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Protein Kinase C Epsilon-Dependent Extracellular Signal-Regulated Kinase 5 Phosphorylation and Nuclear Translocation Involved in Cardiomyocyte Hypertrophy With Angiotensin II Stimulation

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ABSTRACT

Angiotensin II (Ang II) plays a critical role in hypertrophy of cardiomyocytes; however, the molecular mechanism, especially the signaling cascades, in cardiomyocytes remains unclear. In the present study, we examined the mechanism of Ang II in hypertrophy of cardiomyocytes. Ang II rapidly stimulated phosphorylation of protein kinase C epsilon (PKCε) in a time- and dose-dependent manner via Ang II receptor-1 (AT_1). Furthermore, Ang II-induced extracellular signal-regulated kinase 5 (ERK5) phosphorylation and translocation was mediated through a signal pathway that involves AT_1 and PKCε, which resulted in transcriptional activation of myocyte enhancer factor-2C (MEF2C) and hypertrophy. Consequently, inhibiting PKCε or ERK5 by small interfering RNA (siRNA) significantly attenuated Ang II-induced MEF2C activation and hypertrophy of rat cardiomyocytes. These data provide evidence that PKCε-dependent ERK5 phosphorylation and nucleocytoplasmic traffic mediates Ang II-induced MEF2C activation and cardiomyocyte hypertrophy. PKCε and ERK5 may be potential targets in the treatment of pathological vascular hypertrophy associated with the enhanced renin–angiotensin system. J. Cell. Biochem. 109: 653–662, 2010.

KEY WORDS: ANGIOTENSIN II; PKCε; ERK5; CARDIOMYOCYTE; HYPERTROPHY

Myocardial hypertrophy is a risk factor of heart failure. Cardiomyocytes can respond to mechanical load, whether provoked by axial stretch, increased beating frequency, or osmotic swelling [Brede et al., 2003; Yutao et al., 2006]. To compensate for increased mechanical load, cardiomyocytes react in a defined way, by inducing hypertrophic growth [Schlüter and Wenzel, 2008]. Cardiac hypertrophy alters myocyte shape and the extracellular matrix, which results in wall thickening, then chamber dilation, and myocardiac dysfunction. This process is initially compensatory for increased workload, the prolongation of which leads to congestive heart failure, arrhythmia, and sudden death.

Angiotensin II (Ang II) is an octapeptide that exerts inotropic, hypertrophic, and apoptotic effects on cardiomyocytes [Fabris et al., 2007; Mollmann et al., 2007]. Thus, Ang II is central for any process involved in the control of hypertrophy and heart failure. The corresponding signal transduction pathways have been demonstrated in fetal, neonatal, and adult cardiomyocytes. The renin–angiotensin system is an important component of the physiological and pathological responses of the cardiovascular system [Griendling et al., 1996; Joerg and Jeffery, 2006; Mehta and Griendling, 2007]. Through Ang II receptor-1 (AT_1), Ang II carries out its functions, including hypertrophic remodeling of cardiomyocytes, which

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involves various downstream signal transduction mechanisms [Zhan et al., 2005; Mehta and Griendling, 2007]. However, the regulatory molecular mechanisms, specifically the signaling cascades, involved in Ang II-induced cardiomyocyte hypertrophy are not fully understood.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that play a central role in transducing extracellular cues into various intracellular responses [Coulombe and Meloche, 2007]. Activated MAPKs phosphorylate multiple intracellular targets, including numerous transcription factors that induce the reprogramming of gene expression. A new member of the MAPK family, extracellular signal-regulated kinase 5 (ERK5), is activated in response to growth factors and stress. ERK5 is twice the size of ERK1/2 and similar to ERK1/2, has the Thr–Glu–Tyr (TEY) activation motif [Nishimoto and Nishida, 2006]. The pertinent phosphorylation sites of ERK5 have been mapped to Thr218/Tyr220. The kinase domain is encoded in its amino-acid terminus, whereas its unique carboxy terminus encodes two proline-rich regions, a nuclear export domain and a nuclear localization domain [Nishimoto and Nishida, 2006; Wang and Tournier, 2006]. Activated ERK5 translocates from the cytoplasm to the nucleus and then phosphorylates substrates, including myocyte enhancer factor-2C (MEF2C) [Nishimoto and Nishida, 2006].

Several physiological roles of ERK5 have been reported [Nishimoto and Nishida, 2006; Wang and Tournier, 2006]. In pathological conditions, ERK5 is involved in tumor development and hypertrophy [Wang and Tournier, 2006]. All four MAPKs, including ERK5, are activated during the development of cardiac hypertrophy [Nicol et al., 2000]. Recent studies indicated that ERK5 was involved in cardiac hypertrophy and was stimulated by Gq-coupled G protein-coupled receptors in epithelial cells via unknown mechanisms [García-Hoz et al., 2007]. The ERK5 pathway has also been implicated in vascular disorders such as atherosclerosis, a pathological process initiated by injury of endothelial cells due to multiple stimuli (i.e., smoking, diabetes mellitus, hypertension, and hyperlipidemia) [García-Hoz et al., 2007]. More recently, some studies have showed that ERK5 is implicated in Ang II-induced proliferation of vascular smooth muscle cells [Sharma and Goalstone, 2007]. However, little is known about the regulation and role of ERK5 in hypertrophy of cardiomyocytes stimulated by Ang II.

In vivo and tissue-culture experiments with phorbol esters used as general protein kinase C (PKC) agonists have revealed PKC agonists have long been implicated in cell proliferation, survival, and programmed death [Murray et al., 1997]. In cultured cardiomyocytes, PKC agonists regulate contractility and hypertrophy [Dorn and Mochly-Rosen, 2002]. However, PKC has at least 12 different isoforms, and the multiplicity of family members produces varied cellular responses depending on isoform activity and physiological context. In cardiac tissue, PKC isoform expression differs by species, cell type, and developmental stage, with most adult mammalian myocardia expressing PKCα, β1, β2, δ, ε, and λ/ζ [Bowling et al., 1999; Sabri and Steinberg, 2003].

The best-characterized PKC isoform in cardiac hypertrophy is PKCε. PKCε is activated in response to hypertrophic stimuli in cultured myocytes and in vivo [Chen et al., 2001], and overexpression and activation of PKCε results in myocardial hypertrophy [Takeishi et al., 2000]. PKCε is implicated in hypertrophic signaling because it is activated by mechanical stress as well as genetic (Gq) and physiological (pressure overload) hypertrophic stimuli [Gu and Bishop, 1994; D'Angelo et al., 1997]. PKCε was perceived to be a key mediator of maladaptive hypertrophy. However, the downstream signaling molecules, including the particular receptors and specific PKC isoforms, that mediate ERK5 activation in response to specific cellular stimuli in the ERK5 activation pathways remain elusive.

Here, we describe Ang II stimulating PKCε phosphorylation through an AT_1-PKC-dependent signal pathway, which subsequently led to ERK5 phosphorylation, translocation, MEF2C transcriptional activation, and cardiomyocyte hypertrophy. Our findings suggest that PKCε and ERK5 are implicated in Ang II-induced cardiomyocyte hypertrophy.

MATERIALS AND METHODS

MATERIALS

All basic laboratory reagents were from Sigma–Aldrich (St. Louis, MO). Ang II was from Sigma (St. Louis, MO). Anti-phospho-PKCε, anti-PKCε, anti-phospho-ERK5, anti-phospho-MEF2C, anti-MEF2C, and anti-ERK5 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, and anti-p38 antibodies were from Cell Signalling (Boston, MA). SDS–polyacrylamide gels were from Pierce (Rockford, IL); polyvinylidene fluoride (PVDF) and the protein gel apparatus were from Bio-Rad (Hercules, CA). Minimal essential medium (MEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin/streptomycin, non-essential amino-acids and L-glutamine, PD123319 and Go 6983 were from Sigma–Aldrich. Valsartan was obtained from Pro Du (Shandong University, China).

CELL CULTURING

Animals used in these experiments were handled in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Council of the China Physiological Society. Neonatal rat cardiomyocytes from 2- to 3-day-old Sprague–Dawley rats were prepared as previously described [Poizat et al., 2000]. The rats were anesthetized and sacrificed by immersing in 70% (v/v) alcohol. The ventricles were removed and washed three times in D-Hanks balanced salt solution (g/L: 0.4 KCl, 0.06 KH_2PO_4, 8.0 NaCl, 0.35 $NaHCO_3$, and 0.06 Na_2HPO_4 $7H_2O$, pH 7.2) at 4°C, then minced and incubated with 0.25% (w/v) trypsinase for 10 min at 37°C. Addition of an equal volume of cold Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) newborn calf serum was used to terminate the digestion. The supernatant was discarded. Then, cells were incubated with fresh 0.25% trypsinase for 15 min at 37°C, and the supernatant was collected. The latter digestion step was repeated four times. Cells in the supernatant were isolated by centrifugation for 10 min at 1,500 rev/min at room temperature. Cardiomyocytes were cultured as monolayers at 5×10^4 cells/cm^2 and incubated with Ang II at different times and various dosages. Prior to stimulation, all cells were placed in serum-reduced medium (0.1% FBS) for 24 h to maintain quiescence.

WESTERN BLOT ANALYSIS

Cardiomyocytes were lysed in ice-cold lysis buffer (150 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium vandate, 1 mM sodium phosphate, 1% Triton X-100, 0.5% SDS, 10 μg/ml aprotinin, 10 μg/ml leupetin, 50 mM HEPES, pH 7.5). Crude lysates were sonicated and centrifuged at 10,000 rpm for 5 min at 4°C. Total protein concentration from the resulting supernatant was determined by bicinchoninic acid protein assay (Pierce) and normalized to 1 mg/ml. Aliquots of whole cell lysates were dried in a Speedvac Concentrator (Savant, Holbrook, NY), denatured in Laemmli sample buffer, and stored at −20°C until use. Samples were boiled for 10 min, and 30 μg protein per lane was resolved on SDS–PAGE before being transferred to PVDF membranes. Membranes were probed with rabbit or goat polyclonal antibodies specific for phosphorylation, and total proteins were then incubated with goat anti-rabbit or rabbit anti-goat horseradish peroxidase-conjugated polyclonal antibodies. Proteins were detected by use of an electrochemiluminescence detection kit (Amersham, Piscataway, NJ) and quantitated by densitometry, by use of a Bio-Rad Fluor-S MultiImager (Bio-Rad).

IMMUNOFLUORESCENCE STAINING

Cells cultured on plastic dishes were washed with PBS and fixed in 2% paraformaldehyde for 30 min at room temperature, then rinsed twice in PBS. Monolayers were then permeabilized three times for 10 min each, with PBS supplemented with 0.1% (final concentration) Triton X-100 and then blocked twice in PBS with 0.2% BSA for 10 min each at room temperature. Cells were incubated with rabbit polyclonal anti-phospho-ERK5 antibody (diluted 1:100) in blocking solution overnight at 4°C. Coverslips incubated with rabbit polyclonal IgG, instead of primary antiserum, served as negative controls. After 3 washes for 10 min each in PBS with 0.2% BSA, coverslips were incubated with Alexa Fluor 488 (green)-conjugated goat-anti-rabbit secondary antibody for 30 min and washed three times in PBS with 0.2% BSA. Immunolabeled cells were counterstained with DAPI to detect cell nuclei, then slides were mounted. Samples were analyzed by confocal immunofluorescence microscopy with a Zeiss LSM510 system. To avoid interference between fluorescence signals, images were captured under multitracking mode.

SMALL INTERFERING RNA (siRNA) STUDIES

Double-stranded 21-mer siRNA (siR-PKCε) was designed as described [Elbashir et al., 2002]. Rat PKCε siRNA was obtained from Genepharma (Shanghai, China). The PKCε-specific siRNA sequences used were as follows: #1, 5′-AAGATCGAGCTGGC-TGTCTTT-3′; #2, 5′-TTCTAGCTCGACCGACAGAAA-3′. Cardiomyocytes were removed by trypsinization, seeded into 60 mm dishes, and cultured for 24 h. Transfection of cardiomyocytes involved use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol [Vega et al., 2004; Ozgen et al., 2008], with 100 nM non-targeting control siRNA or Smart Pool siRNA for PKCε. After 48 h, cells were exposed to Ang II as indicated, and lysates underwent Western blot analysis. Rat ERK5 siRNA was used to identify the involvement of ERK5 in MEF2C activation and cardiomyocyte hypertrophy. The rat ERK5-specific siRNA sequences were as follows: #1, 5′-CAGUCACUUGUGCCACCUATT-3′; #2, 5′-GGAG-GAAUUCUUAAACCAATT-3′; #3, 5′-GGCCCUGUAUCUCAGACUA-TT-3′ (GenBank Accession No. NM_002749).

MEASUREMENT OF [^{3}H]-LEU INCORPORATION AND CARDIOMYOCYTE DIAMETER

To determine cell hypertrophy, we measured [^{3}H]-Leu incorporation and cardiomyocyte surface area. After being digested, centrifuged, and resuspended in DMEM, cardiomyocytes were counted in at least three dishes at each time point, by use of phase-contrast microscopy. Cardiomyocyte surface area (μm^2) was determined from randomly selected fields from independent cell cultures by use of Quantimet 570 software (Leica, The Netherlands). Ten fields were randomly chosen for every group, and 10 cardiomyocytes were determined for every field.

[^{3}H]-Leu incorporation in cardiomyocytes was assessed as described previously [Luo et al., 2001]. Cardiomyocytes were made quiescent by incubation in serum-free DMEM medium for 24 h. Cells were incubated with 1 μCi/ml [^{3}H]-Leu in the presence or absence of 100 nM Ang II. After 24 h, cells were washed twice with ice-cold PBS, and the proteins were precipitated in 5% trichloroacetic acid. [^{3}H]-Leu incorporation was determined by use of a scintillation counter.

STATISTICAL ANALYSIS

Data were analyzed by SPSS version 12.0 (SPSS, Inc., Chicago, IL). All data were analyzed by Student's *t*-test. A $P < 0.05$ was considered statistically significant. Results are expressed as the mean ± SEM of at least three independent experiments.

RESULTS

ACTIVATION OF MAPKS BY ANG II IN NEONATAL RAT CARDIOMYOCYTES

To determine the signaling molecules that are specifically involved in the hypertrophic response of cells pretreated with Ang II, we first examined the potential role of ERK1/2, P38, and ERK5 in cardiomyocytes in response to Ang II stimulation. Ang II (100 nmol/L) induced phosphorylation of ERK5 after 5 min, with peak phosphorylation between 15 and 30 min, which returned to baseline values after 1 h (Fig. 1A). However, the phosphorylation of ERK1/2 and p38 was earlier than that of ERK5. The phosphorylation response of ERK5 began at 1 nmol/L, with maximal effect at 100 nmol/L (Fig. 1B). With Ang II stimulation, ERK5 protein level did not change (Fig. 1).

AT_1 BUT NOT AT_2 RECEPTOR MEDIATES ANG II–INDUCED ERK5 PHOSPHORYLATION

Ang II exerts its biological effects through binding to the two receptor subtypes AT_1 and AT_2, which belong to the superfamily of G protein-coupled receptors [Touyz and Berry, 2002]. To determine which subtype mediates ERK5 phosphorylation in cardiomyocytes, cells were pretreated for 1 h with valsartan (0.3, 1.0, and 3.0 μmol/L),

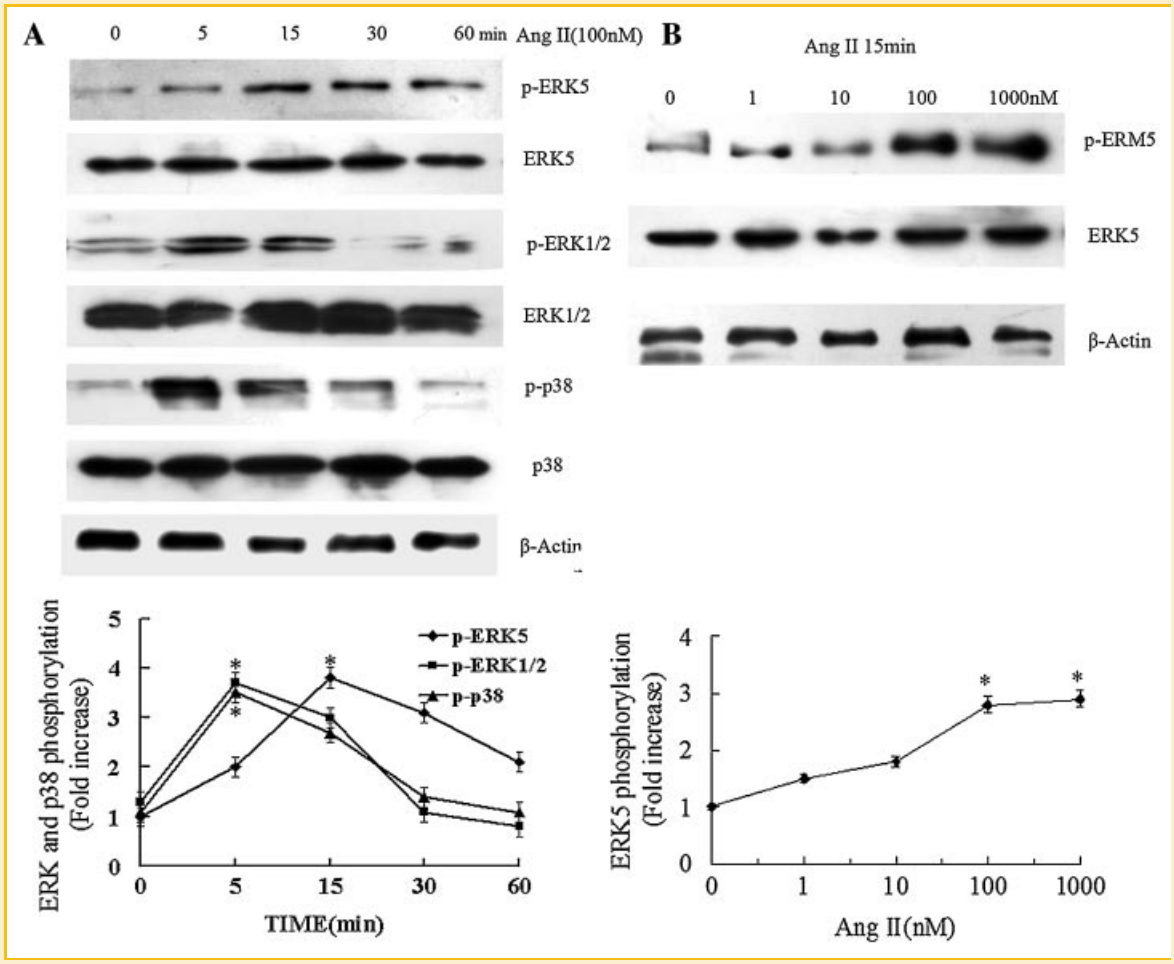

Fig. 1. Ang II stimulates ERK5 phosphorylation in cardiomyocytes. Cardiomyocytes were stimulated with Ang II (100 nM) for various times (A) or at different doses (B). Representative Western blot analysis results of the phosphorylation of ERK5 (p-ERK5), expression of ERK5, and β-actin (as a loading control) in cell lysates (n = 4 experiments). $^{*}P < 0.01$; $^{\#}P < 0.05$.

a specific antagonist for AT_1, or PD123319 (20 μmol/L), an antagonist for AT_2, then stimulated with Ang II (100 nmol/L) for 1 h. Valsartan inhibited Ang II-induced ERK5 activation in a dose-dependent manner, the dose of 3 μmol/L completely blocking ERK5 phosphorylation (Fig. 2B), whereas PD123319 had no effect on Ang II activation of ERK5 (Fig. 2A).

ANG II STIMULATES ERK5 ACTIVATION THROUGH A PKC-DEPENDENT PATHWAY

In vivo and tissue-culture experiments involving phorbol esters as general PKC agonists have implicated PKCs in cell proliferation, survival, and programmed death [Minamino and Komuro, 2007]. In

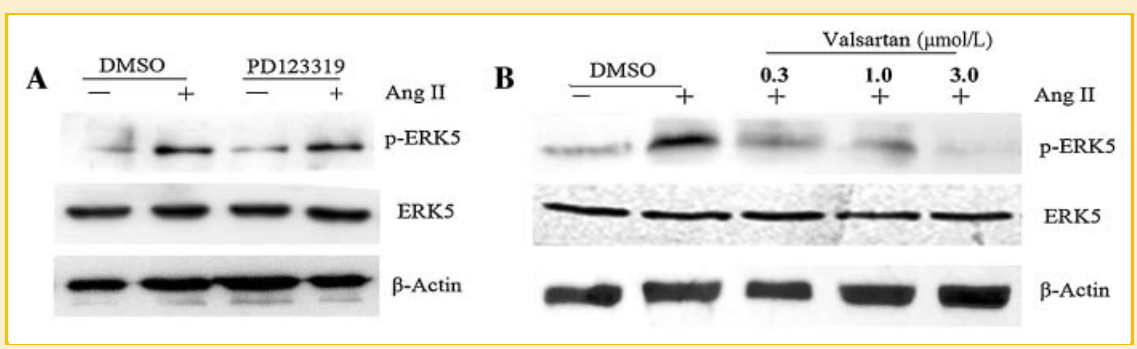

Fig. 2. AT_1-receptor-mediated Ang II-induced ERK5 phosphorylation. Cardiomyocytes were pretreated with PD123319 (20 μmol/L) (A) or valsartan (B) for 30 min and then stimulated with Ang II for 5 min; Western blot analysis (n = 4 experiments) of the phosphorylation of ERK5 and expression of ERK5 and β-actin (as a loading control).

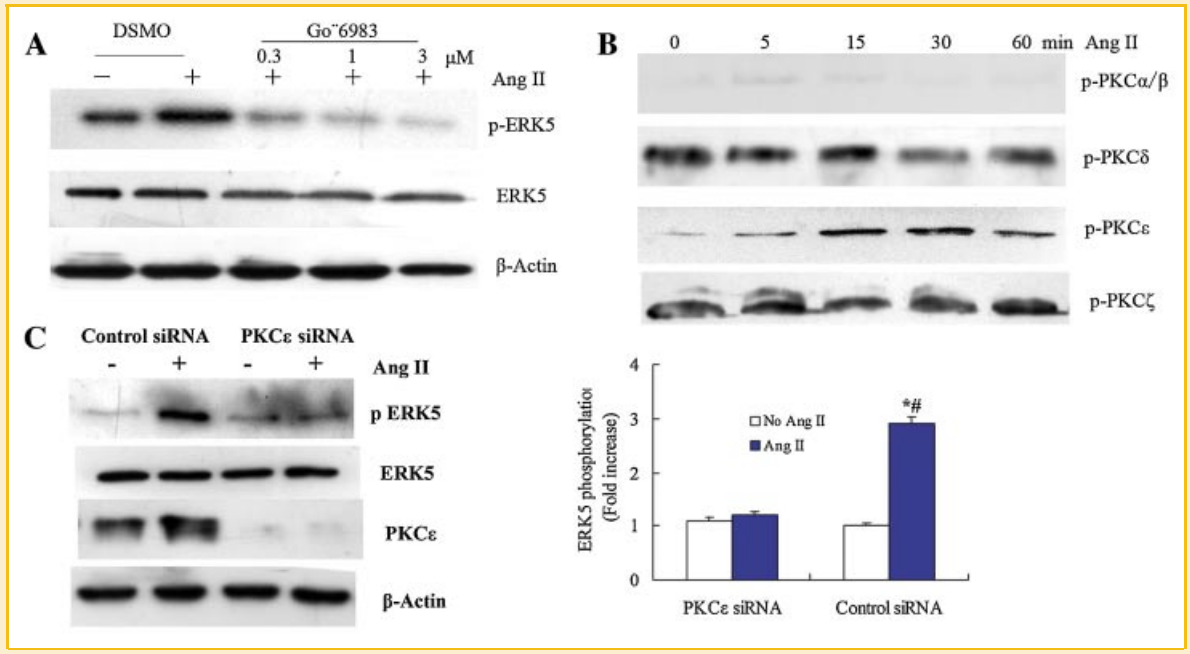

Fig. 3. Ang II stimulates ERK5 phosphorylation through AT_1-PKC in cardiomyocytes. Cardiomyocytes were stimulated with Ang II for 15 min at different doses. Cardiomyocytes were pretreated with the PKC inhibitor Go 6983 (A) for 1 h, then with Ang II for 1 h. Different PKC isozymes were detected on Ang II (100 nM) stimulation for 15 min (B). Cardiomyocytes were transfected with scramble (control) or PKCZ siRNA, then stimulated with Ang II (100 nM) (C). Western blot analysis (n = 3 experiments) of the phosphorylation of ERK5 and expression of ERK5 and β-actin (as a loading control). $^{*}P < 0.01$; $^{\#}P < 0.05$ versus controls.

cultured cardiomyocytes, PKCs regulate contractility and hypertrophy [Dorn and Mochly-Rosen, 2002]. To determine whether PKC activation is involved in Ang II-induced ERK5 phosphorylation in cardiomyocytes, we examined the effect of PKC inhibition on ERK5 phosphorylation. Quiescent cells were pretreated with the general PKC inhibitor Go 6983 (0.3, 1, and 3 μmol/L) for 1 h before being exposed to Ang II (100 nmol/L) for 1 h. Go 6983 blocked ERK5 phosphorylation in a dose-dependent manner (Fig. 3A), which suggests that PKC is involved in Ang II-stimulated ERK5 phosphorylation in cardiomyocytes.

PKCε SPECIFICALLY MEDIATES ANG II–INDUCED ERK5 PHOSPHORYLATION

Next, we attempted to determine which specific isotype of PKC is required for ERK5 activation. Several PKC isoforms, including α, β, δ, ε, and ζ, are expressed in cardiomyocytes [Dorn and Force, 2005], PKCα and PKCβ are upregulated, PKCε is upregulated or is a key mediator of maladaptive hypertrophy, and levels of PKCδ and ζ do not change [Dempsey et al., 2000; Braz et al., 2004; Steinberg, 2008]. Ang II (100 nM) treatment induced marked phosphorylation of PKCε within 15 min (Fig. 3B) but did not induce phosphorylation of PKCα/β, PKCδ, or PKCζ.

To substantiate the role of PKCε in Ang II-induced ERK5 phosphorylation, we knocked down endogenous PKCε in cardiomyocytes using siRNA. Transfection of PKCε siRNA in cardiomyocytes markedly reduced PKCε protein expression without affecting the expression of ERK5 and β-actin (Fig. 3C). Silencing PKCε by siRNA significantly inhibited Ang II-induced ERK5 phosphorylation, which indicates that PKCε is required for ERK5 phosphorylation with Ang II stimulation in cardiomyocytes.

ANG II STIMULATES ERK5 TRANSLOCATION VIA A PKCε-DEPENDENT PATHWAY

To determine the consequences of ERK5 phosphorylation, we studied the effect of Ang II on ERK5 subcellular distribution in cardiomyocytes. Before stimulation with Ang II, ERK5 was located primarily in the cytoplasm of cardiomyocytes (Fig. 4A); ERK5 nuclear entry was seen at 15 min after Ang II stimulation, with a striking translocation from the cytoplasm to the nucleus. At 60 min of Ang II treatment, ERK5 was gradually shuttled back to the cytoplasm.

To gain further insights into the signaling pathways leading to Ang II-induced translocation of ERK5, we studied the effects of PKCε. Knocking down PKCε expression by siRNA greatly attenuated 15 min (100 nM) Ang II-induced translocation of ERK5 in cardiomyocytes (Fig. 4B). The PKC inhibitor Go 6983 (3 μmol/L) also blocked Ang II-induced ERK5 translocation (Fig. 4B), which suggests that an AT_1-PKCε-dependent pathway is involved in the nucleocytoplasmic traffic of ERK5 in cardiomyocytes.

We also demonstrated changes in the amount of total ERK5 content between the nucleus and cytoplasm due to Ang II stimulation. We found a time-dependent increase in nuclear content of total ERK5, with concomitant decrease in cytoplasmic content on both Western blot and graphical analysis (Fig. 4C).

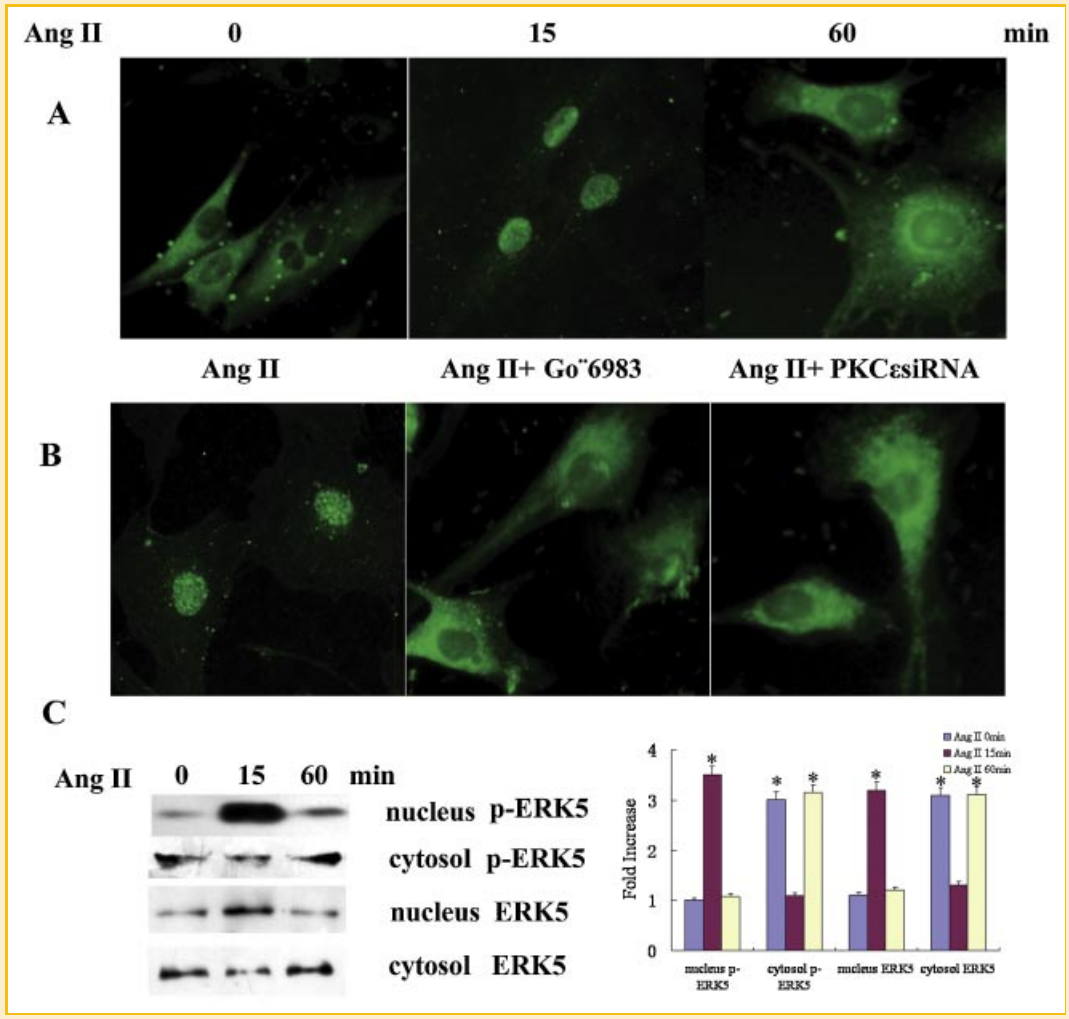

Fig. 4. Ang II stimulates ERK5 nuclear entry through a PKCε pathway. Cardiomyocytes were stimulated with Ang II for different times (A). Cardiomyocytes were transfected with PKCε siRNA or inhibited by Go 6983 (3 μmol/L), then stimulated with Ang II (100 nM) for 15 min (B). Representative fluorescence images show the subcellular localization of the proteins (n = 4 experiments, magnification 40×). $^{*}P < 0.05$ versus Ang II (0 nM). C: Changes in p-ERK5 and total ERK5 between the nucleus and cytoplasm due to Ang II (100 nM) stimulation.

PKCε AND ERK5 ARE INVOLVED IN ANG II-INDUCED ACTIVATION OF MEF2C

Because activated ERK5 phosphorylates MEF2C [Zhao et al., 2009], we examined whether Ang II stimulated the phosphorylation of MEF2C in cardiomyocytes. Ang II significantly stimulated the phosphorylation of MEF2C on treatment with Ang II (100 nM) for 15 min ($P < 0.05$); the phosphorylation returned to basal levels by 60 min (Fig. 5A). Knocking down PKCε or ERK5 expression by siRNA significantly attenuated the activation of MEF2C with Ang II (100 nM) treatment (Fig. 5B).

PKCε AND ERK5 ARE INVOLVED IN ANG II-STIMULATED CARDIOMYOCYTE HYPERTROPHY

Ang II promotes cellular hypertrophy as a result of increased protein synthesis, so we evaluated protein synthesis by measuring [3H]-Leu incorporation, a marker of cell hypertrophy. [3H]-Leu incorporation was significantly greater in cells treated with Ang II (100 and 1,000 nM) than in controls ($P < 0.01$; Fig. 6A). Ang II (100 nM) was therefore used for subsequent measurement of hypertrophy. Moreover, Ang II treatment increased the cell size of cardiomyocytes, as shown by significantly increased cell surface area (Fig. 6A).

To further research the functional role of PKCε and ERK5 in Ang II signaling, we examined whether PKCε and ERK5 are involved in Ang II-stimulated cardiomyocyte hypertrophy. Cardiomyocytes were infected with PKCε or ERK5 siRNA (Fig. 6B). Ang II (100 nM) significantly increased [^{3}H]-Leu incorporation. Inhibiting PKCε by siRNA greatly suppressed Ang II-induced [^{3}H]-Leu incorporation ($P < 0.01$). Similarly, [^{3}H]-Leu incorporation was significantly greater with Ang II (100 nM) and non-targeting ERK5 siRNA treatment (control) than with targeting ERK5 siRNA ($P < 0.01$) (Fig. 6B), which suggests that PKCε and ERK5 play a pivotal role in Ang II-induced cardiomyocyte hypertrophy.

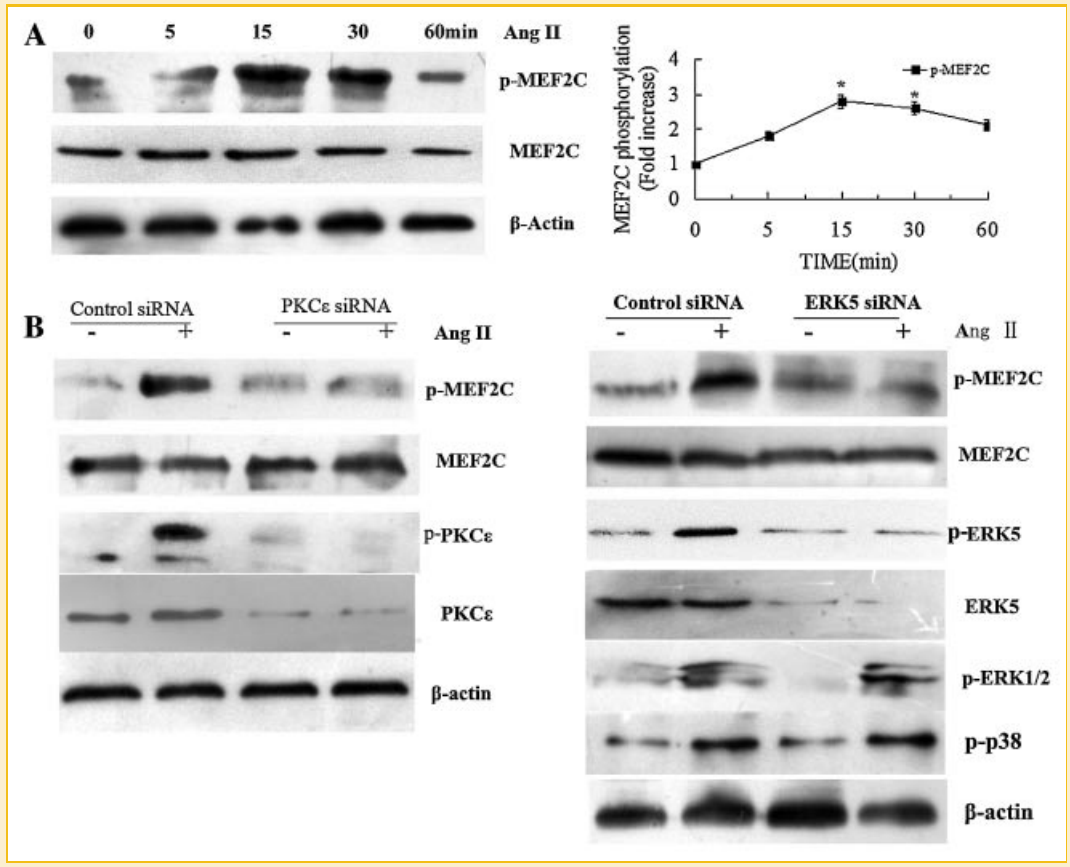

Fig. 5. PKCε and ERK5 are involved in Ang II-induced MEF2C activation. Ang II stimulated MEF2C(A). Cardiomyocytes were transfected with PKCε or ERK5 siRNA, then stimulated with Ang II (B). Western blot analysis of the phosphorylation of MEF2C and expression levels of MEF2C, PKCε, ERK5, and β-actin (as a loading control) with Ang II (100 nM) treatment. $^{*}P < 0.05$ versus Ang II (0 min).

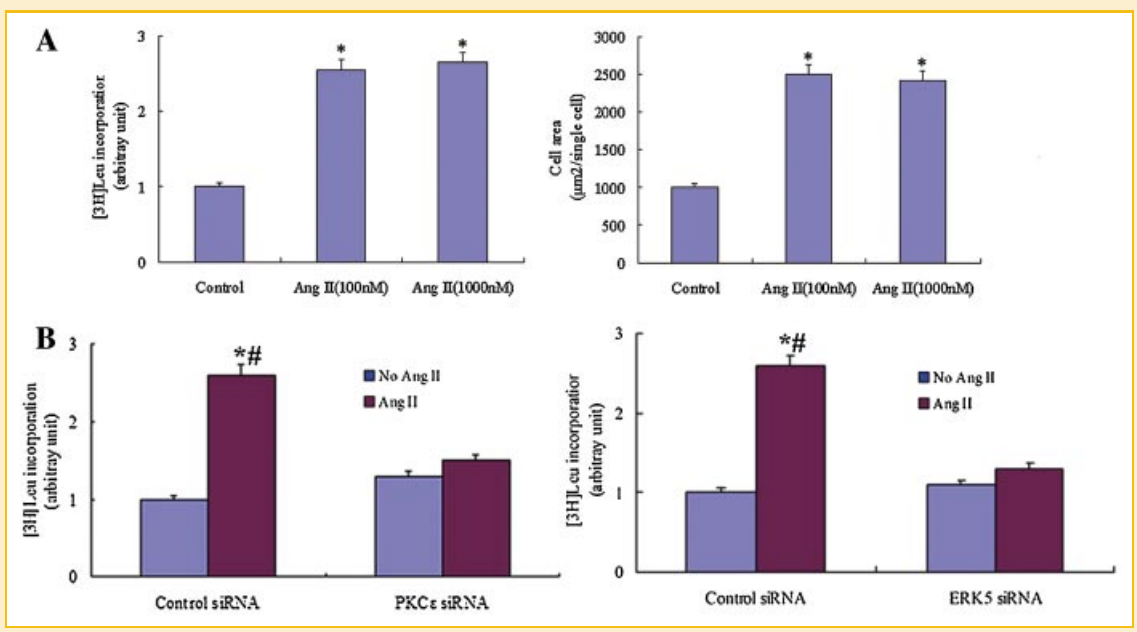

Fig. 6. ERK5 activation is required for induced hypertrophy by Ang II. [^{3}H]-Leu was added to cardiomyocytes, then cells were stimulated with and without Ang II (100 nM) for 15 min; cell area was also detected (A). $^{*}P < 0.01$ versus control. Cardiomyocytes were treated with Ang II (100 nM) for 24 h (B). Bars in figures indicate 20 μm. $^{*}P < 0.05$ versus control. [^{3}H]-Leu incorporation after treatment with non-targeting PKCε or ERK5 siRNA (control) with Ang II (100 nM) stimulation. $^{*,\#}P < 0.05$, $P < 0.01$ versus control.

DISCUSSION

Our results reveal that Ang II induces ERK5-activated nuclear translocation mediated by PKCε in cardiomyocytes, which results in phosphorylation of MEF2C and consequent cardiomyocyte hypertrophy. Ang II stimulated ERK5 phosphorylation in a time- and dose-dependent manner in rat cardiomyocytes. Furthermore, Ang II-induced ERK5 phosphorylation was mediated through a signal pathway that involves the AT_1 receptor and PKCε, and this pathway plays an important role for ERK5 nuclear export and action of MEF2C. Importantly, PKCε- and ERK5-dependent responses contribute to regulation of Ang II-induced [^{3}H]-Leu incorporation into cardiomyocytes to cause cell hypertrophy. These findings reveal a novel role of PKCε and ERK5 in Ang II-induced signal transduction in cardiomyocyte hypertrophy, which may provide new insights into the molecular mechanism of heart remodeling in animal models and in humans.

MAPKs are involved in the regulation of a large variety of cellular processes such as cell growth, differentiation, hypertrophy, cell cycle, death, and survival. The major groups of MAPKs found in cardiac tissue include the ERK1/2, c-Jun NH_2-terminal kinase (JNK), p38-MAPK, and ERK5. Involvement of all three classical MAPK pathways (ERK1/2, JNK, and p38) has been implicated in the mechanisms of cardiac hypertrophy. Accumulating studies have suggested that the reactive oxygen species (ROS) dependent ERK1/2 pathway was involved in cardiac hypertrophy induced by Ang II [Zou et al., 2008; Cai et al., 2009]. The activation of p38-MAPK signaling pathway was associated with the development of hypertrophy in transgenic mice with Ang II-induced cardiac hypertrophy [Pellieux et al., 2000] and in a model of hypertensive cardiac hypertrophy in spontaneously hypertensive stroke-prone rats [Behr et al., 2001]. Studies also documented the possible role of JNK in hypertrophic responses to Gq receptor-coupled hypertrophic agonists. Wang et al. [1998] found that specific activation of JNK pathway led to the induction of hypertrophic responses. Activation of JNK pathway was shown to contribute to the morphological response of neonatal rat cardiomyocytes and to increase the expression of hypertrophy-related genes [Finn et al., 2001]. Possible role of three classical MAPK pathways in hypertrophic responses was demonstrated using adenovirus-mediated gene transfer, whereby constitutive expression of dual-specificity phosphatase MKP-1 in cultured primary myocytes blocked the activation of ERK1/2, JNKs, and p38-MAPK, and prevented the agonist-induced hypertrophy [Bueno et al., 2001].

ERK5, another member of MAPKs, has also been shown to play an important role in cardiomyocyte hypertrophy. Our observations suggest that both ERK5 and other members of the MAPK family are activated by Ang II, but not at the same time. The physiological function of ERK5 signaling is just beginning to be elucidated, as in our study. ERK5 is essential for cardiovascular development and plays a role in cardiomyocyte hypertrophy. The targeted deletion of the ERK5 gene in mice has provided genetic evidence for an essential role of the ERK5 signaling pathway during heart development [Regan et al., 2002; Yan et al., 2003; Wang et al., 2005]. Some recent studies have implicated ERK5 as a potential signal transducer for cardiac hypertrophy [Takahashi et al., 2005; Shin et al., 2008]. Although the pathways activated by Ang II in cardiomyocytes have been explored in detail, no studies have addressed the role of ERK5. In this study, we provide evidence that ERK5 is markedly activated when cardiomyocytes are exposed to Ang II. When MAPKs translocate to the nucleus, they control transcription to regulate many critical cellular processes, including growth, differentiation, survival, apoptosis, and stress-related responses [Nishimoto and Nishida, 2006; Wang, 2007]. The C-terminal region of ERK5 contains a unique sequence and shows transcriptional activation of MEF2C, peroxisome proliferator activated receptor γ1 and members of the activator protein 1 (AP1) family, c-fos and Fra1 [Akaike et al., 2004; Nishimoto and Nishida, 2006]. ERK5 has a potential role in the transcriptional regulation of hypertrophic genes, in particular MEF2C-dependent genes such as c-*jun*, through direct phosphorylation and activation of the transcription factor MEF2C, and governs hypertrophic growth [Takahashi et al., 2005; Zhao et al., 2009]. Consistent with these findings, our study indicated that Ang II-induced ERK5 phosphorylation resulted in MEF2C activation and cardiomyocyte hypertrophy. Knocking down ERK5 by siRNA significantly attenuated MEF2C activation and [^{3}H]-Leu incorporation. Increased [^{3}H]-Leu incorporation could be involved in increased protein synthesis and the resulting cellular hypertrophy caused by Ang II. Our Ang II treatment increased [^{3}H]-Leu incorporation, which was inhibited by siRNA transfection to silence ERK5 (Fig. 6); these results are consistent with Ang II-induced increase in cell size, as shown by increased cell area.

With respect to the subcellular distribution of ERK5, initial reports indicated that analogous to ERK1/2, ERK5 is a cytoplasmic protein that moves to the nucleus on stimulation in NIH3T3 cells [Yan et al., 2005]. However, other reports challenged this concept by showing the nuclear location of ERK5 in HeLa or Rat-1 cells, independent of their stimulation [Raviv et al., 2004]. To analyze whether this different subcellular location of ERK5 was cell-type specific, we analyzed the subcellular distribution of ERK5 in cardiomyocytes and found ERK5 mainly located in the cytoplasm in unstimulated cardiomyocytes and moving to the nucleus on Ang II stimulation. Dual phosphorylation is critical for ERK5 nuclear entry; a form of ERK5 in which the dual phosphorylation site is mutated is unable to enter into the nucleus, even when coexpressed with constitutively active MEK5 [Raviv et al., 2004; Kondoh et al., 2006]. In agreement with this notion, we found that both the PKC inhibitor Go 6983 and PKCε specific siRNA greatly inhibited the phosphorylation of ERK5, with a significant decrease of ERK5 nucleocytoplasmic shuttling. Recently, a mechanism for the nucleocytoplasmic transport of ERK5 has been proposed, whereby three different regions govern the nucleocytoplasmic distribution [Wang and Tournier, 2006]. One region corresponds to the N-terminus and appears to favor cytoplasmic anchoring of ERK5 [Yan et al., 2001]. The other two regions are at the C-terminal region: one acts as a bipartite nuclear localization sequence [Yan et al., 2001; Kondoh et al., 2006], and the other favors the nuclear export of ERK5 [Buschbeck and Ullrich, 2005]. The balance between nuclear import and export determines the subcellular localization of ERK5 [Kondoh et al., 2006].

Many studies have investigated the intracellular molecular mechanisms that regulate developmental and pathological cardiac

hypertrophy. Among the data obtained, PKC isozymes emerged as potential mediators of PKC hypertrophic stimuli. We used multiple approaches to determine and confirm the specific role of PKCε in mediating Ang II-induced ERK5 activation. The general PKC inhibitor Go 6983 blocked Ang II-induced ERK5 activation in a concentration-dependent manner, which suggests that Ang II induces ERK5 activation through a PKC-dependent pathway in cardiomyocytes. Further findings that Ang II induces activation of PKCε in cardiomyocytes (Fig. 4A) and that the siRNA of PKCε blocked Ang II-induced ERK5 activation (Fig. 4B) strongly suggest the functional involvement of PKCε in Ang II-induced ERK5 activation. PKCε is activated in response to hypertrophic stimuli in cultured myocytes and in vivo [Chen et al., 2001], and overexpression and activation of PKCε results in myocardial hypertrophy [Takeishi et al., 2000]. Our results also revealed that PKCε siRNA significantly reduced [^{3}H]-Leu incorporation, a measure of cell hypertrophy, which indicated that PKCε is involved in hypertrophy of cardiomyocytes.

CONCLUSIONS

In summary, we demonstrate that Ang II induces AT_1-PKCε-dependent ERK5 phosphorylation and translocation. As well, PKCε and ERK5 are involved in Ang II-stimulated MEF2C activation and cardiomyocyte hypertrophy. These results provide new insights into the molecular underpinnings of hypertrophy in cardiomyocytes in response to Ang II. Further studies are needed to define the specific genes regulated by the PKCε-ERK5 pathway. Such progress may provide novel targets for therapeutic manipulation through pharmacological or genetic approaches in pathological cardiac remodeling in hypertension, atherosclerosis, and diabetes.

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REFERENCES

Akaike M, Che W, Marmarosh NL, Ohta S, Osawa M, Ding B, Berk BC, Yan C, Abe J. 2004. The hinge-helix 1 region of peroxisome proliferator-activated receptor gamma 1(PPARgamma 1) mediates interaction with extracellular signal-regulated kinase 5 and PPARgamma 1 transcriptional activation: Involvement in flow-induced PPARgamma activation in endothelial cells. Mol Cell Biol 24:8691–8704.

Behr TM, Nerurkar SS, Nelson AH, Coatney RW, Woods TN, Sulpizio A, Chandra S, Brooks DP, Kumar S, Lee JC, Ohlstein EH, Angermann CE, Adams JL, Sisko J, Sackner-Bernstein JD, Willette RN. 2001. Hypertensive end-organ damage and premature mortality are p38 mitogen-activated protein kinase-dependent in a rat model of cardiac hypertrophy and dysfunction. Circulation 11:1292–1298.

Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, Mintze K, Pickard T, Roden R, Bristow MR, Sabbah HN, Mizrahi JL, Gromo G, King GL, Vlahos CJ. 1999. Increased protein kinase C activity and expression of Ca2+-sensitive isoforms in the failing human heart. Circulation 99:384–391.

Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R, Kimball TF, Lorenz JN, Nairn AC, Liggett SB, Bodi I, Wang S, Schwartz A, Lakatta EG, DePaoli-Roach AA, Robbins J, Hewett TE, Bibb JA, Westfall MV, Kranias EG, Molkentin JD. 2004. PKC-alpha regulates cardiac contractility and propensity toward heart failure. Nat Med 10:248–254.

Brede M, Roell W, Ritter O, Wiesmann F, Jahns R, Haase A, Fleischmann BK, Hein L. 2003. Cardiac hypertrophy is associated with decreased eNOS expression in angiotensin AT2 receptor-deficient mice. Hypertension 42:1177–1182.

Bueno OF, De Windt LJ, Lim HW, Tymitz KM, Witt SA, Kimball TR, Molkentin JD. 2001. The dual-specificity phosphatase MKP-1 limits the cardiac hypertrophic response in vitro and in vivo. Circ Res 88:88–96.

Buschbeck M, Ullrich A. 2005. The unique C-terminal tail of mitogen-activated protein kinase ERK5 regulates its activation and nuclear shuttling. J Biol Chem 280:2659–2667.

Cai J, Yi FF, Bian ZY, Shen DF, Yang L, Yan L, Tang QZ, Yang XC, Li H. 2009. Crocetin protects against cardiac hypertrophy by blocking MEK-ERK1/2 signalling pathway. J Cell Mol Med 13:909–925.

Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW II, Mochly-Rosen D. 2001. Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. Proc Natl Acad Sci USA 98(20): 11114–11119.

Coulombe P, Meloche S. 2007. Atypical mitogen-activated protein kinases: Structure, regulation and functions. Biochim Biophys Acta 1773:1376–1387.

D'Angelo DD, Sakata Y, Lorenz JN, Boivin GP, Walsh RA, Liggett SB, Dorn GW II. 1997. Transgenic Galphaq overexpression induces cardiac contractile failure in mice. Proc Natl Acad Sci USA 94:8121–8126.

Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, Messing RO. 2000. Protein kinase C isozymes and the regulation of diverse cell responses. Am J Physiol Lung Cell Mol Physiol 279:L429–L438.

Dorn GW II, Force T. 2005. Protein kinase cascades in the regulation of cardiac hypertrophy. J Clin Invest 115:527–537.

Dorn GW, Mochly-Rosen D. 2002. Intracellular transport mechanisms of signal transducers. Annu Rev Physiol 64:407–429.

Elbashir SM, Harborth J, Weber K, Tuschl T. 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26:199–213.

Fabris B, Candido R, Bortoletto M, Zentilin L, Sandri M, Fior F, Toffoli B, Stebel M, Bardelli M, Belgrado D, Giacca M, Carretta R. 2007. Dose- and time-dependent apoptotic effects by angiotensin II infusion on left ventricular cardiomyocytes. J Hypertens 25:1481–1490.

Finn SG, Dickens M, Fuller SJ. 2001. c-Jun N-terminal kinase/interacting protein 1 inhibits gene expression in response to hypertrophic agonists in neonatal rat ventricular myocytes. Biochem J 358:489–495.

García-Hoz C, Herranz M, Teresa Díaz-Meco M, Moscat J, Ribas C, Mayor F, Jr. 2007. PKC delta mediated GalphaQ stimulation of the ERK5 pathway plays a key role in cardiac hypertrophy. J Mol Cell Cardiol 42:S37–S54.

Griendling KK, Lassegue B, Alexander RW. 1996. Angiotensin receptors and their therapeutic implications. Annu Rev Pharmacol Toxicol 36:281–306.

Gu X, Bishop SP. 1994. Increased protein kinase C and isozyme redistribution in pressure-overload cardiac hypertrophy in the rat. Circ Res 75:926–931.

Joerg H, Jeffery DM. 2006. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 7:589–600.

Kondoh K, Terasawa K, Morimoto H, Nishida E. 2006. Regulation of c-Fos and Fra-1 by the MEK5-ERK5 pathway. Mol Cell Biol 26:1679–1690.

Luo JD, Xie F, Zhang WW, Ma XD, Guan JX, Chen X. 2001. Simvastatin inhibits noradrenaline-induced hypertrophy of cultured neonatal rat cardiomyocytes. Br J Pharmacol 132:159–164.

Mehta PK, Griendling KK. 2007. Angiotensin II cell signaling: Physiological and pathological effects in the cardiovascular system. Am J Physiol Cell Physiol 292:C82–C97.

Minamino T, Komuro I. 2007. Vascular cell senescence: Contribution to atherosclerosis. Circ Res 100(1): 15–26.

Mollmann H, Schmidt-Schweda S, Nef H, Mollmann S, Burtsin JV, Klose S, Elsässer A, Holubarsch CJ. 2007. Contractile effects of angiotensin and endothelin in failing and non-failing human hearts. Int J Cardiol 114:34–40.

Murray NR, Thompson LJ, Fields AP. 1997. The role of protein kinase C in cellular proliferation and cell cycle control. In: Parker PJ, Dekker L, editors. Molecular biology intelligence unit. Austin, Texas, USA: Landes. pp 97–120.

Nicol RL, Frey N, Olson EN. 2000. From the sarcomere to the nucleus: Role of genetics and signaling in structural heart disease. Annu Rev Genomics Hum Genet 1:179–223.

Nishimoto S, Nishida E. 2006. MAPK signalling: ERK5 versus ERK1/2. EMBO Rep 7:782–786.

Ozgen N, Obreztchikova M, Guo J, Elouardighi H, Dorn GW II, Wilson BA, Steinberg SF. 2008. Protein kinase D links Gq-coupled receptors to cAMP response element-binding protein (CREB)-Ser133 phosphorylation in the heart. J Biol Chem 283:17009–17019.

Pellieux C, Sauthier T, Aubert JF, Brunner HR, Pedrazzini T. 2000. Angiotensin II-induced cardiac hypertrophy is associated with different mitogen-activated protein kinase activation in normotensive and hypertensive mice. J Hypertens 18:1307–1317.

Poizat C, Sartorelli V, Chung G, Kloner RA, Kedes L. 2000. Proteasome-mediated degradation of the coactivator p300 impairs cardiac transcription. Mol Cell Biol 20:8643–8654.

Raviv Z, Kalie E, Seger R. 2004. MEK5 and ERK5 are localized in the nuclei of resting as well as stimulated cells, while MEKK2 translocates from the cytosol to the nucleus upon stimulation. J Cell Sci 117:1773–1784.

Regan CP, Li W, Boucher DM, Spatz S, Su MS, Kuida K. 2002. Erk5 null mice display multiple extraembryonic vascular and embryonic cardiovascular defects. Proc Natl Acad Sci USA 99:9248–9253.

Sabri A, Steinberg SF. 2003. Protein kinase C isoform-selective signals that lead to cardiac hypertrophy and the progression of heart failure. Mol Cell Biochem 251:97–101.

Schlüter KD, Wenzel S. 2008. Angiotensin II: A hormone involved in and contributing to pro-hypertrophic cardiac networks and target of anti-hypertrophic cross-talks. Pharmacol Ther 119:311–325.

Sharma G, Goalstone ML. 2007. Regulation of ERK5 by insulin and angiotensin-II in vascular smooth muscle cells. Biochem Biophys Res Commun 354:1078–1083.

Shin SY, Yang JM, Choo SM, Kwon KS, Cho KH. 2008. System-level investigation into the regulatory mechanism of the calcineurin/NFAT signaling pathway. Cell Signal 20:1117–1124.

Steinberg SF. 2008. Structural basis of protein kinase C isoform function. Physiol Rev 88:1341–1378.

Takahashi N, Saito Y, Kuwahara K, Harada M, Tanimoto K, Nakagawa Y, Kawakami R, Nakanishi M, Yasuno S, Usami S, Yoshimura A, Nakao K. 2005. Hypertrophic responses to cardiotrophin-1 are not mediated by STAT3, but via a MEK5-ERK5 pathway in cultured cardiomyocytes. J Mol Cell Cardiol 38:185–192.

Takeishi Y, Ping P, Bolli R, Kirkpatrick DL, Hoit BD, Walsh RA. 2000. Transgenic overexpression of constitutively active protein kinase C epsilon causes concentric cardiac hypertrophy. Circ Res 86(12): 1218–1223.

Touyz RM, Berry C. 2002. Recent advances in angiotensin II signaling. Braz J Med Biol Res 35:1001–1015.

Vega RB, Harrison BC, Meadows E, Roberts CR, Papst PJ, Olson EN, McKinsey TA. 2004. Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol Cell Biol 24:8374–8385.

Wang Y. 2007. Mitogen-activated protein kinases in heart development and diseases. Circulation 116:1413–1423.

Wang X, Tournier C. 2006. Regulation of cellular functions by the ERK5 signalling pathway. Cell Signal 18:753–760.

Wang Y, Su B, Sah VP, Brown JH, Han J, Chien KR. 1998. Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH_2-terminal kinase in ventricular muscle cells. J Biol Chem 273:5423–5426.

Wang X, Merritt AJ, Seyfried J, Guo C, Papadakis ES, Finegan KG, Kayahara M, Dixon J, Boot- Handford RP, Cartwright EJ, Mayer U, Tournier C. 2005. Targeted deletion of mek5 causes early embryonic death and defects in the extracellular signal-regulated kinase 5/myocyte enhancer factor 2 cell survival pathway. Mol Cell Biol 25:336–345.

Yan C, Luo H, Lee JD, Abe J, Berk BC. 2001. Activation of a C-terminal transcriptional activation domain of ERK5 by autophosphorylation. J Biol Chem 276:10870–10878.

Yan L, Carr J, Ashby PR, Murry-Tait V, Thompson C, Arthur JS. 2003. Knockout of ERK5 causes multiple defects in placental and embryonic development. BMC Dev Biol 3:11.

Yan Y, Rubinchik S, Watson PM, Kelley JR, Fraser MM, Wood AL, Dong JY, Gillanders WE, Boylan AM, Watson DK, Cole DJ. 2005. Yan Establishing a murine pancreatic cancer CaSm model: up-regulation of CaSm is required for the transformed phenotype of murine pancreatic adenocarcinoma. Mol Ther 11:363–372.

Yutao X, Geru W, Xiaojun B, Tao G, Aquin M. 2006. Mechanical stretch-induced hypertrophy of neonatal rat ventricular myocytes is mediated by β(1)-integrin-microtubule signalling pathways. Eur J Heart Fail 8: 16–22.

Zhan Y, Brown C, Maynard E, Anshelevich A, Ni W, Ho IC, Oettgen P. 2005. Ets-1 is a critical regulator of Ang II-mediated vascular inflammation and remodeling. J Clin Invest 115:2508–2516.

Zhao Z, Geng J, Ge Z, Wang W, Zhang Y, Kang W. 2009. Activation of ERK5 in angiotensin II-induced hypertrophy of human aortic smooth muscle cells. Mol Cell Biochem 322:171–178.

Zou XJ, Yang L, Yao SL. 2008. Propofol depresses angiotensin II-induced cardiomyocyte hypertrophy in vitro. Exp Biol Med (Maywood) 233:200–208.