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Lysophosphatidic Acid Up-regulates MT1-MMP Expression through a G_i –dependent Pathway in Human Umbilical Vein Endothelial Cells

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ABSTRACT: Lysophosphatidic acid (LPA) is a low molecular weight lysophospholipid (LPL). Through binding to its specific G protein-coupled receptor family, LPA regulates various cellular functions, including proliferation, migration, invasion, and differentiation. Matrix-metalloproteinases (MMPs) are zinc-dependent protease and play important roles in regulating the interaction between cells and extracellular matrix (ECM). Among these MMPs, membrane type 1-metalloproteinase (MT1-MMP) not only degrades ECM protein but also activates metalloproteinase-2 (MMP-2, Gelatinase A), which are important to endothelial cell migration. Our previous study showed that LPA enhances MMP-2 expression and activity in human umbilical vein endothelial cells (HUVECs). In this study, we further revealed that LPA also induce MT1-MMP mRNA and protein expressions in HUVECs through real-time PCR and Western blotting, respectively. Furthermore, by applying chemical inhibitors, we found that LPA-induced MT1-MMP expression is mainly through a G_i- and partially through a G_q-dependent pathway. Our results provide new evidence that LPA might modulate ECM through regulating the expression of MT1-MMP.

KEY WORDS: LPA, MT1-MMP, MMP-2, HUVECs, ECM.

INTRODUCTION

Lysophosphatidic acid (1-acyl-2-hydroxy-sn-glycero-3-phosphate, LPA), the simplest phospholipid, was first identified as a mediator of de novo glycerolphospholipid synthesis (Vandenbo, 1974). The first discovered source of LPA was platelets, which release LPA upon phospholipase C (PLC) or thrombin treatment (Mauco, 1987). Recently, many studies suggested that LPA is also produced by other cell types, including adipocytes, neuronal cells, fibroblasts, and cancer cells (Westermann, 1998; Xie, 2002). LPA was further demonstrated to be a bioactive factor, which regulates migration, adhesion, proliferation, and cell differentiation (Gaits, 1997; Moolenaar, 2000). LPA triggers several cellular signaling events through binding to the specific family of the G protein-coupled receptors (Edgs). To the present, at least four LPA receptors have been identified, termed $LPA_{1\text{-}4}$. Generally, LPA receptors associate with three subtypes of G protein families, including G_i, G_q, and G_{12}. Furthermore, LPA receptors have been suggested to regulate various cellular functions via cross-talk between their downstream signaling events (Anliker, 2004).

Endothelial cells (ECs) are widely used to investigate angiogenesis and normally form a tight monolayer by interacting with the underlying extracellular matrix (ECM). Interaction between ECs and ECM are important determinants of EC migration and signaling. In addition, EC migration plays important roles in neovasculralization and angiogenesis. Furthermore, it was shown that EC migration is enhanced by LPA treatment, and matrix metalloproteinases (MMPs) play critical roles in this process.

MMPs are zinc-dependent proteases, and at least twenty MMPs have been identified in human. The major function of MMPs is to modify the ECM, which involved in regulating cell behaviors and modulating cell morphology (Nagase, 2006). Based on enzyme activities and structures, MMPs are classified into six categories as follows: collagenases MMPs; gelatinases MMPs; stromelysins MMPs, matrilysins MMPs; membrane-type MMPs (MT-MMPs); and other MMPs. Basically, MMPs are produced as zymogens and their active domains are covered by a fragment of pro-peptides. Most MMPs can be activated either by themselves or by other proteases (Visse, 2003; Nagase, 2006). Several studies have demonstrated that MMP-2, a crucial regulator of cell migration and angiogenesis, is activated by MT1-MMP (Strongin, 1995; Zucker, 2003; Wu, 2005). Moreover, MT1-MMP not only associated with MMP-2 activation but also involved in tumor invasion, cell migration, and angiogenesis (Seiki, 2002; Langlois, 2004; Genis, 2007; Sato, 2007).

Our previous study indicated that LPA induces MMP-2 expression in HUVECs. Moreover, we also

Table 1. Chemical inhibitors.

Inhibitors	Inhibit target	Stock	Treatment c.	Pretreat-time
Pertusiss toxin	G_i	100 ng/μL	15 ng/mL	O/N
U73122	PLC	10 mM	1 μM	1h
Ki16425	LPA antagonist		10 μM	1h

PLC, phospholipase C; LPA, lysophosphatidic acid.

Table 2. Human primer sets.

Primer		Sequence	Product size (bp)	Tm (℃)
GAPDH	Sense	AAGGTGAAGGTCGGAGTC	121	60
	Anti-sense	TGTAGTTGAGGTCAATGAAGG		
MT1-MMP	Sense	TGGTTCTGGCGGGTGAGG	104	60
	Anti-sense	CTCTCGTAGGCAGTGTTGATGG		

Tm, melting temperature.

demonstrated that MMP-2 plays an important role in LPA-induced human EC invasion and migration (Wu, 2005). Since MMP-2 activity is regulated by MT1-MMP, we intend to investigate whether MT1-MMP is also modulated by LPA in hECs. Herein, we showed that both mRNA and protein expression of MT1-MMP is regulated by LPA in HUVECs. Furthermore, we demonstrated that LPA-induced MT1-MMP expression is mediated through G_i protein. These results implied that LPA enhancement of MMP-2 activity is likely mediated through induction of MT1-MMP expression in HUVECs.

MATERIALS AND METHODS

Reagents

LPA, gelatin, perussis toxin (PTx) (Table 1), and monoclonal rabbit anti-human MT1-MMP antibody (M3927) were products of Sigma-Aldrich (St. Louis, MO). HUVEC culture media, including medium 199 and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT) and endothelial growth medium (EGM) was from cell application (San Diego, CA). Goat anti-rabbit IgG-HRP (sc2004) antibody was ordered from Santa Cruz (Santa Cruz, CA) and rabbit anti-goat IgG-HRP (31402) antibody was from Pierce (Rockford, IL). The PLC inhibitor, U73122 (Table 1), was obtained from Tocris Cookson (Bristol, UK). Penicillin-Streptomycin and Trypsin-EDTA were purchased from Invitrogen Corporation (Grand Island, NY).

Cell culture

Human umbilical cords were kindly provided from National Taiwan University Hospital and Tong-Kun Wu Obstetrics and Gynecology Clinic (Taipei, Taiwan). Primary cultures of HUVECs were described previously (Lee, 2006) and those cells at passages 2-5 were used in all experiments. Cells were incubated in a humidified incubator containing 5% CO_2 at 37°C.

RNA extraction and reverse transcription

After treatment, HUVECs were washed three times with cord buffer, RNA was then extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). After quantification, 0.5 μg of RNA was mixed with 1 μL dNTP (Viogene, Sunnyvale, CA) and 1 μL oligo-dT (Purigo, Taipei, Taiwan) for 5 min at 65°C. Then, complementary (c) DNA was acquired by reverse transcriptase at 42°C for 50 min (Thermo Scientific, Waltham, MA).

Real-time PCR

Real-time PCR was carried out using the iCycler iQ real-time detection system (Bio-Rad Laboratories, Hercules, CA) with ABsolute SYBR Green Fluorscein (Thermo Scientific, Waltham, MA) as the fluorescent dye. Briefly, cDNA was subjected to the real-time PCR using the primer pairs listed in table 2. Cycling conditions were 95°C for 13.5 min, followed by 40 cycles at 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s. For quantification, the target gene was normalized to GAPDH gene. Oligonucleotide primers for PCR were designed using Beacon Designer 2 software (Premier Biosoft International, Palo Alto, CA).

SDS polyacrylamide gel electrophoresis (PAGE) and immunoblotting

After treatment, 60 μg protein were separated by 4~10% SDS-PAGE and then transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA). Transferred blots were blocked with 5% BSA and were incubated with antibodies against human MT1-MMP (1:5000), and human β-actin (1:5000), followed by incubating with various HRP-conjugated secondary antibodies. Blots were incubated in enhanced chemiluminescence (ECL) substrate (Western LightingTM) for 1 min at room temperature, and detected using X-ray film. All data were quantified with TotalLab version 2.01 (Durham, NC).

Statistical analysis

Significant differences between the control and treatment groups were analyzed using one-way analysis of

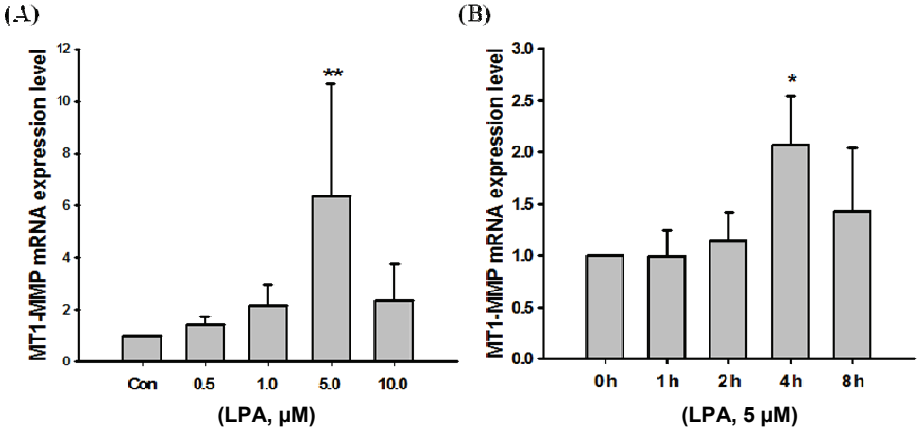

Fig. 1. Lysophosphatidic acid (LPA) induces membrane type 1 (MT1)-matrix metalloproteinase (MMP) mRNA expressions in dose- and time-dependent experiments. Human umbilical vein endothelial cells (HUVECs) were starved with serum-free M199 medium overnight. Starved HUVECs were then (A) treated with 0.5, 1, 5, and 10 μM of LPA for 4 h at 37°C, or (B) treated with 5 μM of LPA at for 1, 2, 4, and 8 h at 37°C. After treatment, RNA was isolated and was reverse-transcribed into cDNA. Subsequently, mRNA expression of MT1-MMP was detected through real-time PCR and normalized by GAPDH gene expression. Each bar of histogram represents at least three experiments, and the data were shown as the mean ± SD (* p< 0.05; ** p<0.01, compared to the control).

variance (ANOVA) (StatView, Abacus Concept, Berkeley, CA). Each experiment was repeated at least three times. A value of $p < 0.05$ was considered statistically significant difference.

RESULTS

LPA induces MT1-MMP mRNA expression in HUVECs

Since MMP-2 expression is regulated by LPA (Wu, 2005), we further verify whether MT1-MMP is also regulated by LPA in HUVECs. In dose- and time-dependent experiments, various concentrations of LPA were applied to HUVECs at different time intervals. Real-time PCR results showed that LPA-induced MT1-MMP mRNA expression reached its maximal level at 5 μM and then decreased at higher concentration (Fig. 1A). Moreover, a significant difference of MT1-MMP mRNA expression was observed following 4 h treatment with 5 μM LPA (Fig. 1B). These results indicated that LPA induces MT1-MMP mRNA expression in HUVECs.

LPA induces MT1-MMP protein expression in HUVECs

Next, Western blotting assay was conducted to test whether MT1-MMP protein expression is also regulated by LPA. Similar to mRNA results, protein expression of MT1-MMP was up-regulated by LPA at 5 μM (Fig. 2A) in dose-dependent experiment. In addition, LPA induced MT1-MMP protein expression at 8 h and sustained till 12 h after treatment (Fig. 2B) in time-course experiments. These results indicated that LPA induces MT1-MMP protein expression with a concentration- and time-dependent manner in HUVECs.

LPA induces MT1-MMP expression mainly through a Gi-dependent pathway

Since only LPA_1 and LPA_3 are highly expressed in HUVECs (Lee, 2006), their downstream inhibitors were applied to investigate which pathways are involved in LPA-induced MT1-MMP expression. As shown in Fig 3, LPA-induced MT1-MMP expression was inhibited by treatment with PTx, a chemical inhibitor of the G_i. On the other hand, we showed that pretreatment with U73122, an inhibitor of G_q downstream signaling, partially down-regulates LPA-induced MT1-MMP mRNA expression (Fig. 3). Moreover, the LPA-induced MT1-MMP mRNA expression was attenuated by Ki16425, a functional blocker of LPAR, in HUVECs (Fig. 4). These results suggested that LPA-induced MT1-MMP expression is mostly G_i-dependent.

DISCUSSION

LPA is a well-known modulator of cell migration, cell invasion, wound healing, cell proliferation, cell growth, and angiogenesis in various cell types (Vanblitterswijk, 1987; Lee, 2000; Panetti, 2000; Sauer, 2004). LPA-induced cell behaviors and functions are mediated through binding to their receptors (Anliker, 2004; Ishii, 2004; Skoura, 2009). In addition, accumulating evidences have revealed that MMPs are critical to cancer development, cell behaviors, and angiogenesis (Chang, 2001; Sternlicht, 2001; Rundhaug, 2005). These observations suggested that LPA may be involved in

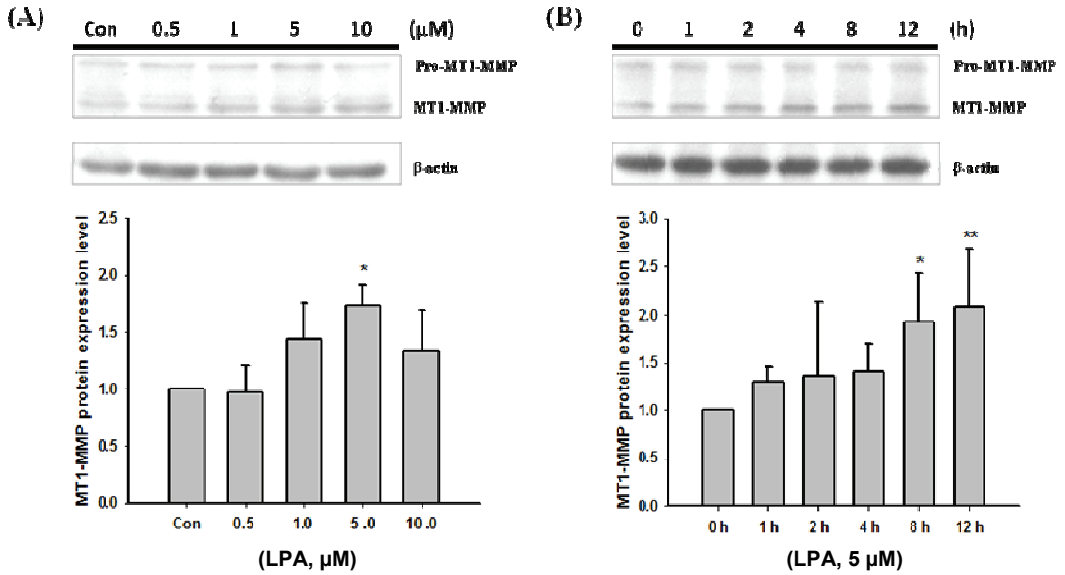

Fig. 2. Lysophosphatidic acid (LPA) induces membrane type 1 (MT1)-matrix metalloproteinase (MMP) protein expressions in dose- and time-dependent experiments. Human umbilical vein endothelial cells (HUVECs) were starved with serum-free M199 medium overnight. Starved HUVECs were then (A) treated with 0.5, 1, 5, and 10 μM of LPA at for 4 h 37°C, or (B) treated with 5 μM of LPA for 1, 2, 4, 8, and 12 h at 37°C. After treatment, protein was extracted and MT1-MMP expression was detected by Western blotting. Subsequently, the expression of MT1-MMP was normalized by β-actin (42 kDa). Total MT1-MMP expression is determined by both of pro-MT1-MMP (65 kDa) and MT1-MMP (60 kDa). Each bar of histogram represents at least three experiments, and the data were shown as the mean ± SD (* p< 0.05; ** p<0.01, compared to the control).

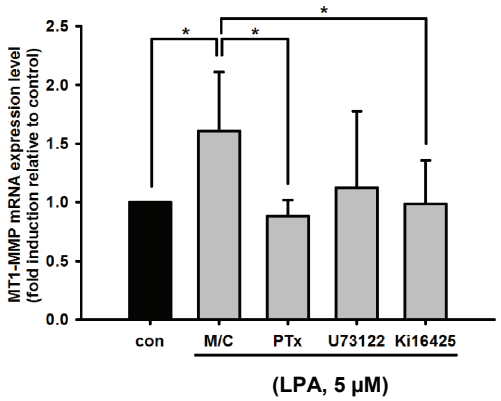

Fig. 3. Lysophosphatidic acid (LPA)-induced membrane type 1 (MT1)-matrix metalloproteinase (MMP) mRNA expressions is mainly mediated through the G_i-dependent pathway. Human umbilical vein endothelial cells (HUVECs) were starved with serum-free M199 medium overnight, then pretreated with the following inhibitors, including 15 ng/ml PTx overnight, 1 μM of U73122 for 1 h, and 10 μM Ki16425 for 1 h at 37°C. Pretreated HUVECs were then incubated with 5 μM of LPA for 4 h at 37°C. After treatment, RNA was extracted and was reverse-transcribed into cDNA. Subsequently, the expression of MT1-MMP was detected using real-time PCR and normalized by GAPDH gene. Methanol/ chloroform (M/C) is the solvent of LPA. The MT1-MMP induction fold presented here is relative compared between LPA-treated and -untreated cells. Each bar of histogram represents at least three experiments, and the data were shown as mean ± SD (* p< 0.05, compared to the control).

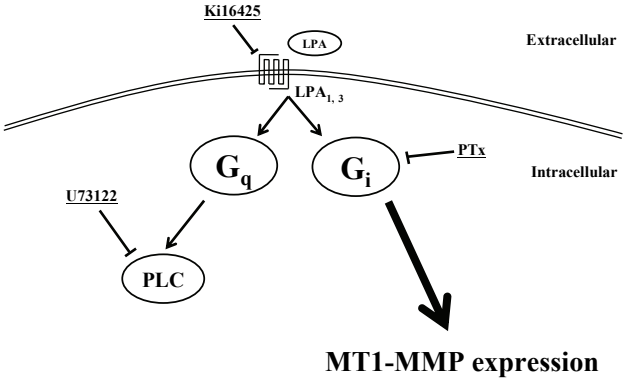

Fig. 4. Proposed molecular mechanism of lysophosphatidic acid (LPA)-induced membrane type 1 (MT1)-matrix metalloproteinase (MMP) mRNA expressions is primarily mediated through the G_i- and partially by the G_q -dependent pathway in HUVECs.

MMP-related functions. Indeed, it was reported that LPA regulates MT1-MMP-mediated tumor cell invasion (Fisher, 2006). Additionally, LPA also plays important roles in ECM remodeling, an essential process of cell migration, through regulating the expressions of MMPs.

Our previous study revealed that LPA increases MMP-2 expression and activity in both wild-type HUVECs and EAhy926, a human endothelial cell line (Wu, 2005). Herein, we further showed that LPA induces MT1-MMP expression in HUVECs. As shown in Fig. 1,

MT1-MMP mRNA expression is induced by 5 μM LPA after 4 h stimulation. Furthermore, we demonstrated that 5 μM LPA induces MT1-MMP protein expression after 8 h treatment (Fig. 2). Our results are well corresponded with previous studies showing that MMP-2 activity is modulated by MT1-MMP in fibrosarcoma (Strongin, 1995).

As mentioned above, LPA receptors are indispensable mediator of LPA- regulated pathways. According to our previous study, LPA_1 and LPA_3 are highly expressed in HUVECs (Lee, 2006). Since G_i and PLC are downstream mediators of LPA_1 and LPA_3 (Anliker, 2004; Ishii, 2004), we introduced their chemical inhibitors, including PTx (Gi inhibitor) and U73122 (PLC inhibitor) to test the involvement of G_i and PLC on LPA-induced MT1-MMP expression. We showed that only PTx had a significant inhibitory effect on LPA-induced MT1-MMP expression. This result is similar to Devine and Smicun's study (2008) in ovarian cancer cell line, indicating that LPA-induced MT1-MMP expression is inhibited by PTx. On the other hand, U73122 only partially suppressed the LPA effect on MT1-MMP mRNA induction in HUVECs (Fig. 3). In MCF-7 cells, N-terminal parathyroid hormone-related protein (PTHrp)-induced MT1-MMP expression is independent of PLC (Torricelli, 2006). In contrast, Luo et al. (2004) reported that PTH-induced MT1-MMP expression is mediated through PLC in MG-63 cells. Taken together, these results indicate that LPA-induced MT1-MMP expression in endothelial cells is mainly through the G_i- and may only partially through the G_q-dependent pathways.

In conclusion, our results suggest that LPA up-regulates MT1-MMP expression through the G_i pathway. Moreover, the G_q pathway is partially involved in LPA-induced MT1-MMP expression. Our results provide new evidence that LPA might modulate ECM through regulating MMPs.

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Lysophosphatidic Acid 經由 G_i 蛋白相關路徑在人類內皮細胞促使 Membrane Type-1 Metalloproteinase 之表現

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摘要：Lysophosphatidic acid (LPA) 是小分子量的水解磷酸脂。LPA 藉由接合到專一的 G 蛋白受器影響許多細胞的功能，包括細胞增生、細胞遷移、細胞入侵和細胞分化。Matrix-metalloproteinases (MMPs) 是一群需要鋅離子才有活性的蛋白酶，MMPs 在細胞和細胞基質間 (Extracellular matrix, ECM) 的交互作用中扮演著重要的角色。再者，membrane type 1-metalloproteinase (MT1-MMP) 不只切除 ECM 也會幫助 metalloproteinase-2 (MMP-2, Gelatinase A) 的活化，MMP-2 已被證實在細胞遷移中扮演著重要的角色。我們之前的研究指出 LPA 會促進 MMP-2 在人類臍帶靜脈內皮細胞 (Human umbilical vein endothelial cells, HUVECs) 中的表現以及活性。在這份研究中，利用即時聚合酶放大技術 (real-time PCR) 以及西方點墨法 (Western blotting)，我們進一步發現 LPA 也會在 HUVECs 中促使 MT1-MMP 的訊息核醣核酸以及蛋白質表現增加。再者，藉由使用一些化學抑制物，我們發現 LPA 促使 MT1-MMP 的表現增加主要是經由 G_i 路徑，且部分經由 G_q。這結果提供了 LPA 可能經由調節 MT1-MMP 的表現來調控 ECM 的新證據。

關鍵詞：LPA、MT1-MMP、MMP-2、人類臍帶靜脈內皮細胞、細胞基質。