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Reactive Oxygen Species Modulate Angiotensin II-Induced β-Myosin Heavy Chain Gene Expression via Ras/Raf/Extracellular Signal-Regulated Kinase Pathway in Neonatal Rat Cardiomyocytes

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Angiotensin II (Ang II) causes cardiomyocytes hypertrophy. Cardiac β-myosin heavy chain (β-MyHC) gene expression can be altered by Ang II. The molecular mechanisms are not completely known. Reactive oxygen species (ROS) are involved in signal transduction pathways of Ang II. However, the role of ROS on Ang II-induced β-MyHC gene expression remains unclear. Here we found that Ang II increased β-MyHC promoter activity and it was blocked by Ang II type 1 receptor antagonist losartan. Ang II dose-dependently increased the intracellular ROS. Cardiomyocytes cotransfected with a dominant negative mutant of Ras (RasN17), Raf-1 (Raf301), or a catalytically inactive mutant of extracellular signal regulated kinase (mERK2) inhibited Ang II-induced β-MyHC promoter activity, indicating Ras/Raf/ERK pathway was involved. Antioxidants such as catalase or *N*-acetylcysteine decreased Ang II-activated ERK phosphorylation and inhibited Ang II-induced β-MyHC promoter activity. These data indicate that Ang II increases β-MyHC gene expression in part via the generation of ROS.

Key Words: **angiotensin II; cardiomyocyte; reactive oxygen species; Ras/Raf/ERK signaling pathway; myosin heavy chain gene.**

Angiotensin II (Ang II) has been identified in various tissues including the heart (1). In neonatal cardiac myocytes (2, 3) and adult hearts (4, 5), Ang II stimulates early signals of the cardiac growth and hypertrophy, independently of the load *in vivo.* This response depends on translocation of protein kinase C (PKC) from cytosol to membrane fraction and is blocked specifically by the Ang II type 1 (AT_1) receptor antagonist losartan (2, 4, 5, 6, 7). Ang II is also known to rapidly activate early proto-oncogenes as well as later structural genes (2).

A number of growth factors signaling through G-protein-coupled receptors, including Ang II, induce the expression of β-myosin heavy chain (β-MyHC) gene in cardiac cells (3, 8, 9, 10). The cardiac β-MyHC gene encodes one of the major sarcomeric proteins responsible for contractility as well as the hypertrophic response (11). The molecular signals involved in β-MyHC transcriptional control are not well defined.

Recent evidence indicates that reactive oxygen species (ROS) may function as intracellular messengers to modulate signaling pathways (12, 13). The changes of intracellular ROS have been detected in a variety of cells stimulated with cytokines, growth factors, and agonists of receptors such as Ang II (13–15).

Studies have demonstrated that Ang II causes hypertrophy in part via the generation of ROS in cardiomyocytes (15). However, the role of ROS in Ang II-induced β-MyHC gene expression in cardiomyocytes has not yet been determined. In the present study, we clearly demonstrate that ROS modulate Ang II-induced β-MyHC gene expression via Ras/Raf/extracellular signal regulated kinase (ERK) pathway in cardiomyocytes.

MATERIALS AND METHODS

Materials. The catalytic inactive mutant of ERK2 (mERK) was a gift from Dr. R. J. Davis, University of Massachusetts Medical School

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(Worcester, MA). RasN17, RasL61, and Raf310 were previously described (16). 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was obtained from Serva Co. (Heidelberg, Germany). H_2O_2 was purchased from Acros Organics (Pittsburgh, PA). Ang II, catalase, *N*-acetylcysteine (NAC), and all other chemicals of reagent grade, were obtained from Sigma.

Culture of cardiac myocytes. Primary cultures of neonatal rat ventricular myocytes were prepared as previously described (13). Myocytes cultures thus obtained were >80% pure based on their contractile characteristics under light microscopy. Serum-containing medium for these cultured myocytes was replaced with serum-free medium before the cells were exposed to agents indicated.

Transfections. Cardiac cells were transiently transfected with different expression vectors by the calcium phosphate method as previously described (13). The chimaeric construct β-MyHCCAT contained a 1.2 kb *Hin*dIII-*Pst*I fragment of the 5′-flanking sequence of β-MyHC gene linked to the prokaryotic chloramphenicol acetyltransferase (CAT) reporter gene. In some experiments, mERK, RasN17, RasL61, or Raf310 were cotransfected with β-MyHCCAT. DNA concentration for all samples was adjusted to equal amount with empty vector pSR[IBMS1]α in each experiment.

Chloramphenicol acetyltransferase (CAT) assays and β-galactosidase assays. The CAT and β-galactosidase assays were performed. The relative CAT activity was determined by normalizing the CAT value to its respective β-galactosidase activity. Cotransfected β-galactosidase activity was observed to vary by 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 thymidine kinase promoter) and pBLCAT3 (without promoter) were included in every assay.

Detection of intracellular ROS. ROS was measured by the previously described method (17). Prior to the chemical or Ang II treatment, cardiomyocytes were incubated in culture medium containing DCFH-DA of 30 μM for 1 h to establish a stable intracellular level of the probe. The same concentration of DCFH-DA was maintained during the chemical or Ang II treatment. Subsequently, the cells were washed with PBS, and removed from petri dishes by scraping, then measured for the 2′,7′-dichlorofluorescein (DCF) fluorescence intensity. DCFH-DA penetrating the cells is initially converted into DCFH by cellular esterase, and DCFH is in turn oxidized to DCF in the presence of ROS. The DCF fluorescence intensity of the cells is an index of intracellular levels of ROS; and it can be determined by fluorescence spectrophotometry with excitation and emission wavelengths at 475 and 525 nm, respectively. The cell number in each sample was counted and utilized to normalize the fluorescence intensity of DCF.

Assay of ERK phosphorylation. ERK phosphorylation was assayed. Briefly, cardiomyocytes were lysed with buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). Cells were disrupted by repeated aspiration through a 21-gauge needle. For detection of ERK phosphorylation in cardiac myocytes, the cell lysates were collected and boiled. Total cell lysates (100 μg of protein) were separated by SDS–PAGE (12% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-μm pore size). The membrane was then incubated with anti–active ERK1/ERK2 antibody (Promega Inc.). Immunodetection was performed using the Western-Light chemiluminescent detection system (Tropix, Inc.).

Statistical analysis. Data were presented as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) or the unpaired Student's *t*-test. *P*-values less than 0.05 were considered to be statistically significant.

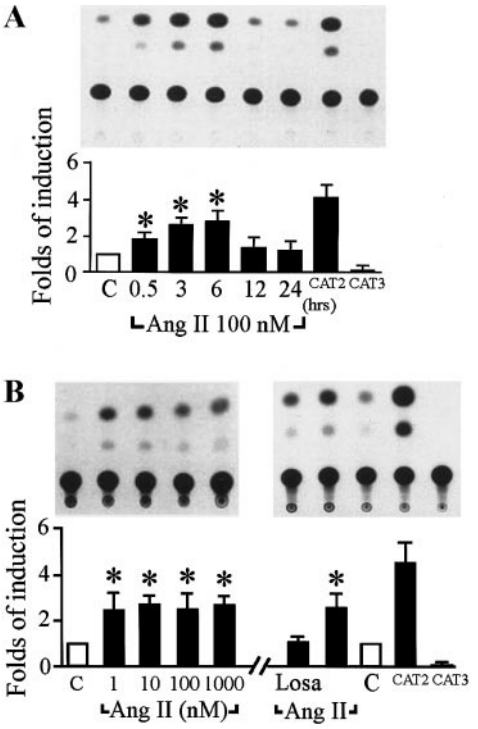

FIG. 1. Effect of Ang II on β-MyHC promoter activity. (A) Time course of Ang II-increased β-MyHC promoter activity. Cultured cardiomyocytes were transfected with chimeric CAT fusion genes followed by treatment with Ang II (100 nM) for the time indicated. (B) Induction of β-MyHC promoter activity by different concentration of Ang II and effect of nonpeptide Ang II receptor antagonist (losatan; Losa) on Ang II-induced β-MyHC gene expression. Cardiomyocytes were pretreated with losartan (100 nM) for 30 min and then stimulated with Ang II (1 nM). Cells were harvested and CAT activities were measured as described under Materials and Methods. C (control), no drugs; CAT2 and CAT3 are shown as positive and negative control. CAT activities are shown as % incorporation after normalizing to that of β-galactosidase activities. Data are represented as fold increase relative to control groups. The results are show as mean ± SEM. *$P < 0.05$ vs control; #$P < 0.05$ vs Ang II alone. The experiment was repeated three times with reproducible results.

RESULTS

Ang II Increases β-MyHC Gene Expression

Recent data show Ang II causes cardiac myocyte hypertrophy (2). Because cardiac hypertrophy is also characterized by the increased expression of β-MyHC gene, we then examined the effect of Ang II on the β-MyHC gene expression. As shown in Fig. 1A, the induced expression of β-MyHC gene was found within 30 min following the addition of Ang II (100 nM), reaching a maximum at the interval between 3 to 6 h, and thereafter subsided to control levels after 24 h. The successive addition of Ang II into culture medium at each 6 h interval will sustain the maximal promoter activity of β-MyHC gene (data not shown). These data indicate that a substantiating effect of Ang II is required for a hypertrophic response in cardiomyocytes.

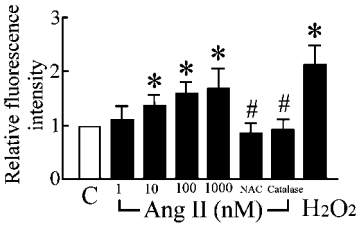

FIG. 2. Induction of ROS generation due to Ang II exposure. Cardiomyocytes treated with Ang II increased intracellular ROS levels as revealed by fluorescent intensities of DCF. Cardiomyocytes preincubated with DCF-DA for 1 h and then treated with Ang II for 1 h. Ang II induced ROS generation in a dose-dependent manner (1, 10, 100, and 1000 nM; columns 2–5). Cardiomyocytes from either control (C; column 1) or treated with Ang II (100 nM) in presence of 10 mM NAC (column 6) or 350 U/ml catalase (column 7) for 1 h. H_2O_2 (25 μM) treated cells (column 8) are shown as positive controls. Fluorescence intensities of cells are shown as relative intensity of experimental groups compared to untreated control cells. The results show mean ± SEM. $*P < 0.05$ vs control; $\#P < 0.05$ vs Ang II (100 nM) treated cells. The experiment was repeated six times with reproducible results.

Ang II dose-independently increased β-MyHC promoter activity (Fig. 1B). Higher concentration of Ang II from l to 1000 nM gave similar induction. The effect of losartan on the Ang II-induced β-MyHC gene expression was also investigated (Fig. 1B). Losartan is known to be an AT_1 receptor blocker. The cultured cardiomyocytes were pretreated with losartan (100 nM) for 30 min and then stimulated with Ang II for 6 h. The Ang Il-induced β-MyHC gene expression was inhibited by losartan.

Dose-Dependent Increase in Fluorescence by Exposure to Ang II

Cardiomyocytes incubated with Ang II for 1 h showed an increase of DCF fluorescence intensity in a dose-dependent manner (Fig. 2). To examine whether the ROS were responsible for the oxidation of DCF in cardiomycytes, we examined whether antioxidants prevent the oxidation of DCF. Cardiomyocytes were treated with Ang II (100 nM) in the presence of catalase (350 U/ml) or NAC (10 mM). The addition of either catalase or NAC to cultured cardiomyocytes completely inhibited Ang II-induced ROS levels as measured at 1 h after Ang II treatment (Fig. 2). These findings clearly demonstrate that Ang II treatment to cardiomyocytes increases intracellular ROS levels.

Ang II–Induced β-MyHC Expression Is Mediated via the Ras/Raf/ERK Pathway

In order to identify the signaling pathway involved in Ang II-induced β-MyHC expression, we transiently transfected cardiomyocytes with various dominant negative mutants of Ras (RasN17), Raf-1 (Raf301), or a catalytically inactive mutant of ERK2 (mERK), all of which are associated with the Ras/Raf/ERK pathway. Cardiomyocytes cotransfected with the empty vector PSRα as a control, revealed no effect on Ang II-induced β-MyHC promoter activity (Fig. 3). However, cotransfection with RasN17, Raf301, or mERK resulted in a significant inhibition in Ang II-induced β-MyHC promoter activity. In contrast, cardiomyocytes cotransfected with a dominant positive mutant of Ras (RasL61) or MEK1 greatly increased their β-MyHC promoter activity. These results suggest that the Ras/Raf/ERK signaling pathway is involved in Ang II-induced β-MyHC gene expression in cardiomyocytes.

Antioxidants Inhibit Ang II–Activated ERK1/2 Phosphorylation in Cardiomyocytes

Given that the ERK signaling pathway is involved in Ang II-induced β-MyHC expression, we further investigated whether antioxidants inhibit ERK pathway in Ang II-treated cardiomyocytes. We examined the phosphorylation of ERK1/2 in cardiomyocytes exposed to Ang II (100 nM) in the presence of catalase (350 U/ml) or NAC (10 mM). As shown in Fig. 4, cardiomyocytes exposure to Ang II for 30 min rapidly activated phosphorylation of ERK1/2. However, cardiomyocytes pretreated with catalase or NAC showed significantly decreased Ang II-induced ERK1/2 phosphorylation. On the other hand, cardiomyocytes treating with H_2O_2 (25 μM) showed increased ERK1/2 phosphorylation (Fig. 4). These findings imply that ROS mediate Ang II-activated ERK1/2 signaling pathway in cardiomyocytes.

ROS Modulate AII-Induced β-MyHC Gene Expression

To further determine whether the ROS modulate the Ang II-induced β-MyHC gene expression, the β-MyHC

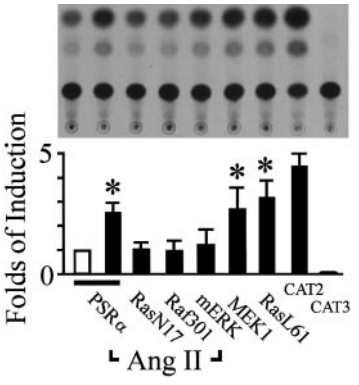

FIG. 3. Ang II-induced β-MyHC gene expression mediated via Ras/Raf/ERK signaling pathway. Cardiomyocytes were transfected with either pSRα-empty vector (5 μg), or an expression plasmid encoding the RasN17 (5 μg), Raf301, or dominant negative mutant mERK was cotransfected with 15 μg of β-MyHCCAT. Cardiomyocytes cotransfected with an expression plasmid encoding MEK1 (5 μg) or RasL61 (5 μg) were used as positive controls. Results are shown as mean ± SEM. $*P < 0.05$ vs pSRα-transfected control. The experiment was repeated three times with reproducible results.

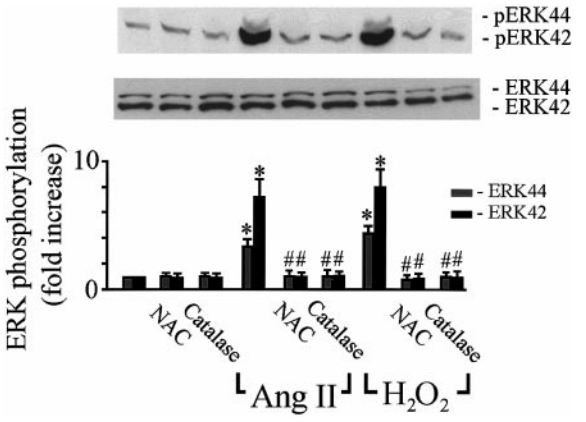

FIG. 4. Inhibitory effect of antioxidants on Ang II-activated ERK phosphorylation. Cardiomyocytes were in control condition (C) or exposed to Ang II (100 nM) for 30 min. After Ang II treatment, cardiomyocytes were lysed, and the phosphorylation of ERK was determined by using Western blot analysis as described under Materials and Methods. Antibody to the active form of ERK42/ERK44 was used. Some cells were pretreated with NAC or catalase for 30 min. Cardiomyocytes were then treated with Ang (100 nM) or H_2O_2 (25 μM) for 30 min. Data are represented as fold increase relative to control groups. Results are shown as mean ± SEM from three independent experiments. *$P < 0.05$ vs control; #$P < 0.05$ vs Ang II (or H_2O_2) alone.

promoter activity was examined by Ang II or H_2O_2 stimulation. As demonstrated in Fig. 5, either catalase or NAC alone had no effect on the basal β-MyHC promoter activity. However, cardiomyocytes treated with Ang II (100 nM) and H_2O_2 (25 μM) for 6 h led to a 2.1- and 2.8-fold increases in CAT activity, respectively, as compared to unstimulated control cells (Fig. 5). In the presence of catalase (or NAC), Ang II-increased β-MyHC promoter activities were significantly inhibited. These data clearly indicate that ROS modulate the Ang II-induced β-MyHC promoter activity in cardiomyocytes.

DISCUSSION

The major new finding of this work is that ROS modulate Ang II-induced β-MyHC gene expression via Ras/Raf/ERK pathway in cultured neonatal rat cardiac myocytes. Our hypothesis that Ang II increases β-MyHC gene expression via the generation of ROS is supported by the following observations. First, Ang II increased β-MyHC promoter activity in cultured cardiomyocytes. Second, Ang II generated ROS in a dose-dependent manner. Third, either catalase or NAC inhibited the phosphorylation of ERK1/2 or the increase of β-MyHC promoter activity induced by Ang II.

It is well known that Ang II causes hypertrophy of cardiac myocytes (2), and multiple intracellular pathways in Ang II signaling have been reported. Among the reported effects are the activation of PKC (18), ERK (6), or c-Jun N-terminal kinase (19, 20), the induction of immediate-early genes (2, 18), the elevation in intracellular calcium (18), and the formation of ROS (15). Ang II has been shown to activate many signaling pathways through its AT_1 receptor. In cultured cardiac cells, the activation of the AT_1 receptor leading to the activation of phospholipase C, PKC (21, 22), and mitogen-activated protein (MAP) kinases has been documented (23). We characterized the response to Ang II by using agents that implicated the AT_1 receptor (losartan) and the requirement of ROS generation (catalase and NAC). In the present work, we provide evidence suggesting a role for myocardial AT_1 receptors in Ang II-induced β-MyHC gene expression. Indeed, myocardial-specific overexpression of the human AT_1 receptors produces initially cardiomyocyte hypertrophy (3). We also showed that Ang II generated ROS in the same dose used in an earlier study (15).

Cotransfection with dominant negative mutants RasN17, Raf301, and mERK implicates Ras/Raf/ERK activation in the signaling pathway. Ras is known to be involved in mediating the effects of Ang II in several cell types, although one study has reported that Ras was not activated by Ang II in cultured cardiac myocytes (6). In further Western analysis, we found that ERK1/2 phosphorylation was increased by Ang II and inhibited by either NAC or catalase, suggesting that it could be a potential site of action for these drugs;

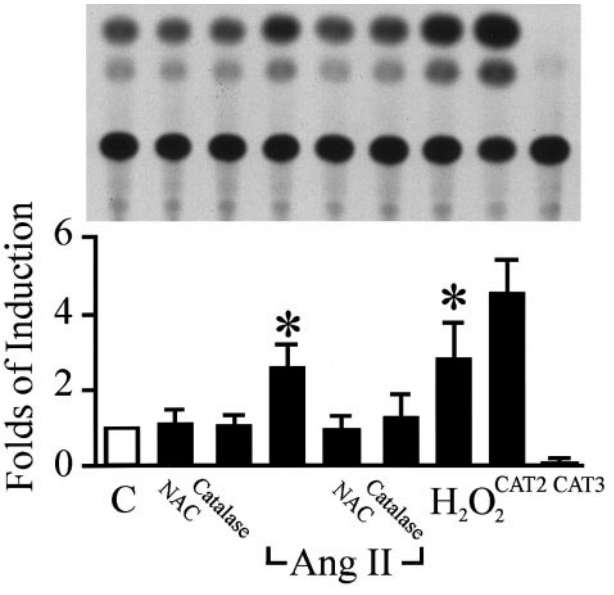

FIG. 5. Modulation of Ang II-increased β-MyHC promoter activity by ROS. Cardiomyocytes were transfected with 15 μg of β-MyHCCAT chimeric gene for 24 h. Some cells were pretreated with NAC or catalase for 30 min. Cardiomyocytes were then treated with Ang II (100 nM) for 6 h. H_2O_2 (25 μM) treated cells (column 7) are shown as positive controls. CAT2 (lane 8) and CAT3 (lane 9) are shown as positive and negative controls of a CAT assay system. The results are show as mean ± SEM. *$P < 0.05$ vs control; #$P < 0.05$ vs Ang II alone. The experiment was repeated three times with reproducible results.

although any site upstream of ERK1/2 also could be implicated. Whether ERK1/2 is the sole site of action for these drugs remains to be clarified since both c-Jun N-terminal kinases and p38 MAPK activation were also inhibited by antioxidants (24), and these pathways are generally believed to be independent of ERK1/2. A role for ROS influencing the MAP kinase pathway has been suggested, with available data implicating superoxide anion as a positive effector of MAP kinase activation (25). Ang II, by virtue of activating the NADPH oxidase system, produces the superoxide anion, which triggers the kinase cascade and promotes cell proliferation (26). If the above finding is true, scavengers of free radicals such as the superoxide anion would inhibit activation. NAC has been suggested as a direct scavenger of free radicals in studies where it reduced the activation of MAP kinase phosphorylation in response to PDGF (27) and lowered MAP kinase activity and Ras activation in response to lactosylceramide (28). In this study, we also treated cardiomyocytes with catalase, a big molecule that is unlikely to diffuse into cells, appears to clear rapidly the intracellularly generated H_2O_2, which freely permeates the cell membrane (13). Our findings that pretreatment of the cardiac myocytes to NAC (or catalase) inhibits the activation of ERKs in response to Ang II are consistent with the literatures mentioned above (24, 27, 28).

In our studies, NAC and catalase inhibit Ang II-induced β-MyHC gene expression. The molecular signals involved in β-MyHC transcriptional control are not well defined. Previous work that focused on functional analyses of the β-MyHC gene promoter suggests the interplay of *cis* and *trans* factors in the regulation of β-MyHC gene expression (29–34). Most of these studies have focused on the proximal 400 bp of the promoter sequence. This region contains several binding sites such as a PKC response element (30) and a GATA motif (34). At this point, it can only be speculated as to how Ang II or ROS affects β-MyHC promoter activity. The PKC response element located in the β-MyHC promoter (−215/−196) (30), and studies have shown that PKC could be activated by ROS (35). Inhibition of the ERK cascade with PD098059 blocking the phenylephrine-induced increase in the DNA binding ability and the phosphorylation of GATA protein (36) suggest ROS could affect GATA protein to stimulate the gene expression via influencing the ERK cascade.

In our studies, antioxidants inhibit the effects of Ang II on the activation of an essential signaling system. It is plausible that the Ang II signaling pathway consists of redox-sensitive steps and that antioxidants pretreatment could modulate the redox state of the cell. In summary, our data show that ROS modulate Ang II-induced β-MyHC gene expression via Ras/Raf/ERK pathway in cardiomyocytes. These findings support that ROS may play an important role in signal transduction underlying the cardiomyocyte hypertrophy.

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