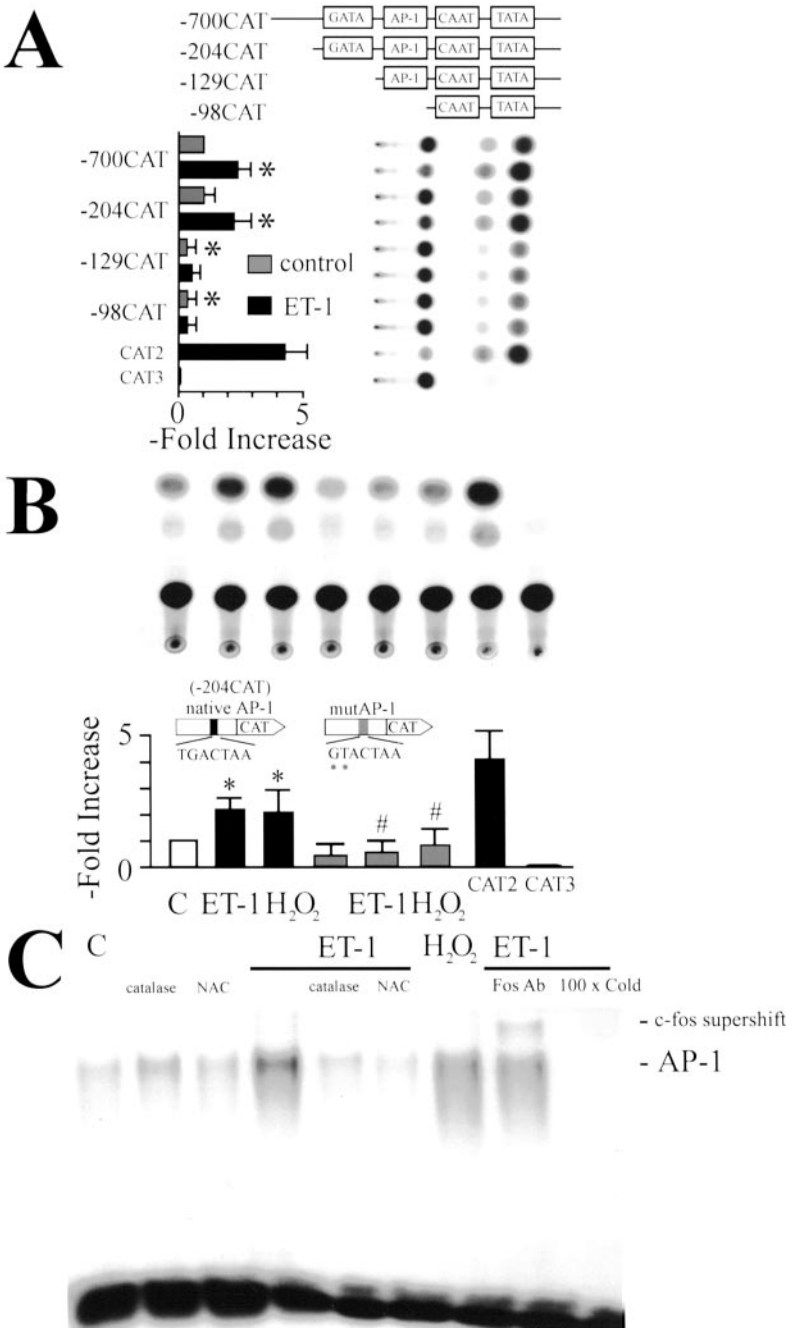

Fig. 7. Identification of ET-1–responsive elements in the ET-1 promoter. In A, and B, the results are shown as the mean ± S.E.M. ($n = 3$). *, $p < 0.05$ versus control. #, $p < 0.05$ versus ET-1 alone. A, a series of deletion mutants of ET-1 promoter gene plasmids were cotransfected into cardiac fibroblasts. Transfected cells were stimulated with ET-1 (10 nM) for 24 h, and CAT activities were measured. B, wild-type (204 bp) or AP-1 mutants of the plasmids for the ET-1 promoter and CAT were cotransfected into cardiac fibroblasts. Cells were stimulated with ET-1 (10 nM) for 24 h. The mutation of AP-1 strongly abolished the responsiveness to ET-1. C, NAC or catalase attenuated the ET-1–stimulated AP-1 binding activity in cardiac fibroblasts. This binding activity was measured by using the electrophoretic mobility shift assay. Cells were preincubated with catalase (350 U/ml) or NAC (10 mM) for 30 min after incubation with 10 nM ET-1 for 6 h. *C-Fos Ab* denotes the anti-c-Fos antibody. *100 × Cold* denotes a 100-fold molar excess of unlabeled oligonucleotide relative to the radiolabeled probe; this was added to the binding assay for competition with the unlabeled oligonucleotide. The experiment was repeated two times with reproducible results.

pathway is involved in the transcriptional activation of the ET-1 by ET-1 because all inhibited the ET-1 promoter activity induced by ET-1 (Fig. 6F). In comparison, the empty vectors had no effect. The ET-1 promoter contains the AP-1 element (Lee et al., 1991), which could be activated by ROS. Truncation and mutational analysis of the ET-1 gene promoter showed that AP-1 was an important *cis*-element for ET-1–induced expression of the ET-1 gene. AP-1 is one of the best-characterized transcription factors known to be influenced by the cellular oxidation-reduction (redox) state (Wung et al., 1997). Evidence suggests that ROS serve as messengers in AP-1 activation (Wung et al., 1997). The AP-1 element binds the protooncogene products jun and fos (Paul et al., 1995), whereas it is well known that ROS activates the genes for *jun* and *fos* (Guyton et al., 1996; Cheng et al., 1999). Because ET-1 induced intracellular ROS production, we investigated whether ET-1–induced ROS activated AP-1 in cardiac fibroblasts. Using gel mobility shift assays, we demonstrated that ET-1 increased binding of AP-1 to the AP-1 site of the ET-1 gene promoter in a redox-sensitive manner. These findings indicated that the activation of AP-1 pathway in response to ET-1 is necessary for ET-1 induction. The primary target in AP-1 activation by ROS seems to be the activation of MAPK pathways and the induction of the c-*fos* and *c-jun* family of protooncogenes; these ultimately activate AP-1 through phosphorylation.

In conclusion, ET-1–related increases in intracellular ROS levels via the $ET\text{-}1_A$ receptor, and these ROS were at least partly involved in ET-1–induced proliferation and increased activation of ERK, JNK, and p38 MAPK pathways in cardiac fibroblasts. Moreover, we showed that ERK is crucial in ET-1–stimulated expression of the ET-1 gene. ROS-MAPK (ERK)–mediated AP-1–dependent transcription plays an important role in ET-1–induced proliferation and ET-1 gene expression in cardiac fibroblasts. The mechanism by which ROS activates various signaling pathways remains undetermined, and it should be clarified. The fact that antioxidants inhibit DNA synthesis in cardiac fibroblasts suggests that ROS may be an important endogenous regulator of fibroblast proliferation in the heart. The cardioprotective effect of ET_A antagonists, ECE inhibitors, or antioxidants may occur by limiting fibroblast proliferation; therefore, they might reduce fibrosis in patients with hypertension.

Acknowledgments

We thank Dr. Xin Chen (Division of Biotechnology and Pharmaceutical Research, National Health Research Institute, Taiwan) for the critical reading of the manuscript and the insightful comments.

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