Journal of Cellular Biochemistry 103:1718–1731 (2008)

Specific LPA Receptor Subtype Mediation of LPA-Induced Hypertrophy of Cardiac Myocytes and Involvement of Akt and NFκB Signal Pathways

Jinghai Chen,[1] Yuefeng Chen,[2] Weiquan Zhu,[1] Yu Han,[1] Bianmei Han,[1] Ruixia Xu,[1] Linzi Deng,[1] Yan Cai,[1] Xiangfeng Cong,[1] Yuejing Yang,[2] Shengshou Hu,[1]* and Xi Chen[1]*

[1]Research Center for Cardiovascular Regenerative Medicine,
the Ministry of Health, Cardiovascular Institute and Fuwai Hospital,
Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China
[2]Department of Cardiology, Cardiovascular Institute and Fuwai Hospital,
Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China

Abstract Lysophosphatidic acid (LPA) is a bioactive phospholipid with diverse functions mediated via G-protein-coupled receptors (GPCRs). In view of the elevated levels of LPA in acute myocardial infarction (MI) patients we have conducted studies aimed at identifying specific LPA receptor subtypes and signaling events that may mediate its actions in hypertrophic remodeling. Experiments were carried out in cultured neonatal rat cardiomyocytes (NRCMs) exposed to LPA and in a rat MI model. In NRCMs, LPA-induced hypertrophic growth was completely abrogated by DGPP, an LPA1/LPA3 antagonist. The LPA3 agonist OMPT, but not the LPA2 agonist dodecylphosphate, promoted hypertrophy as examined by ^{3}[H]-Leucine incorporation, ANF-luciferase expression and cell area. In in vivo experiments, LPA1, LPA2 and LPA3 mRNA levels as well as LPA1 and LPA3 protein levels increased together with left ventricular remodeling (LVRM) after MI. In addition, LPA stimulated the phosphorylation of Akt and p65 protein and activated NF-κB-luciferase expression. Inhibitors of PI3K (wortmannin), mTOR (rapamycin), and NF-κB (PDTC or SN50) effectively prevented LPA-induced ^{3}[H]-Leucine incorporation and ANF-luciferase expression. Furthermore, ERK inhibitors (U0126 and PD98059) suppressed LPA-stimulated activation of NF-κB and p65 phosphorylation whereas wortmannin showed no effect on NF-κB activation. Our findings indicate that LPA3 and/or LPA1 mediate LPA-induced hypertrophy of NRCMs and that LPA1 and LPA3 may be involved in LVRM of MI rats. Moreover, Akt and NF-κB signaling pathways independently implicate in LPA-stimulated myocardial hypertrophic growth. J. Cell. Biochem. 103: 1718–1731, 2008.

Key words: lysophosphatidic acid; receptor; myocardial infarction; hypertrophy; signaling pathway

Lysophosphatidic acid (LPA) is a simple and natural lipid which has emerged as a potent mediator with a broad range of cellular actions, including stimulation of cell contraction and proliferation, protection from apoptosis, reorganization of cytoskeletal, and regulating cellular migration [Moolenaar, 1999; Mills and Moolenaar, 2003]. Within the cardiovascular system, LPA may be involved in angiogenesis and in the pathogenesis of atherosclerosis [Tigyi and Parrill, 2003]. Moreover, we recently reported that serum LPA level is significantly elevated (maximal ~sevenfold) in serum LPA concentration in acute myocardial infarction (MI) patients [Chen et al., 2003]. In addition, in ex vivo study, LPA promotes cardiomyocytes hypertrophy [Goetzl et al., 2000; Hilal-Dandan et al., 2004] and prevents myocyte apoptosis

Grant sponsor: National Natural Science Foundation of China; Grant number: 30170374; Grant sponsor: National 973 Program; Grant number: 2007CB512108; Grant sponsor: National Outstanding Young Foundation; Grant number: 30125039; Grant sponsor: Specialized Research Fund for the Doctoral Program of Higher Education of China; Grant number: 20050023016.

*Correspondence to: Xi Chen, PhD and Shengshou Hu, MD, Research Center for Cardiovascular Regenerative Medicine, the Ministry of Health, Cardiovascular Institute and Fu Wai Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, 167 Beilishilu, Beijing 100037, P.R.China. E-mail: chenxipumc@yahoo.com.cn

Received 17 January 2007; Accepted 8 August 2007

DOI 10.1002/jcb.21564

[Karliner et al., 2001]. These findings indicate that after MI, LPA may be involved in left ventricular (LV) remodeling, which is characterized by infarct expansion, compensative hypertrophy of non-infarcted myocardium and alteration of LV geometry.

It has been shown that LPA receptors belong to the endothelial differentiation genes (EDG) family of receptors with LPA1, LPA2, and LPA3, respectively corresponding to Edg2, Edg4, and Edg7. These are G-protein-coupled receptors (GPCRs) that mediate pleiotypic and specific action of LPA [Contos et al., 2000; Fukushima and Chun, 2001]. LPA1 is widely expressed in various normal tissue, whereas LPA2 and LPA3 show a more restricted expressed [Anliker and Chun, 2004]. Alteration of LPA receptor expression or the activation of a specific LPA receptor subtype under pathological condition may be an important event in the development of the disease. In this regard, LPA2 mRNA level is significantly high in colorectal cancers [Shida et al., 2004] while LPA1 receptor is implicated in pancreatic cancer [Yamada et al., 2004] and cancer metastasis to bone [Boucharaba et al., 2006], LPA1 and LPA3 contribute to thrombus formation leading to cardiovascular diseases [Rother et al., 2003]. At present, however, it is not clear as to which receptor subtype mediate the LPA-induced hypertrophy in cardiomyocytes as well as whether LPA receptors is specifically up-regulated in process of hypertrophic remodeling after MI.

Cardiomyocytes hypertrophy is an adaptive pathophysiological process in response to MI that may involve a complex network of cellular events. For instance, it is reported that under certain conditions, activation of the ERK, PI3K, and potentially NF-κB pathways may play an important role in the development of cardiac hypertrophy [Purcell et al., 2001; Matsui et al., 2003]. It has been reported that LPA induces hypertrophy of neonatal cardiomyocytes through co-activation of Gi/ERK and Rho pathways [Hilal-Dandan et al., 2004]. However, the signaling pathways of LPA-stimulated cardiac hypertrophy have not been satisfactorily examined. Given the fact that cardiac hypertrophy is regulated by a complex signaling network rather than a single pathway, it would be of value to fully elucidate the signaling events that mediate LPA-induced cardiomyocyte hypertrophy. In particular, identifying not only the receptors involved but also the key signaling molecules affected and their downstream targets would be crucial for a better understanding of the cellular mechanisms that regulate hypertrophy. The aims of the present studies therefore were to clarify the involvement of LPA receptor subtypes in cardiac hypertrophy in vitro and in vivo, and to gain further insight into the transductional and transcriptional signaling pathways that may mediate the hypertrophic actions of LPA.

MATERIALS AND METHODS

Materials

LPA (oleoyl C: 18:1), OMPT (1-oleoyl-2-Omethyl-rac-glycero-phosphothionate), BrdU, Phalloidin-TRITC and Rapamycin, Trypan blue and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-terzolium bromide (MTT) were from Sigma. DGPP [dioctylglycerol pyrophosphate (8:0)] was from Avanti Polar Lipids. Dodecylphosphate, Wortmannin, U0126, SN50 were from Biomol Research Labs. PD98059 and PDTC were from Calbiochem. Nppa promoter-luciferase reporter construct (ANF-Luc) was as a gift from professor Issei Komuro. 2 × NF-κB-Luciferase reporter vector (NF-κB-Luc) was from Clontech. pRL-CMV Renilla luciferase vector and Dual-Luciferase Reporter (DLR) Assay kit were from Promega, Trizol, and Lipofectamine 2000 were from Invitrogen. Antibodies were from suppliers as indicated: anti-LPA1 antibodies (Calbiochem); anti-LPA2, anti-LPA3, anti-p65 (Santa Cruz Biotechnology); anti-β-Actin antibodies (Sigma); anti-phospho-p65 and anti-Akt, anti-phospho-Akt (Cell Signaling Inc.). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology.

Cell Culture of Neonatal Ventricular Myocytes

Neonatal rat cardiomyocytes (NRCMs) were cultured as previous described [Chen et al., 2002] from 1 to 3-day-old SD rats. Cardiomyocytes obtained were plated at a density of 50,000 cells/cm^2 supplemented with DMEM containing 10% fetal bovine serum, penicillin/streptomycin (1000 U/ml each) with 100 μM BrdU added to inhibit the growth of cardiac fibroblasts. Subconfluent cardiomyocytes were starved overnight in serum-free medium prior to the experiments.

Cell Viability Assay

Cell viability was tested by MTT assay and trypan blue dye exclusion assay as previous

described [Wang et al., 2006]. For MTT assay, cells were seeded in 96-well plates with different reagents for 44 h. MTT was added to each well at a final concentration of 0.5 mg/ml and incubated for another 4 h. The supernatants were removed and 100 μl of DMSO was added to each well to dissolve the blue formazan product. Quantitative colorimetric assay at 570 nm was measured on a 96-well scanning spectrophotometer. For trypen blue assay, cells in six-well were treated with reagents for 48 h. Then, cells were harvested by trypsinization, washed with PBS, pelleted, and resuspended in PBS. The cell suspension was mixed with an equal volume of 0.4% Trypan blue solution and incubated for 3 min at room temperature. The number of unstained cells (living cells) and the total number of cells were counted on a haemacytometer under a microscope.

Animals and Acute Myocardial Infarction

Acute MI model was established by the ligation of the left anterior descending (LAD) artery in randomly selected female Sprague–Dawley (SD) rats (body weight 210–270 g; Charles River Laboratories, Inc.) as previously reported [Yang et al., 2003]. Twenty-four hours after the operation, survivors were observed after 4 weeks. In the sham-operated groups, rats were randomly selected and a suture was tied loosely around the left coronary artery, but the vessel was not ligated. These animals were assigned as control group.

Evaluation of LV Remodeling and LV Function

Hemodynamic measurements and LV remodeling analysis were performed as previous described [Yang et al., 2003] at the end of 4 weeks. The hearts were arrested by intravenous injection of 10% KCl, rapidly excised and randomly divided to two groups. One group was preserved in liquid nitrogen for analysis of expression of LPA receptor subtypes. LV relative weight (LV weight/body weight) was determined in the second group and subsequently fixed for examination of LV remodeling including septal thickness (STh), left ventricular transverse diameter (c), left ventricular cross-sectional area (LVCSA).

Preparation of RNA and RT-PCR Analysis

Total RNA was extracted from the left ventricles of adult rat hearts using Trizol according to the manufacturer's instructions and quantified by ultraviolet (UV) spectrophotometry. Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed as our previously described [Chen et al., 2006] using the following gene specific extron primers: LPA1/Edg2: 5′-TCTTCTGGGCCATTTTCA A-3′ and 5′-GCCGTTGGGGTTCTCGTT-3′; LPA2/Edg4: 5′-CCTACCTCTTCCTCATGTTC-3′ and 5′-AATGATGACAACCGTCTTGACTA-3′; LPA3/Edg7: 5′-TGT CAACCGCTGGCTTCT-3′ and 5′-CAGTCATCACCGTCTCATTAG-3′; GAPDH: 5′-CCATGGAGAAGGCTGGG -3′ and 5′-CAAAGTTGTCATGGATGACC-3′. PCR products were run on 1.5% agarose gels and bands visualized with a UV transiluminator (Bio-Rad) followed by computerized densitometric analysis with the image analysis system TotalLab1.1. The mRNA levels were expressed as the ratios of densitometric scan values of LPA receptor subtypes to GAPDH.

Protein Extraction and Western Blot Analysis

Total protein was extracted from LV tissue using T-PER® Tissue Protein Extraction

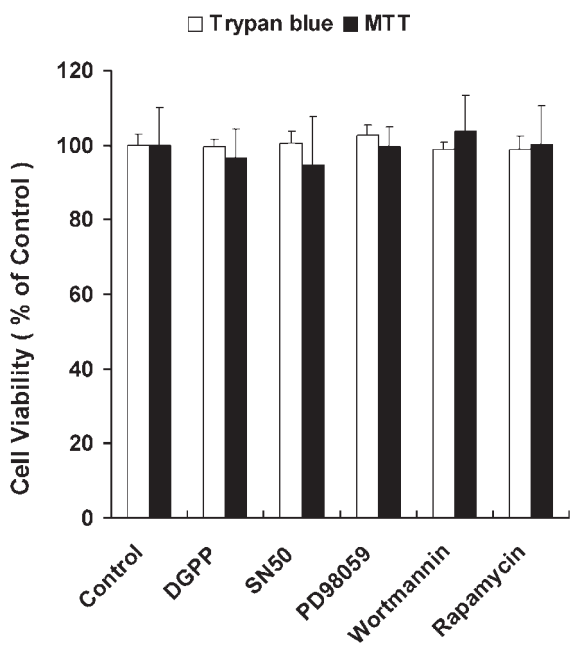

Fig. 1. Effect of inhibitors on cardiomyocytes viability. Cardiomyocytes were incubate with or without 50 μM DGPP, 50 μg/ml SN50, 50 μM PD98059, 100 nM Wortmannin, 100 nM Rapamycin for 48 h. Cell viability as measured by trypan blue dye exclusion assay and MTT assay described under Materials and methods. Data are expressed as the mean ± SD. For this and all other illustration in this paper, representative results from three independent experiments are shown.

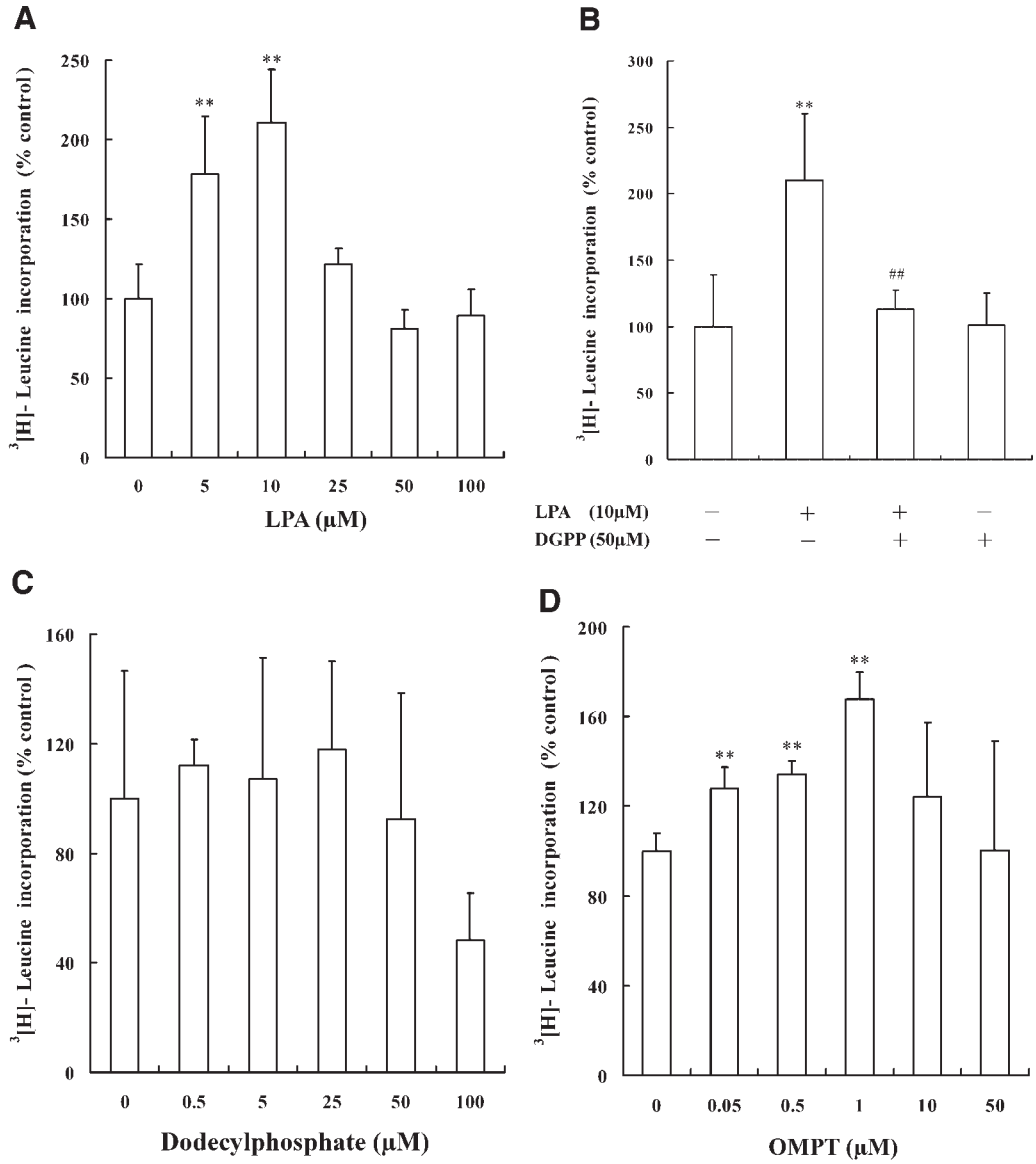

Fig. 2. Specific LPA receptor subtypes mediate the ^{3}[H]-Leucine incorporation of LPA-induced primary cardiomyocytes hypertrophy. Cardiomyocytes were treated with the agents indicated on each panel for 48 h. (**A**) LPA dose-dependently induced ^{3}[H]-Leucine incorporation in primary cardiomyocytes. (**B**) Specific LPA1/LPA3 receptor antagonist, DGPP completely blocked the LPA-induced ^{3}[H]-Leucine incorporation (**C**) Specific LPA2 receptor agonist, Dodecylphosphate showed no contribution to ^{3}[H]-Leucine incorporation. (**D**) Specific LPA3 receptor agonist OMPT effectively stimulated the ^{3}[H]-Leucine incorporation. The incorporation of ^{3}[H]-Leucine in cardiomyocytes was measured as described in section Materials and Methods. Data are expressed as the mean ± SD. $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.01$ versus LPA alone.

Reagent (Pierce) according to the manufacturer's instructions. Cultured cells were rinsed twice with ice-cold PBS and then lysed at 4°C in buffer containing 1% TritonX-100, 20 mmol/L HEPES (pH 7.5), 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol(DTT), 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), and 1 mg/ml each of leupeptin, aprotinin, and pepstatin for 30 min. Fifty micrograms of total protein was mixed with 5 × SDS sample buffer, boiled for 5 min and subjected to SDS–PAGE electrophoresis. Protein bands were visualized by enhanced chemiluminescence and exposed to radiographic film. Band intensities were quantified by scanning with a color scanner (EPSON Perfection) and

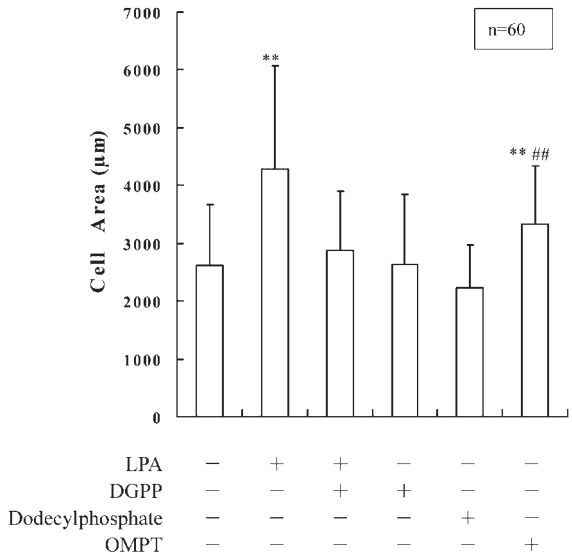

Fig. 3. Effects of specific LPA antagonist and agonist on LPA-induced hypertrophic changes in cell area. Cardiomyocytes were treated with 50 μM Dodecylphosphate (LPA2 agonist) or 1 μM OMPT (LPA3 agonist) for 48 h, or pretreated with 50 μM DGPP (LPA1/LPA3 antagonist) 1 h and then exposure to 10 μM LPA for 48 h. Cardiomyocytes were stained with TRITC-conjugated phalloidin. Cell area was measured and calculated as detailed in Materials and Methods. Data are expressed as the mean ± SD. $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.01$ versus LPA alone.

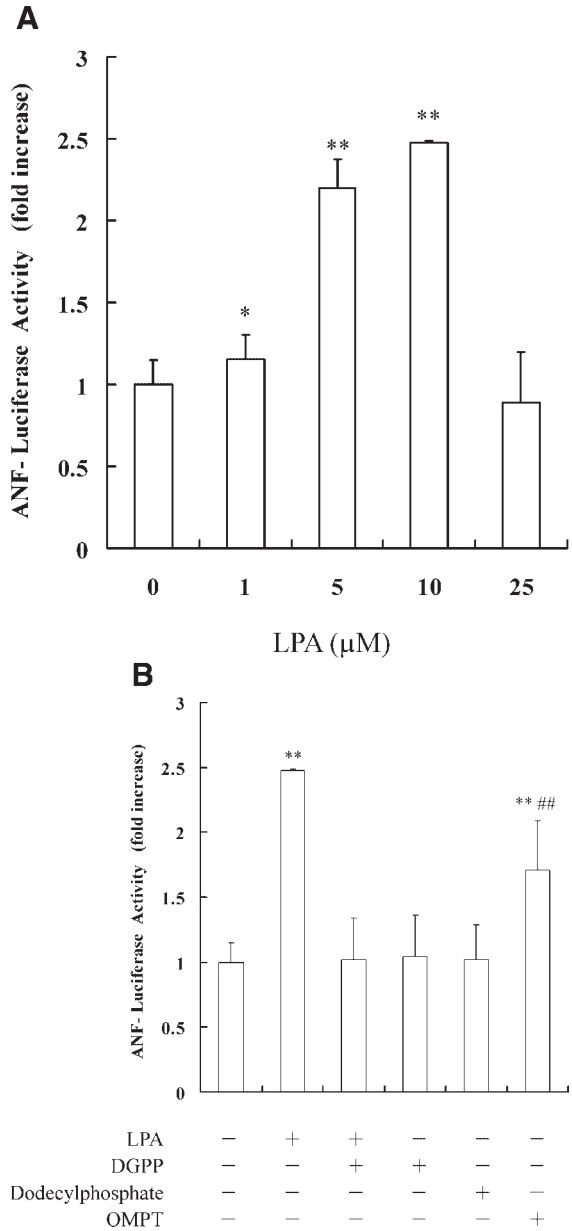

Fig. 4. Specific LPA receptor subtypes mediate the LPA-induced ANF-Luc gene expression in primary cardiomyocytes. Cardiomyocytes were transfected and then treated with different agents as indicated in Materials and Methods. (**A**) LPA dose-responsedly induced ANF-luc expression. (**B**) 50 μM DGPP completely prevents LPA-induced expression of ANF-Luc. Stimulation of 1 μM OMPT was weaker than 10 μM LPA. 50 μM dodecylphosphate has no effect on expression of ANF-Luc. Data are expressed as the mean ± SD. $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.01$ versus LPA alone.

analyzed with the image analysis system-TotalLab1.1.

Measurement of Protein Synthesis

Serum-deprived cells were stimulated with dodecylphosphate or OMPT for 48 h. Then cells were pre-incubated with other drugs used for 1 h prior to stimulation with LPA for 48 h. ^{3}[H]-Leucine was added to the serum-free medium at a final concentration of 1 μCi/ml (Institute of High Energy Physics, Chinese Academy of Sciences) during the last 6 h of stimulation. Experiments were terminated by rinsing cells 3× with PBS followed by incubation with ice-cold 10% TCA for 30 min at 4°C. Precipitated proteins were solubilized in 1% SDS/0.1 M NaOH overnight at room temperature. Radioactivity of SDS-soluble protein was quantified by liquid scintillation spectrometry.

Measurement of Cardiomyocytes Area

Serum-deprived NRCMs exposed for 48 h to an LPA receptor subtypes agonist or LPA in the continued presence of a select antagonist were fixed for 15 min in 3.7% paraformaldehyde and subsequently permeabilized with 0.1% Triton X-100 for 10 min before staining in the dark with a 2 μg/ml TRITC-conjugated phalloidin solution for 2 h at room temperature. Cell profile was visualized using a laser confocal microscope system (Leica TCS NT) and a computer that

TABLE I. Hemodynamics LV Function and Analysis of LV Remodeling after MI

Hemodynamics and LV Function after MI		
	Sham(n = 10)	MI(n = 13)
LVSP(mmHg)	161.21 ± 17.78	139.74 ± 20.63*
LVEDP(mmHg)	1.25 ± 2.74	19.86 ± 12.76**
+dp/dt(mmHg/s)	7750.10 ± 1568.97	5689.54 ± 1036.98***
−dp/dt(mmHg/s)	6353.80 ± 1795.58	4812.77 ± 934.76**
LV Remodeling after MI		
	Sham(n = 5)	MI(n = 7)
LVRW(mg/g)	1.95 ± 0.24	2.57 ± 0.39***
STh(mm)	2.74 ± 0.15	3.47 ± 0.14***
LVTD (mm)	14.04 ± 1.80	20.37 ± 2.66**

*$P < 0.05$
**$P < 0.01$
***$P < 0.001$ vs sham-operated control group.
MI, myocardial infarction; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; ± dp/dt, maximum rise and fall rate of left ventricular pressure. LVRW, left ventricular relative weight (heart weight/body weight); STh, septal thickness; LVTD, left ventricular transverse diameter.

allowed direct calculation of the cardiomyocytes area using image analysis software (Leica confocal software).

Cell Transfection and Luciferase Assays

Sub-confluent NRCMs seeded in 24-well plates were transiently transfected with ANF-Luc reporter vector (0.8 μg/well) or NF-κB-Luc reporter vector (1.0 μg/well) with pRL-CMV (0.05 μg/well) used as an internal control vector. Transfections were carried out using Lipofectamine2000 (2 μl/well) in culture medium without serum or antibiotics for 12–16 h. Transfected cells were maintained in serum-free medium with various agents for 48 h and luciferase activity examined by the Promega's Veritas™ Microplate Luminometer in combination with DLR Assay according to the supplied protocol.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD) of at least three independent experiments unless stated otherwise. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were evaluated using Student's *t*-test. A value of $P < 0.05$ was considered as significantly different.

RESULTS

Effects of Antagonist and Inhibitors on Cell Viability

As shown in Figure 1, a dose of 50 μM DGPP, 50 μg/ml SN50, 50 μM PD98059, 100 nM Wortmannin and 100 nM Rapamycin had no effect on the viability of cardiomyocytes. It is reported that 50 μM PDTC [Hattori et al., 1997; Gupta et al., 2002] and 10 μM U0126 [Yue et al., 2000] were selected for the optimal inhibition without damaging the cardiomyocytes. These agents at the indicated concentrations are used in this study.

LPA1 and LPA3 Mediate LPA-Induced Cultured Neonatal Cardiomyocyte Hypertrophy

Treatment of cardiomyocytes with LPA for 48 h caused a dose-dependent change in ^{3}[H]-Leucine incorporation (Fig. 2A). Protein synthesis increased by 2.2-fold at 10 μM but decreased to control level at higher concentration (25–100 μM). As shown in Figure 2B, DGPP (50 μM), which acts as an effective antagonist against LPA1/LPA3 [Fischer et al., 2001], completely inhibited the incorporation of ^{3}[H]-Leucine induced by LPA (10 μM). OMPT, which was recently described as a selective agonist at the LPA3 receptor [Hasegawa et al., 2003], significantly stimulated protein synthesis at 50 nM to 1 μM (Fig. 2D). However, Dodecylphosphate (0.5–100 μM), a specific LPA2 agonist [Virag et al., 2003], had no effect on the protein synthesis (Fig. 2C).

As shown in Figure 3, treatment of cells with LPA (10 μM) resulted in a 1.64-fold enlargement ($P < 0.01$ vs. control) in cell size which was blocked by DGPP(50 μM). OMPT (1 μM) was weaker than LPA, causing only a 1.27-fold increase in cell size ($P = 0.0026$ vs. control,

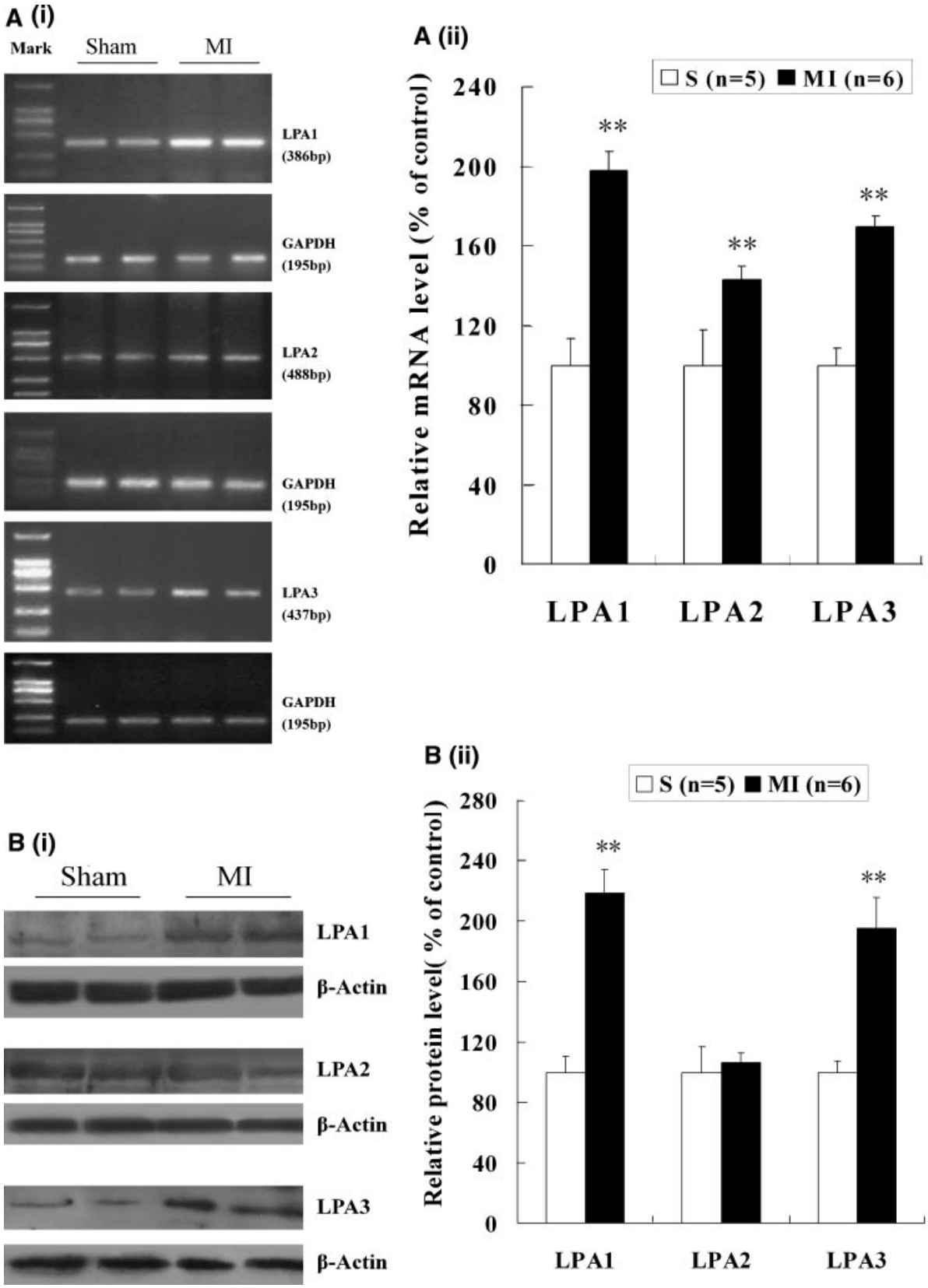
A (i)
Mark
Sham
MI
LPA1
(386bp)
GAPDH
(195bp)
LPA2
(488bp)
GAPDH
(195bp)
LPA3
(437bp)
GAPDH
(195bp)
A (ii)
S (n=5)
MI (n=6)
Relative mRNA level (% of control)
240
200
160
120
80
40
0
**
**
**
LPA1
LPA2
LPA3
B (i)
Sham
MI
LPA1
β-Actin
LPA2
β-Actin
LPA3
β-Actin
B (ii)
S (n=5)
MI (n=6)
Relative protein level(% of control)
280
240
200
160
120
80
40
0
**
**
LPA1
LPA2
LPA3

Fig. 5.

$P = 0.0055$ vs. LPA) while dodecylphosphate (50 μM) was without significant effects.

Expression of ANF-Luc was dose-dependently induced by LPA (Fig. 4A) and this was reversed to control level by the LPA1/LPA3 receptor antagonist DGPP (50 μM). Consistent with the other findings OMPT (10 μM) was less effective in promoting ANF-Luc gene expression while dodecylphosphate was again without effect (Fig. 4B).

Up-Regulated LPA1/LPA3 Expression in LV Remodeling After MI

Data shown in Table I strongly indicated that the rat MI model was established and that rat left ventricle occurred hypertrophic remodeling, which is consistent with our previous study [Yang et al., 2003].

Semi-quantitative RT-PCR revealed that mRNA expression of LPA1 and LPA3 were significantly enhanced ($198 \pm 10\%$ and $169 \pm 5\%$ of control respectively) with that for LPA2 showing a modest increase ($142 \pm 7\%$ of control) in MI groups when compared with sham controls (Fig. 5A). To further determine whether LPA receptor subtypes are indeed up-regulated, the expression profile of receptor protein was determined by Western blot analysis. The data obtained revealed that compared to sham group, the MI group showed significantly elevated protein levels of LPA1 ($219 \pm 15\%$ of control) and LPA3 ($195 \pm 20\%$ of control) with LPA2 protein expression remaining virtually unchanged ($106 \pm 6\%$ of control, $P > 0.05$) (Fig. 5B). These findings are consistent with in vitro results, which further confirms the involvement of LPA1 and LPA3 receptors in hypertrophy of LV remodeling, (Figs. 2–4).

LPA-Induced Cardiomyocyte Hypertrophy Is Mediated by NF-κB

As shown in Figure 6A, LPA dose-dependently induced l NF-κB luciferase activity in cardiomyocytes with a maximal activation of 4.2-fold at 10 μM. In parallel, LPA also time-dependently caused a significant increase in p65 (ser276) phosphorylation which peaked 30 min after activation before declining (Fig. 6B). Both these effects of LPA were blocked by NF-κB inhibitor PDTC (50 μM; Fig. 6A,B). More importantly, 50 μM PDTC also prevented the uptake of ^{3}[H]-Leucine (Fig. 6C) and both kinds of NF-κB inhibitors, PDTC (50 μM) and SN50 (50 μg/ml) suppressed the increased expression of ANF-luc induced by LPA (Fig. 6D).

Because LPA activates ERK1/2 in cardiomyocytes [Hilal-Dandan et al., 2004], we examined whether ERK1/2 play a role in the regulation of LPA-induced NF-κB activation. The latter was completely abolished by two ERK1/2 inhibitors, U0126 (10 μM) and by PD98059 (50 μM) (Fig. 7A). Western blot analysis also demonstrated that U0126 (10 μM) clearly blocked the phosphorylation of p65 stimulated by LPA (Fig. 7B) suggesting that ERK1/2 may be upstream of NF-κB signaling in LPA induced hypertrophic response.

PI3K/Akt/mTOR Pathways Implicated in LPA-Induced Cardiac Hypotrophy Response

Phosphorylation of Akt was observed at 15 min and peaked at 60 min in Western blot analysis of cardiomyocytes treated with LPA (10 μM). This was blocked by 100 nM wortmannin (PI3K/Akt inhibitior) (Fig. 8A) and both wortmannin (100 nM) and rapamycin (100 nM)(p70S6K activation inhibitor), significantly abrogated the uptake of ^{3}H-Leucine (Fig. 8B,C) and ANF-luciferase gene expression (Fig. 8D,E) induced by LPA(10 μM). In addition, inhibition of PI3K/Akt with wortmannin (100 nM) only marginally altered NF-κB activation and phosphorylation of p65 induced by LPA (Fig. 8F,G), suggesting that NF-κB is not downstream of PI3K/Akt pathway.

Fig. 5. Expression of LPA receptor subtypes after MI. (**A**) (i) RT-PCR analysis of mRNA expression was performed as detailed in Materials and Methods. Mark represents a DL2000 (Takara Bio Inc.) molecular weight marker (from top to bottom lane, 2000, 1000, 750, 500, 250, 100 bp, respectively). (**A**) (ii) Relative intensities of PCR bands relative to those GAPDH bands amplified in the experiment were determined and summarized. (**B**) (i) Protein expression of LPA receptor subtypes after MI were examined with Western blot as described in Materials and Methods. The β-Actin was as loading control. Specific antibodies recognized ~45 kDa LPA1, 45–50 kDa LPA2 and 35–40 kDa LPA3 protein respectively and Monoclonal Anti-β-Actin labeled at band of 42 kDa. (**B**) (ii) Relative intensities of hybridization signals of LPA1-3 Western blot bands relative to β-Actin bands in the experiment were determined and summarized. Data are expressed as the mean ± SD. $^{**}P < 0.01$, versus sham-operated control (S).

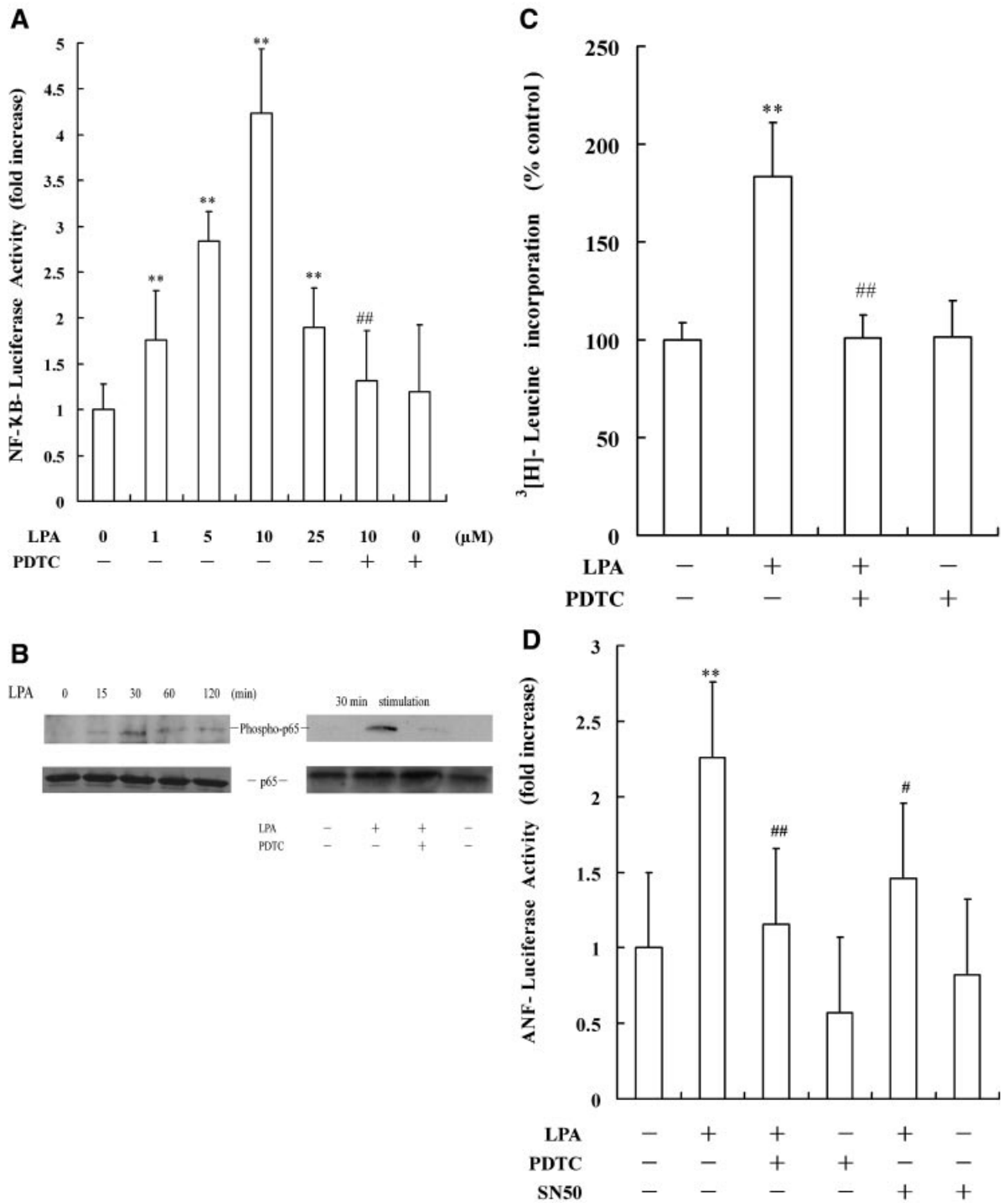

Fig. 6. Activation of NF-κB signaling pathway by LPA in cardiomyocyte hypertrophy. Cardiomyocytes were pretreated with inhibitors 1 hr prior to addition of LPA. The NF-κB-Luc with pRL-CMV transfection and luciferase assay and examination of uptake of ^{3}[H]-Leucine were performed as described in Materials and Methods. (**A**) LPA dose-dependently induced NF-κB-Luc expression and NF-κB inhibitor: PDTC (50 μM) blocked such expression. (**B**) Analysis of time course of LPA-induced activation of NF-κB by phosphorylation of p65 at ser 276 and effect of PDTC (50 μM) were detected by Western blot. (**C**) Effect of NF-κB inhibitor: PDTC (50 μM) on incorporation of ^{3}[H]-Leucine in LPA-induced hypertrophic growth. (**D**) Effect of NF-κB inhibitors: PDTC (50 μM) and SN50 (50 μg/ml) on ANF-luc expression induced by LPA (10 μM). Data are expressed as the mean ± SD. $^{*}P < 0.05$ $^{**}P < 0.01$ versus control; $\#P < 0.05$ $\#\#P < 0.01$ versus LPA.

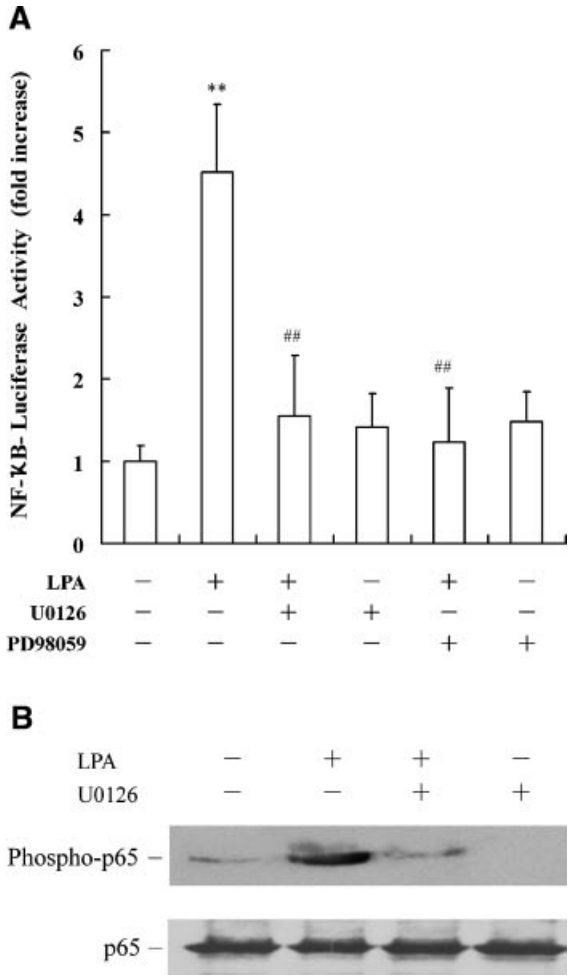

Fig. 7. Regulation of ERK1/2 signaling pathway in LPA-induce NF-κB activity. NF-κB-Luc and pRL-CMV transfection assays were performed as described in Materials and Methods. Inhibitors were incubated 1 h prior to exposure to LPA for 48 h (luciferase assay) and 30 min (phospho-p65 assay) respectively. (**A**) Effect of ERK inhibitors U0126 (10 μM) and PD98059 (50 μM) on 10 μM LPA-induced activation of NF-κB by detecting luciferase expression. Data are expressed as the mean ± SD. $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.05$ versus LPA. (**B**) Effect of U0126 (10 μM) on LPA-stimulated phosphorylation of p65 (ser276).

DISCUSSION

LPA, which is released by activated platelets under pathological conditions, is considerably relevant to cardiovascular disease, such as atherosclerosis [Rother et al., 2003] and vascular remodeling in vivo [Yoshida et al., 2003]. In addition, our previous study has documented that serum LPA level increased significantly after acute MI [Chen et al., 2003]. However, little is known about the role of LPA receptors on cardiac hypertrophy during LV remodeling after MI. The present study demonstrated for the first time that specific LPA receptor subtypes mediate LPA-induced hypertrophy in primary cultured cardiomyocytes and that specific expression of LPA receptor subtypes involve in LV remodeling in rat MI model. Moreover our data has extended the current understanding of the intracellular signal pathway implicated in LPA-induced cardiomyocyte hypertrophy by identifying key signaling molecules that may be involved in this process.

Increasing evidence indicates that LPA receptor subtypes are implicated in various diseases. mRNA level of LPA receptors are upregulated in various cancers([Schulte et al., 2001; Fang et al., 2002; Kitayama et al., 2004]). LPA1 and LPA3 contribute to the thrombosis associated with plaque rupture [Rother et al., 2003]. Selective blockade of LPA3 receptor protects against renal ischemia-reperfusion injury [Okusa et al., 2003] and inhibition of LPA1 receptor is a therapeutic target in cancer for metastasis to bone [Boucharaba et al., 2006]. In the present in vitro experiments, we showed that specific LPA receptors mediate the actions of LPA. Using the selective LPA1/LPA3 antagonist DGPP, we were able to elucidate that LPA1 and/or LPA3 but not LPA2 involved in hypertrophy. This was further confirmed by the fact that LPA3 selective agonist OMPT, but not the LPA2 selective agonist dodecylphosphate, induced cardiomyocyte hypertrophy. In addition, the hypertrophic effect of LPA was more robust than that of OMPT, strongly suggesting that LPA1 may also mediate cardiomyocyte hypertrophy. Our data obtained from in vivo study are consistent with those in vitro. In early phase of LV remodeling (48 hour after MI), both mRNA and protein levels of LPA1, but not LPA2 and LPA3, were enhanced. In the late phase of LV remodeling (4 week after MI), mRNA and protein expression of both LPA1 and LPA3 were significantly elevated while mRNA level of LPA2 increased moderately yet with no change in protein level. These in vivo findings further suggest that the LPA1 and LPA3 receptors are the major receptors that mediate cardiac hypertrophy during remodeling after MI. Since we have demonstrated that LPA1 and LPA3 are involved in hypertrophic responses both in vitro and in vivo, it is feasible to speculate that antagonists of specific LPA receptor subtype

may provide a new strategy towards preventing the progress of hypertrophic remodeling after MI.

Multiple cellular signaling pathways contribute to hypertrophy in cardiomyocytes, including activation of the MAPK cascades, NF-κB, the PKC, and PI3K pathways [Li et al., 2005]. Previous studies have shown that the mechanism by which a hypertrophic response is triggered by LPA may be very distinct from conventional GPCR agonist-induced cardiomyocyte hypertrophy such as that seen with endothelin or phenylephrine. Instead of involving Gq/PLC/PKC and downstream activation of ERK, the LPA-induced cardiac hypertrophy is mediated through Gi/ERK and concomitant activation of Rho [Hilal-Dandan et al., 2004]. However little is known about other signaling pathway associated with cardiac hypertrophic growth. Activation of NF-κB may be required for hypertrophic response both in vitro [Purcell et al., 2001; Gupta et al., 2002; Cook et al., 2003]

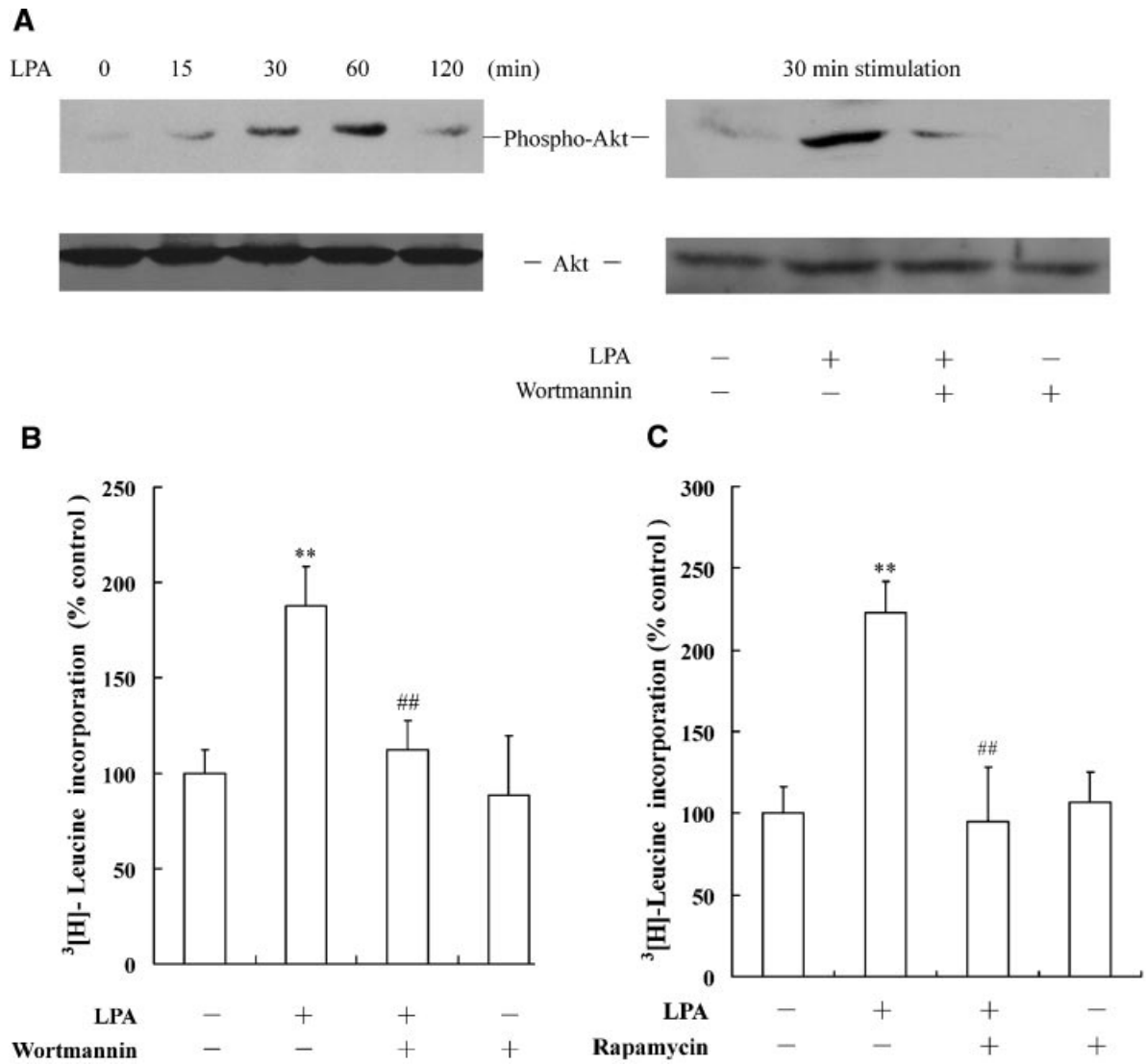

Fig. 8. Role of PI3K/Akt signaling pathway in LPA-induced cardiomyocytes hypertrophy. Cardiomyocytes were pretreated with inhibitors 1 hr prior to addition of LPA. The ANF-luc, pRL-CMV transfection and luciferase assay together with the uptake of ^{3}[H]-Leucine were performed as detailed in the Materials and Methods. (**A**) shows Western blot of changes in Akt phosphorylation. (**B**) and (**C**) show effects of wortmannin (100 nM, PI3K/Akt inhibitior) and rapamycin (100 nM p70S6K activation inhibitor) on incorporation of ^{3}[H] Leucine induced by LPA(10 μM). (**D**) and (**E**) show effect of wortmannin (100 nM) and rapamycin (100 nM) on ANF-luc expression induced by LPA (10 μM). (**F**) and (**G**) shows effects of wortmannin (100 nM) on LPA-induced activation of NF-κB-Luc expression and on phosphorylation of p65 respectively. Data are expressed as the mean ± SD. $^{**}P < 0.01$ versus control; $^{\#\#}P < 0.01$ versus LPA.

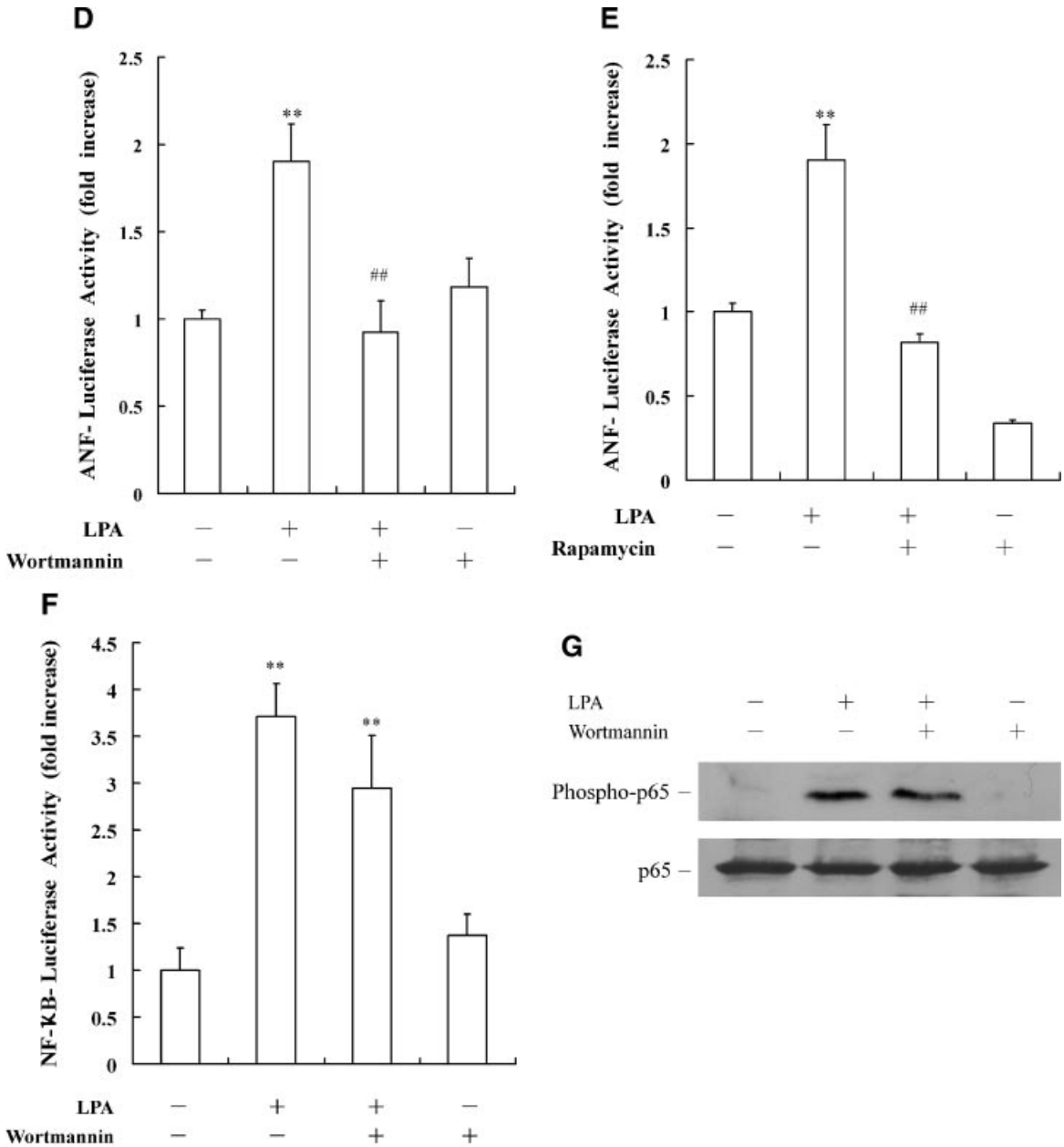

Fig. 8. *(Continued)*

and in vivo [Li et al., 2004]. We therefore investigated whether NF-κB was activated by LPA indeed. Our results show that LPA dose-dependently stimulated the activation of NF-κB and phosphorylation of p65. Both processes were inhibited by PDTC, which also attenuated the expression of ANF luciferase gene and suppressed protein synthesis induced by LPA. These findings indicate that activation of NF-κB is essential in LPA-stimulated hypertrophy in primary neonatal cardiomyocytes. The activation of NF-κB by LPA appears to be linked to ERKs that are known to be involved in the activation of NF-κB [Funakoshi-Tago et al., 2003]. Our data with U0126 and PD98059 confirmed the above speculation since both compounds completely abolished LPA-stimulated activation of NF-κB and U0126 also significantly blocked LPA-stimulated phosphorylation of p65 subunit.

A large body of evidence has implicated the PI3k/Akt pathway in mediating cardiomyocyte

hypertrophy [Matsui et al., 2003]. However it is not clear if LPA stimulates the PI3K pathway in primary cultured neonatal cardiomyocytes and whether activation of PI3K/Akt pathway is involved in LPA-induced hypertrophy. Our data revealed that LPA activates the PI3K pathway through Akt (PKB) activation as wortmannin, a PI3K specific inhibitor which blocked Akt phosphorylation, prevented LPA-induced hypertrophy. In relation to this, rapamycin, an immunosuppressive macrolide that inhibits mTOR signaling to p70S6kinase [Brown et al., 1995], also prevented LPA- stimulated hypertrophy. This is therefore consistent with reports that mTOR plays an important role in mediating the effects of Akt on cardiomyocyte growth and the hypertrophic response [Shioi et al., 2002, 2003].

Given that PI3K plays a role in LPA-induced activation of MAPK [Daub et al., 1997; Xu et al., 2000] and that activation of PI3K/Akt signaling stimulates NF-κB activation [Romashkova and Makarov, 1999], we speculated that PI3K/Akt might be upstream of NF-κB and investigated this possibility. The data obtained showed that inhibition of PI3K/Akt did not attenuate the expression of NF-κB-luciferase, nor did it suppress phosphorylation of the p65 subunit suggesting that although both NF-κB and PI3K/Akt participate in LPA-stimulated cardiac hypertrophy, these signaling events may function independently. This is in contrast to the established regulation of NF-κB activation by PI3K/Akt which commonly occurs in cell survival in preventing apoptosis [Romashkova and Makarov, 1999; Matsui et al., 2003].

In summary, we have demonstrated that LPA1 and LPA3 receptors play an important role in hypertrophic remodeling in vitro, as well as in vivo. Both receptor subtypes may mediate hypertrophy of cardiomyocytes responsing to LPA. This finding draws attentions for LPA and its specific receptors implicated in post-infarction and provide a new therapeutic strategy with specific receptor antagonist to prevent the risk of LV remodeling which may lead to heart failure. Furthermore, our data indicate that PI3K/Akt/mTOR and NF-κB signal pathways are involved in the process of LPA-induced hypertrophy but that these pathways may act in parallel with NF-κB being a downstream target for ERK1/2 but not for Akt.

ACKNOWLEDGMENTS

The authors are indebted to Professor Anwar R Baydoun, Dr. Rodney J. Dilley and Dr. Xiaojun Du for expert reviews of the manuscript.

REFERENCES

Anliker B, Chun J. 2004. Cell surface receptors in lysophospholipid signaling. Semin Cell Dev Biol 15:457–465.

Boucharaba A, Serre CM, Guglielmi J, Bordet JC, Clezardin P, Peyruchaud O. 2006. The type 1 lysophosphatidic acid receptor is a target for therapy in bone metastases. Proc Natl Acad Sci U S A 103:9643–9648.

Brown EJ, Beal PA, Keith CT, Chen J, Shin TB, Schreiber SL. 1995. Control of p70 s6 kinase by kinase activity of FRAP in vivo. Nature 377:441–446.

Chen X, Cui Z, Zhang F, Chang W, Chen L, Liu L. 2002. Angiotensin II and cAMP regulate AT(1)-mRNA expression in rat cardiomyocytes by transcriptional mechanism. Eur J Pharmacol 448:1–9.

Chen X, Yang XY, Wang ND, Ding C, Yang YJ, You ZJ, Su Q, Chen JH. 2003. Serum lysophosphatidic acid concentrations measured by dot immunogold filtration assay in patients with acute myocardial infarction. Scand J Clin Lab Invest 63:497–503.

Chen J, Han Y, Zhu W, Ma R, Han B, Cong X, Hu S, Chen X. 2006. Specific receptor subtype mediation of LPA-induced dual effects in cardiac fibroblasts. FEBS Lett 580:4737–4745.

Contos JJ, Ishii I, Chun J. 2000. Lysophosphatidic acid receptors. Mol Pharmacol 58:1188–1196.

Cook SA, Novikov MS, Ahn Y, Matsui T, Rosenzweig A. 2003. A20 is dynamically regulated in the heart and inhibits the hypertrophic response. Circulation 108:664–667.

Daub H, Wallasch C, Lankenau A, Herrlich A, Ullrich A. 1997. Signal characteristics of G protein-transactivated EGF receptor. Embo J 16:7032–7044.

Fang X, Schummer M, Mao M, Yu S, Tabassam FH, Swaby R, Hasegawa Y, Tanyi JL, LaPushin R, Eder A, Jaffe R, Erickson J, Mills GB. 2002. Lysophosphatidic acid is a bioactive mediator in ovarian cancer. Biochim Biophys Acta 1582:257–264.

Fischer DJ, Nusser N, Virag T, Yokoyama K, Wang D, Baker DL, Bautista D, Parrill AL, Tigyi G. 2001. Short-chain phosphatidates are subtype-selective antagonists of lysophosphatidic acid receptors. Mol Pharmacol 60:776–784.

Fukushima N, Chun J. 2001. The LPA receptors. Prostaglandins Other Lipid Mediat 64:21–32.

Funakoshi-Tago M, Sonoda Y, Tanaka S, Hashimoto K, Tago K, Tominaga S, Kasahara T. 2003. Tumor necrosis factor-induced nuclear factor kappaB activation is impaired in focal adhesion kinase-deficient fibroblasts. J Biol Chem 278:29359–29365.

Goetzl EJ, Lee H, Azuma T, Stossel TP, Turck CW, Karliner JS. 2000. Gelsolin binding and cellular presentation of lysophosphatidic acid. J Biol Chem 275:14573–14578.

Gupta S, Purcell NH, Lin A, Sen S. 2002. Activation of nuclear factor-kappaB is necessary for myotrophin-induced cardiac hypertrophy. J Cell Biol 159:1019–1028.

Hasegawa Y, Erickson JR, Goddard GJ, Yu S, Liu S, Cheng KW, Eder A, Bandoh K, Aoki J, Jarosz R, Schrier AD, Lynch KR, Mills GB, Fang X. 2003. Identification of a phosphothionate analogue of lysophosphatidic acid (LPA) as a selective agonist of the LPA3 receptor. J Biol Chem 278:11962–11969.

Hattori Y, Nakanishi N, Kasai K. 1997. Role of nuclear factor kappa B in cytokine-induced nitric oxide and tetrahydrobiopterin synthesis in rat neonatal cardiac myocytes. J Mol Cell Cardiol 29:1585–1592.

Hilal-Dandan R, Means CK, Gustafsson AB, Morissette MR, Adams JW, Brunton LL, Heller Brown J. 2004. Lysophosphatidic acid induces hypertrophy of neonatal cardiac myocytes via activation of Gi and Rho. J Mol Cell Cardiol 36:481–493.

Karliner JS, Honbo N, Summers K, Gray MO, Goetzl EJ. 2001. The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. J Mol Cell Cardiol 33:1713–1717.

Kitayama J, Shida D, Sako A, Ishikawa M, Hama K, Aoki J, Arai H, Nagawa H. 2004. Over-expression of lysophosphatidic acid receptor-2 in human invasive ductal carcinoma. Breast Cancer Res 6:R640–R646.

Li Y, Ha T, Gao X, Kelley J, Williams DL, Browder IW, Kao RL, Li C. 2004. NF-kappaB activation is required for the development of cardiac hypertrophy in vivo. Am J Physiol Heart Circ Physiol 287:H1712–H1720.

Li HL, Wang AB, Huang Y, Liu DP, Wei C, Williams GM, Zhang CN, Liu G, Liu YQ, Hao DL, Hui RT, Lin M, Liang CC. 2005. Isorhapontigenin, a new resveratrol analog, attenuates cardiac hypertrophy via blocking signaling transduction pathways. Free Radic Biol Med 38:243–257.

Matsui T, Nagoshi T, Rosenzweig A. 2003. Akt and PI 3-kinase signaling in cardiomyocyte hypertrophy and survival. Cell Cycle 2:220–223.

Mills GB, Moolenaar WH. 2003. The emerging role of lysophosphatidic acid in cancer. Nat Rev Cancer 3:582–591.

Moolenaar WH. 1999. Bioactive lysophospholipids and their G protein-coupled receptors. Exp Cell Res 253:230–238.

Okusa MD, Ye H, Huang L, Sigismund L, Macdonald T, Lynch KR. 2003. Selective blockade of lysophosphatidic acid LPA3 receptors reduces murine renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 285:F565–F574.

Purcell NH, Tang G, Yu C, Mercurio F, DiDonato JA, Lin A. 2001. Activation of NF-kappa B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. Proc Natl Acad Sci U S A 98:6668–6673.

Romashkova JA, Makarov SS. 1999. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 401:86–90.

Rother E, Brandl R, Baker DL, Goyal P, Gebhard H, Tigyi G, Siess W. 2003. Subtype-selective antagonists of lysophosphatidic Acid receptors inhibit platelet activation triggered by the lipid core of atherosclerotic plaques. Circulation 108:741–747.

Schulte KM, Beyer A, Kohrer K, Oberhauser S, Roher HD. 2001. Lysophosphatidic acid, a novel lipid growth factor for human thyroid cells: Over-expression of the high-affinity receptor edg4 in differentiated thyroid cancer. Int J Cancer 92:249–256.

Shida D, Watanabe T, Aoki J, Hama K, Kitayama J, Sonoda H, Kishi Y, Yamaguchi H, Sasaki S, Sako A, Konishi T, Arai H, Nagawa H. 2004. Aberrant expression of lysophosphatidic acid (LPA) receptors in human colorectal cancer. Lab Invest 84:1352–1362.

Shioi T, McMullen JR, Kang PM, Douglas PS, Obata T, Franke TF, Cantley LC, Izumo S. 2002. Akt/protein kinase B promotes organ growth in transgenic mice. Mol Cell Biol 22:2799–2809.

Shioi T, McMullen JR, Tarnavski O, Converso K, Sherwood MC, Manning WJ, Izumo S. 2003. Rapamycin attenuates load-induced cardiac hypertrophy in mice. Circulation 107:1664–1670.

Tigyi G, Parrill AL. 2003. Molecular mechanisms of lysophosphatidic acid action. Prog Lipid Res 42:498–526.

Virag T, Elrod DB, Liliom K, Sardar VM, Parrill AL, Yokoyama K, Durgam G, Deng W, Miller DD, Tigyi G. 2003. Fatty alcohol phosphates are subtype-selective agonists and antagonists of lysophosphatidic acid receptors. Mol Pharmacol 63:1032–1042.

Wang Y, Chen X, Zhu W, Zhang H, Hu S, Cong X. 2006. Growth inhibition of mesenchymal stem cells by aspirin: involvement of the WNT/beta-catenin signal pathway. Clin Exp Pharmacol Physiol 33:696–701.

Xu YJ, Ouk Kim S, Liao DF, Katz S, Pelech SL. 2000. Stimulation of 90- and 70-kDa ribosomal protein S6 kinases by arginine vasopressin and lysophosphatidic acid in rat cardiomyocytes. Biochem Pharmacol 59:1163–1171.

Yamada T, Sato K, Komachi M, Malchinkhuu E, Tobo M, Kimura T, Kuwabara A, Yanagita Y, Ikeya T, Tanahashi Y, Ogawa T, Ohwada S, Morishita Y, Ohta H, Im DS, Tamoto K, Tomura H, Okajima F. 2004. Lysophosphatidic acid (LPA) in malignant ascites stimulates motility of human pancreatic cancer cells through L PA1. J Biol Chem 279:6595–6605.

Yang YJ, Tang YD, Ruan YM, Zhang P, Zhou YW, Wang PH, Gao RL, Chen JL, Chen ZJ. 2003. Comparative effects of carvedilol and losartan alone and in combination for preventing left ventricular remodeling after acute myocardial infarction in rats. Circ J 67:159–162.

Yoshida K, Nishida W, Hayashi K, Ohkawa Y, Ogawa A, Aoki J, Arai H, Sobue K. 2003. Vascular remodeling induced by naturally occurring unsaturated lysophosphatidic acid in vivo. Circulation 108:1746–1752.

Yue TL, Gu JL, Wang C, Reith AD, Lee JC, Mirabile RC, Kreutz R, Wang Y, Maleeff B, Parsons AA, Ohlstein EH. 2000. Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. J Biol Chem 275:37895–37901.