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Mechanism of phospholipid induction of cell migration

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ABSTRACT

Lysophosphatidic acid (LPA) is a potent bioactive lipid component of oxidized low density lipoproteins (oxLDL). High concentrations of LPA have been detected in human atherosclerotic plaques. Our data has shown that LPA highly induces smooth muscle cell (SMC) migration. Cyr61, a matricellular protein, which also accumulates in human atherosclerotic plaques, has been implicated in the injury-induced neointimal formation. Smooth muscle cell migration is a key event in the development of atherosclerosis, and it contributes to the progressive growth of atherosclerotic lesions. Data generated by this study demonstrate that LPA markedly induces Cyr61 expression in mouse aortic smooth muscle cells (MASMC). We hypothesized that LPA-induced matricellular Cyr61 mediates LPA-induced MASMC migration. To date, little is known about the relationship between LPA and Cyr61 in smooth muscle cells; the signaling pathway leading to LPA-induced Cyr61 is unknown. Furthermore, whether Cyr61 contributes to LPA-induced cell migration is unrevealed. Our study demonstrates that LPA, by binding to LPA1 receptor, activates the intracellular signaling pathway leading to the activation of PKCδ, which in turn contributes to the increased expression of Cyr61 in MASMCs. Interestingly, we found that after LPA-induced Cyr61 mRNA has been translated into its protein intracellularly, the de novo synthesized proteins promptly accumulate in the Golgi apparatus and then translocalize to the extracellular matrix. Importantly, our data reveal a novel LPA/Cyr61 pathway in controlling MASMC migration. Understanding

the mechanism underlying LPA induction of Cyr61 provides new insight into pathogenesis of atherosclerosis.

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INTRODUCTION

Cardiovascular diseases are the No.1 cause of death in the United States. Atherosclerosis is a major component of cardiovascular disease, the primary cause of heart disease and stroke. It is the underlying cause of about 50% of all deaths in westernized societies. Over the past years, the mechanism of atherosclerosis has been more understood. Atherosclerosis is a chronic inflammatory condition, which causes a progressive disease characterized by the accumulation of lipids, cells and fibrous elements in the large arteries [1].

NORMAL ARTERIES

The basic components of blood vessels are cells, mostly endothelial cells (ECs) and smooth muscle cells (SMCs), and extracellular matrix, which includes collagen, elastin and glycosaminoglycans [2].

There are three concentric layers (intima, media, and adventitia) in the arterial walls. The intima consists of a single layer of ECs with little underlying subendothelial connective tissue and is bordered by internal elastic lamina. The endothelial cells form a firm barrier between the vessel lumen and the stroma of the arterial wall. Research has revealed that endothelial cells regulate a wide set of functions in the arterial wall, including vascular tone and structure, and they exhibit anticoagulant, antiplatelet, and fibrinolytic properties [3].

The most prominent constituent of the media layer is smooth muscle cells. These

cells are held together by an extracellular matrix which consists mostly of elastic fibers and collagen. These cells may also be attached together by junctional complexes. The smooth muscle cell is the main contributor to production of the extracellular matrix [4]. It is well known that the vascular smooth muscle has the function of controlling vascular tone through contraction or relaxation. The vascular smooth muscle has also been shown to be involved in the regulation of cell growth, death, and migration, as well as matrix modulation and inflammation. These functions of vascular smooth muscle play important roles in beneficial physiological vascular functions, such as vascular remodeling, and also in pathological disorders, such as atherosclerosis, restenosis, transplant vasculopathy, and other vascular diseases [5].

The outer limit of the media is defined by the external lamina. The outermost layer of the artery is the adventitia, consisting of the connective tissue with nerve fibers and the vasa vasorum [6]. The adventitia is thought to be a major source of vasoactive factors and a pivotal participant in vascular remodeling. Over the past years the researchers noted that the changes occurring in the adventitia may be a signal of impending vascular disease, with typical hypercellularity, increased connective tissue production, and clear signs of inflammation [7].

ATHROSCLEROSIS

Atherosclerosis is a progressive disease caused by the accumulation of lipids and fibrous elements in the large arteries. (1) The intimal lesions are called atheromas or fibrofatty plaques, which protrude into and obstruct vascular lumina, weaken the

underlying media, and finally cause serious complications. Atherosclerosis mostly affects elastic arteries and large and medium muscular arteries. As a result, acutely or chronically diminished or stopped arterial perfusion leads to lots of severe consequences, including mesenteric occlusion, chronic ischemic heart disease, sudden cardic death and encephalopathy.

Due to the overwhelming clinical importance of atherosclerosis, over the past decades scientists have put enormous efforts into discovering the mechanism behind the diseases. The current concept, called the response to injury hypothesis, considers that atherosclerosis is a chronic inflammatory response of the arterial wall initiated by injury to the endothelium. Hyperlipidemia, and other risk factors, cause chronic endothelia injury, which yields increased permeability, leukocyte adhesion and thrombotic potential. As a result, lipoproteins, mostly Low-density Lipoprotein (LDL), accumulate in the subendothlial matrix. Meanwhile, blood monocytes adhere to the endothelium and migrate into the intima and then are transformed into macrophages.

Under the induction of free redicals generated by macrophages or EC, accumulated LDL is modified through oxidation, proteolysis, lipolysis and aggregation [2][8]. Distinct from native LDL, oxidized LDL can be ingested by macrophages through the scavenger receptor, leading to the formation of the foam cells. It can also stimulate the overlying ECs to produce a number of proinflammatory molecules, including adhesion molecules and growth factors. In the lumen, platelets adhere to the endothelial cells and become activated. After being activated, platelets release numerous imflammatory factors, and they, together with other factors released

by other cells cause the smooth muscle cells to migrate from the media into the intima Finally, SMC proliferation and the extracellular matrix that SMCs deposit in the intima convert a fatty steak into a mature firbrofatty atheroma, leading to the progressive growth of atherosclerosis lesions [2]. Most importantly, in the development of atherosclerosis SMC migration and proliferation are key events .

RISK FACTORS:

Over the past years, epidemiological studies have revealed numerous risk factors for atherosclerosis. The well-established familial predisposition to causes of atherosclerosis is polygenic. Age, along with other factors, including hyperlipidemia, smoking, diabetes mellitus, homocystinemia, contribute to the development of the atherosclerosis [2]. Among the risk factors, low density lipoprotein is one of the most important risk factors. And the most pathogenic form of LDL is the oxidized LDL.

LYSOPHOPHATIDIC ACID (LPA):

Oxidized low density lipoprotein (LDL) is an important contributor in the pathogenesis of atherosclerosis and its thrombotic complications, such as stroke and myocardial infarction. It has been found that lysophosphatidic acid (LPA) is formed during mild oxidation of LDL and is the active compound in mildly oxidized LDL and minimally modified LDL. LPA also initiates platelet activation and stimulates endothelial cell stress-fiber and gap formation. Furthermore, LPA is the primary platelet-activating lipid of atherosclerotic plaques, and the amount of LPA within the

human carotid atherosclerotic lesion is highest in the lipid-rich core, which is the region most thrombogenic and most prone to rupture [9].

LPA, a potent bioactive lipid, elicits a variety of cellular responses in various cell types including proliferation, migration, prevention of apoptosis, cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, and neurite retraction. LPA has multiple effects on vessel wall cells and blood platelets. Our group has reported that LPA induces the expression of transcription factor, Early Response Gene-1 (EGR-1) and coagulation initiator, tissue factor (TF) in aortic smooth muscle cells, and promotes interleukin 6 secretion from aortic smooth muscle cells. These results imply that LPA might play a role in vascular inflammation and thrombosis [10][11][44]. Our recent data and previous studies demonstrate that LPA induces smooth muscle cell migration and proliferation. Given the fact that smooth muscle cell migration is an important future of atherogenesis, it is of great clinic importance to understand the molecular mechanism of LPA induction of smooth muscle cell migration.

LPA RECEPTORS:

LPA is an extracellular ligand for a family of G protein-coupled receptors. Through binding to the LPA receptors, LPA activates intracellular signaling pathways, which have been shown to be involved in many essential cellular processes [12]. At least 5 LPA receptors have been identified. They are LPA_{1-5}, which are widely expressed in various cells and mammalian organ systems, such as cardiovascular,

nervous and reproductive systems [13].

$LPA_{1,}$ also called EDG2, was the first identified LPA receptor [14]. It is highly expressed in many organ tissues including brain, heart, lung, thymus, testis, stomach, small intestine, spleen, and skeletal muscle in adult mice [15] and has also been detected in numerous human tissues such as brain, heart, placenta, spleen, kidney, colon, small intestine, prostate, testis, ovary, pancreas, thymus, and skeletal muscle [16]. It is reported that LPA_1 is involved in cell proliferation, serum-response element (SRE) activation, MAPK activation, PLC/PKC activation, Akt activation, and Rho activation through three types of G proteins: Gi/o, Gq, and G12/13 [17] [18].

LPA_2, also called EDG4, was the second LPA receptor identified by sequence homology searches using LPA_1 [16] [19]. It shows gene express in embryonic brain, testis, kidney, lung, thymus, spleen, and stomach in mice [15]. LPA_2 has also been detected in human tissues including testis, pancreas, prostate, thymus, spleen, and peripheral blood leukocytes [16]. Like LPA_1, LPA_2 signaling is also via three G proteins: Gi/o, Gq, and G12/13 [19] [20]. Within MEFs, LPA_2 has been shown to have redundant functions in mediating PLC activation, proliferation, JNK activation, Akt activation, and stress fiber formation [21].

LPA_3, also called EDG7, was identified by degenerate polymerase chain reaction (PCR)-based cloning and homology searches [22][23]. LPA_3 is found in kidney, lung, testis, small intestine, heart, thymus, brain, oviduct, placenta, and uterus in adult mice [15]. It is also detected in heart, pancreas, prostate, testis, lung, ovary, and brain in humans [22][23]. LPA_3 couples to Gi/o and Aug protein, but unlike LPA_1 and LPA_2, it

does not couple to G12/13 [18]. LPA_3 signaling induces PLC activation, Ca2+mobilization, AC inhibition/activation, and MAPK activation [18][22][23].

LPA_4, also called p2y9/GPR23, was identified during a “de-orphaning” project of G protein-coupled receptors. It is found in human ovary, kidney, skeletal muscle [24] and has also been detected in brain, heart, lung, thymus, kidney, skeletal muscle, ovary, uterus, and placenta in adult mice. LPA4 signaling couples with Gq/11 and Gs proteins [25].

LPA_5 also called GPR92 was a GPCR closely related to LPA_4 [26], sharing at most 35% sequence identity with LPA4 and lower identities compared with $LPA_{1–3}$. It is highly expressed in small intestine, and is also detected in spleen and stomach of adult mice. It has been demonstrated that LPA_5-mediated signaling is relevant to normal function, most likely in concert with previously identified receptors [27].

CYSTEINE-RICH PROTEIN 61:

Although the extracellular matrix was considered a benign scaffold for arranging cells within connective tissues for many years, it is now being redefined as a dynamic, mobile, and flexible regulator of cellular behaviour [28]. The ECM can regulate the bioavailability and activity of growth factors, chemokines, cytokines and extracellular enzymes. Moreover, ECM proteins can also directly interact with cell surface receptors, triggering the activation of signal transduction cascades, and regulating diverse cellular functions [29].

The CCN proteins are an important family of matricellular regulatory factors

involved in internal and external cell signaling, in angiogenesis, chondro-genesis, and osteogenesis, and they probably participate in the control of cell proliferation and differentiation [30].

Cysteine-rich 61 (Cyr61, CCN1) is a **Error! Reference source not found.** that is encoded by the Cyr61 gene [31]. As a member of the CCN protein family, CCN1/Cyr61 plays important roles in cell proliferation, adhesion, differentiation, angiogenesis and extracellular matrix production. Moreover, CCN1/Cyr61 also has many potential functions in tumorigenesis, development, embryo implantation, as well as formation of endometriotic lesions. Various agents, including cytokines, growth factors, steroid hormones, and some drugs, regulate expression of CCN1/Cyr61 through several signaling transduction pathways. As a result, CCN1/Cyr61 is not only able to regulate the growth of epithelial cells and fibroblasts, but also to induce or suppress apoptosis in a specific cell type [32].

Cyr61 regulates SMC proliferation, adhesion, migration, differentiation, apoptosis, extracellular matrix production, which are important steps in the initiation and progression of atherosclerosis, therefore Cyr61 could be one important regulator in the process of atherosclerosis.

MATERIAL AND METHODS

MATERIALS:

LPA (1-Acyl-2-hydroxy-sn-glycerol-3-phosphate) in this study was purchased from Avanti Polar Lipids, Inc; transient chambers (6.5 mm diameter, with 8.0 μm pore size polycarbonate membranes) were from Corning Costar Corp; and phosphate buffer, Harris hematoxylin solution, and eosin Y solution were from Sigma; PD98059 (PD), SB203580 (SB), U0126 (U0), GF109203X (GF), GO6976, GO6983, SP600125 (SP), Wortmannin, LY294002 (LY) and Rottlerin were from Enzo Life Science. Resveratrol was from EMD. Antibody against actin was from Sigma; Antibodies against Mouse Cyr61 was from R&D System; Antibodies against adaptin was from BD Transduction Laboratories; Antibodies against Egr-1, p-JNK, p-AKT, p-ERK, p-PKD, p-PKCδ, p-PKCθ, p-P38, p-PKC, p-PKC p-PKCαβ, PKD_1, PKD_2, and MEK were from Cell signaling; Rhodamine Red-X-conjugated AffiniPure goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories. Goat anti-sheep IgG Alexa Fluor 488 was from Invitrogen; SelectFX Nuclear Labeling Kit and SlowFade Gold antifade reagent were from Invitrogen; mouse PKCδ siRNA, mouse PKCθ siRNA, mouse PKD_1 siRNA and mouse PKD_2 siRNA were from Qiagen; VECTASTAIN ABC kit and peroxidase substrate kit DAB were from Vector Laboratories, INC. Hybond-N membranes were from GE Healthecare. Trizol reagent was from Invitrogen. GoTaq Flexl DNA Polymerase and Reverse Transcription system were from Promega. RNeasy Mini Kit was from Qiagen. Recombinant Cyr61 protein was from Abcam.

Fast Optical 96-Well Reaction Plaste with Barcode, Taqman Gene Expression Master MixReal time, Mouse $LPA_{1\text{-}5}$ receptors primers for Real time PCR reagents were from Applied Biosystem.

CELL CULTURE:

Mouse aorta smooth muscle cells (MASMCs) were prepared from explants of excised aortas of mice and rats as described previously [33]. Cells between 10 and 20 were used in these studies. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% glutamine. For Western blot assays, cells were starved for 48 hours prior to addition of LPA or other reagents. For cell migration assay, prior to the cell migration assay, cells were starved for 24 hours.

SMOOTH MUSCLE CELLS WESTERN BLOT ANALYSIS

Mouse aortic SMCs (MASMCs) were rinsed with cold PBS and then lysed in cell lysis buffer (50 mM Tris–HCl, pH 6.8, 8 M urea, 5%-mercaptoethanol, 2% SDS, and protease inhibitors) with sonication for 30 s on ice. After addition of 4X SDS sample buffer and incubation at 65 ℃ for 20 minutes, samples were subjected to 10% SDS polyacrylamide gel electrophoresis and were transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membranes were then probed with the specific first antibodies, after being washed in TBST 4 times (5 minutes for each), the membranes were incubated in specific second antibodies, which depended on the source of the first antibody used, and finally the bands were visualized by ECL Plus

(GE Healthcare) as described previously [11].

CELL MIGRATION ASSAY

Cell migration was performed using transwell migration plates purchased from Corning. Mouse smooth muscle cells were trypsinized and plated onto transwell plates for migration assays. A volume of 200 μl media containing 2×10^5 cells was added to the upper chamber. Cells were allowed to migrate through filters (8 μm pore size), which had been precoated on both sides with gelatin, in the presence of either medium (600 μl) alone or medium with LPA at designated concentrations in the lower chamber. Cell migration was carried out at 37 ℃ in 5% CO2 for 6 h. Cells remaining on the upper surface of the filter were carefully removed by mechanical scraping. The upper chambers were rinsed with PBS, and the cells were fixed with methanol and then stained with Harris hematoxylin and eosinY. The number of cells that had migrated to the lower surface of the filter was counted in 4 random objective fields (200×magnification) using a Nikon Eclipse E600 microscope.

IMMUNOFLUORESCENCE

MASMCs grown in 6-well chamber coverglass slides were fixed in 4% ice cold paraformaldehyde solution for 30 minutes, permeablized with or without 0.3% triton X-100 in PBS for 5 minutes at room temperature, blocked with 5% goat serum (Sigma) plus 0.1% Tween-20 in PBS for 1 hour, and then incubated with Cyr61 antibody and adaptin in 1/100 dilution overnight at 4 ℃. After being washed with

mild shaking with PBS 3 times (5 minutes for each), the cells were incubated with the secondary antibody, goat anti-sheep IgG Alexa Fluor 488 or Rhodamine Red-X-conjugated AffiniPure goat anti-mouse IgG for 2 hours at room temperature. Then the cells were washed with PBS 4 times (5 minutes for each) at room temperature, and then incubated with DAPI for 2 minutes and washed with PBS 3 times (5 minutes for each) at room temperature. Subsequently, the coverslips were mounted on slides with permanent aqueous mounting medium (Biogenex), and the labeled cells were analyzed by fluorescence microscopy with a Nikon Eclipse E600 microscope.

IMMUNOPRECIPITATION

After being stimulated with 5 μM LPA for 3, 5, 12, or 24 hours, MASMCs were harvested and conditioned media was collected. Secreted Cyr61 was immunoprecipitated from conditioned media using the sheep anti-mouse Cyr61 antibody from R&D System. To detect intracellular Cyr61 protein with the immunoprecipitation, cells were homogenized with buffer A (20 mM HEPES, PH7.4, 50 mM KCl, 2 mM EDTA, protease inhibitor cocktail from ROCHE) by passing through 20-gauge needles for 15 times after freezing and thawing 5 times in liquid nitrogen and at 37 ℃ water bath. The homogenized samples were then centrifuged at 800 x g for 10 minutes to separate the post nuclear supernatant (PNS) from the unbroken cells and nuclei. PNS was further subjected to centrifugation at 20,000 x g for 1 hour and the pellet was solubilized in buffer B (50 mM PIPES, pH7.0, 150 mM

KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and protease inhibitor cocktail with 1% CHAPSO) for 1 hour at 4 ℃, and then centrifuged again at 20,000 x g for 30 minutes. After the supernatant was incubated with the Cyr61 antibody and protease inhibitor cocktail for 3 hours with rotation at 4 ℃, Protein A sepharose beads were added and incubated overnight with rotation. The immunocomplex was washed with Buffer B four times and then lysed with PARP buffer, the samples were subjected to 10% SDS-PAGE gel.

ADENOVIRAL INFECTION OF MASMCs

Adenoviruses encoding mouse PKCδ subtype was constructed as previously described [34]. MASMCs were infected for 24 h with either wild type or dominant negative PKCδ adenovirus, then starved for 48 hours followed by treatment either with or without LPA.

SMALL INTERFERING RNA (siRNA) TRANSFECTION:

MASMCs were cultured in RPMI 1640 medium with 10% fetal bovine serum. Signal Silence Control siRNA (non-silencing siRNA, 40 nM), Mouse PKCδ siRNA (40 nM), Mouse PKCθ siRNA (40 nM), Mouse PKD_1 siRNA (40 nM), mouse PKD_2 siRNA (40 nM) were transfected according to the manufacturer's instructions (Cell Signaling Technology). The Signal Silence Control siRNA was used as a negative control. Forty-eight hours after transfection, the cells were trypsinized and transfected with siRNA again. After forty-eight hours the cells were starved for 48 h followed by treatment either with or without LPA.

PREPARATION OF CELL LYSATES AND ECM

MASMCs were grown in 60 mm dishes and treated as described before. After removal of the culture medium and rinsed with PBS, cells were detached from the dish by incubation with 1 mM EDTA. The dishes were then rinsed twice with EDTA to remove remaining cells. Cellular fractions were lysed with PARP buffer, sonicated on ice, added with 4x loading buffer, and incubated in 65 ℃ for 15 min prior to analysis. Extracellular material remaining on the dishes after removal of the cellular components was extracted by scraping at 90 ℃ in 1 x Laemmli sample buffer (I x = 60 mM Tris -HCI, pH 6.8, 2% SDS, 5 % fl-mercaptoethanol, 5% glycerol) . These fractions were designated ECM.

NORTHERN BLOTTING ANALYSIS FOR DETECTION OF MASMCS CYR61 mRNA:

Total cellular mRNA was isolated according to the manufacturer's instruction. Total RNA (6-8 μg) was subjected to denaturing electrophoresis in formaldehyde/agarose gels and was blotted onto Hybond-N membranes. Hybridization was carried out using ^{32}p-labeled Cyr61 cDNA probes. A 0.7 kb fragment of mouse Cyr61 cDNA was used to detect Cyr61 mRNA. 18S and 28S ribosomal RNA was used as an internal control.

RT-PCR ASSAY:

Expression of mRNA was evaluated by RT-PCR. Total RNA was isolated from MASMCs and mouse tissues using a Trizol Reagent. The first strand of cDNA was reverse transcribed using the reverse transcription system. The cDNA products were amplified using GoTaq Flexl DNA Polymerase. The amplification conditions were as follows: 5min at 95 ℃; 27 to 33 cycles of 30 s at 95 ℃, 30 s at 55 ℃, and 1 min at 72 ℃; this was followed by a final extension for 10 min at 72 ℃. The primers were used as follows: LPA1, 5′-AGC TGC CTC TAC TTC CAG C-3′(forward) and 5′-TTG CTG TGA ACT CCA GCC AG-3′ (reverse); LPA2, 5′-ATG GGC CAG TGC TAC TAC AAC G-3′ (forward) and 5′ AGG GTG GAG TCC ATC AGT G-3′ (reverse); LPA3, 5′-GAC AAG CGC ATG GAC TTT-3′ (forward); 5′-CAT GTC CTC GTC CTT GTA CG-3′ (reverse)); LPA4, 5′-GTT GTA TTC ATC CTG GGT CT-3′ (forward); 5′-AGC GAC TCC ATC CTT ATA TG-3′ (reverse); LPA4, 5′-TGC TCT GAC CTT GTT GTT CC-3′ (forward); 5′-AGC AAC CCA TAT ACA GCC AGC G-3′ (reverse)). The PCR products were analyzed by electrophoresis on a 1.0% agarose gel.

STATISTICS:

Results are means ± SE. Comparisons between multiple groups were performed using one-way ANOVA with post-hoc t-tests. Single comparisons were made using two-tailed, unpaired Student's t-tests. A p value of 0.05 was considered to be statistically significant.

RESULTS

1. Cyr61 is markedly induced by LPA in MASMCs.

1.1 LPA markedly induces Cyr61 protein expression:

When MASMCs were stimulated with various concentrations of LPA after the cells were starved for 48 hours, Cyr61 protein expression increased in response to LPA stimulation in a concentration dependent manner with the maximal induction in 25-200 μM (Figure 1). We also observed that LPA induction of Cyr61 protein was transient, it increased from 30 minutes and peaked at 3 hours, then declined dramatically after 3 to 6 hours (Figure 2). Our data reveal for the first time that LPA induces Cyr61 expression in SMCs.

1.2 Induction of Cyr61 mRNA expression by LPA:

To determine whether LPA induction of Cyr61 protein expression is due to the accumulation of Cyr61 mRNA, we examined the effect of LPA on Cyr61 mRNA levels. We observed that LPA significantly increased Cyr61 mRNA accumulation in quiescent MASMCs. The induction is also transient. The induction peak time is at around 1 hour, and declined rapidly after 3 hours (Figure 3).

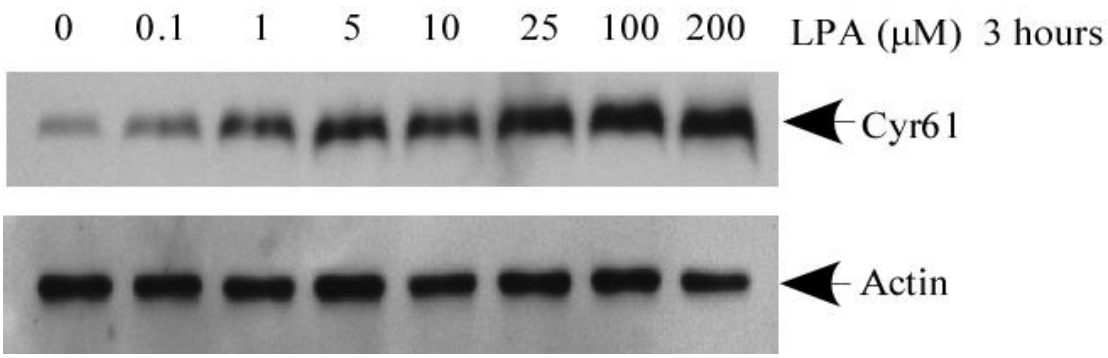


Figure 1. Induction of Cyr61 protein in response to LPA in MASMC. LPA was added to quiescent MASMCs at the concentrations indicated above each lane for 3 hours. Cyr61 protein level was determined by western blotting with 10% SDS PAGE gel. Actin was used as the loading control.

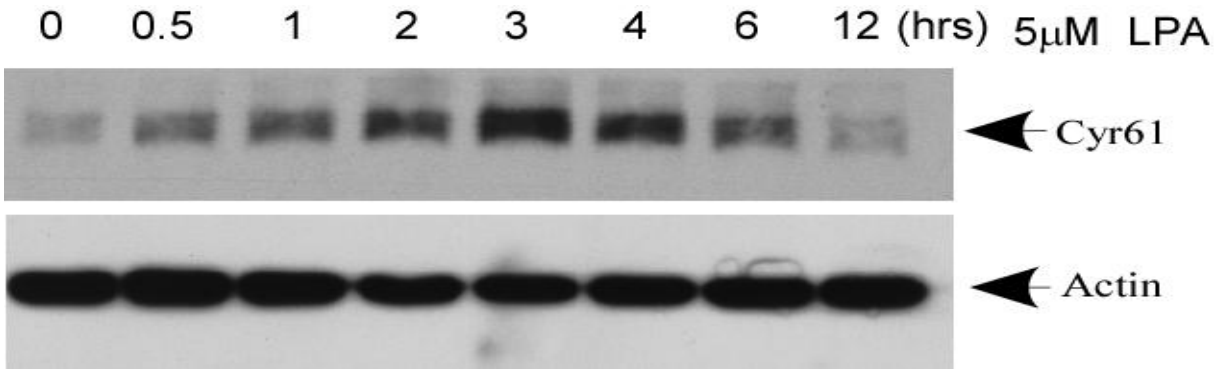


Figure 2. Time course of LPA induction of Cyr61 protein in MASMCs. 5 μM LPA was added to quiescent MASMCs for various times indicated. Cyr61 protein level was determined by western blotting with 10% SDS PAGE gel. Actin was used as the loading control.

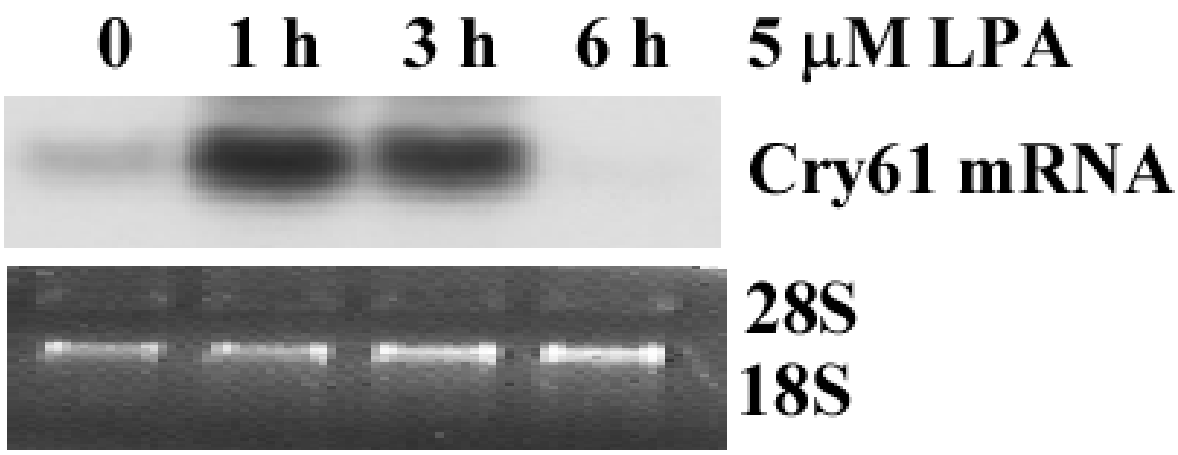


Figure 3. Time course of LPA induction of Cyr61 mRNA in MASMCs. 5 μM LPA was added to quiescent MASMCs for various times indicated. Total mRNA was isolated, and Cyr61 mRNA levels were determined by Northern blot analysis. A mouse Cyr61 cDNA fragment was used as a probe. 18S rRNA was visualized by ethidium bromide staining.

1.3 LPA receptor expression in MASMCs, mouse aorta and carotid arteries.

To determine the expression levels of LPA_{1-5} receptors in MASMCs, mouse aorta and carotid arteries, total mRNA was extracted and RT-PCR was performed. We observed that the expression levels of each type of LPA receptors in these cells and tissues were similar, mainly because the major component of mouse aorta and carotid arteries is the smooth muscle cells. As shown in figure 4, receptors LPA_1, LPA_2 and LPA_4 were highly expressed, LPA_3 was expressed at a very low level and there is no LPA_5 receptor expressed in these cells and tissues.

1.4 The expression of LPA_{1-5} receptors in WT, $LPA_1^{-/-}$, $LPA_2^{-/-}$ and $LPA_3^{-/-}$ MASMCs.

The above data showed LPA receptor expression in WT MASMCs, but it is still unclear whether up-regulation or down-regulation of the other LPA receptor genes occurred when certain LPA receptors were knockout. We have received LPA1 +/- mice from Dr. Jerold Chun, The Scripps Research Institute and successfully established LPA_1-/- and LPA_2-/- mice and isolated smooth muscles cells from these knockout mice. It was reported that LPA_3 receptor was dramatically upregulated in LPA1 knockout (KO) MASMCs [35], however, it was also reported that in $LPA_1^{-/-}$ adult brain, the change in transcript levels of LPA_2 and LPA_3 gene can not be observed [36]. To determine whether LPA_3 receptor was upregulated in $LPA_1^{-/-}$ or $LPA_2^{-/-}$ MASMCs, we extracted total mRNA from each cell type and performed real

time PCR. As shown in figure 5, there was no significant difference in the expression levels of LPA_3 in the LPA1 or LPA2 knockout cells compared to wild type, indicating that there is no compensation of LPA_3 in LPA_1 or LPA_2 knockout cells. Our Northern blot data and Western blot data confirm this conclusion, as shown in figure 6, there is no obvious changes in expression levels of LPA3 in $LPA_{1\text{-}2}$ knockout MASMCs. We next determined whether LPA stimulates LPA3 gene expression in WT and $LPA_1^{-/-}$ MASMCs. As shown in figure 7, the expression of LPA_3 was not changed after LPA stimulation, all of the above data strongly supported that LPA_3 was not upregulated in LPA1-/-, LPA2-/- MASMCs, and LPA stimulation has no effect on LPA3 receptor expression, either.

We also examined whether expression levels of $LPA_{1.2.4.5}$ receptors were upregulated in LPA1-/-, LPA2-/-, LPA3-/- MASMCs. As shown in Figure 5, 6, 8 and 9, there was no compensation phenomenon was observed in any of the LPA receptors in LPA receptor knockout SMCs.

1.5 LPA receptor LPA_1, but not PPARγ, mediates LPA-induced Cyr61 protein expression.

To explore the intracellular pathways through which LPA exerts its functions, we first evaluated the involvement of LPA receptors. Since LPA_1, LPA_2 and LPA_4 are predominantly expressed in MASMCs, we examined which LPA receptor mediated LPA-induced Cyr61 protein expression. We stimulated quiescent wild-type LPA_1-/- and LPA_2-/- MASMCs with LPA for 3 hours, and Cyr61 expression was detected by

Western blot analysis. We observed that genetic depletion of LPA1 nearly completely shut down LPA-induced Cyr61 protein expression; however, genetic depletion of LPA2 has no effect on LPA induction of Cyr61 expression, indicating that LPA_1 mediates Cyr 61 expression (Figure 11). To examine whether genetic depletion of LPA receptors affect other stimulus-initiated signaling pathway, we determined the influence of LPA1-/- and LPA2-/- on the EGF signaling pathway. Quiescent wild-type, LPA_1-/- or LPA_2-/- MASMCs were stimulated with 100 ng/ml EGF for 5 minutes and the phosphorylation of MAPKs was detected by Western blot analysis, the results showed genetic depletion of LPA1 and LPA2 receptors had no effect on EGF-induced phosphorylation of MAPKs (Figure 12). Besides LPA-specific plasma membrane receptors, nuclear receptor PPAR-γ has also been reported to work as an intracellular receptor and to transmit the LPA signal to downstream molecules [37]. To examine whether PPAR-γ has a role in LPA-induced Cyr61 protein expression in MASMCs, we determined the effect of the PPAR-γ antagonist GW-9662 on LPA-induced Cyr61 protein expression. We observed that pretreatment of MASMCs with various concentrations of GW-9662 (0.1–10 μM) for 45 min had no effect on the expression levels of Cyr61 induced by LPA (Figure 10). The range of the concentrations used is based on the information reported previously that 1 μM of GW-9662 efficiently blocks PPAR-γ activation in vascular SMCs [38]. Our results indicate that the PPAR-γ pathway is not involved in the LPA induction of Cyr61 protein expression.

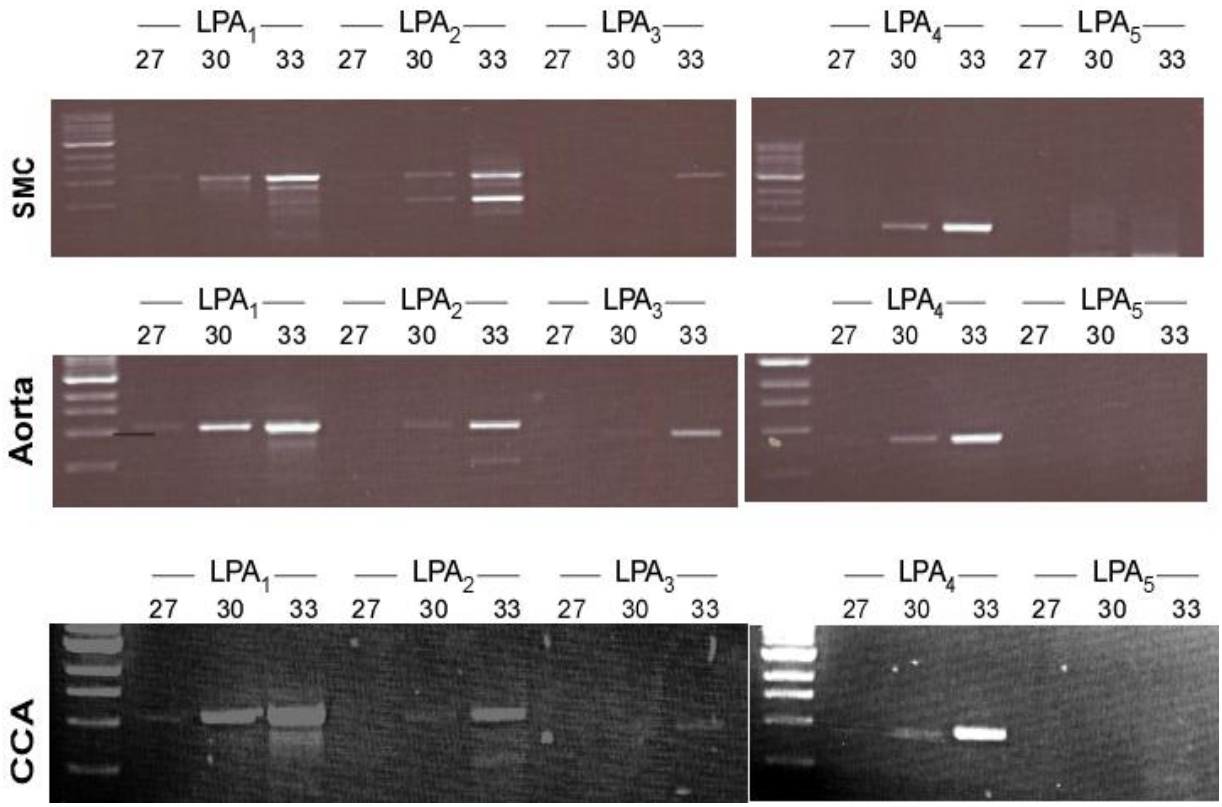


Figure 4. R-T PCR results, LPA1-5 receptor expression in MASMCs, mouse aortas and carotid arteries. Total mRNA from MASMC, mouse aorta and carotid arteries was extracted with trizol reagent. After reverse transcritption, cDNA was used to perform RCR analysis with Mouse $LPA_{1\text{-}5}$ primers.

Ratio	$LPA_1^{-/-}$/WT	$LPA_2^{-/-}$/WT
LPA_1	-	0.87
LPA_2	0.89	-
LPA_3	0.73	0.67
LPA_4	0.93	1.10
LPA_5	-	-

Figure. 5. Real time PCR data, LPA1-5 receptor expression in MASMCs. Total mRNA from MASMCs was extracted with RNeasy mini kit. After reverse transcription, cDNA was used to perform Real time RCR analysis with Mouse $LPA_{1\text{-}5}$ primers.

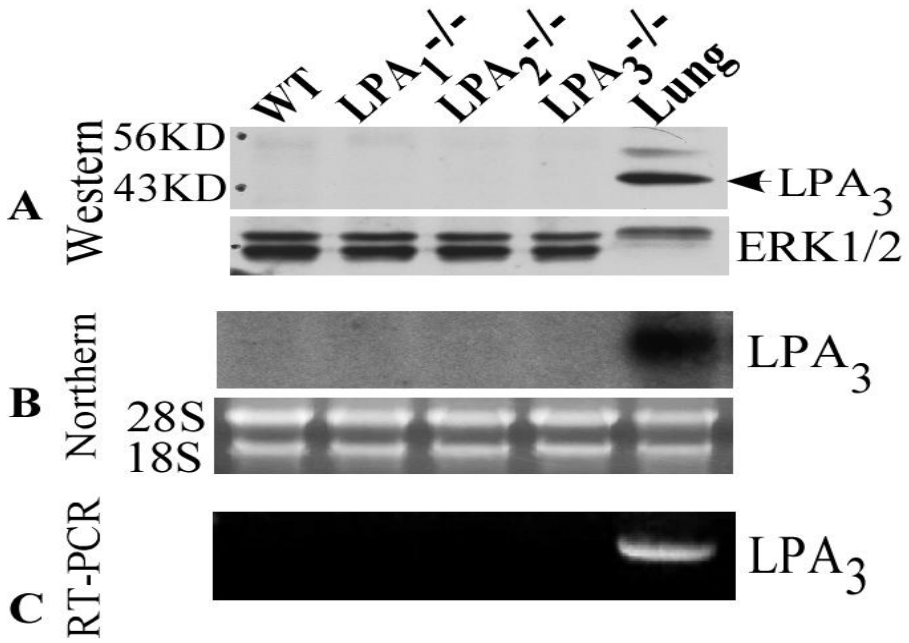


Figure 6. A. Western blot analysis showing LPA3 receptor expression in WT, LPA1-/-, LPA2-/- and LPA3-/- MASMCs. The tissue samples from mouse lung were used as a positive control, Erk has been used as a loading control. **B**. Northern blot analysis showing LPA3 receptor expression in WT, LPA1-/-,LPA2-/- and LPA3-/- MASMCs. Tissue samples from mouse lung were used as a positive control, 28S and 18S rRNA were visualized by ethidium bromide staining. **C**. Total mRNA of MASMCs, mouse aorta and carotid arteries was extracted with trizol reagent. After reverse transcritption, cDNA was used as template DNA, which was amplified 30 cycles with mouse $LPA_{1\text{-}5}$ primer pairs. Tissue samples from mouse lung were used as positive controls.

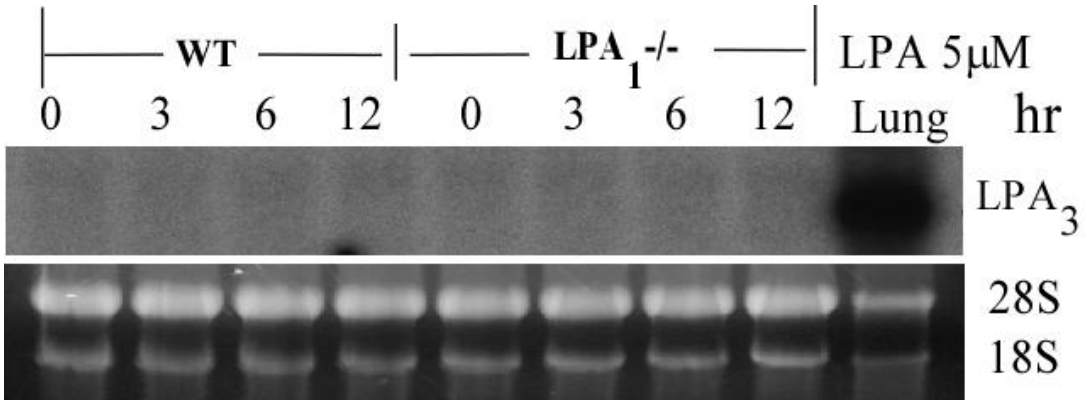


Figure 7. Northern blot analysis data: LPA_3 expression levels in WT, $LPA_1^{-/-}$MASMCs after stimulation with LPA. WT and $LPA_1^{-/-}$MASMCs were stimulated with LPA for the time periods indicated above, the samples from mouse lung has been used as a positive control, 28S and 18S rRNA were visualized by ethidium bromide staining.

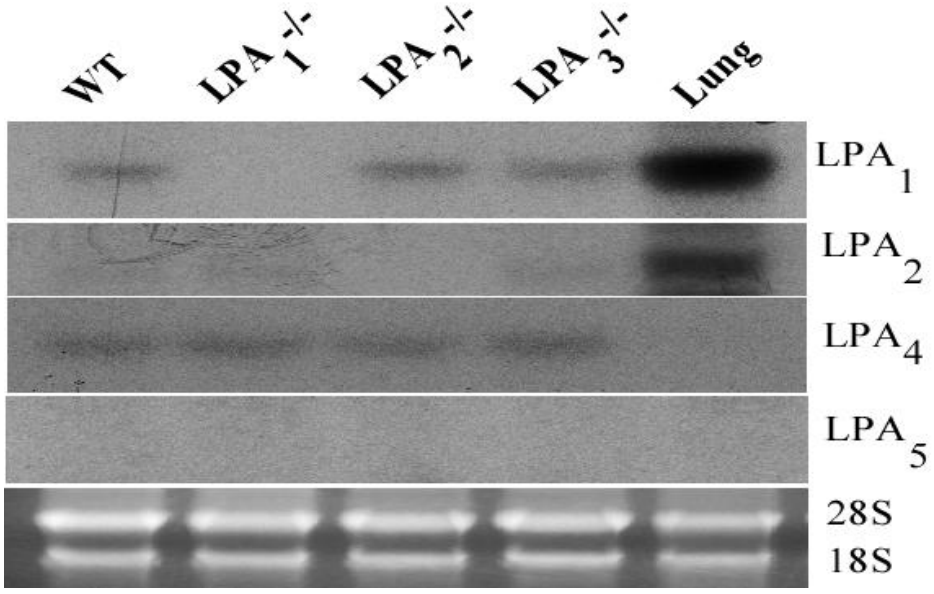


Figure 8. Northern blot analysis, the expression of LPA1, LPA2, LPA4 and LPA5 in WT, LPA1-/-, LPA2-/- and LPA3-/- MASMCs. The tissue from mouse lung was used as a positive control for $LPA_{1.2}$, 28S and 18S rRNA were visualized by ethidium bromide staining.

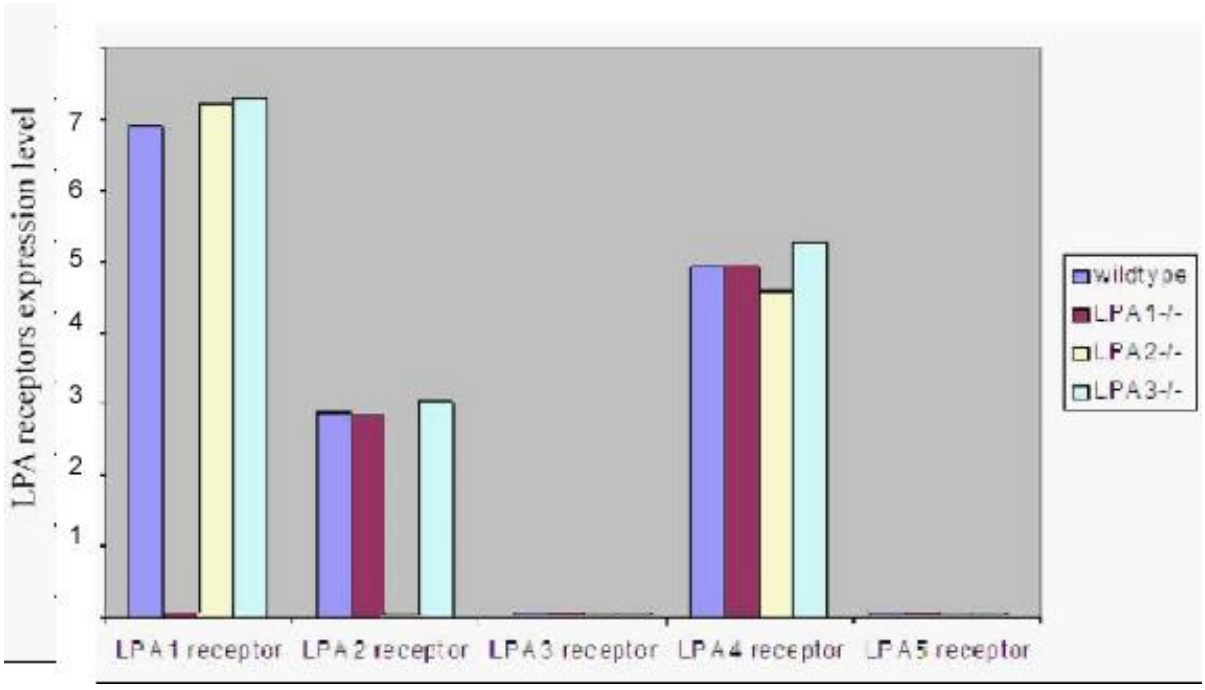


Figure 9. A diagram showing the relative expression levels of $LPA_{1.2.3.4.5}$ in WT, $LPA_1^{-/-}$, $LPA_2^{-/-}$ and $LPA_3^{-/-}$ MASMCs.

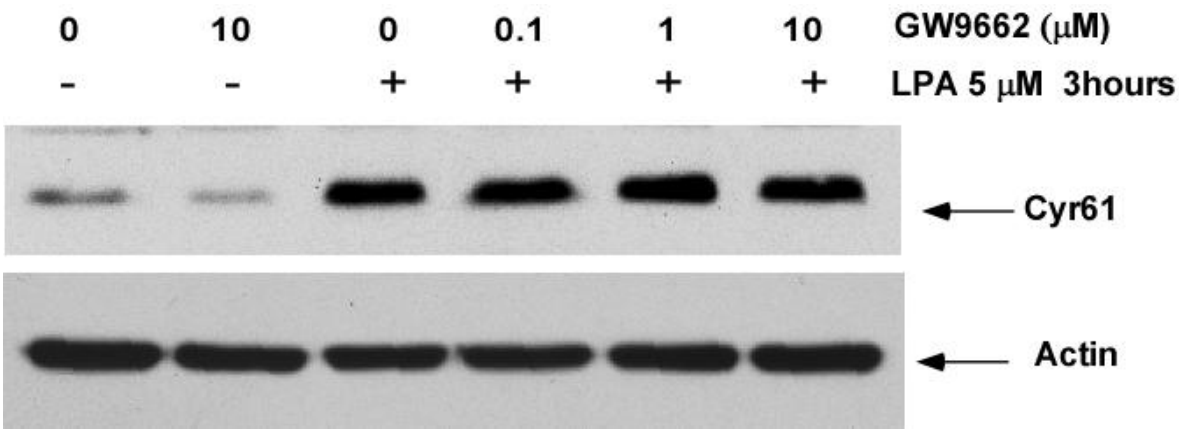


Figure 10. GW9662 has no effect on LPA-induced Cyr61 protein expression. Quiescent MASMCs were pretreated with GW9662 at the concentrations indicated above for 45 minutes, then 5 μM LPA was added for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin was used as the loading control.

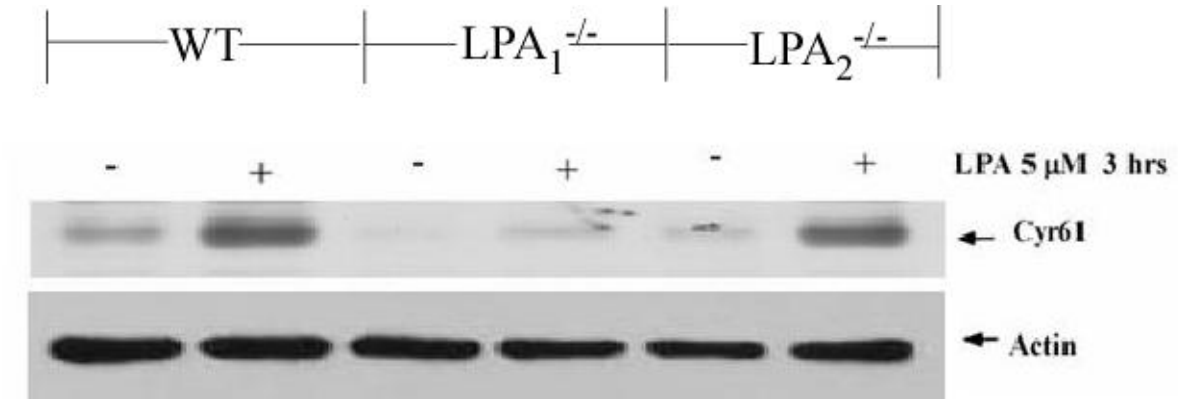


Figure 11. LPA_1, but not LPA_2 mediates LPA-induced Cyr61 protein expression. 5 μM LPA was added to quiescent wild-type, $LPA_1^{-/-}$ and $LPA_2^{-/-}$ MASMCs for 3 hours, Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin was used as the loading control.

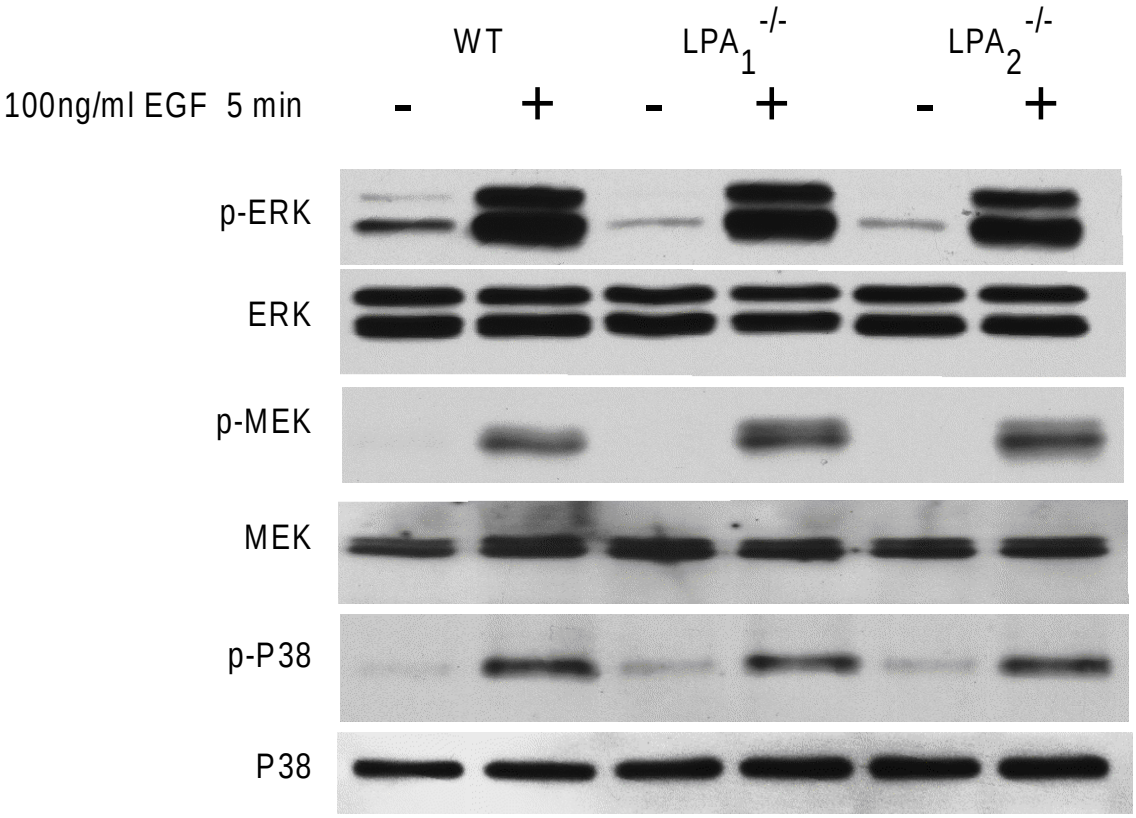


Figure 12. LPA receptors do not mediate EGF-induced signaling pathway. 100 ng/ml EGF was added to quiescent wild-type, $LPA_1^{-/-}$ and $LPA_2^{-/-}$ MASMCs for 5 minutes, p-ERK, p-MEK, and p-P38 were determined by Western blotting with 10% SDS-PAGE gel. The expression of ERK, MEK and p38 were used as loading controls.

1.4 MAPKs are not involved in LPA-induced Cyr61 expression.

Mitogen-activated protein kinases (MAPKS) are serine/threonine-specific protein kinases, which respond to extracellular stimuli and regulate numerous cellular activities, such as gene expression, cell differentiation and proliferation. MAPKS have several subfamilies, such as extracellular signal-regulated kinases (ERKs), p38, c-Jun N-terminal kinases (JNKs). Our data showed that LPA significantly induced the phosphorylation of ERK, P38 and JNK in a time dependent manner (Figure 13), these results prompted us to determine whether LPA-induced MAPKs play roles in LPA induced Cyr61 protein production. MASMCs were pretreated with different concentration of ERK inhibitors U0126 and PD98059 for 45 minutes, then 5 μM LPA was applied to every dish and stayed for 3 hours. The cell lysates were analyzed by 10% Tris/Glycine SDS-PAGE gel followed by Western blotting with anti-mouse Cyr61 and p-ERK antibody. As shown in Figures 14 and 15, treatment with these inhibitors didn't block LPA-induced Cyr61 protein expression, indicating that ERK is not involved in LPA induction of Cyr61 protein. Using the same strategy, we examined the effects of SB203580, a specific p38 inhibitor and SP600125, a specific JNK inhibitor on LPA induction of Cyr61 protein expression, as shown in figure 16 and figure 17, none of those inhibitors had any effect on LPA-induced Cyr61 expression. This result excludes the possibility that p38 or JNKs plays a role in LPA-induced Cyr61 expression.

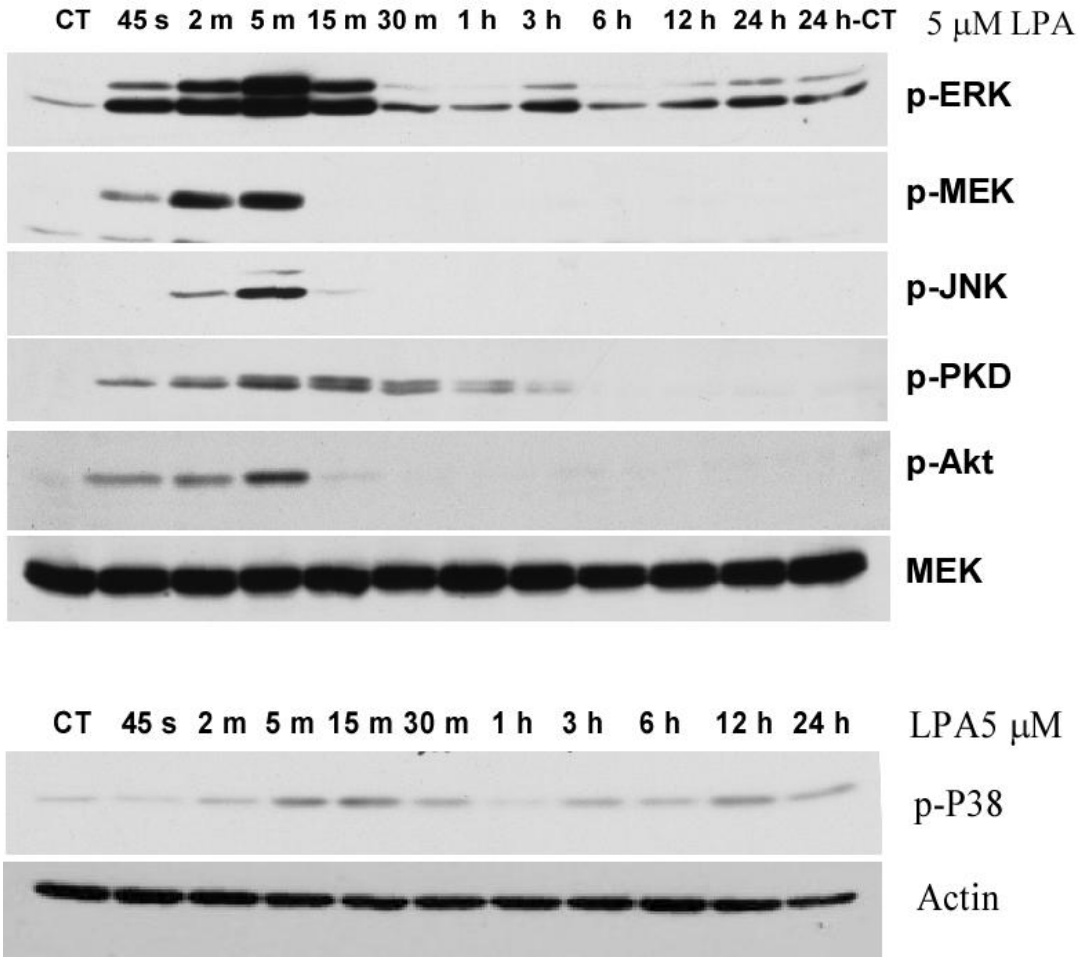


Figure 13. LPA induces the activation of MAPKs, Akt, and PKD in MASMC. 5 μM LPA was added to quiescent MASMC. At the time points indicated above, cells were lysed. The phosphorylation of MAPKs, AKT and PKD was determined by Western blotting with 10% SDS PAGE gel. The expression of MEK and actin was used as the loading control.

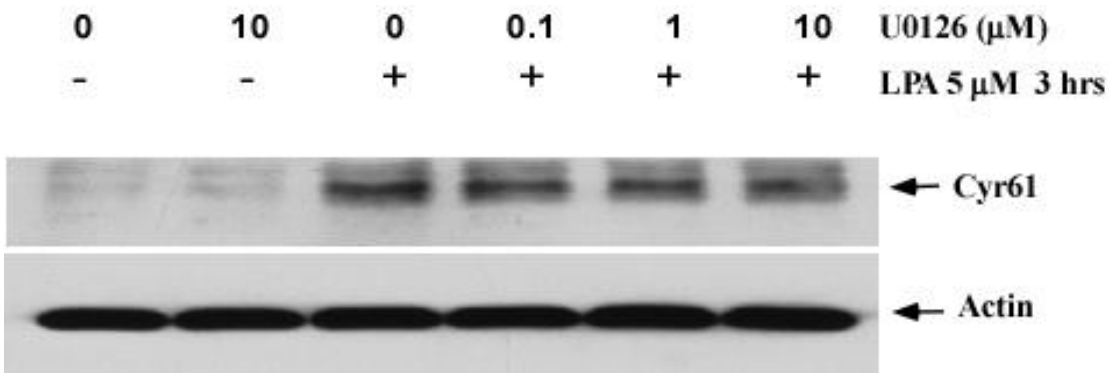


Figure 14. U0126 doesn't block LPA-induced Cyr61 protein expression. Quiescent MASMCs were pretreated with U0126 at the concentrations indicated above for 45 minutes, then 5 μM LPA was added and stayed in the medium for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin was used as the loading control.

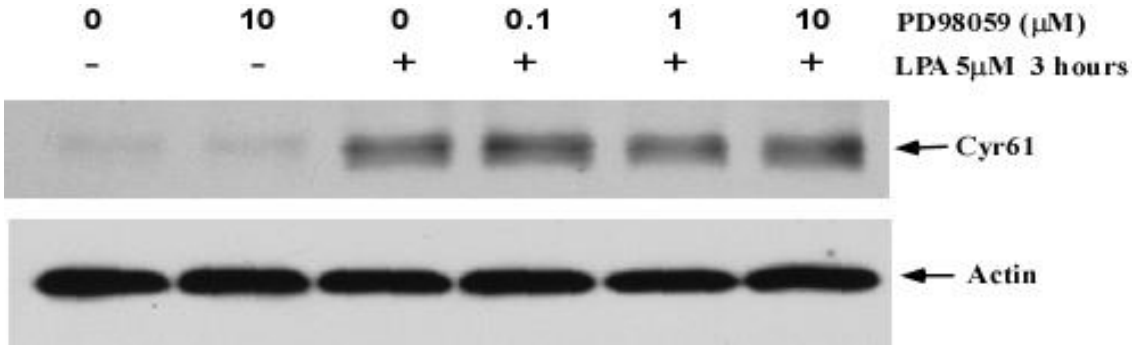


Figure 15. PD98059 doesn't block LPA-induced Cyr61 protein expression. Quiescent MASMCs were pretreated with PD98059 at the concentrations indicated above for 45 minutes, then 5μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. The expression of Actin was used as the loading control.

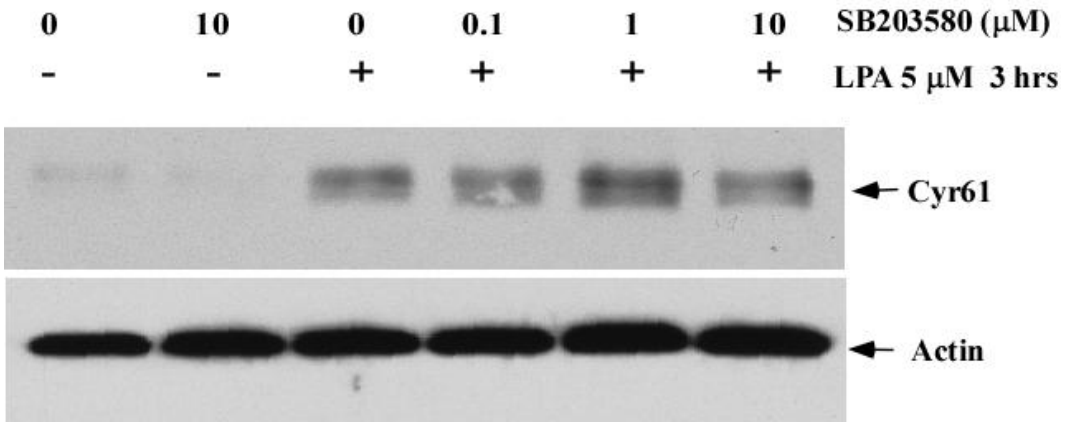


Figure 16. SB203580 doesn't block LPA-induced Cyr61 protein expression. Quiescent MASMCs was pretreated with SB203580 at the concentrations indicated above for 45 minutes, then 5 μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. The expression of Actin was used as the loading control.

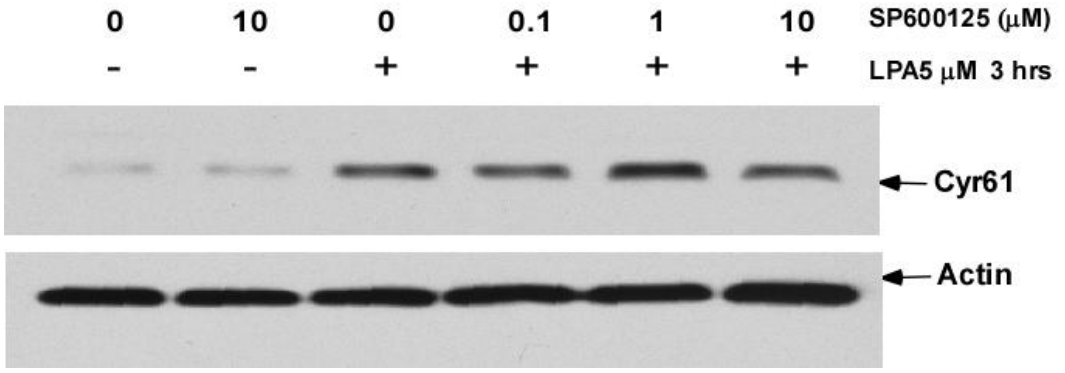


Figure 17. SP600125 doesn't block LPA-induced Cyr61 protein expression. Quiescent MASMCs were pretreated with SP600125 for 45 minutes, then 5 μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin was detected as the loading control.

1.5 PKD is not involved in LPA-induced cyr61 expression.

One of the earliest responses of many cell types to extracellular stimuli is an increase in the synthesis of DAG, and protein kinase D (PKD) which is a DAG regulated protein kinase that plays a role in mediating some of the cellular responses initiated by DAG-producing receptors [39]. Our group has reported thrombin rapidly induces PKD phosphorylation [40], angiotensin II induces protein kinase D activation [41] and PKD2 regulates LPC-induced PC3 cell migration [42]. LPA also induces phosphorylation of PKD in MASMCs (Figure 13), therefore, we determined whether PKD is involved in LPA-induced Cyr61 expression. MASMCs were pretreated with different doses of Resveratrol, a PKD inhibitor, then 5 μM LPA was added, as shown in the figure 18, Resveratrol doesn't block LPA-induced Cyr61 expression, suggesting PKD is not involved in Cyr61 protein accumulation induced by LPA. Furthermore, siRNA transfection experiment has been performed to confirm this result. MASMCs were tranfected with PKD1 and PKD2 siRNA for 48 hours, the cells were starved for 48 h followed by LPA treatment. As shown in figure 19, treatment with neither PKD1 nor PKD2 siRNA blocked the induction of Cyr61 expression by LPA. Taken together, these results demonstrate that LPA-induced Cyr 61 expression is not mediated by PKD.

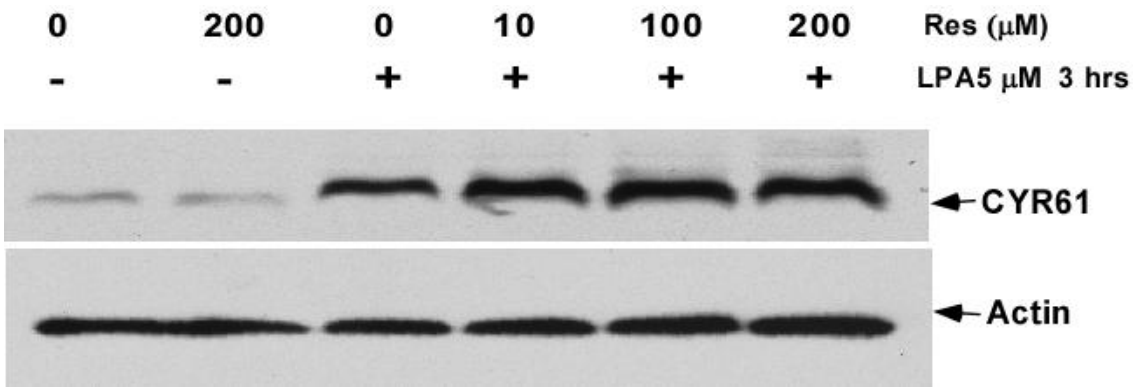


Figure 18. Resveratrol doesn't block LPA-induced Cyr61 protein expression. Quiescent MASMCs were pretreated with Resveratrol at the concentrations indicated above for 45 minutes, then 5 μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin level was used as the loading control.

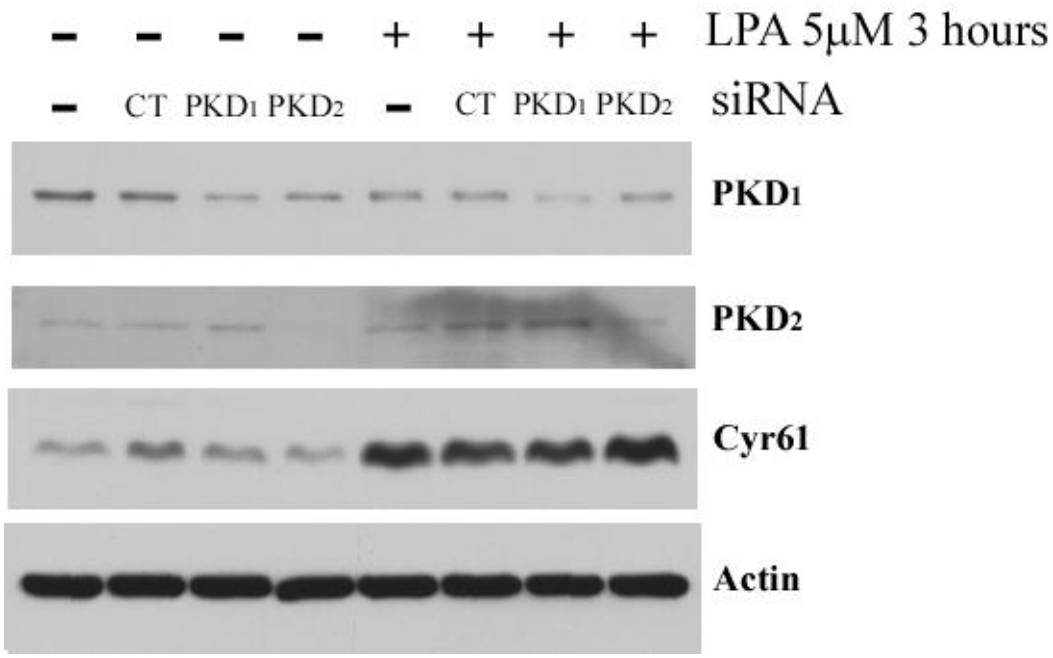


Figure 19. Transfection of PKD1 and PKD2 siRNA doesn't block LPA-induced Cyr61 protein expression in MASMCs. MASMCs were transfected with PKD1 and PKD2 siRNA for 48 hours, after being starved for 48 hours, the cells were stimulated with or without LPA, the expression levesl of PKD1 & PKD2 were determined with specific antibodies to monitor the knockdown level of Cyr61. Cyr61 protein level was determined by western blotting with 10% SDS-PAGE gel.

1.6 LPA-induced Cyr61 expression is not mediated by PI3Ks.

Phosphoinositide 3-kinases (PI 3-kinases or PI3Ks) include a family of enzymes involved in cellular functions such as cell growth, differentiation, motility, proliferation, survival and intracellular trafficking. The PI3K pathway is implicated in human diseases including diabetes and cancer [43] . Our previous results showed LPA significantly induced the phosphorylation of Akt (Figure 13), which is downstream of PI3Ks. To determine whether PI3Ks pathway is involved LPA induction of Cyr61, we pretreated MSMCs with various concentrations of PI3Ks inhibitor LY294002 and Wortmanin for 45 minutes, then incubated the cells with 5 μM LPA for 3 hours, as shown in Figures 20 and 21, Both inhibitors didn't have any effect on LPA-induced Cyr61 protein expression, implying that PI3Ks does not play a role in Cyr61 production induced by LPA.

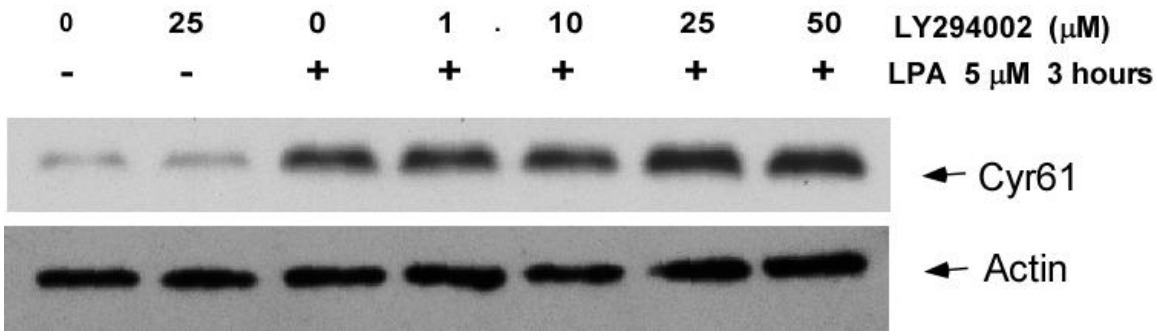


Figure 20. LY294002 doesn't block LPA-induced Cyr61 protein expression. Quiescent MASMCs were pretreated with LY294002 at the concentrations indicated above for 45 minutes, then 5 μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by western blotting with 10% SDS-PAGE gel. Actin was detected as the loading control.

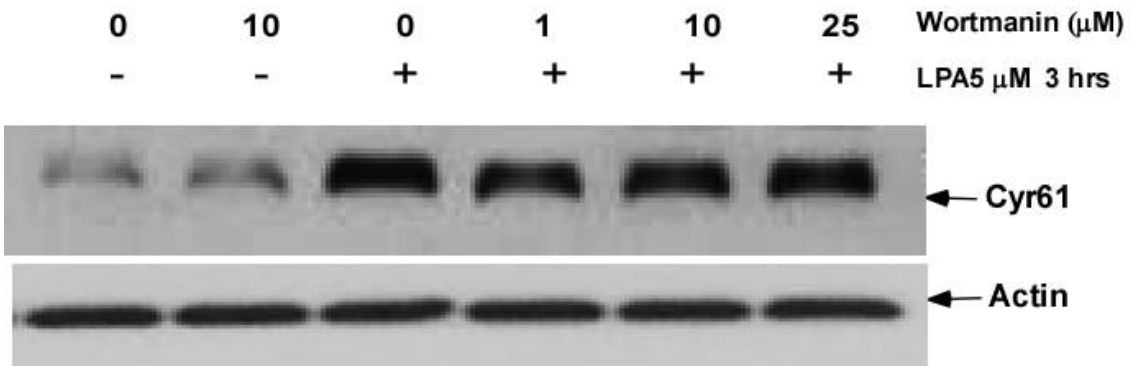


Figure 21. Wortmannin doesn't block LPA-induced Cyr61 protein expression. Quiescent MASMCs was pretreated with **Wortmannin** at the concentrations indicated above for 45 minutes, then 5 μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin was detected as the loading control.

1.7 LPA-Induced Cyr61 Expression is mediated by PKCδ.

We have excluded the possibility that MAPKs, PKD and PI3Ks were involved in LPA induction of Cyr61, so next we want to determine which kinase(s) mediates LPA-induced Cyr61 expression. **Protein kinase C (**PKC) is a family of enzymes, which play important roles in many signal transduction pathways by mediating the functions of many proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid resides. Our group has reported histamine induces Egr-1 expression in human aortic endothelial cells via the H1 receptor-mediated protein kinase Cdelta-dependent ERK activation pathway [44]. To investigate whether PKC mediates LPA-induced Cyr61 protein expression, we pretreated MSMCs with indicated concentrations of pan PKC inhibitor GF109203X and GO6983 for 45 minutes, then incubated the cells with 5 μM LPA for 3 hours, as shown in Figures 22 and 23, Both of those inhibitors dose-dependently block LPA-induced Cyr61 protein expression, strongly supporting that PKC mediates LPA induced Cyr61 expression. The PKC family consists at least ten isozymes [45], and they are divided into three subfamilies, conventional, novel, and atypical based on their second messenger requirements. It is unknown which PKC subtype is involved in LPA-induced Cyr61 expression; our result shows LPA highly induces the phosphorylation of PKCδ (Figure 24).

To examine whether PKCδ mediates LPA-induced Cyr61 expression, we pretreated MSMCs with various concentrations of PKCδ inhibitor, Rottlerin and PKCαβ2 inhibitor, GO6976 for 45 minutes, then incubated cells were treated with 5

μM LPA for 3 hours, as shown in figure 25, Rottlerin markedly blocked LPA-induced Cyr61 protein expression in a dose-dependent manner. In contrast, PKCαβ2 inhibitor, GO6976 had no effect on LPA-induced Cyr61 protein expression (Figure 26). These data strongly support a key role of PKCδ in mediating LPA-induced Cyr61 protein expression.

To further confirm the above results, we performed small siRNA knockdown exprements, MASMCs were tranfected with PKCδ siRNA for 48 hours; then the cells were starved for 48 h followed by treatment of LPA. As shown in figure 27, PKCδ siRNA completely blocks PKCδ expression and LPA-induced Cyr61 expression. In addition, we infected MASMCs with PKCδ dominant-negative adenovirus and wildtype adenovirus, we observed that infection of PKCδ dominant negative adenovirus blocked LPA-induced Cyr61 expression in MASMCs. As a positive control, LPA highly induces Cyr61 expression after wildtype PKCδ adenovirus infection in MASMCs (Figure 28). All of those data support the conclusion that PKCδ mediates LPA-induced Cyr61 protein expression.

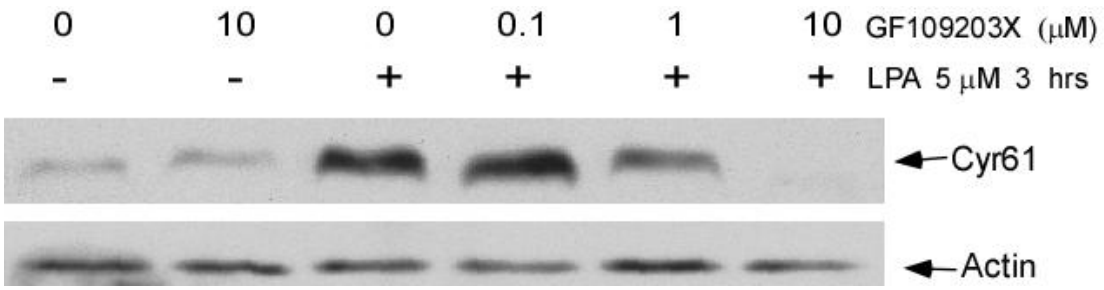


Figure 22. PKC inhibitor GF109203X blocks LPA-induced Cyr61 protein expression. Quiescent MASMCs were pretreated with GF109203X at the concentrations indicated above for 45 minutes, then 5μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by western blotting with 10% SDS-PAGE gel. Actin was detected as the loading control.

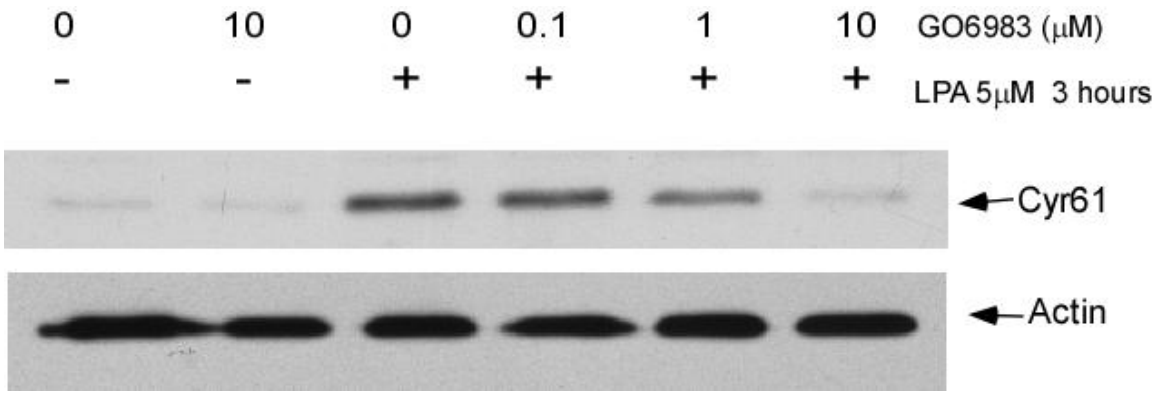


Figure 23. Go6983 blocks LPA-induced Cyr61 protein expression. Quiescent MASMCs was pretreated with GO6983 at the concentrations indicated above for 45 minutes, then 5 μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin was detected as the loading control.

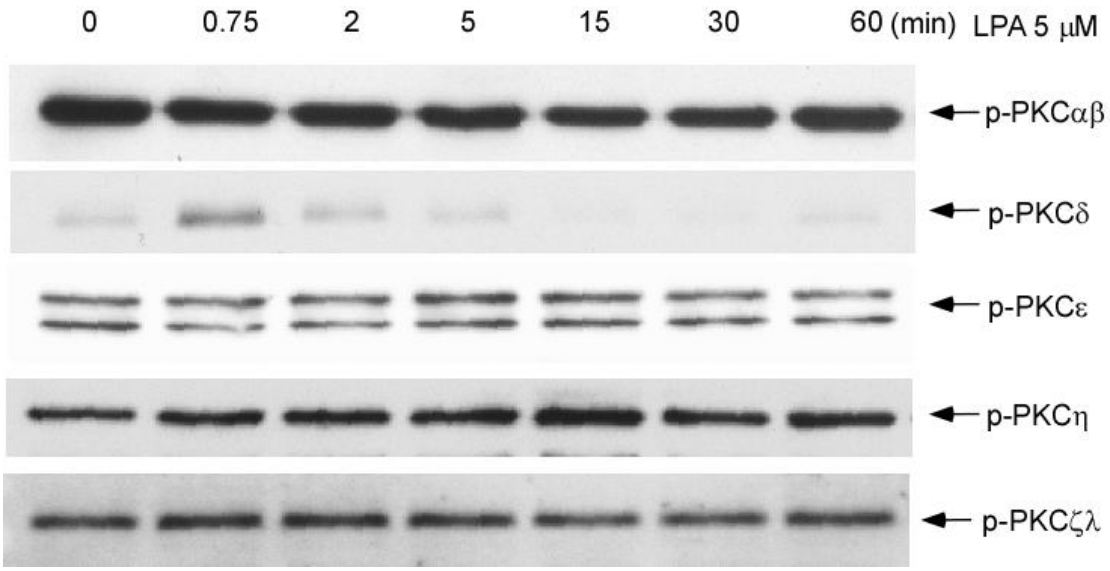


Figure 24. LPA induces phosphorylation of PKCδ, but not other PKC subtypes in MASMCs.

5 μM LPA was added to quiescent MASMC at the time points indicated above. Phosphorylation of PKC subtypes were determined by Western blotting with 10% SDS PAGE gel.

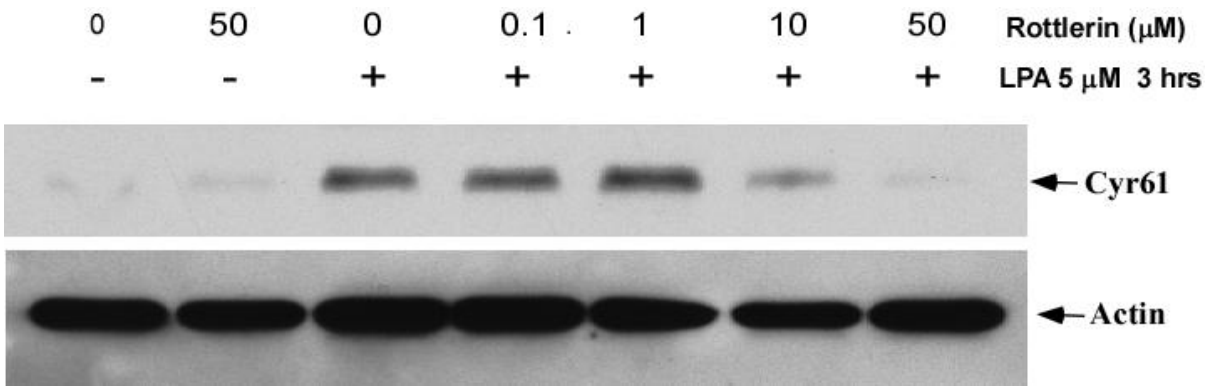


Figure 25. Rottlerin blocks LPA-induced Cyr61 protein expression. Quiescent MASMCs were pretreated with Rottlerin at the concentrations indicated above for 45 minutes, then 5 μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin was detected as the loading control.

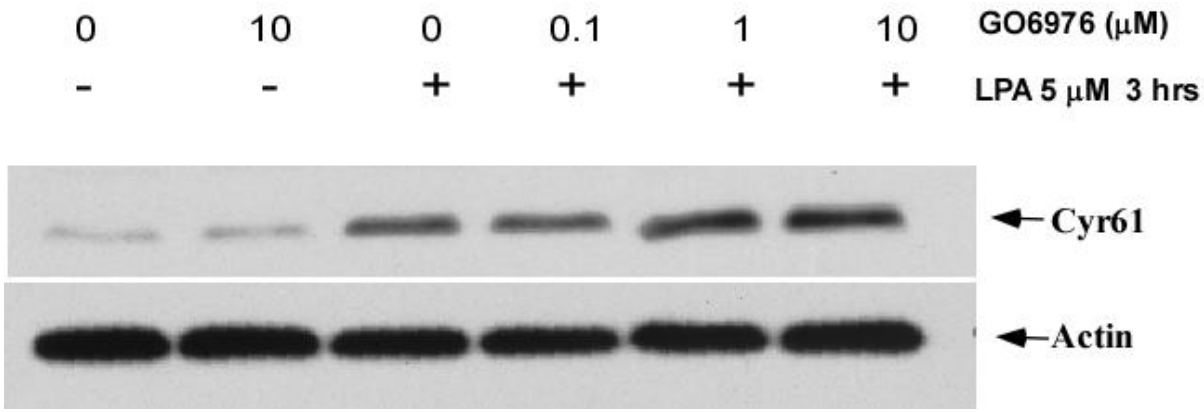


Figure 26. GO6976 doesn't block LPA-induced Cyr61 protein expression. Quiescent MASMCs were pretreated with G06976 at the concentrations indicated above for 45 minutes, then 5 μM LPA was added in the medium for 3 hours. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin was detected as the loading control.

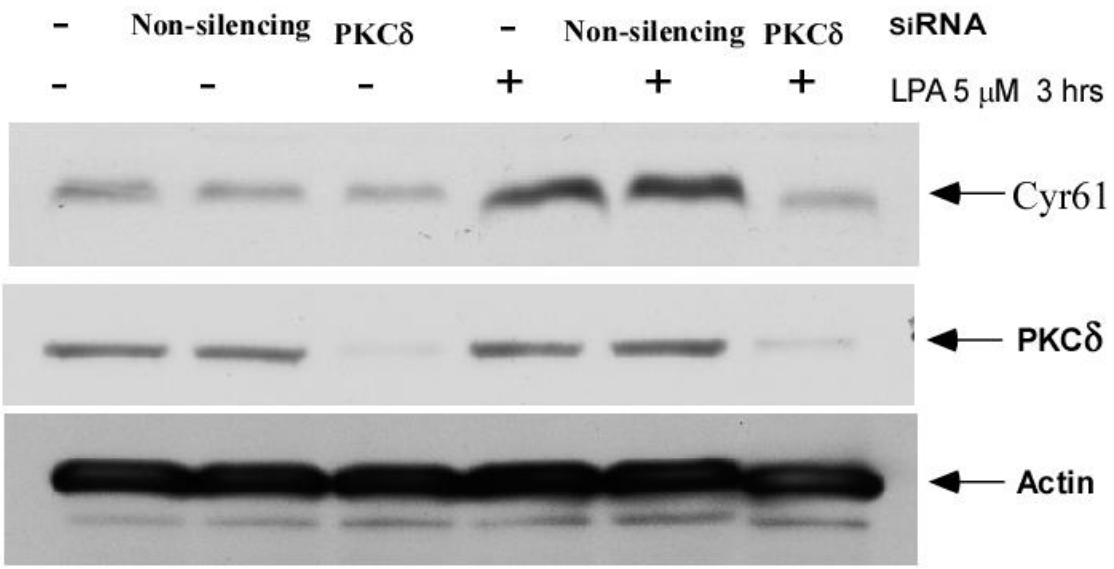


Figure 27. Transfection of PKCδ siRNA blocks LPA-induced Cyr61 protein expression in MASMCs. MASMCs were transfected with PKCδ siRNA for 48 hours, after being starved for 48 hours, the cells were stimulated with or without LPA. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. Actin was detected as the loading control, PKCδ was detected with the specific antibody to show the knockdown level.

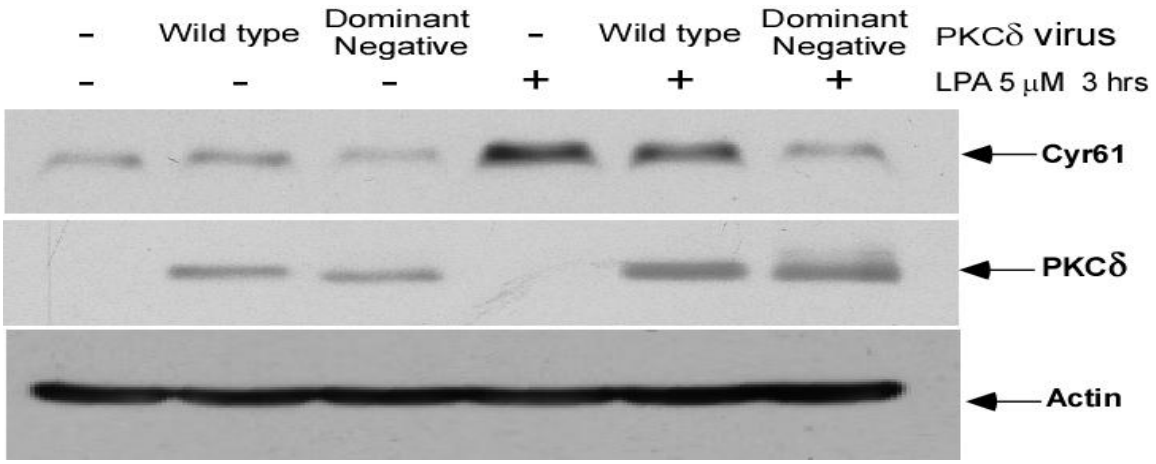


Figure 28. Infection of PKCδ dominant negative adenovirus blocks LPA-induced Cyr61 protein expression in MASMCs. MASMCs were infected with type and dominant negative PKCδ viruses for 12 hours, after being starved for 48 hours, the cells were stimulated with or without LPA. Cyr61 protein level was determined by Western blotting with 10% SDS-PAGE gel. PKCδ was detected with the specific antibody to show the efficiency of infection.

2. The dynamic localization of LPA-induced Cyr61 protein in MASMCs.

2.1 LPA markedly induced Cyr61 protein intracellularly and the induced Cry61 is deposited in the extracellular matrix.

Cyr61 is an extracellular matrix protein, it exerts its function mostly in the extracellular matrix, and the above data showed that LPA markedly induces Cyr61 protein expression. To investigate the localization of LPA-induced Cyr61 protein, we stimulated quiescent MASMCs with 5 μM LPA for various time periods, both cell lysates and extracellular matrix were collected and detected with a specific Cyr61 antibody, as shown in figure 29, after 20 min stimulation, LPA rapidly induces Cyr61 protein expression intracellularly, however, the increased Cyr61 protein level can only be detected in the extracellular matrix after LPA stimulation for 1 hour. All of those data demonstrate that LPA rapidly induces Cyr61 protein expression intracellularly and the induced Cyr61 translocates outside the plasma membrane and is deposited in the extracellular matrix.

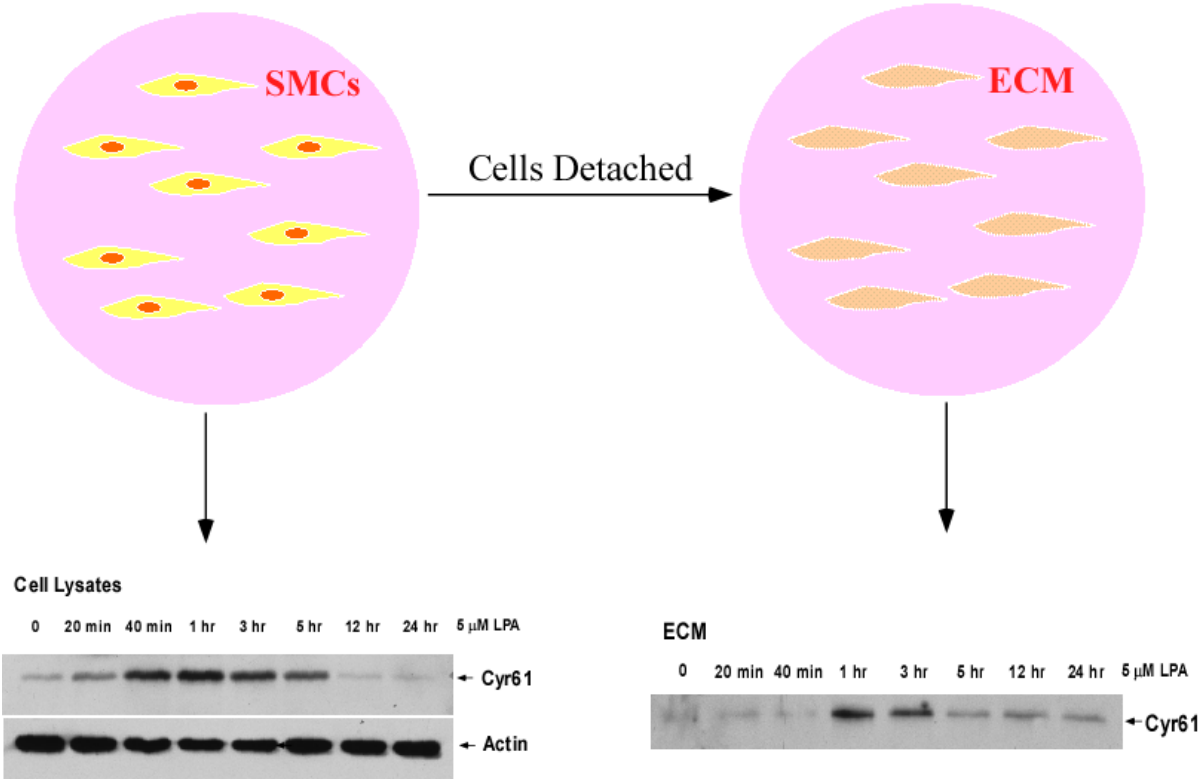


Figure 29. LPA highly induces Cyr61 protein expression intracellularly and LPA-induced Cyr61 protein is deposited in the extracellular matrix. MASMCs were detached from the dishes with 1mM EDTA and lysed in the lysis buffer, and ECM remaining on the culture dishes was extracted in Laemmli sample buffer. Cyr61 protein level was determined by Western blotting with 10% SDS PAGE gel. Actin was used as an internal control.

2.2 The dynamic localization of LPA-induced Cyr61 protein in MSMCs.

Although the above data gave us a brief view the localization of LPA-induced Cyr61 protein, it is still unclear that through which subcellular compartments, LPA-induced Cyr61 protein is translocated to the extracellular matrix. Immunofluorescence analysis has been performed to further explore this question. Quiescent MASMCs were stimulated with LPA for various time periods as shown in figure 30, after paraformaldehyde fixation, the cells were treated with 0.3% triton X-100 for permeabilization of the plasma membrane and then the cells were immunostained with the specific antibodies against Cyr61, DAPI (a specific nuclear marker) and Adaptin (a specific Golgi apparatus marker). The results showed that intracellular Cyr61 protein was highly induced after 1 hour LPA treatment, moreover, the image of LPA-induced Cyr61 protein and Golgi apparatus marker adaptin merged together perfectly, indicating that after Cyr61 mRNA being translated into Cyr61 protein, these proteins immediately accumulate in the Golgi apparatus for further processing prior to the secretion into the extracellular matrix. We also observed that the intracellular Cyr61 protein induced by LPA decreased after 1 hour, whereas the extracellular Cyr61 increased, strongly supporting that LPA-induced Cyr61 proteins first accumulated in the Golgi apparatus and then were secreted to the extracellular matrix. We also used another approach to directly observe the LPA-induced Cyr61 deposition on the extracellular matrix. We treated the cells without Triton X100 and then immunostained the cells with Cyr61 antibody. As shown in figure 31, Cyr61

protein in the extracellular matrix was highly induced by LPA after 3 hr stimulation.

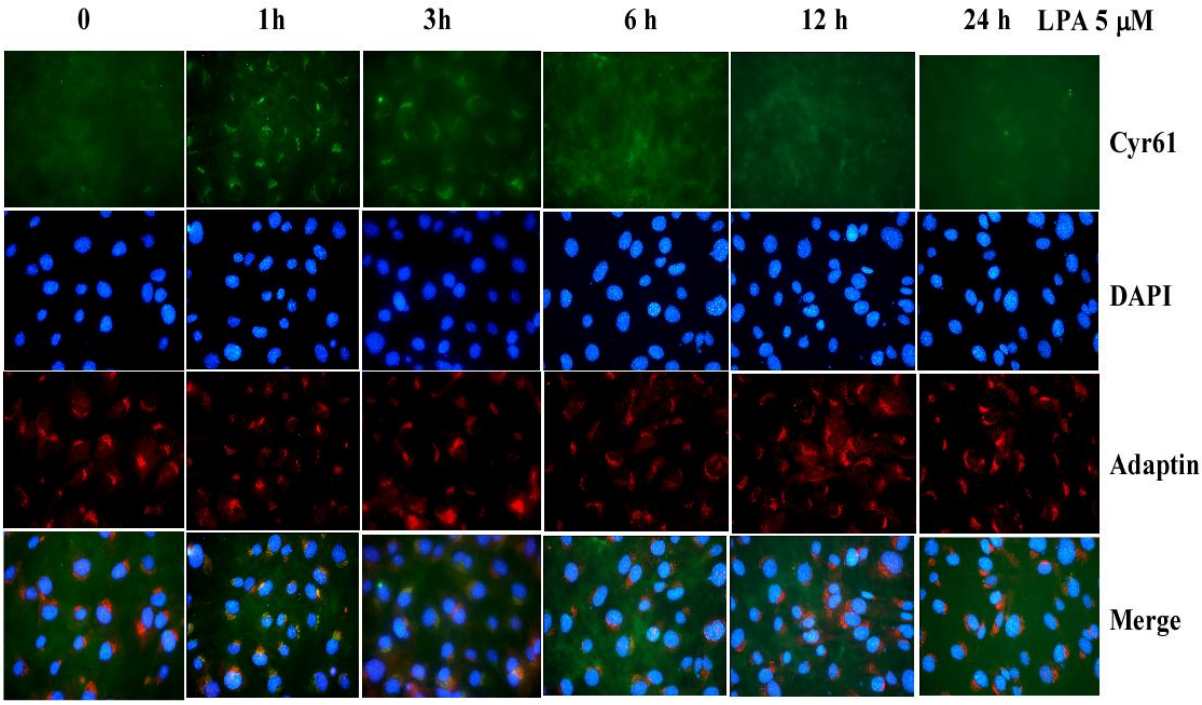


Figure 30. Dynamic localization of Cyr61 protein induced by LPA. MASMCs were cultured on microscope cover glasses, after 48 hours starvation, cells were stimulated with LPA for various time periods indicated above; then cells were immunostained with the Cyr61 antibody, DAPI (nuclear marker) antibody and Adaptin (Golgi Apparatus marker) antibody after triton X-100 treatment.

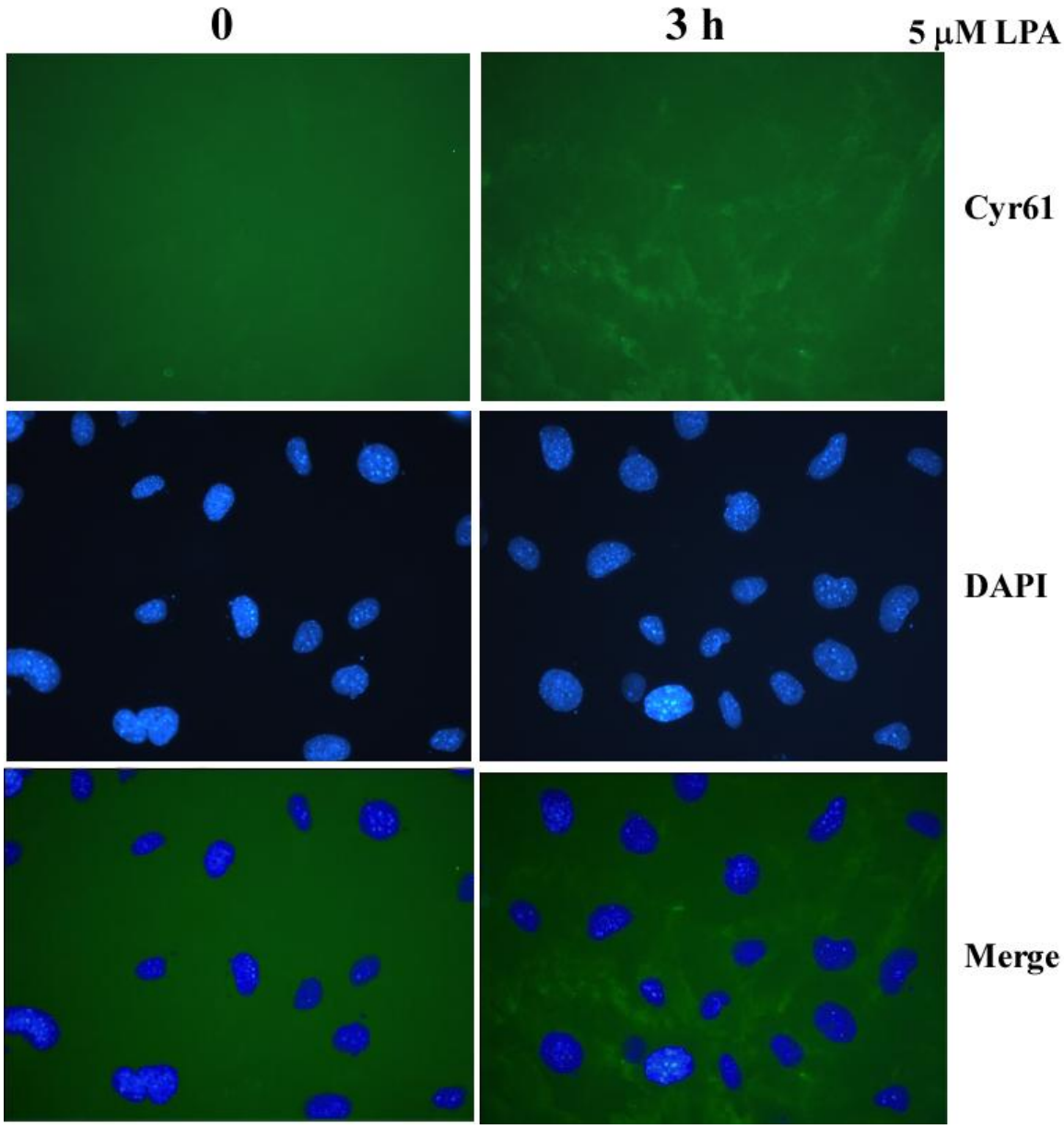


Figure 31. Localization of LPA-induced Cyr61 protein in the extracellular matrix. MASMCs were cultured on microscope cover glasses, after 48 hours starvation, cells were stimulated with LPA for 3 hours, and then fixed with paraformaldehyde solution followed by immunostaining with the specific Cyr61 antibody, DAPI (nuclear marker) antibody, Adaptin (Golgi Apparatus marker) antibody without triton X-100 treatment.

3. Cyr61 mediates LPA-induced MASMC migration.

3.1 Cyr61 is involved in LPA-induced MASMC migration.

As shown above, our data demonstrate that LPA markedly induces Cyr61 expression and secretion into extracellular matrix. High concentrations of LPA and Cyr61 have been found in atherosclerotic lesions [9][46]. We hypothesize that LPA-induced Cyr61 mediates LPA signaling leading to cell migration. To date, whether Cyr61 contributes to LPA-induced cell migration is unknown.

To determine whether LPA-induced MASMC migration is mediated by Cyr61, we applied an antibody neutralization approach. We pretreated MASMC with the specific Cyr61 antibody for 45 minutes and stimulated the cells with LPA, the cells were then subjected to the migration assay, and we observed that LPA-induced cell migration was reduced about 80% after the Cry61 antibody treatment, suggesting Cyr61 mediates LPA-induced MASMC migration induced by LPA (Figure 29).

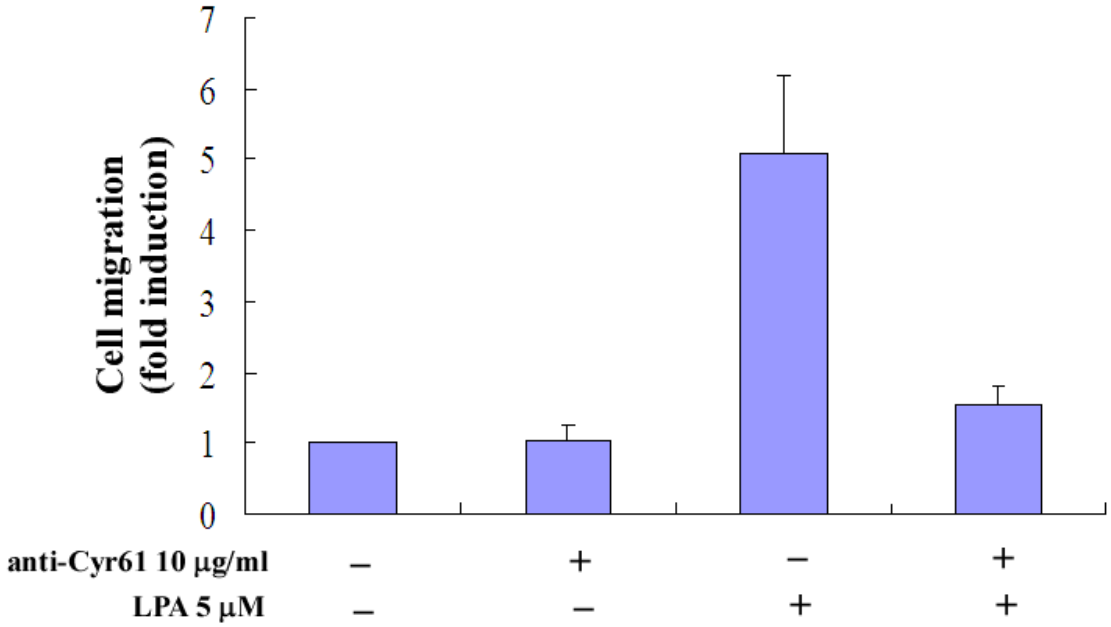


Figure 32. Cyr61 is involved in LPA-induced MASMC migration. MASMCs were pretreated with or without Cyr61 antibody for 45 minutes and then subjected to a migration assay in response to 5 μM LPA.

3.2 LPA_1 receptor and PKC mediate LPA-induced MASMC migration.

As demonstrated above, LPA-induced Cyr61 expression was through LPA_1 receptor and mediated by PKC, and Cyr61 was involved in LPA-induced MASMC migration. These data implied that LPA_1 receptor and PKC may also play roles in the MASMC migration. To determine which LPA receptor mediates cell migration, we performed cell migration assay using wildtype, $LPA_1^{-/-}$, $LPA_2^{-/-}$ MAMSCs stimulated with LPA. We found that genetic deletion of LPA1, but not LPA2 blocked LPA-induced cell migration, indicating that LPA_1 but not LPA_2 mediates LPA-induced MASMC migration (Figure.30). We next determined whether PKC mediated LPA-induced MASMC migration, quiescent MASMCs were stimulated with 5 μM LPA after being pretreated with pan-PKC inhibitor GF109203X for 45 minutes, and then subjected to the cell migration assay. As shown in figure 31, PKC specific inhibitor GF109203X completely blocked LPA-induced MASMC migration, these data indicate that LPA_1- and PKC-mediated LPA-induced Cyr61 protein expression is a key event in LPA-induced MASMC migration.

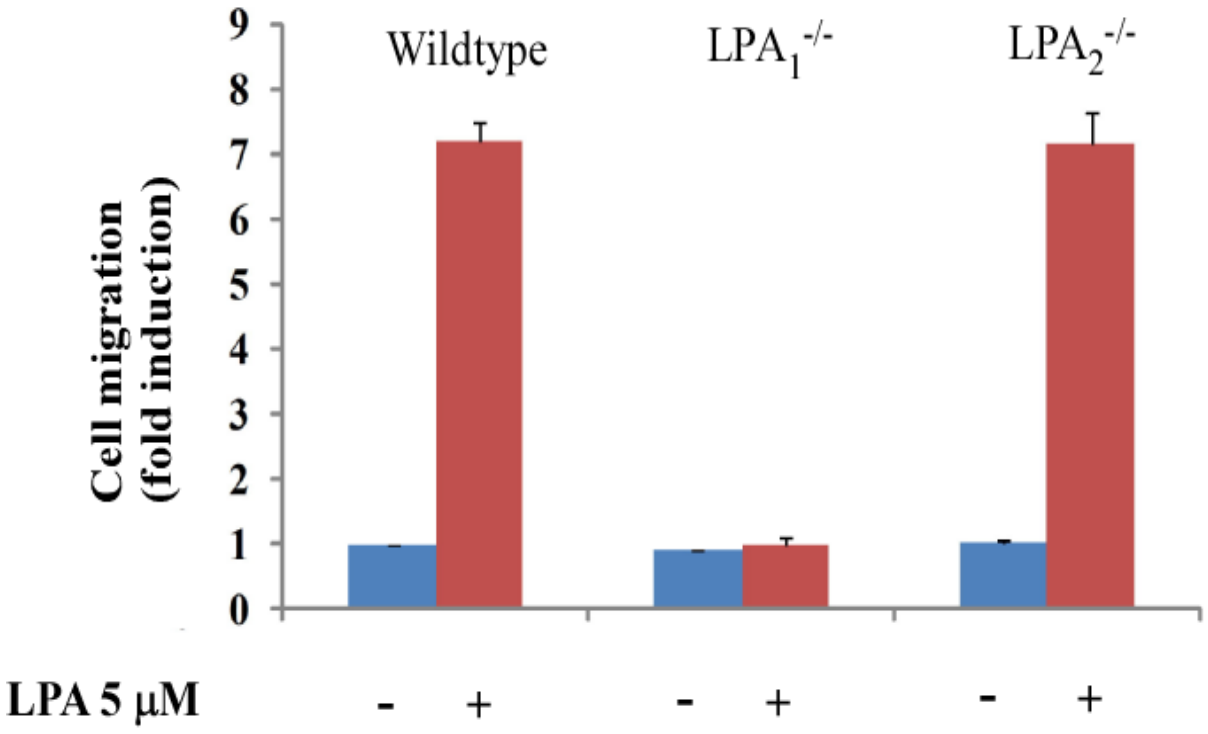


Figure 33. LPA induces the MASMC migration, and the migration is mediated by LPA_1 receptor, but not LPA_2 receptor. Quiescent wildtype, $LPA_1^{-/-}$, $LPA_2^{-/-}$ MASMCs were stimulated with 5 μM LPA, and then subjected to a migration assay.

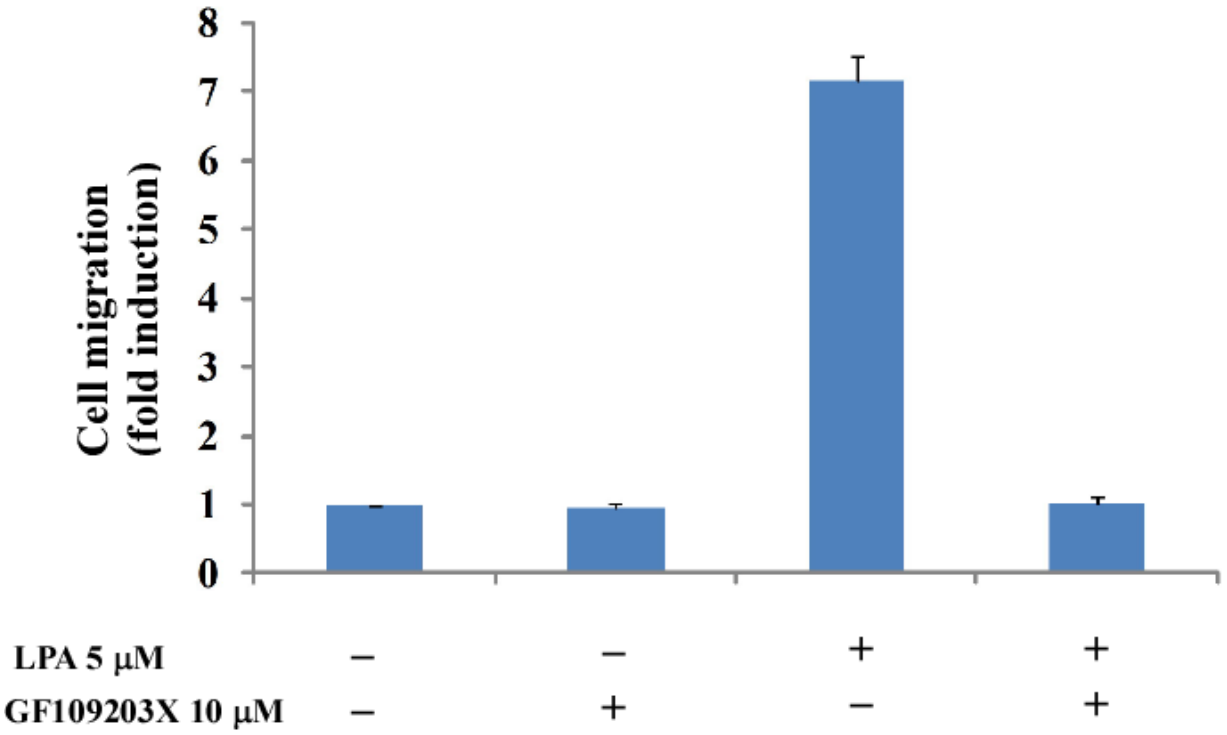


Figure 34. The effect of GF109203X on LPA-induced MASMC migration. MASMCs were pretreated either with or without PKC inhibitor GF109203X for 45 minutes and then subjected to a migration assay in response to 5 μM LPA.

DISCUSSION

One of the important risk factors in the pathogenesis of atherosclerosis is oxidized low density lipoprotein (LDL). LPA is one of the most bioactive lipid components in oxidized LDL. LPA plays a very important role in initiating platelet activation and stimulating endothelial cell stress-fiber and gap formation. It has been found that LPA is the most important platelet-activating lipid of atherosclerotic plaques and also highly accumulated in lipid-rich core in the atherosclerotic lesion. Our group has reported that LPA increased TF mRNA, TF protein, and TF pathway activity via transcription factor, Egr-1 [35]. In addition, LPA also prominently induces the secretion of IL-6 and monocyte chemoattractant protein (MCP)-1 from human aortic SMCs (HASMCs) [47]. These data indicated the important roles of LPA in the initiation and progression of atherosclerosis.

Cyr61 has been shown to be highly expressed in human atherosclerotic plaques, correlating with the degree of stenosis and plaque histopathology [46]. The upregulation of Cyr61 in VSMCs was first identified in the screening for genes that are differentially expressed in response to Ang II stimulation in vascular smooth muscle cells [48]. Cyr61 is an extracellular matrix-associated angiogenic inducer that promotes cell adhesion, migration, and proliferation. Aberrant expression of Cyr61 is

associated with wound healing, and vascular diseases such as atherosclerosis [49]. Whether Cyr61 plays a role in LPA-induced cell migration is currently unknown. In this study, we determined the relationship between LPA and Cyr61 in VSMCs, and their roles in vascular function. Our Western blot analysis and Northern blot analysis demonstrate that LPA markedly induces Cyr61 protein and mRNA expression. The induction is LPA dose- and time-dependent in MASMCs.

LPA is well known as an important signaling molecule to produce many cellular responses. These responses were explained by both non-receptor and receptor-mediated mechanisms. LPA is a direct agonist of the nuclear transcription factor PPARgamma [50]. Here our results showed PPARgamma was not involved in LPA-induced Cyr61 expression. Currently, there are at least 5 LPA receptors that couple with several types of G proteins, Gs, Gi/o, Gq, and G12/13. By activation of these G proteins, LPA induces cell proliferation, cell migration, MAPK activation, PLC activation, PKC activation, Ca2+ mobilization, Akt activation, and Rho activation [17][51][52][53]. It was reported that LPA stimulates prostaglandin E2 production in cultured stromal endometrial cells through LPA1 [54]. In vascular systems, our RT-RCR data clearly showed that LPA1, LPA2 and LPA4 receptors were highly expressed. Our group has published the data showing that LPA induces IL-6 secretion from aortic smooth muscle cells via an LPA1-regulated pathway [49], and LPA1 receptor also mediates prostate cancer PC3 cell migration [55]. In this study, our Western blot analysis demonstrated that LPA-induced Cyr61 expression was mediated by LPA1 receptor, but not LPA2 receptor. Up to date, the signaling pathway

involved in LPA induced-Cyr61 expression was unknown. LPA was shown to activate MAPKs, Akt and PKD, which are frequently involved in LPA-induced G-protein coupled signaling pathways. It has been found that LPA stimulates gastric cancer cell proliferation via Erk dependent upregulation of sphingosine kinase 1 transcription [56]. Chiu, TT et al., reported in 2007 that protein kinase D2 mediates lysophosphatidic acid-induced interleukin 8 production in nontransformed human colonic epithelial cells through NF-kappaB [57]. Singla, A et al. reported in 2009 that LPA-mediated stimulation of Cl(-)/OH(-) exchange activity was depending on activation of phosphatidylinositol 3-kinase/Akt signaling pathway. In this study, our Western blot analysis data showed that none of those kinases were involved in LPA-induced Cyr61 expression, indicating that an unveiled specific signaling pathway leads to LPA-induced Cyr61 expression.

It was reported that LPA-induced NF-kappaB activation and cytokine production were mediated by PKCs but not by JNK, p38 or Erk MAP kinase. Our previous data also showed that histamine activates the phosphorylation of PKC and that PKCδ mediates histamine-induced Egr-1 expression in RASMCs and human endothelial cells [44]. To investigate whether PKC pathway mediated LPA-induced Cyr61 expression, we used PKC specific inhibitors, the dominant negative virus constructs and siRNA to block the functions of PKCδ and to knockdown of PKCδ expression. Our Western blotting results clearly demonstrated that LPA-induced Cry61 expression was blocked by each of these approaches, strongly supporting our conclusion that PKCδ mediated LPA-induced Cry61 expression.

Another interesting finding in this study is the dynamic localization of LPA induced-Cyr61 protein. Cyr61 is a matricellular protein. It is important to know the transportation of Cyr61 protein after Cyr61 mRNA is translated. Our Western blotting data demonstrated that the intracellular Cyr61 protein was highly induced by LPA stimulation within 20 minutes, and then via Golgi, Cyr61 was secreted into extracellular matrix. The peak time difference in Cyr61 protein accumulation in intracellular compartment and extracellular matrix suggests a dynamic transportation of LPA-induced Cry61 protein from the intracellular compartments to the ECM. Our immunocytochemistry results confirmed the Western blot results.

Atherosclerosis is a chronic arterial disease where vascular smooth muscle cells (VSMCs), inflammatory cells, lipids, cholesterol and cellular products like cytockines work together to produce a fibro-fatty plaque and initiate neointima formation [58]. It has been shown that the neointimal SMCs are derived from the media after balloon endothelial denudation or cholesterol-induced injury [59]. The importance of smooth muscle cell migration in neointima formation of atherosclerosis is well appreciated. Studies have been done to support the role of LPA in the smooth muscle cell migration (citation). Zhou et al in 2009 reported LPA induced vascular smooth muscle migration via p38 mitogen-activated protein kinase pathway activation [60]. Lin BR et al. in 2007 reported that Cyr61 enhanced transendothelial cell migration by concomitantly up-regulating chemokine receptor 1 and 2 [61]. It was also reported that recombinant Cyr61 protein induces SMC migration in a dose-dependent manner [62]. But it was unknown whether Cyr61 played a role in LPA induced MASMC

migration. In this study, we found that Cyr61 neutralizing antibody significantly blocked LPA-induced MASMCs migration, indicating Cyr61 mediates LPA-induced MASMC migration. In addition, our cell migration analysis data clearly demonstrated that LPA-induced MASMC migration is mediated by LPA_1 and PKC kinase. LPA1 and PKC are responsible for LPA-induced Cyr61 expression.

Taken together, the above results demonstrate that LPA, via LPA_1 receptor, activates a specific signaling pathway leading to PKCδ activation, which in turn, mediates LPA-induced Cyr61 protein in MASMCs. LPA-induced Cyr61 proteins first accumulated in the Golgi apparatus then were secreted into extracellular matrix. Our data further show that LPA-induced Cyr61 is responsible for LPA-induced SMC migration, which may contribute to the development of atherosclerosis. The identification of the specific LPA receptor and PKCdelta, especially the novel LPA/Cyr61 pathway in controlling smooth muscle cell migration, provides new insight into mechanisms underlying the pathogenesis of atherosclerosis.

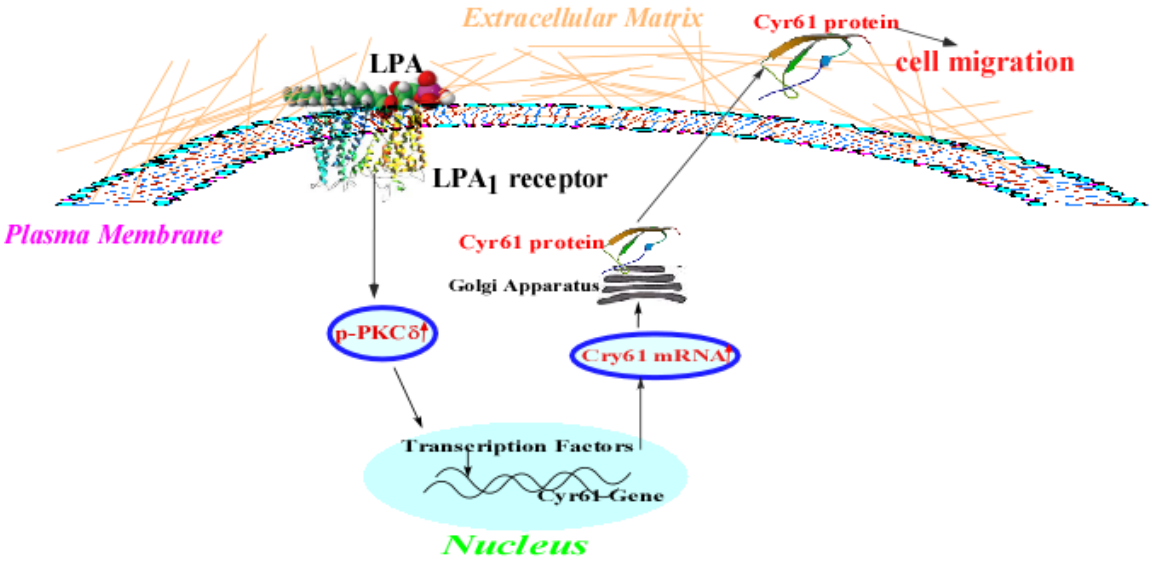


Figure 36. A diagram showing the signaling pathway by which LPA induces Cyr61 and MASMC migration.

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