ARTICLE
Journal of Cellular Biochemistry 106:823–834 (2009)

Journal of Cellular Biochemistry

# PPARδ Activation Inhibits Angiotensin II Induced Cardiomyocyte Hypertrophy by Suppressing Intracellular $Ca^{2+}$ Signaling Pathway

Kuy-Sook Lee,[1] Jin-Hee Park,[1] Seahyoung Lee,[2] Hyun-Joung Lim,[1] and Hyun-Young Park[1*]

[1]*Division of Cardiovascular and Rare Diseases, Center for Biomedical Sciences, National Institute of Health, Seoul, Korea*

[2]*Institute of Polymer Research, GKSS Research Center Geesthacht, Teltow, Germany*

## ABSTRACT

Peroxisome proliferator-activated receptors δ (PPARδ) is known to be expressed ubiquitously, and the predominant PPAR subtype of cardiac cells. However, relatively less is known regarding the role of PPARδ in cardiac cells except that PPARδ ligand treatment protects cardiac hypertrophy by inhibiting NF-κB activation. Thus, in the present study, we examined the effect of selective PPARδ ligand L-165041 on angiotensin II (AngII) induced cardiac hypertrophy and its underlying mechanism using cardiomyocyte. According to our data, L-165041 (10 μM) inhibited AngII-induced [$^3$H] leucine incorporation, induction of the fetal gene atrial natriuretic factor (ANF) and increase of cardiomyocyte size. Previous studies have implicated the activation of focal adhesion kinase (FAK) in the progress of cardiomyocyte hypertrophy. L-165041 pretreatment significantly inhibited AngII-induced intracellular $Ca^{2+}$ increase and subsequent phosphorylation of FAK. Further experiment using $Ca^{2+}$ ionophore A23187 confirmed that $Ca^{2+}$ induced FAK phosphorylation, and this was also blocked by L-165041 pretreatment. In addition, overexpression of PPARδ using adenovirus significantly inhibited AngII-induced intracellular $Ca^{2+}$ increase and FAK expression, while PPARδ siRNA treatment abolished the effect of L-165041. These data indicate that PPARδ ligand L-165041 inhibits AngII induced cardiac hypertrophy by suppressing intracellular $Ca^{2+}$/FAK/ERK signaling pathway in a PPARδ dependent mechanism. J. Cell. Biochem. 106: 823–834, 2009. 

**KEY WORDS:** PPARδ; FOCAL ADHESION KINASE; CALCIUM; CARDIOMYOCYTE HYPERTROPHY

Cardiac hypertrophy is an adaptive response of the heart to variety of intrinsic and extrinsic stimuli, such as hypertension, myocardial infarction, valvular diseases. Although hypertrophy starts as a compensatory response, prolonged hypertrophy can be deleterious and result in cardiomyopathy, heart failure, and sudden death [Levy et al., 1990; Lorell and Carabello, 2000; Heineke and Molkentin, 2006].

Angiotensin II (AngII) is the main effector molecule of the rennin-angiotensin system responsible for the pressure and volume homeostasis. Being mediated by the AT1 receptor, AngII can induce cardiac hypertrophy [Sadoshima and Izumo, 1993; Miyata and Haneda, 1994; Booz and Baker, 1996]. Activation of AT1 receptors leads to an increase in tyrosine phosphorylation of several signal molecules such as Shc and PLC, and as well as several non-receptor tyrosine kinases including focal adhesion kinase (FAK), JAK2 and Src that are known to activate cellular migration, adhesion and mitogenic and hypertrophic processes [Leduc and Meloche, 1995; Sadoshima and Izumo, 1996; McWhinney et al., 1997; Hunt et al., 1999].

Previous studies have demonstrated that FAK signaling plays a key role in cardiomyocyte hypertrophy, not only in response to mechanical stress, but also in response to soluble factors such as the adrenergic agonist PE, ET-1, and AngII [Eble et al., 2000; Taylor et al., 2000; Torsoni et al., 2003; Qin and Liu, 2006].

In particular, AngII promotes cell migration and induces changes of the cell shape and volume by activating FAK dependent signaling cascades [Leduc and Meloche, 1995]. FAK is a 125 kDa protein tyrosine kinase localized to focal adhesion complexes and widely expressed in different tissues and organs [Hildebrand et al., 1993]. Autophosphorylation of Tyr397, a critical residue in FAK-mediated signaling [Schlaepfer et al., 1999], leads to recruitment of SH2 and SH3 domain-containing kinases such as Fyn and Src, and

Grant sponsor: Korea National Institute of Health; Grant number: 4800-4845-300-210.
*Correspondence to: Dr. Hyun-Young Park, MD, PhD, Center for Biomedical Sciences, National Institute of Health, Seoul, Korea. E-mail: hypark65@nih.go.kr
Received 22 September 2008; Accepted 2 December 2008 • DOI 10.1002/jcb.22038 • 2009 Wiley-Liss, Inc.
Published online 17 February 2009 in Wiley InterScience (www.interscience.wiley.com).

subsequent phosphorylation of other cytoplasmic plaque proteins such as paxillin, tensin, and p130CAS [Turner et al., 1995; Parsons, 1996; Zhu et al., 1998]. FAK is activated by phosphorylation of one or more tyrosine residues in response to stimuli such as activation of G-protein-coupled receptors that subsequently elevate intracellular $Ca^{2+}$ level or activate PKC and Src [Weng and Shukla, 2002; Yin et al., 2003]. Furthermore, ERK or JNK MAP kinase cascades can be resulted by FAK phosphorylation in cardiac hypertrophy [Schlaepfer et al., 1999; Govindarajan et al., 2000; Weng and Shukla, 2002].

The ligand-activated nuclear receptor peroxisome proliferator-activated receptors δ (PPARδ) is the predominant PPAR subtype in cardiac cells [Gilde et al., 2003] and acts as an important regulator of genes related with cardiac lipid metabolism.

Recently, it has been reported that cardiomyocyte-restricted deletion of PPARδ downregulated constitutive expression of key fatty acid oxidation genes in mice and these mice developed cardiac dysfunction, cardiac hypertrophy and congestive heart failure with reduced survival rate [Cheng et al., 2004]. In addition, PPARβ/δ activation inhibited PE-induced cardiac hypertrophy and LPS-induced NF-κB activation [Planavila et al., 2005]. Nevertheless, relatively less is known regarding the role of PPARδ in cardiac cells. Thus, in the present study, we examined the effect of selective PPARδ ligand L-165041 on AngII-induced cardiac hypertrophy and our data suggest that PPARδ inhibits AngII induced cardiac hypertrophy by suppressing intracellular $Ca^{2+}$/FAK/ERK signaling pathway.

## MATERIALS AND METHODS

### MATERIALS

Cell culture reagents and oligo(dT)$_{12-18}$ were from Invitrogen (Carlsbad, CA). AngII and mouse monoclonal antibody against β-actin were purchased from Sigma (St. Louis, MO). L-165041 was purchased from Tocris Bioscience (Bristol, UK). Peroxidase-conjugated anti-rabbit secondary antibody and anti-mouse secondary antibody were from Jackson ImmunoReserach (West Grove, PA). Accupower RT premix and Accupower PCR premix were purchased from Bioneer (Seoul, Korea). Calcium Green™-1, AM and Alexa Flour® 594-conjugated goat anti-mouse IgG were from Molecular Probes (Eugene, OR). RNeasy mini kit was from Qiagen (Valencia, CA). Phospho-FAK antibody was from BD Biosciences (San Jose, CA).

### CELL CULTURE

The embryonic rat-heart derived H9c2 cells were purchased from ATCC and were maintained in DMEM containing 10% FBS. Cells were starved for 4 h in DMEM containing 0.1% FBS before any experimental treatment.

Neonatal rat cardiomyocytes were prepared as described elsewhere with minor modification [Wang et al., 1999]. Briefly, hearts harvested from 1 to 3 days old Sprague–Dawley rat (obtained from Orient, Seoul, Korea) were washed in PBS ($Mg^{2+}$, $Ca^{2+}$ free). Hearts were minced with collagenase type II (1 mg/ml, 262 U/mg, GibcoBRL) in sterile HBSS and incubated for 5 min. The pellet was collected in HBSS containing collagenase type II and incubated for 5 min. The supernatant was collected in α-MEM containing 10% FBS and digestion steps were repeated up to 12 times. Cardiomyocytes were purified from fibroblasts using repeated centrifugation step. Finally, cardiomyocytes were resuspended in α-MEM containing 10% FBS and 0.1 mM BrdU. Cells were then cultured in α-MEM containing 10% FBS at 37°C for 2–3 days in a humidified atmosphere containing 5% $CO_2$ and 95% air. More than 90% of cells were cardiomyocytes (positive for Troponin I and beating feature).

### [$^3$H]-LEUCINE INCORPORATION ASSAY

Protein synthesis assays were performed using cells plated in 96-well plates at a density of $3.5 \times 10^3$ cells/well. Cells were starved in DMEM with 0.1% FBS for 4 h, then preincubated with L-165041 before AngII treatment. Twenty-four hours after AngII treatments, 1 μCi/ml of [$^3$H]-leucine was added and the cells were incubated for additional 4 h before transferred to a filtermat (PerkinElmer, MA) using a Tomtec havester 96 (Hamden, CT). The amount of [$^3$H]-leucine incorporated was measured by a Wallac microbeta Trilux 1450 counter (PerkinElmer).

### IMMUNOCYTOCHEMISTRY

Cultured cells were stained by an indirect immunofluorescence method. Cultured cells were fixed with 4% paraformaldehyde at room temperature for 30 min and washed three times with PBS. Anti-α-actinin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a primary antibody in 1% BSA–PBS. The primary antibody was detected with Alexa Flour® 594-conjugated goat anti-mouse IgG (Molecular Probes). Immunofluorescence was visualized using a confocal laser fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Cell sizes were measured using Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

### REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT–PCR) AND QUANTITATIVE REAL-TIME PCR (qRT–PCR) ANALYSIS

Total RNA was extracted with RNeasy mini kit (Qiagen) according to the manufacturer's recommendations. From 500 ng of total RNA, cDNAs were synthesized using oligo(dT)$_{12-18}$ primer and Accupower RT premix. PCR was done using Accupower PCR premix. Primers used are the following: rat ANF, 5′-TCC TCT TCC TGG CCT TTT GGC-3′ and 5′-AGA CGG GTT GCT TCC CCA GTC-3′; rat MCD, 5′-TGG CCT CTT CCA CAT CAG-3′ and 5′-TTC AGA CGG AGG GCA CTC TTT C-3′; rat PDK4, 5′-AGC TGC TGG ACT TCG GTT CAG-3′ and 5′-GAG GAT GTG CTG GTT CAT GAG C-3′; rat MCPT-1, 5′-TTC ACT GTG ACC CCA GAC GGG-3′ and 5′-AAT GGA CCA GCC CCA TGG AGA-3′; rat β-actin, 5′-CCC ATT GAA CAC GGC ATT GTC-3′ and 5′-CGC ACG ATT TCC CTC TCA GC-3′.

For qRT-PCR, high capacity cDNA reverse transcription kits and one-step SYBR GREEN PCR master mix kits (Applied Biosystems, Foster City, CA) were used. qRT-PCR analysis was performed using an ABI prism 7900 (Applied Biosystems), and the primer sequences are listed in Table I. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was used as a calibrator for the calculation of relative mRNA levels of individual genes.

TABLE I. Primers Used for Quantitative Real-Time PCR Analysis

| Name | Sequence | Accession no. |
|---|---|---|
| ANF | | |
| Sense | aaa ctg agg gct ctg ctc gct g | NM_012612 |
| Anti-sense | tgt tgc agc cta gtc cgc tct g | |
| MCD | | |
| Sense | atg gac atg aag cgg cgt gtg | AF304865 |
| Anti-sense | tgt cac cgg tca gag cca cat g | |
| PDK4 | | |
| Sense | atg aag gca gcc cgt ttc | NM_053551 |
| Anti-sense | tgt gaa ccg aag tcc agc ag | |
| MCPT-1 | | |
| Sense | gac cag cca tgc cac caa gat c | NM_013200 |
| Anti-sense | tga att gtg gct ggc aca ctg g | |
| GAPDH | | |
| Sense | ctg gaa agc tgt ggc gtg atg | NM_017008 |
| Anti-sense | ttc agc tct ggg atg acc ttg c | |

### WESTERN BLOT ANALYSIS

Cells were starved for 4 h in DMEM containing 0.1% FBS before any experimental treatment. Equal amounts of cell lysates were separated by SDS–PAGE gel. Membranes were incubated with appropriate primary antibodies, and the immunopositive bands were visualized by ECL system (Amersham Biosciences, Buckinghamshire, England). Each experiment was triplicated at least.

### INTRACELLULAR $CA^{2+}$ IMAGING

Cells starved in DMEM containing 0.1% FBS for 4 h were loaded with 1 μM fluorescence probe Calcium Green™-1, AM (Molecular Probes) for 3 h and then treated with AngII for 1 h. Cells were washed with PBS twice and the immunofluorescence was detected using a confocal laser fluorescence microscope (Carl Zeiss, Inc.).

### ADENOVIRUS INFECTION

Adenovirus carrying PPARδ was a kind gift from E. J. Lee (Yonsei University, College of Medicine, Seoul, Korea). Cells were transfected with 10–100 multiplicity of infection (MOI) of adenovirus. After 5 h, medium was changed to DMEM containing 0.1% FBS. Cells were incubated for 48 h after infection and then treated with AngII.

### siRNA TRANSFECTION

For transfection, the cells were grown to 20–30% confluence in DMEM containing 10% FBS. FAK or PPARδ siRNA was delivered into H9c2 cells using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, RT-PCR and Western blot were used to evaluate the silencing effect of siRNA on FAK or PPARδ expression. Rat siRNA sequences are following; FAK, sense 5′-GAA GCU GAU UGG AGU CAU UAC AGdA dG-3′, anti-sense 5′-CUC UGU AAU GAC UCC AAU CAG CUU CAC-3′ and PPARδ, sense 5′-CCA CAA CGC UAU CCG CUU UGG AAdG dG-3′, anti-sense 5′-CCU UCC AAA GCG GAU AGC GUU GUG GGA-3′.

### STATISTICAL ANALYSIS

Data are represented as means ± SEM of more than three separate experiments. Significant difference between the respective control and each experimental test condition was assessed by using Student's *t*-test for each paired experiments. *P* value $<0.05$ was considered statistically significant.

## RESULTS

### PPARδ LIGAND, L-165041 INHIBITS ANGII-INDUCED CARDIAC HYPERTROPHY AND ASSOCIATED GENE EXPRESSION

We examined the effect of selective PPARδ ligand L-165041 on AngII induced cardiac hypertrophy using cardiomyocyte. After 1 h of 10 μM L-165041 pretreatment, cardiomyocytes were stimulated with 100 nM AngII for additional 24 h. As shown in Figure 1A, [$^3$H]-leucine incorporation was significantly increased by AngII and this was inhibited by L-165041. Immunostaining of α-actinin showed 100 nM of AngII increased cell size, and this increase was also blocked by pretreatment of L-165041 (Fig. 1B). AngII-induced fetal gene expression such as atrial natriuretic factor (ANF) was also suppressed by L-165041 pretreatment at the mRNA level (Fig. 1C). Since decrease of fatty acid oxidation is also an important molecular characteristic of cardiac hypertrophy, we further examined the mRNA levels of those genes involved in fatty acid metabolism. According to our data, AngII treatment decreased or significantly inhibited fatty acid metabolism related gene expressions such as malonyl-CoA decarboxylase (MCD), pyruvate dehydrogenase kinase 4 (PDK-4) and muscle-type carnitine palmitoyltransferase-1 (MCPT-1). However, this decrease of expression induced by AngII was completely abolished with L-165041 pretreatment (Fig. 1D).

### L-165041 INHIBITS ANGII-INDUCED PHOSPHORYLATION OF FAK

Activation of FAK has been implicated both in the progress of cardiomyocyte hypertrophy and in promoting expression of the hypertrophic response genes ANP and brain natriuretic peptide (BNP) [Kovacic-Milivojevic et al., 2001]. We also examined the effect of L-165041 on the phosphorylation of FAK induced by AngII. H9c2 cells were stimulated with 100 nM of AngII for 10–120 min and subjected to Western blot using p-FAK antibody. Our data show that FAK phosphorylation was increased with AngII treatment and L-165041 attenuated this increase (Fig. 2A). However, L-165041 treatment without subsequent AngII treatment had no significant effect on FAK phosphorylation (Fig. 2B).

### L-165041 ABROGATES ANGII-INCREASED INTRACELLULAR $CA^{2+}$ LEVEL IN CARDIOMYOCYTES

To identify which signaling pathway was involved in the observed effect of L-165041, H9c2 cells were incubated with AngII for 2 h in the presence of several key signaling pathway inhibitors (intracellular $Ca^{2+}$ chelator: BAPTA-AM 10 μM, PKC inhibitor: chelerythrine 1 μM and PKC inhibitor peptide 10 μM, Src inhibitor: PP2 10 μM). Among these inhibitors, only BAPTA-AM inhibited AngII induced FAK phosphorylation suggesting $Ca^{2+}$ signaling pathway was involved (Fig. 3A). In addition, BAPTA-AM (10 μM) also inhibited AngII-induced ANF mRNA gene expression and reversed the decrease of fatty acid oxidation related gene (MCD, PKD4, and MCPT-1) expression in cultured H9c2 cells (Fig. 3B).

To examine the effect of L-165041 on the intracellular $Ca^{2+}$ level, the cells were loaded with 1 μM fluorescence probe Calcium Green™-1, AM for 3 h and then treated with AngII for 1 h. AngII

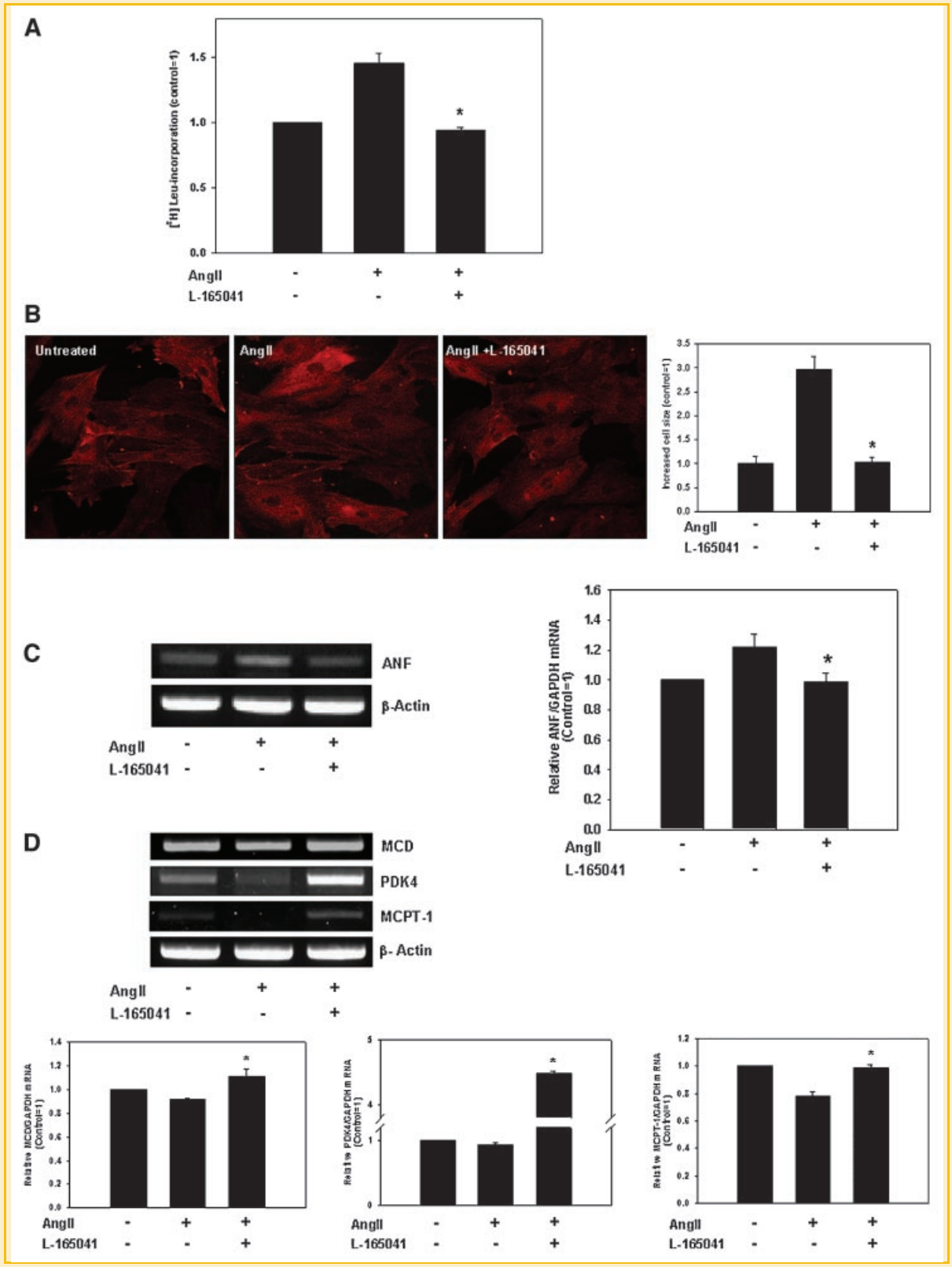


Fig. 1. L-165041 inhibits AngII-induced cardiac hypertrophy and associated gene expression. H9c2 cells were stimulated with 100 nM AngII for 24 h. For L-165041 group, the cells were pretreated with 10 μM of L-165041 for 1 h before the AngII treatment. A: Effect of L-165041 on protein synthesis induced by AngII was assessed using [$^3$H]-leucine incorporation assay. B: Immunofluorescent microscopy using specific antibody to α-actinin. C,D: Total mRNAs were prepared and the mRNA expressions of ANF, MCD, PDK4, and MCPT-1 were evaluated by RT-PCR using specific primers. Also the results of qRT-PCR analysis are represented in a bar graph format. Data are represented as the average of at least triplicated independent experiments ± SEM; $^*P < 0.05$.

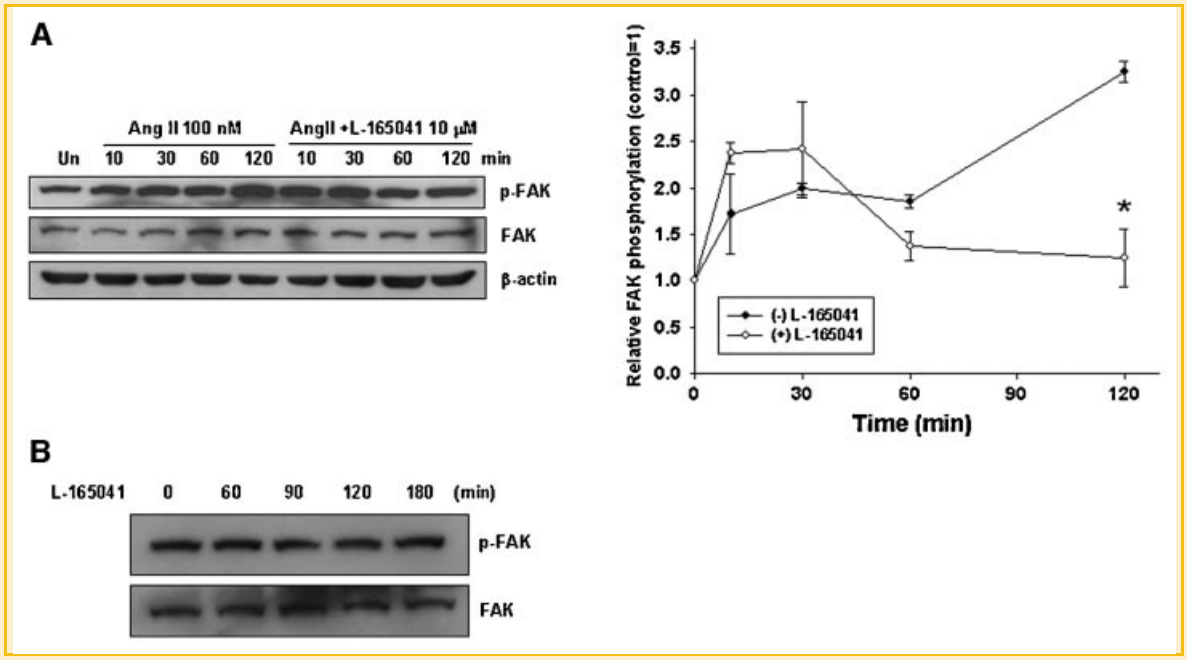


Fig. 2. L-165041 inhibits phosphorylation of FAK. A: Left panel: H9c2 cells were stimulated by 100 nM AngII with or without 1 h pretreatment of 10 μM L-165041, and collected at the time indicated. The cell lysates were subjected to Western blot using p-FAK antibody. Right panel: The intensity of the corresponding p-FAK immunopositive bands is quantified using densitometry. Data are represented as the average of at least triplicated independent experiments ± SEM; $^{*}P < 0.05$. B: H9c2 cells were stimulated with 10 μM of L-165041 treatment for the indicated time periods. The cell lysates were subjected to Western blot using p-FAK antibody.

increased intracellular $Ca^{2+}$ level and this was significantly abrogated by L-165041 pretreatment (Fig. 3C). We further examined whether this increase of $Ca^{2+}$ was responsible for the activation of FAK signaling pathway using $Ca^{2+}$ ionophore A23187. According to our data, 1 μM of A23187 induced phosphorylation of FAK as early as 5 min and this was suppressed by L-165041 pretreatment (Fig. 3D).

### ANGII-INDUCED ERK ACTIVATION IS ATTENUATED BY L-165041 TREATMENT IN CULTURED CARDIOMYOCYTES

Previous studies have shown that phosphorylation of FAK affects downstream signal molecules such as ERK or JNK in cardiac hypertrophy [Govindarajan et al., 2000]. Thus, we also examined whether L-165041 or BAPTA-AM can inhibit AngII-induced ERK or JNK activation. Our Western blot data show that AngII-induced ERK activation was inhibited by both L-165041 and BAPTA-AM pretreatment in cultured cardiomyocytes. However, JNK pathway was not affected by neither AngII nor L-165041 (Fig. 4A,B). We further examined the effect of FAK siRNA (10–100 nM) on ERK phosphorylation. First, 10 and 50 nM (or higher) of FAK siRNA treatment decreased FAK expression approximately 57% and 75%, respectively (Fig. 4C). Furthermore, AngII-induced ERK phosphorylation was significantly decreased in cells transfected with 50 nM of FAK siRNA for 48 h (Fig. 4D).

### PPARδ SIGNIFICANTLY INHIBITED ANGII-INDUCED FAK PHOSPHORYLATION AND INTRACELLULAR $CA^{2+}$ INCREASE

To determine whether the observed effect of L-165041 was PPARδ dependent, we performed further experiments using PPARδ siRNA and adenovirus carrying PPARδ. Cardiomyocytes were transfected with adenoviral LacZ or PPARδ for 48 h (10–100 MOI) and then the cells were treated with AngII (100 nM) for 2 h. As shown in Figure 5A, AngII-induced FAK phosphorylation was significantly inhibited by PPARδ overexpression at 100 MOI compared to non-transfected cells or cells transfected with adenoviral LacZ. Overexpression of PPARδ also inhibited AngII-induced increase of intracellular $Ca^{2+}$ level in cardiomyocytes (Fig. 5B). Furthermore, as shown in Figure 5C, the effect of L-165041 was blocked in the cells transfected with PPARδ siRNA (100 nM) for 48 h.

To examine whether this observed effect of L-165041 was a non-genomic effect or a genomic effect involving transcription step, we used a transcription inhibitor actinomycin D. Our data show that actinomycin D pretreatment failed to reverse or abolish the effect of L-165041 suggesting the observed effect of L-165041 was a non-genomic effect (Fig. 5D).

### PPARδ ATTENUATED ANGII-INDUCED CARDIAC HYPERTROPHY THROUGH THE INHIBITION OF INTRACELLULAR $CA^{2+}$ INCREASE AND SUBSEQUENT FAK PHOSPHORYLATION IN PRIMARY CARDIOMYOCYTES

To eliminate the possibility that embryonic heart derived cell H9c2 behaves differently from physiological cardiomyocyte in response to PPARδ, we performed additional experiments using cultured primary cardiomyocytes.

AngII treatment induced fetal gene ANF expression in primary cardiomyocytes, and this was again significantly decreased by L-165041 pretreatment. L-165041 pretreatment reversed the AngII induced decrease of fatty acid oxidation related gene (MCD, PKD4,

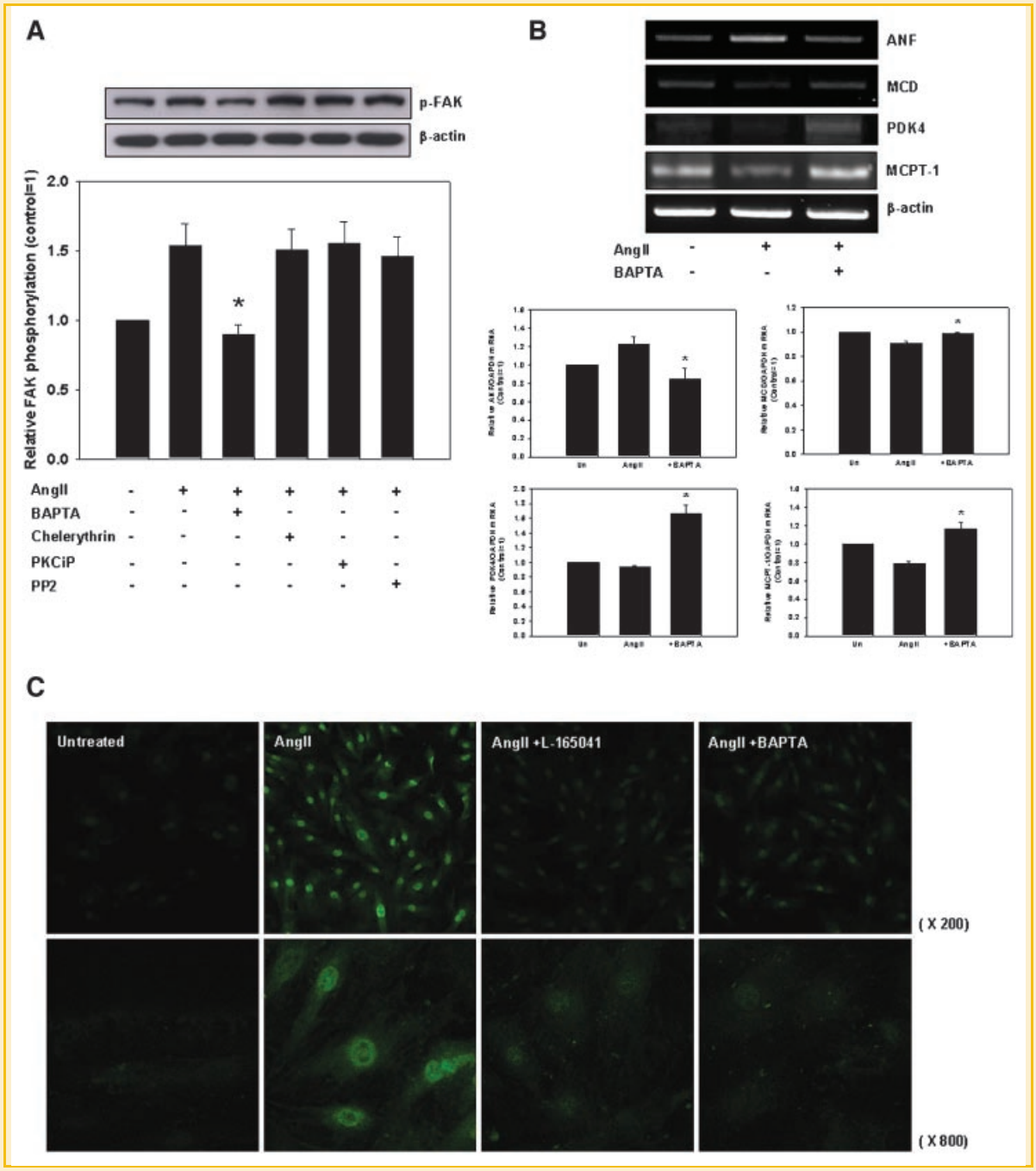


Fig. 3. L-165041 abrogates AngII-increased intracellular $Ca^{2+}$ level and FAK phosphorylation. A: H9c2 cells were stimulated by AngII (100 nM) with or without 1 h pretreatment of various inhibitors (10 μM of BAPTA-AM; 1 μM of chelerythrine, 10 μM of PKC inhibitor peptide, 10 μM of PP2). The cells were collected 2 h after AngII stimulation and subjected to Western blot using p-FAK antibody. Data are represented as the average of at least triplicated independent experiments ± SEM; $^{*}P < 0.05$. B: H9c2 cells were stimulated with 100 nM of AngII for 24 h. For BAPTA-AM group, the cells were pretreated with 10 μM of BAPTA-AM for 1 h before the AngII treatment. Total mRNAs were prepared and the mRNA expressions of ANF, MCD, PDK4, and MCPT-1 were evaluated by RT-PCR using specific primers. The results of qRT-PCR analysis are represented in a bar graph format. C: Starved H9c2 cells were loaded with 1 μM of fluorescence probe Calcium Green™-1, AM for 3 h and then stimulated by 100 nM of AngII for 1 h with or without pretreatment of 10 μM of L-165041 or 10 μM of BAPTA-AM depend on the experimental group. Immunofluorescence was detected using a confocal laser fluorescence microscope. D: H9c2 cells were stimulated by $Ca^{2+}$ ionophore, 1 μM of A23187 with or without 1 h pretreatment of 10 μM of L-165041, and collected at the time indicated. The cell lysates were subjected to Western blot using p-FAK antibody. The relative intensity of the corresponding p-FAK immunopositive bands are represented in a line graph format. Data are represented as the average of at least triplicated independent experiments ± SEM; $^{*}P < 0.05$.

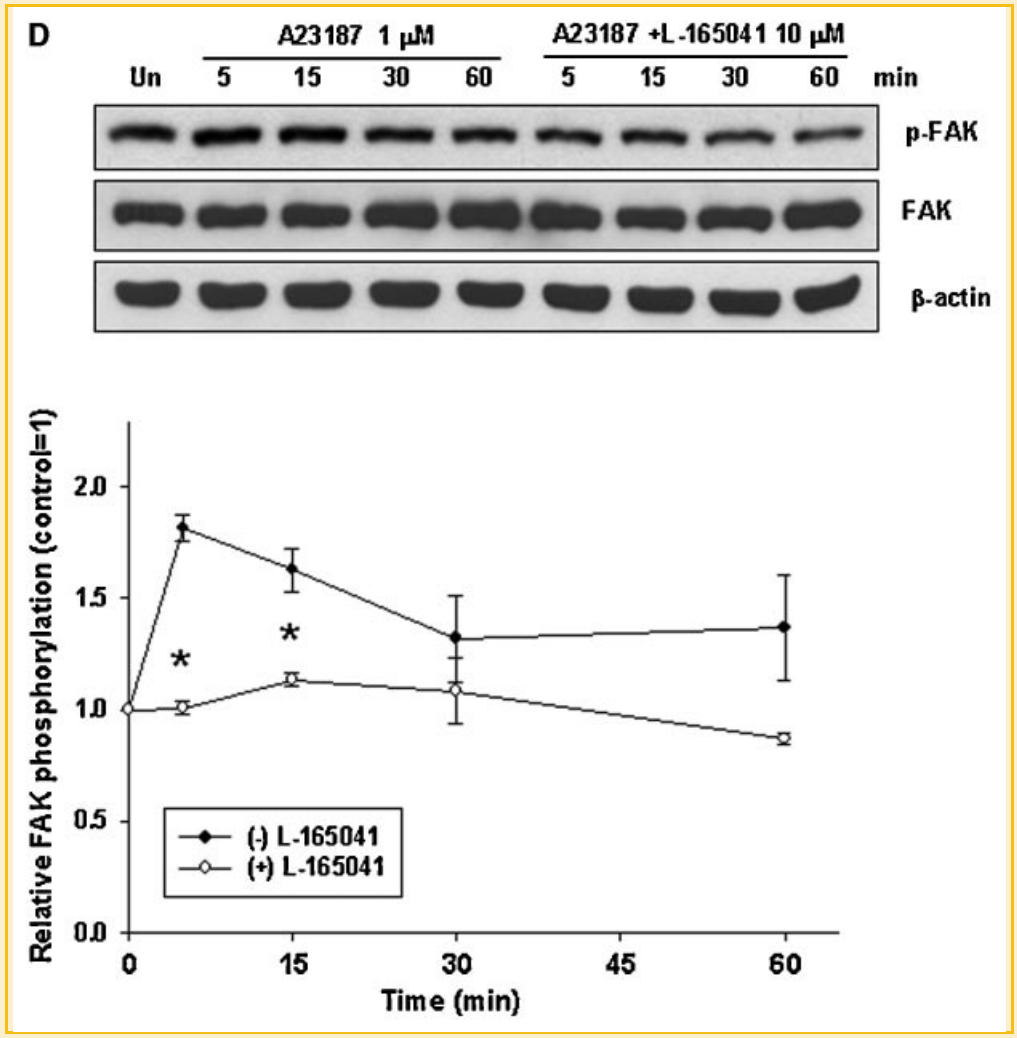


Fig. 3. (*Continued*)

and MCPT-1) expressions also (Fig. 6A). Furthermore, AngII induced FAK phosphorylation and subsequent ERK phosphorylation was also significantly decreased by L-165041 or BAPTA-AM pretreatment (Fig. 6B). Lastly, AngII induced intracellular $Ca^{2+}$ level was also suppressed by L-165041 or BAPTA-AM pretreatment (Fig. 6C).

## DISCUSSION

It has been reported that PPARδ may play an important role in cardiovascular diseases as a dominant isoform of PPAR in cardiac cells [Gilde et al., 2003]. However, relatively less is known regarding its role in cardiac cells except that PPARδ ligand treatment protects cardiac hypertrophy by inhibiting NF-κB activation [Planavila et al., 2005]. Thus, in this study we tried to elucidate the effect of PPARδ on AngII induced cardiac hypertrophy and its underlying mechanism. Our data show that PPARδ activation attenuates cardiac hypertrophy through the inhibition of intracellular $Ca^{2+}$ increase and subsequent FAK phosphorylation.

Once cardiac hypertrophy sets in motion, it induces various molecular characteristic changes such as cell size increase, re-expression of fetal genes such as ANF, and downregulation of fatty acid oxidation related genes [Sugden, 2002]. In the present study, these typical characteristics of hypertrophy were induced by AngII treatment and this was suppressed by PPARδ ligand L-165041. L-165041 is a cell-permeable phenoxyacetic acid derivative described as a potent and selective PPARδ ligand [Berger et al., 1999]. Although L-165041 has been reported to have weak PPARγ activity in the micromolar concentration [Berger et al., 1999; Seimandi et al., 2005], we could not detect PPARγ mRNA in neonatal cardiomyocyte and H9c2 cells (data not shown). Therefore, we eliminated the possibility that the observed anti-hypertrophic effect of L-165041 on cardiomyocytes was PPARγ-dependent.

Next, we tried to elucidate its underlying mechanism especially focusing on $Ca^{2+}$ signaling. It is well known that hypertrophic stimuli such as AngII and phenylephrine cause an increase of intracellular $Ca^{2+}$ levels in cardiomyocytes and this, in turn, results in $Ca^{2+}$-dependent activation of various signal molecule such as FAK, calcineurin, PKC [Eble et al., 2000; Wilkins and Molkentin,

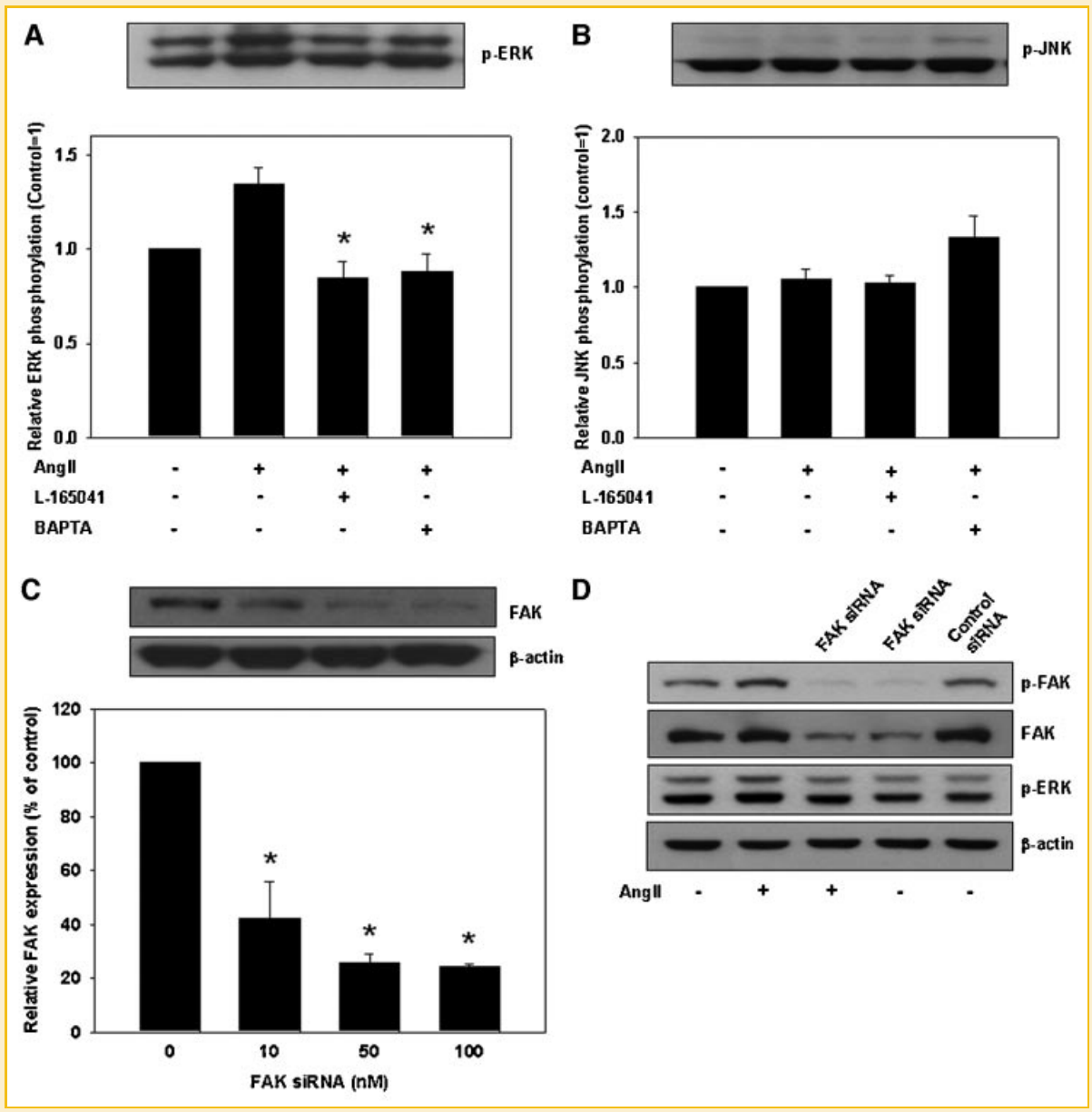


Fig. 4. AngII-induced ERK activation is attenuated by L-165041 treatment. The cells were pretreated with either 10 of μM L-165041 or 10 μM of BAPTA-AM for 1 h depend on the experimental group before the stimulation with 100 nM of AngII for 2 h. The cell lysates were subjected to Western blot using p-ERK antibody (A) and p-JNK antibody (B). Data are represented as the average of at least triplicated independent experiments ± SEM; $^{*}P < 0.05$. C: FAK siRNA (10–100 nM) was delivered into H9c2 cells. The cells were collected 48 h after transfection and subjected to Western blot using FAK antibody. The relative intensity of the corresponding FAK immunopositive bands are represented in a bar graph format. Data are represented as the average of at least triplicated independent experiments ± SEM. D: After 48 h of transfection (FAK siRNA, 50 nM), the cells were stimulated with 100 nM of AngII. The cell lysates were subjected to Western blot using p-FAK and p-ERK antibody.

2004]. According to our data, AngII treatment increased intracellular $Ca^{2+}$ level and this increase was significantly suppressed by L-165041 pretreatment. Furthermore, our results show that AngII treatment increased FAK phosphorylation, which regulates myocyte hypertrophy associated gene expressions such as ANP, BNP [Kovacic-Milivojevic et al., 2001], and this was also attenuated by L-165041 pretreatment.

Since AngII-induced FAK phosphorylation (Tyr 397) can be affected by various signal mediators such as $Ca^{2+}$, PKC, and Src [Yin et al., 2003], we used inhibitors of such signal molecules to further clarify the underlying mechanism of AngII-induced FAK activation. Our data clearly show that only intracellular $Ca^{2+}$ chelator BAPTA-AM significantly inhibited AngII-induced FAK phosphorylation (Tyr 397) while PKC and Src inhibitors failed to do so. This suggests that $Ca^{2+}$ signaling is the mainly responsible for linking AngII treatment and subsequent FAK activation in our experimental settings. These findings are also consistent with the previous studies showed that GPCR agonist induced FAK activation in a Src independent manner [Salazar and Rozengurt, 2001], and AngII induced FAK phosphorylation did not require PKC [Li et al., 1998]. In

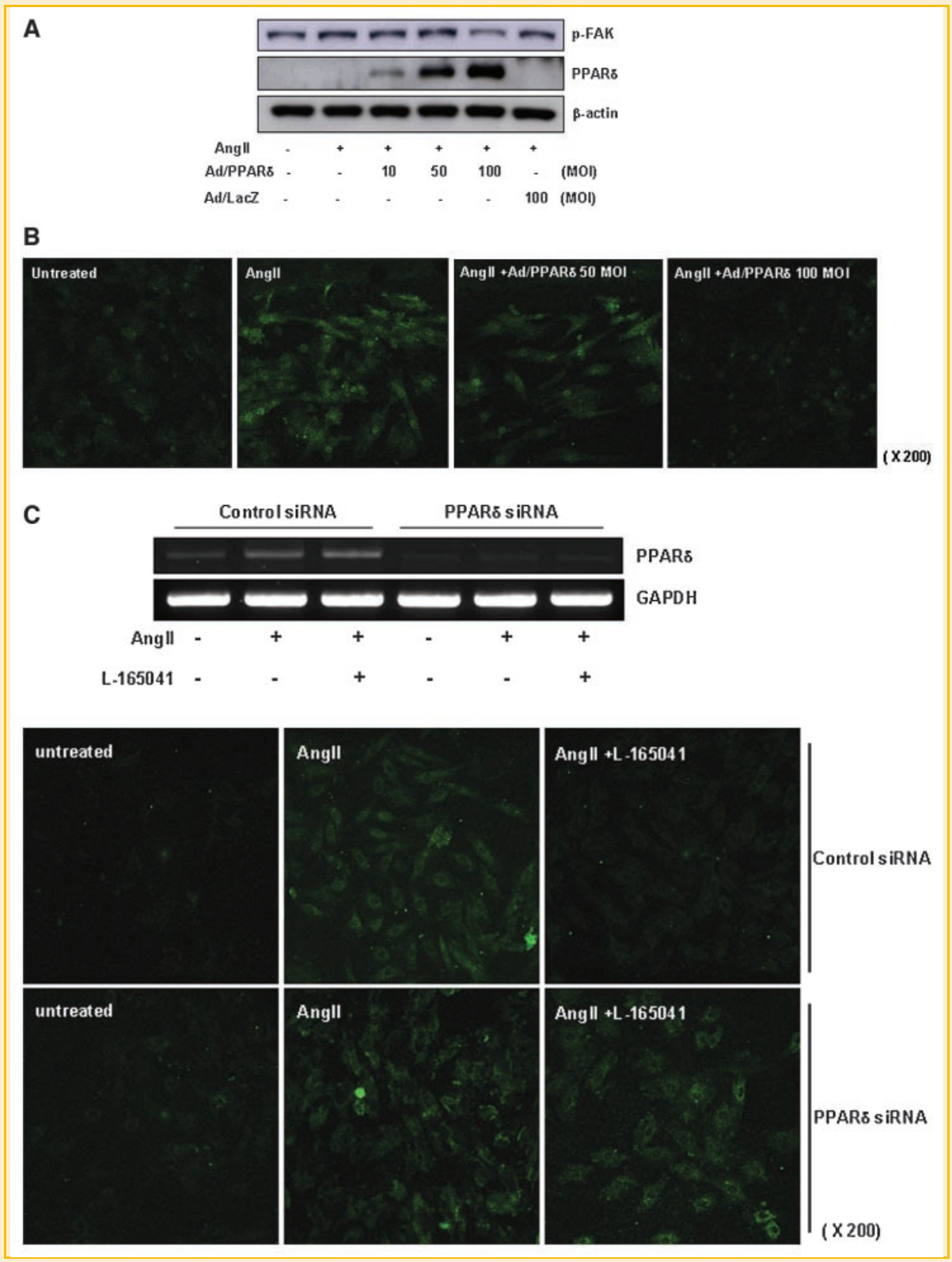


Fig. 5. Overexpression of PPARδ significantly inhibits AngII-activated FAK expression and intracellular $Ca^{2+}$ increase. H9c2 cells were transfected with adenoviral PPARδ (10–100 MOI) or PPARδ siRNA. After 48 h, cells were treated with 100 nM of AngII. A: The cell lysates were subjected to Western blot using PPARδ and p-FAK antibody. B: The cells were stained with Calcium Green™-1, AM and then immunofluorescence was detected using a confocal laser fluorescence microscope. C: The result of RT-PCR using PPARδ specific primer. Also the cells were stained with Calcium Green™-1, AM. D: The cells were pretreated with actinomycin D (ActD; 5 μg/ml) for 1 h. And the cells were stimulated with AngII (100 nM) in the presence or absence of 10 μM L-165041. The cells were stained Calcium Green™-1, AM and the cell lysates were subjected to Western blot using p-FAK antibody.

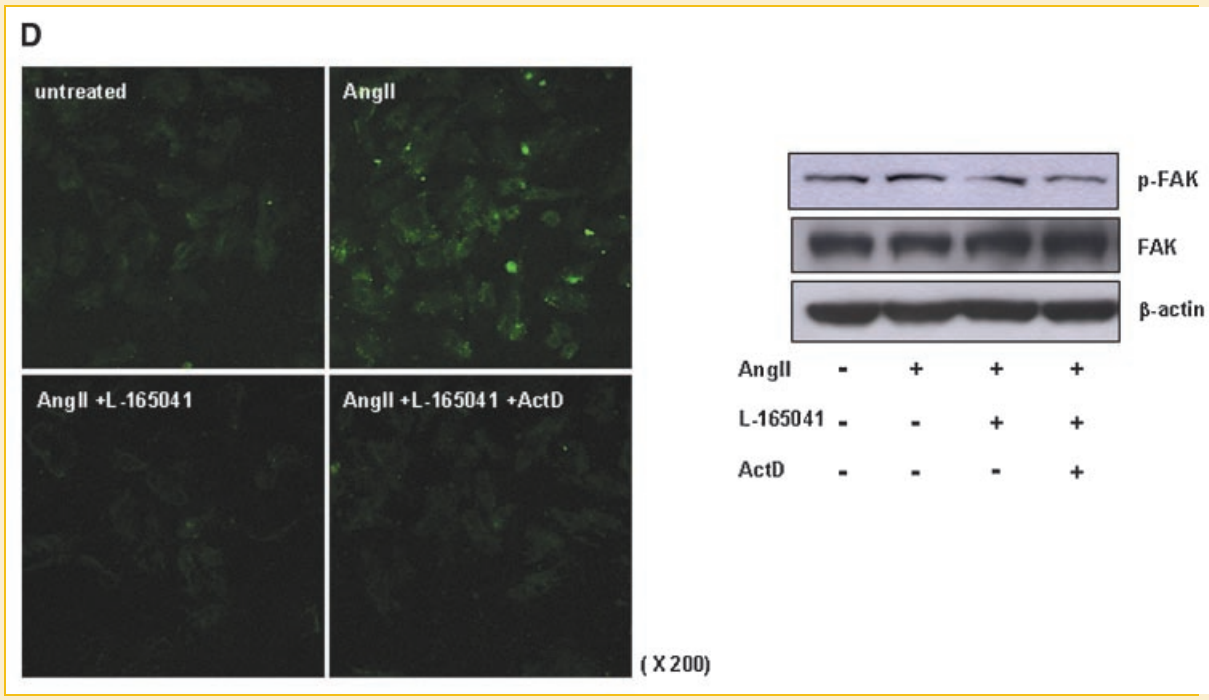


Fig. 5. (*Continued*)

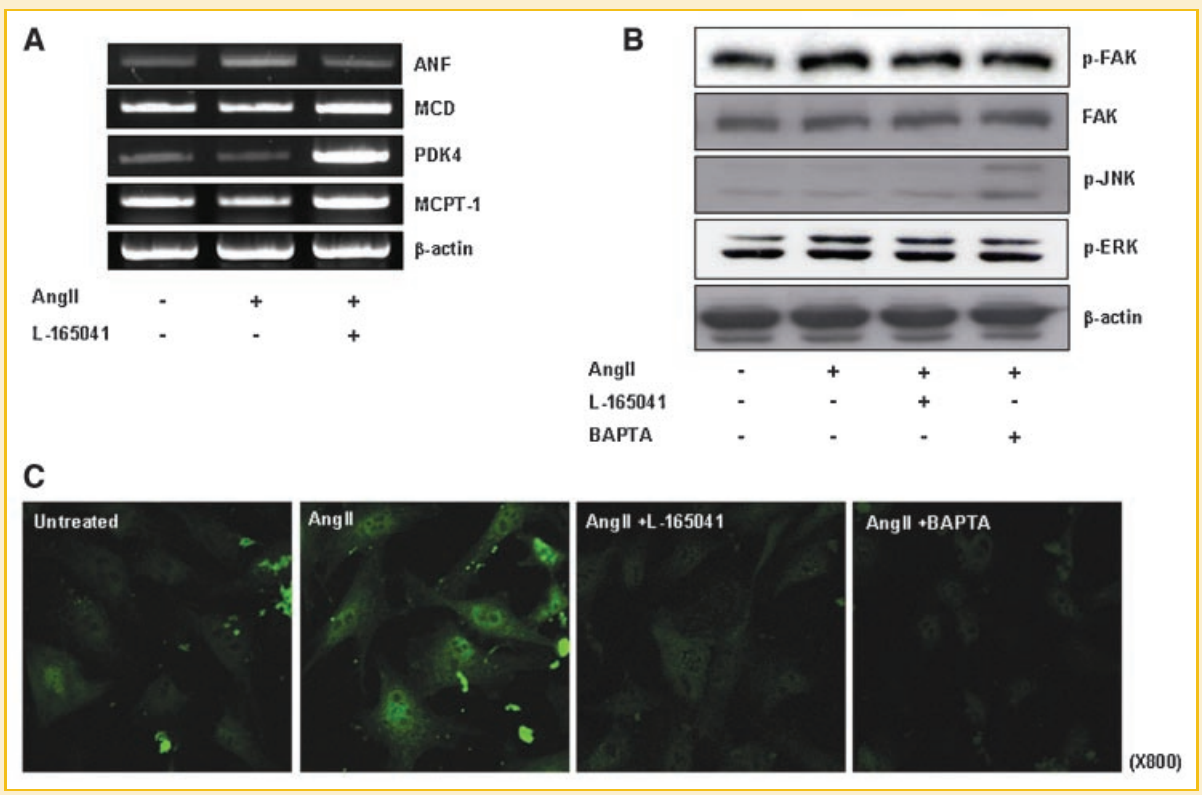


Fig. 6. L-165041 attenuates AngII-induced cardiac hypertrophy through the inhibition of intracellular $Ca^{2+}$ increase and subsequent FAK phosphorylation in primary cardiomyocytes. A: Primary cardiomyocytes were stimulated with 100 nM of AngII for 24 h with or without 1 h of L-165041 (10 μM) pretreatment. Total mRNAs were prepared and the mRNA expressions of ANF, MCD, PDK4, and MCPT-1 were evaluated by RT-PCR using specific primers. B: Primary cardiomyocytes were stimulated by AngII (100 nM) with or without 1 h pretreatment of L-165041 (10 μM) or BAPTA-AM (10 μM). The cells were collected 2 h after AngII stimulation and subjected to Western blot using p-FAK, p-ERK, and p-JNK antibody. C: Primary cardiomyocytes were stained with Calcium Green™-1, AM and then immunofluorescence was detected using a confocal laser fluorescence microscope.

addition, the fact that $Ca^{2+}$ chelating can block the activation of adhesion kinases including FAK has been reported [Sabri et al., 1998].

Enhanced FAK tyrosine phosphorylation is an important intermediary link between integrin or AngII receptors and the activation of downstream targets such as the ERK and JNK MAP kinase cascades [Schlaepfer et al., 1999; Govindarajan et al., 2000; Weng and Shukla, 2002]. However, in our experimental settings, only ERK pathway was significantly affected by FAK activation while JNK pathway was not. In further experiments, FAK siRNA transfection significantly suppresses AngII-induced ERK activation, and both L-165041 and BAPTA-AM pretreatment inhibit AngII-induced ERK activation suggesting L-165041 most likely blocks AngII induced $Ca^{2+}$ increase thereby blocks subsequent FAK activation and ERK activation.

The effect of L-165041 was not affected by a transcription inhibitor actinomycin D treatment. Thus, the observed effect of L-165041 is a PPARδ dependent but non-genomic effect, which does not require the action of PPARδ as a transcription factor. In fact, a number of studies have reported that the nuclear receptor PPAR can affect various cytosolic molecules rendering non-genomic effects [Takeda et al., 2001; Gardner et al., 2005; Han et al., 2008].

One thing should be pointed out is that even the overexpression of PPARδ itself without L-165041 treatment was sufficient to suppress AngII induced cardiomyocyte hypertrophy. Although we are not able to provide supporting data at this point, we can still speculate that this might indicate that there exist sufficient amount of endogenous PPARδ ligand in our experimental system so that it can exert its effect once additional PPARδ is introduced. It will be worth to test this hypothesis for the future study.

In the present study, we demonstrate that PPARδ ligand L-165041 inhibits AngII-induced cardiac hypertrophy and associated gene expressions. Our data strongly suggest that PPARδ ligand L-165041 blocks AngII induced intracellular $Ca^{2+}$ increase, thereby blocks subsequent FAK and ERK activation. The present study further warrants future studies and suggests a therapeutic potential of PPARδ ligand in the treatment of cardiac hypertrophy.

## ACKNOWLEDGMENTS

This work was supported by the Korea National Institute of Health intramural research grant (4800-4845-300-210).

## REFERENCES

Berger J, Leibowitz MD, Doebber TW, Elbrecht A, Zhang B, Zhou G, Biswas C, Cullinan CA, Hayes NS, Li Y, Tanen M, Ventre J, Wu MS, Berger GD, Mosley R, Marquis R, Santini C, Sahoo SP, Tolman RL, Smith RG, Moller DE. 1999. Novel peroxisome proliferator-activated receptor (PPAR) gamma and PPAR-delta ligands produce distinct biological effects. J Biol Chem 274:6718–6725.

Booz GW, Baker KM. 1996. Role of type 1 and type 2 angiotensin receptors in angiotensin II-induced cardiomyocyte hypertrophy. Hypertension 28:635–640.

Cheng L, Ding G, Qin Q, Huang Y, Lewis W, He N, Evans RM, Schneider MD, Brako FA, Xiao Y, Chen YE, Yang Q. 2004. Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. Nat Med 10:1245–1250.

Eble DM, Strait JB, Govindarajan G, Lou J, Byron KL, Samarel AM. 2000. Endothelin-induced cardiac myocyte hypertrophy: Role for focal adhesion kinase. Am J Physiol Heart Circ Physiol 278:H1695–H1707.

Gardner OS, Dewar BJ, Graves LM. 2005. Activation of mitogen-activated protein kinases by peroxisome proliferator-activated receptor ligands: An example of nongenomic signaling. Mol Pharmacol 68:933–941.

Gilde AJ, van der Lee KA, Willemsen PH, Chinetti G, van der Leij FR, van der Vusse GJ, Staels B, van Bilsen M. 2003. Peroxisome proliferator-activated receptor (PPAR) alpha and PPARbeta/delta, but not PPARgamma, modulate the expression of genes involved in cardiac lipid metabolism. Circ Res 92:518–524.

Govindarajan G, Eble DM, Lucchesi PA, Samarel AM. 2000. Focal adhesion kinase is involved in angiotensin II-mediated protein synthesis in cultured vascular smooth muscle cells. Circ Res 87:710–716.

Han J-K, Lee H-S, Yang H-M, Hur J, Jun S-I, Kim J-Y, C C-H, Koh G-Y, Peters JM, Park K-W, Cho H-J, Lee H-Y, Kang H-J, Oh B-H, Park Y-B, Kim H-S. 2008. Peroxisome proliferator-activated receptor-δ agonist enhances vasculogenesis by regulating endothelial progenitor cells through genomic and nongenomic activations of the phosphatidylinositol 3-kinase/Akt pathway. Circulation 118:1021–1033.

Heineke J, Molkentin JD. 2006. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 7:589–600.

Hildebrand JD, Schaller MD, Parsons JT. 1993. Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. J Cell Biol 123:993–1005.

Hunt RA, Bhat GJ, Baker KM. 1999. Angiotensin II-stimulated induction of sis-inducing factor is mediated by pertussis toxin-insensitive G(q) proteins in cardiac myocytes. Hypertension 34:603–608.

Kovacic-Milivojevic B, Roediger F, Almeida EA, Damsky CH, Gardner DG, Ilic D. 2001. Focal adhesion kinase and p130Cas mediate both sarcomeric organization and activation of genes associated with cardiac myocyte hypertrophy. Mol Biol Cell 12:2290–2307.

Leduc I, Meloche S. 1995. Angiotensin II stimulates tyrosine phosphorylation of the focal adhesion-associated protein paxillin in aortic smooth muscle cells. J Biol Chem 270:4401–4404.

Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP. 1990. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. N Engl J Med 322:1561–1566.

Li X, Lee JW, Graves LM, Earp HS. 1998. Angiotensin II stimulates ERK via two pathways in epithelial cells: Protein kinase C suppresses a G-protein coupled receptor-EGF receptor transactivation pathway. EMBO J 17:2574–2583.

Lorell BH, Carabello BA. 2000. Left ventricular hypertrophy: Pathogenesis, detection, and prognosis. Circulation 102:470–479.

McWhinney CD, Hunt RA, Conrad KM, Dostal DE, Baker KM. 1997. The type I angiotensin II receptor couples to Stat1 and Stat3 activation through Jak2 kinase in neonatal rat cardiac myocytes. J Mol Cell Cardiol 29:2513–2524.

Miyata S, Haneda T. 1994. Hypertrophic growth of cultured neonatal rat heart cells mediated by type 1 angiotensin II receptor. Am J Physiol 266: H2443–H2451.

Parsons JT. 1996. Integrin-mediated signalling: Regulation by protein tyrosine kinases and small GTP-binding proteins. Curr Opin Cell Biol 8:146–152.

Planavila A, Rodriguez-Calvo R, Jove M, Michalik L, Wahli W, Laguna JC, Vazquez-Carrera M. 2005. Peroxisome proliferator-activated receptor beta/delta activation inhibits hypertrophy in neonatal rat cardiomyocytes. Cardiovasc Res 65:832–841.

Qin J, Liu ZX. 2006. FAK-related nonkinase attenuates hypertrophy induced by angiotensin-II in cultured neonatal rat cardiac myocytes. Acta Pharmacol Sin 27:1159–1164.

Sabri A, Govindarajan G, Griffin TM, Byron KL, Samarel AM, Lucchesi PA. 1998. Calcium- and protein kinase C-dependent activation of the tyrosine kinase PYK2 by angiotensin II in vascular smooth muscle. Circ Res 83:841–851.

Sadoshima J, Izumo S. 1993. Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. Circ Res 73:413–423.

Sadoshima J, Izumo S. 1996. The heterotrimeric G q protein-coupled angiotensin II receptor activates p21 ras via the tyrosine kinase-Shc-Grb2-Sos pathway in cardiac myocytes. EMBO J 15:775–787.

Salazar EP, Rozengurt E. 2001. Src family kinases are required for integrin-mediated but not for G protein-coupled receptor stimulation of focal adhesion kinase autophosphorylation at Tyr-397. J Biol Chem 276:17788–17795.

Schlaepfer DD, Hauck CR, Sieg DJ. 1999. Signaling through focal adhesion kinase. Prog Biophys Mol Biol 71:435–478.

Seimandi M, Lemaire G, Pillon A, Perrin A, Carlavan I, Voegel JJ, Vignon F, Nicolas JC, Balaguer P. 2005. Differential responses of PPARalpha, PPARdelta, and PPARgamma reporter cell lines to selective PPAR synthetic ligands. Anal Biochem 344:8–15.

Sugden PH. 2002. Signaling pathways activated by vasoactive peptides in the cardiac myocyte and their role in myocardial pathologies. J Card Fail 8:S359–S369.

Takeda K, Ichiki T, Tokunou T, Iino N, Takeshita A. 2001. 15-Deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ and thiazolidinediones activate the MEK/ERK pathway through phosphatidylinositol 3-kinase in vascular smooth muscle cells. J Biol Chem 276:48950–48955.

Taylor JM, Rovin JD, Parsons JT. 2000. A role for focal adhesion kinase in phenylephrine-induced hypertrophy of rat ventricular cardiomyocytes. J Biol Chem 275:19250–19257.

Torsoni AS, Constancio SS, Nadruz W, Jr., Hanks SK, Franchini KG. 2003. Focal adhesion kinase is activated and mediates the early hypertrophic response to stretch in cardiac myocytes. Circ Res 93:140–147.

Turner CE, Pietras KM, Taylor DS, Molloy CJ. 1995. Angiotensin II stimulation of rapid paxillin tyrosine phosphorylation correlates with the formation of focal adhesions in rat aortic smooth muscle cells. J Cell Sci 108(Pt 1): 333–342.

Wang GW, Schuschke DA, Kang YJ. 1999. Metallothionein-overexpressing neonatal mouse cardiomyocytes are resistant to $H_2O_2$ toxicity. Am J Physiol 276:H167–H175.

Weng YI, Shukla SD. 2002. Angiotensin II activation of focal adhesion kinase and pp60c-Src in relation to mitogen-activated protein kinases in hepatocytes. Biochim Biophys Acta 1589:285–297.

Wilkins BJ, Molkentin JD. 2004. Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. Biochem Biophys Res Commun 322: 1178–1191.

Yin G, Yan C, Berk BC. 2003. Angiotensin II signaling pathways mediated by tyrosine kinases. Int J Biochem Cell Biol 35:780–783.

Zhu T, Goh EL, LeRoith D, Lobie PE. 1998. Growth hormone stimulates the formation of a multiprotein signaling complex involving p130(Cas) and CrkII. Resultant activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). J Biol Chem 273:33864–33875.