Cell Type–Specific Angiotensin II–Evoked Signal Transduction Pathways

Critical Roles of Gβγ Subunit, Src Family, and Ras in Cardiac Fibroblasts

Yunzeng Zou, Issei Komuro, Tsutomu Yamazaki, Sumiyo Kudoh, Ryuichi Aikawa, Weidong Zhu, Ichiro Shiojima, Yukio Hiroi, Kazuyuki Tobe, Takashi Kadowaki, Yoshio Yazaki

Abstract—Angiotensin II (Ang II) induces hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. To determine the molecular mechanism by which Ang II displayed different effects on cardiac myocytes and fibroblasts, we examined signal transduction pathways leading to activation of extracellular signal–regulated kinases (ERKs). Ang II–induced ERK activation was abolished by pretreatment with pertussis toxin and by overexpression of the Gβγ subunit–binding domain of the β-adrenergic receptor kinase 1 in cardiac fibroblasts but not in cardiac myocytes. Inhibition of protein kinase C strongly inhibited activation of ERKs by Ang II in cardiac myocytes, whereas inhibitors of tyrosine kinases but not of protein kinase C abolished Ang II–induced ERK activation in cardiac fibroblasts. Overexpression of C-terminal Src kinase (Csk), which inactivates Src family tyrosine kinases, suppressed the activation of transfected ERK in cardiac fibroblasts. Ang II rapidly induced phosphorylation of Shc and association of Shc with Grb2. Cotransfection of the dominant-negative mutant of Ras or Raf-1 kinase abolished Ang II–induced ERK activation in cardiac fibroblasts. Overexpression of Csk or the dominant-negative mutant of Ras had no effects on Ang II–induced ERK activation in cardiac myocytes. These findings suggest that Ang II–evoked signal transduction pathways differ among cell types. In cardiac fibroblasts, Ang II activates ERKs through a pathway including the Gβγ subunit of G protein, tyrosine kinases including Src family tyrosine kinases, Shc, Grb2, Ras, and Raf-1 kinase, whereas Gq and protein kinase C are important in cardiac myocytes. (Circ Res. 1998;82:337–345.)

Key Words: angiotensin II ■ cardiac fibroblast ■ extracellular signal–regulated kinase ■ G protein ■ Src

The heart is composed of cardiac myocytes and several kinds of nonmyocytes, including fibroblasts, smooth muscle cells, and endothelial cells. Nonmyocytes, most of which are fibroblasts, constitute two thirds of the cell population of the heart. Although cardiac myocytes lose their proliferative ability soon after birth, nonmyocytes can still proliferate even in the adult heart. When the heart is exposed to various stresses, such as hemodynamic overload and myocardial infarction, cardiac myocytes increase in size, whereas cardiac fibroblasts increase in number and produce ECM proteins, such as collagens and fibronectins. The abnormal proliferation of cardiac fibroblasts with excessive accumulation of ECM proteins is one of the features of left ventricular remodeling, which leads ultimately to cardiac dysfunction. To elucidate the molecular mechanism of proliferation of cardiac fibroblasts is thus very important.

Ang II is a potent inducer of the cardiac hypertrophy and remodeling following sustained hypertension or myocardial infarction. Many laboratories, including our own, have reported that Ang II induces cardiomyocyte hypertrophy in vitro and in vivo and that it also plays a critical role in mechanical stress–induced cardiac hypertrophy. In cardiac fibroblasts, Ang II has also been demonstrated to stimulate proliferation and induce the expression of genes for collagens, fibronectin, and integrins. Ang II exerts its hypertrophic and hyperplastic effects by activating a number of intracellular signal transduction pathways through AT1Rs. Many studies have indicated that Ang II stimulates phosphatidylinositol–specific PLC through a heterotrimeric guanine nucleotide–binding protein (G protein). The activation of PLC induces the generation of DG and IP3, which, respectively, induce activation of PKC and the release of Ca2+ from intracellular stores in many cell types. It has recently been reported that Ang II also activates tyrosine kinases, including Src family tyrosine kinases, and Ras in cardiac myocytes, cardiac fibroblasts, and smooth muscle cells. Many lines of evidence have suggested that Ras, Src family tyrosine kinases, and Gβγ subunit act downstream of AT1R to mediate Ang II–evoked signal transduction. The roles of other signal transduction pathways such as the phosphatidylinositol 3-kinase–Akt pathway, mitogen-activated protein kinase (MAPK) pathway, and extracellular signal–regulated kinase (ERK) pathway have also been studied extensively in many cell types. ERKs are a key component of the MAPK cascade, which is involved in cell proliferation, differentiation, and apoptosis. Many studies have suggested that ERKs mediate Ang II–induced signaling in cardiac myocytes and fibroblasts. In this study, we examined which signal transduction pathway is mediated by ERK in cardiac fibroblasts and cardiac myocytes by using various signaling modifiers.
Angiotensin II–Evoked Signal in Cardiac Fibroblast

Selected Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>βARK1</td>
<td>β-adrenergic receptor kinase 1</td>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>Anti-HA</td>
<td>anti-hemagglutinin</td>
</tr>
<tr>
<td>AT1R, AT2R</td>
<td>Ang II type-1 and -2 receptor</td>
</tr>
<tr>
<td>Csk</td>
<td>C-terminal Src kinase</td>
</tr>
<tr>
<td>D.N.Raf-1, D.N.Ras</td>
<td>dominant-negative mutants of Raf-1 and Ras</td>
</tr>
<tr>
<td>DG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal–regulated kinase</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol trisphosphate</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase/ERK kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol 13-acetate</td>
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Evidence has suggested that ERKs function as integrators for mitogenic and differentiation signals originating from several distinct classes of cell surface receptors, such as receptor tyrosine kinases and G protein–coupled receptors.26–28 In cardiac myocytes, activation of ERKs is also required for phenylephrine-induced expression of specific genes, such as the atrial natriuretic factor, c-fos, and myosin light chain 2 genes.29 Although ERK activation is not sufficient for full promotion of cardiac hypertrophy,30 a recent study using an antisense oligodeoxynucleotide has shown that ERKs are necessary for phenylephrine-induced sarcomerogenesis and increase in cardiac cell size.31 We have recently demonstrated that in cardiac myocytes, PKC, but not tyrosine kinases or Ras, plays an essential role in Ang II–induced activation of ERKs.32 On the other hand, in smooth muscle cells, Ang II has been shown to activate ERKs through Src/Ras-dependent pathways.33 These observations suggest that Ang II–induced signal transduction pathways may differ among cell types.

To clarify the molecular mechanism of proliferation of cardiac fibroblasts, we determined the Ang II–evoked signal transduction pathways leading to activation of ERKs, which are required for DNA synthesis in cardiac fibroblasts. In the present study, we show that in cardiac fibroblasts, Ang II activates ERKs through a pathway consisting of the G\textsubscript{i}G\textsubscript{ir} subunit of the G protein, tyrosine kinases including Src family tyrosine kinases, Shc, Grb2, Ras, and Raf-1, which differs considerably from the pathway in cardiac myocytes.32

Materials and Methods

Materials

\( ^{[32P]}\text{ATP} \) was purchased from Du Pont-New England Nuclear Co. DMEM, FBS, tyrphostin (A25), and genistein were from GIBCO-BRL Co. Pertussis toxin was from List Biological Laboratories, Inc. Calphostin C was from BIOMOL. Anti-HA polyclonal antibody was from Mitsubishi Biochemical Laboratories. Anti-Shc and anti-Grb2 polyclonal antibodies and anti-phosphotyrosine monoclonal antibody (PY20) were from Santa Cruz Biotechnology, Inc. Anti-rabbit IgG conjugated with horseradish peroxidase and the enhanced chemiluminescence reaction system were from Amersham. CV-11974, an active form of TCV-116, was a gift from Takeda Chemical Industries, Ltd (Osaka, Japan). PD123319 was a gift from Parke-Davis (Ann Arbor, Mich). PD98059 was purchased from New England Biolabs, Inc. Ang II, TPA, MBP, and other reagents were from Sigma Chemical Co.

cDNA Plasmids

HA-tagged ERK2 (HA-ERK2) in the SV40 promoter was a kind gift from M. Karin, School of Medicine, University of California at San Diego, La Jolla.35 The cDNA encoding carboxyl-terminal β subunit of the G\textsubscript{i}G\textsubscript{ir} subunit–binding domain of the bovine βARK1 residues Gly–495 to Leu–689 was provided by K. Tsuchiya, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC.35 The dominant-negative mutant (AN-17) of Ras (D.N.Ras) and the dominant-negative mutant (Ala–375) of Raf-1 (D.N.Raf-1), both of which are driven by the cytomegalovirus promoter, were provided by Y. Takai, Kobe (Japan) University School of Medicine,36 and T. Kadowaki, Faculty of Medicine, University of Tokyo (Japan),36 respectively. Wild-type Csk and the kinase-negative mutant of Csk (Csk\textsuperscript{−}−) were provided by H. Sabe, Rockefeller University, New York, NY. All plasmid DNA was prepared using QIAGEN plasmid DNA preparation kits.

Cell Culture

Cardiac myocytes and fibroblasts from ventricles of 1-day-old Wistar rats were isolated as previously described.38 To selectively enrich cardiac myocytes, dissociated cells were plated onto 100-mm culture dishes for 30 minutes, which permitted preferential attachment of fibroblasts to the bottom of the dish. Nonadherent cells were plated at a cell density of \( 1 \times 10^5 \) cells/cm\(^2\) on 35-mm culture dishes with 2 mL of culture medium (DMEM with 10% FBS) as a cardiomyocyte-rich culture. Cardiac fibroblasts were obtained from adherent cells on the preplating dish and split twice before use. Cells were grown to subconfluence in DMEM +10% FBS, and the culture medium was changed to serum-free DMEM at 48 hours before treatment.

\( ^{[3H]}\text{Thymidine Incorporation} \)

DNA synthesis was assessed by measuring \( ^{[3H]}\)thymidine incorporation as previously described.39 Cardiac fibroblasts were serum-deprived for 2 days and then incubated for 24 hours with Ang II or vehicle. \( ^{[3H]}\)Thymidine (1.25 μCi/mL) was added 2 hours before the harvest. Plates were then placed on ice, quickly washed three times with 1 mL of ice-cold PBS, incubated 10 minutes with 1 mL of 10% trichloroacetic acid, and washed twice with 1 mL of 10% trichloroacetic acid and three times with 1 mL of 95% ethanol. Precipitates were solubilized for 1.5 hours in 800 μL of 0.2N NaOH and neutralized, and radioactivity was measured by liquid scintillation spectrometry.

Transfection

Twenty-four hours after plating the cardiac myocytes or fibroblasts on 35-mm culture dishes, DNA was transfected by the calcium phosphate method as previously described.\( ^{32} \) For each dish, 2.5 μg of HA–ERK2 plasmid DNA was transfected with or without 7.5 μg of other relevant plasmids such as βARK1, Csk, D.N.Ras, or D.N.Raf-1. After 20 hours of transfection, the culture medium was removed, and cells were washed with PBS twice and then maintained in serum-free DMEM for 48 hours before stimulation with Ang II. The transfection efficiency of each experiment was \( \approx 1% \) in cardiac myocytes and 5% to 10% in fibroblasts as assessed by LacZ staining after transfection of a LacZ-containing expression plasmid.

Assay of ERK Activity

ERK activities were measured using MBP-containing gel as previously described.40 In brief, cell lysates were electrophoresed on an SDS-polyacrylamide gel containing 0.5 mg/mL MBP. ERKs in the gel were denatured in guanidine HCl and renatured in Tris–HCl containing Trition X–100 and 2-mercaptoethanol. Phosphorylation activities of ERKs were assessed by incubating the gel with \( ^{[32P]}\text{ATP} \). After incubation, the gel was washed extensively and subjected to autoradiography.
Kinase Assay of Transfected HA-Tagged ERK (MBP Assay)
The activity of transfected HA-tagged ERK2 was assayed as previously described. In brief, after transfection of HA-tagged ERK2, cell lysates were incubated with anti-HA polyclonal antibody (1:1000) for 1 hour at 4°C. After incubation, the immunocomplex was precipitated using protein A-Sepharose beads, washed, resuspended, and incubated with MBP and [γ-32P]ATP for 10 minutes. The sample was subjected to SDS-PAGE, and the phosphorylated MBP band was visualized by autoradiography.

Phosphorylation of Shc and Its Association With Grb2
Tyrosine phosphorylation of Shc was examined by Western blot analysis. After treatment, cell lysates were incubated with anti-Shc polyclonal antibody (1:1000) for 1 hour at 4°C. After precipitation with protein A-Sepharose beads, the immunocomplex was subjected to SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Association of Shc with Grb2 was examined by incubating cell lysates with anti-Grb2 antibody (1:1000) for 1 hour at 4°C. After blocking with 30% nonfat dry milk, immunoblots were incubated with anti-phosphotyrosine monoclonal antibody (PY20) (1:1000) or anti-Shc polyclonal antibody (1:1000) for 1 hour at 37°C. After it was washed, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG, and immunoreactivity was detected using the enhanced chemiluminescence system according to the manufacturer’s direction.

Statistics
Statistical comparison of the control group with treated groups was carried out using the paired-sample t test with P values corrected by the Bonferroni method. The accepted level of significance was P < 0.05.

Results
Activation of ERKs Is Required for Ang II–Induced DNA Synthesis in Cardiac Fibroblasts Through AT1R
When 10–6 mol/L Ang II was added to the culture medium of cardiac fibroblasts obtained from neonatal rats, thymidine incorporation into cells was increased by ≈40% (Fig 1). This Ang II–induced increase in thymidine incorporation was completely inhibited by an AT1R antagonist, CV-11974, but not by an AT2R antagonist, PD123319 (Fig 1), consistent with the findings of previous studies. A growing body of evidence has suggested that ERKs play key roles in transducing cytoplasmic signals to the nucleus and in proliferation and differentiation of many types of cells. Therefore, we examined whether ERKs are required for Ang II–induced DNA synthesis in cardiac fibroblasts with the use of a MEK1 inhibitor, PD98059, which specifically inhibits activation of ERKs. When 5×10–5 mol/L PD98059 was added to the culture medium at 30 minutes before the addition of Ang II, the Ang II–induced increase in thymidine incorporation was completely suppressed (Fig 1), suggesting that MEK and thus probably ERKs play a pivotal role in Ang II–induced DNA synthesis in cardiac fibroblasts.

Ang II Activates ERKs Through AT1R in Cardiac Fibroblasts
To determine the molecular mechanism of Ang II–induced proliferation of cardiac fibroblasts, we examined the signal transduction pathways leading to the activation of ERKs. We first examined whether Ang II activates ERKs in cardiac fibroblasts. When 10–6 mol/L Ang II was added to the culture medium, ERKs were strongly activated at 2 minutes, and activity reached a peak at 8 minutes (Fig 2A and 2B). Activity decreased sharply thereafter and returned to unstimulated levels by 30 minutes after stimulation with Ang II. The time course of ERK activation by Ang II in cardiac fibroblasts was almost the same as that previously reported for cardiac myocytes. It has been reported that two subtypes of Ang II receptors, AT1R and AT2R, are present in cardiac fibroblasts of neonatal rats and that both receptors are involved in proliferative responses to Ang II. To determine which receptor is involved in Ang II–induced ERK activation in cardiac fibroblasts, we stimulated cardiac fibroblasts with Ang II (10–6 mol/L) after pretreatment with CV-11974 (10–6 mol/L) or PD123319 (10–8 mol/L) for 30 minutes. As was the case for DNA synthesis, Ang II–induced ERK activation was completely suppressed by pretreatment with CV-11974 but not by that with PD123319 (Fig 2C), suggesting that Ang II–induced ERK activation in cardiac fibroblasts is mediated through AT1R.

G Protein and Gbg, Subunit Are Required for Ang II–Induced ERK Activation in Cardiac Fibroblasts
AT1R is a prototypical G protein–coupled receptor with seven transmembrane–spanning regions. Which G proteins are coupled to AT1R depends on cell type. It has been reported that Ga and Gbg subunits are coupled to AT1R in cardiac myocytes and vascular smooth muscle cells and that Ga couples to AT1R in hepatocytes. To examine whether Ang II–induced ERK activation occurs via G protein, we preincubated cardiac fibroblasts and myocytes with 100 ng/mL pertussis toxin for 24 hours and treated them with 10–6 mol/L Ang II. As shown in Fig 3, the activity of ERK2 was strongly suppressed by pretreatment with pertussis toxin, suggesting that Gbg subunits are required for Ang II–induced ERK activation.
hours and then treated them with $10^{-6}$ mol/L Ang II for 8 minutes. Activation of ERKs by Ang II was completely suppressed by pretreatment with pertussis toxin in cardiac fibroblasts (Fig 3A) but not in cardiac myocytes (Fig 3B). Pertussis toxin also suppressed Ang II–induced thymidine incorporation into cardiac fibroblasts (Fig 1). These findings suggest that pertussis toxin–sensitive Gi protein mediates Ang II–induced activation of ERKs and an increase in DNA synthesis in cardiac fibroblasts, whereas in cardiac myocytes, a pertussis toxin–insensitive G protein, possibly Gq protein, is involved in Ang II–induced ERK activation.

It has recently been reported that stimulation of Gi protein–coupled receptors activates ERKs by the $\beta y$ subunit complex (G$_{\beta y}$ subunit) but not by the $\alpha$ subunit.34–36 The carboxyl-terminal 195 amino acids of $\beta$ARK1 (Gly–495 to Leu–689), which include pleckstrin homology domains, have been reported to bind G$_{\beta y}$ subunit and inhibit G$_{\beta y}$ subunit–dependent activation of a wide variety of cell regulatory processes.37–40 We therefore examined the role of the G$_{\beta y}$ subunit in Ang II–induced ERK activation by overexpressing a minigene construct encoding $\beta$ARK$_{495–689}$ polypeptides in cultured cardiac fibroblasts (Fig 4A) and cardiac myocytes (Fig 4B) to almost the same extent. Although overexpression of a vector alone did not affect Ang II–induced ERK2 activation in either cell type (data not shown), expression of $\beta$ARK$_{495–689}$ polypeptide abolished Ang II–induced activation of ERK2 in cardiac fibroblasts (Fig 4A) but not in cardiac myocytes (Fig 4B). These findings suggest that Ang II activates ERKs via the G$_{\beta y}$ subunit–dependent pathway in cardiac fibroblasts but that in cardiac myocytes, Ang II–induced activation of ERKs is independent of the G$_{\beta y}$ subunit.

**Ang II–Induced Activation of ERKs Requires Tyrosine Kinases but Not PKC in Cardiac Fibroblasts**

We have recently demonstrated that Ang II activates ERKs through PKC–dependent pathways in cultured cardiac myocytes.32 We therefore examined the role of PKC in Ang II–induced ERK activation in cardiac fibroblasts. When PKC was downregulated by incubation with $10^{-7}$ mol/L TPA for 24 hours, readdition of $10^{-7}$ mol/L TPA did not activate ERKs in cardiac fibroblasts (data not shown). With the same pretreatment, however, Ang II increased the activity of the transfected ERK2 in both cardiac fibroblasts (Fig 5A) and cardiac myocytes (Fig 5B) to almost the same extent. Although overexpression of a vector alone did not affect Ang II–induced ERK2 activation in either cell type (data not shown), expression of $\beta$ARK$_{495–689}$ polypeptide abolished Ang II–induced activation of ERK2 in cardiac fibroblasts (Fig 5A) but not in cardiac myocytes (Fig 5B). These findings suggest that Ang II activates ERKs via the G$_{\beta y}$ subunit–dependent pathway in cardiac fibroblasts but that in cardiac myocytes, Ang II–induced activation of ERKs is independent of the G$_{\beta y}$ subunit.

**Figure 2.** Time course of Ang II–induced ERK activation in cardiac fibroblasts. A, Cultured cardiac fibroblasts were exposed to $10^{-6}$ mol/L Ang II for various periods of time. Activities of ERKs were assayed by the “in-gel” method. Cell lysates were electrophoresed on an SDS-polyacrylamide gel containing 0.5 mg/mL MBP. ERKs in the gel were denatured in guanidine HCl and renatured in Tris–HCl containing Triton X-100 and 2-mercaptoethanol. Phosphorylation activities of ERKs were assayed by incubating the gel with [$\gamma$–$^{32}$P]ATP. After incubation, the gel was washed extensively and subjected to autoradiography. A representative autoradiogram is shown. B, Relative kinase activities were determined by scanning each band with a densitometer. Results are presented as means±SE of four independent experiments. Activities of ERK1 (solid bar) and ERK2 (hatched bar) are expressed relative to those of ERK1 obtained in unstimulated control (1). C, Cardiac fibroblasts were pretreated with either AT1R antagonist (10^{-6} mol/L CV-11974) or AT2R antagonist (10^{-6} mol/L PD123319) for 30 minutes and stimulated with $10^{-6}$ mol/L Ang II for 8 minutes. Activities of ERKs were assayed by the “in-gel” method as described above.

**Figure 3.** The effect of pertussis toxin on Ang II–induced ERK activation in cardiac fibroblasts and myocytes. Cardiac fibroblasts (A) or myocytes (B) were preincubated with 100 ng/mL pertussis toxin (islet-activating protein [IAP]) for 24 hours. The cells were then treated with $10^{-6}$ mol/L Ang II for 8 minutes. Activities of ERKs were assayed as described in Fig 2. Representative autoradiograms from three independent experiments are shown.

**Ang II–Induced Activation of ERKs Requires Tyrosine Kinases but Not PKC in Cardiac Fibroblasts**

We have recently demonstrated that Ang II activates ERKs through PKC–dependent pathways in cultured cardiac myocytes.32 We therefore examined the role of PKC in Ang II–induced ERK activation in cardiac fibroblasts. When PKC was downregulated by incubation with $10^{-7}$ mol/L TPA for 24 hours, readdition of $10^{-7}$ mol/L TPA did not activate ERKs in cardiac fibroblasts (data not shown). With the same pretreatment, however, Ang II strongly activated ERKs at levels almost the same as without pretreatment (Fig 5A). A highly specific PKC inhibitor, calphostin C ($10^{-7}$ mol/L), also had no effects on Ang II–induced ERK activation in cardiac fibroblasts (Fig 5A), suggesting that unlike cardiac myocytes, in cardiac
fibroblasts, Ang II activates ERKs through PKC-independent pathways.

Activation of receptor and nonreceptor tyrosine kinases activates ERKs in many types of cells, and it has recently been reported that Ang II activates tyrosine kinases in several cell types, including hepatocytes, smooth muscle cells, and cardiac fibroblasts. We therefore examined whether tyrosine kinases are involved in the activation of ERKs induced by Ang II in cardiac fibroblasts.

Cultured cardiac fibroblasts were pretreated with two chemically and mechanistically dissimilar tyrosine kinase inhibitors, tyrphostin A25 (5 × 10⁻⁵ mol/L), which is a potent inhibitor of the epidermal growth factor receptor–associated protein tyrosine kinase, and genistein (2 × 10⁻⁵ mol/L), for 30 minutes and then stimulated with Ang II (10⁻⁶ mol/L) for 8 minutes. Aliquots of the reaction mixture were subjected to SDS-PAGE, and the gel was washed, dried, and subjected to autoradiography. A representative autoradiogram from three independent experiments is shown.

Src Family Protein Tyrosine Kinases Are Required for Ang II–Induced Activation of ERKs in Cardiac Fibroblasts

It has been reported that Ang II activates Src family protein tyrosine kinases in cardiac myocytes and smooth muscle cells. We therefore examined the role of Src family protein tyrosine kinases in Ang II–induced ERK activation by overexpressing Csk in cardiac fibroblasts and myocytes. Csk has been reported to phosphorylate the tyrosine residue in the carboxyl terminus of Src family protein kinases and thereby inactivate their function. We transfected Csk or kinase domain–deleted Csk (Csk⁺) with HA-ERK2 into cells and determined the activity of transfected ERK2 after stimulation with Ang II. Although cotransfection of Csk⁺, which has no effect on Src family tyrosine kinases, did not affect Ang II–induced ERK activation (Fig 6A), overexpression of Csk completely inhibited activation of ERK2 by Ang II in cardiac fibroblasts (Fig 6B). In contrast, in cardiac myocytes, overexpression of Csk had no effects on Ang II–induced ERK activation (Fig 6B). Overexpression of Csk, however, partially suppressed insulin-induced ERK activation in cardiac myocytes (data not shown). These findings suggest that Src family tyrosine kinases play a pivotal role in Ang II–induced ERK activation in cardiac fibroblasts but not in cardiac myocytes.
Ras and Raf-1 Are Required for Ang II–Induced ERK Activation in Cardiac Fibroblasts

Association of Grb2 with the membrane fraction, resulting in activation of Ras.

We therefore next examined whether Ras is involved in Ang II–induced ERK activation in cardiac fibroblasts. Although Ras is not required for Ang II–induced ERK activation in cardiac myocytes,32 Ang II–induced activation of transfected ERK2 was completely suppressed by overexpression of D.N.Ras in cardiac fibroblasts (Fig 8A), suggesting that activation of Ras is required for ERK activation by Ang II in cardiac fibroblasts.

Raf-1 has been reported to be activated by Ras and to activate dual protein kinase MEK.33 MEK in turn activates ERKs by phosphorylating their threonine and tyrosine residues.26,27 We finally examined whether Raf-1 is required for Ang II–induced ERK activation in cardiac fibroblasts. After transfection of HA-ERK2 with or without D.N.Raf-1, cardiac fibroblasts were exposed to Ang II (10−6 mol/L) for 8 minutes. Activation of transfected ERK2 by Ang II was abolished by cotransfection of D.N.Raf-1 (Fig 8B), indicating that activation of Raf-1 is critical for activation of ERKs by Ang II in cardiac fibroblasts as well as in cardiac myocytes.32

Discussion

Two-thirds of the cells in the heart are nonmyocytes, the vast majority of which (>90%) are fibroblasts.1,2 Cardiac fibroblasts enhance the production of ECM proteins when the heart is exposed to a variety of injuries, such as myocardial infarction and myocarditis. Increase in the number of cardiac fibroblasts and the content of ECM proteins during cardiac remodeling is one of the major causes of cardiac dysfunction.7,8 Therefore, elucidation of the mechanism of proliferation of cardiac fibroblasts is of great importance. Ang II has been reported to be a potent inducer of both cardiac fibroblast hyperplasia and cardiomyocyte hypertrophy.12,13,17,18 In the present study, we compared the Ang II–induced signal transduction pathways leading to proliferation of cardiac fibroblasts with the pathways leading to cardiomyocyte hypertrophy. Ang II activated ERKs and enhanced DNA synthesis in cardiac fibroblasts of neonatal rats. The activation of ERKs was critical for the enhancement of DNA synthesis in cardiac fibroblasts, as is the case for many other types of cells.26–28 In cardiac fibroblasts, Ang II activated ERKs via a pathway through an AT1R, a Gαs subunit derived from Gs, Src family tyrosine kinases, Shc, Ras, and Raf-1; this
pathway differs considerably from the pathway in cardiac myocytes, in which G and PKC play critical roles. Two major subtypes of the Ang II receptor, AT1R and AT2R, are each seven-membrane serpentine receptors and elicit intracellular signals through heterotrimeric G proteins. Ang II activated ERKs and enhanced DNA synthesis through AT1R in cardiac fibroblasts, as previously reported. It has also been reported that AT1R couples to G in cardiac myocytes and smooth muscle cells and G in hepatocytes. In cardiac fibroblasts, pretreatment with pertussis toxin abolished the Ang II–induced activation of ERKs and the increase in DNA synthesis, whereas pertussis toxin had no effects on cardiac myocytes, indicating that AT1R may couple to G in cardiac fibroblasts and to G in cardiac myocytes. Thus, which G proteins couple to AT1R depends on cell type, according to these findings. However, there is another possibility that there are unidentified factors determining signal transduction pathways downstream from G proteins and leading to ERK activation. It has been reported that although lysophosphatidic acid receptors couple to both pertussis toxin–sensitive and –insensitive G proteins and that the latter G protein induces phosphoinositide hydrolysis with subsequent Ca mobilization and stimulation of PKC, the pertussis toxin–insensitive G protein does not activate ERKs in fibroblasts. Thus, we cannot rule out the possibility that AT1R couples to both G and G proteins in both cardiac myocytes and fibroblasts, but the G predominantly leads to ERK activation in cardiac myocytes, whereas the AT1R signals to ERKs predominantly via G in the cardiac fibroblasts. A growing body of evidence has suggested that the G subunit derived from pertussis toxin–sensitive G protein regulates many effectors within the cell. Stimulation of many receptors, such as β-adrenergic, M, muscarinic acetylcholine, D, dopamine, and A1 adenosine receptors, induces Ras-dependent ERK activation by G subunits in COS-7 cells. In addition, overexpression of G subunits, but not of constitutively activated G or G, mutants, has been demonstrated to be sufficient for activation of ERKs in COS-7 cells. By overexpressing the βARK1–66 polypeptide mimogene, we showed that the G subunit is required for Ang II–induced ERK activation in cardiac fibroblasts. Although the precise mechanism of ERK activation by the G subunit remains to be determined, the G subunit has been reported to activate ERKs through Src family tyrosine kinases. It has also been reported that the G subunit activates PI3K, which in turn activates Ras through Src and Shc. Quite recently, it has been proposed that PI3K mediates signals from G to Src family protein tyrosine kinases. We have recently observed that PI3K plays a critical role in the Ang II–induced activation of p70S6 kinase and the increase in protein synthesis in cardiac myocytes (authors’ unpublished data, 1997). Further studies will be needed to determine whether PI3K is involved in G subunit–induced Src activation by Ang II in cardiac fibroblasts.

AT1R stimulates PLC, thereby generating two major second messengers, IP3 and DG. IP3 leads to the release of Ca from intracellular Ca stores, and DG activates PKC. In cardiac myocytes, Ang II activates ERKs through the PKC-dependent pathway. In cardiac fibroblasts, however, tyrosine kinases, but not PKC, play a critical role in Ang II–induced ERK activation.

Zou et al 343
on cell type and G protein. In cardiac myocytes, in which AT1R couples to Gq, PKC, but not Ras or tyrosine kinases, is critical for Ang II–induced Raf-1/ERK activation.\(^3\) On the other hand, in cardiac fibroblasts, in which AT1R couples to Gq, Ras, but not PKC, is a key component of signal transduction pathways leading to the activation of ERKs induced by Ang II. In vascular smooth muscle cells, AT1R has been reported to couple to pertussis toxin–insensitive G protein (G\(_q\)) and activate ERKs through Ca\(^{2+}\), tyrosine kinases, and the Ras-dependent pathway.\(^2,4\) These results collectively suggest that AT1R-evoked signal transduction pathways are quite different among cell types. G proteins coupling to AT1R may be different, or signal transduction pathways downstream from G proteins leading to ERK activation may be different among cell types. Distinctive signaling pathways may result in different effects of Ang II: hypertrophy in cardiac myocytes, hyperplasia in cardiac fibroblasts, and hypertrophy and hyperplasia in smooth muscle cells.

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