水解磷酸脂於內皮細胞與癌細胞結合作用之研究

計畫類別：個別型計畫
計畫編號：
執行期間：93年08月01日至94年07月31日
執行單位：國立臺灣大學生命科學系

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報告類型：精簡報告
處理方式：本計畫可公開查詢

中華民國94年05月30日
Investigation of the Effects of Lysophospholipids on
Cancer-Endothelial Interaction

Abstract
Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are both low molecular weight lysophospholipids (LPLs), which promote cell proliferation, migration, and invasion via interaction with a family of specific G protein-coupled receptors. Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes, which are involved in degradation of the extracellular matrix, and which play critical roles in endothelial cell migration and matrix remodeling during angiogenesis. Among these MMPs, MMP-2 is known to trigger cell migration. In our present study, we examined the effects of LPA and S1P on MMP-2 expression in human endothelial cells. We showed that LPA and S1P enhanced MMP-2 expression in mRNA, protein levels, and also enzymatic activity of cells of the EAhy926 human endothelial cell line. The enhancement effects occurred in concentration- and time-dependent manners. Results from real-time PCR, Western Blots and substrate gels indicated that these enhancement effects were mediated through MEK/ERK-, NF-κB-, and calcium...
influx-dependent pathways. Furthermore, we show that endothelial cell invasion of the gel was enhanced by LPLs, and the induction could be prevented by an MMP inhibitor, GM6001. These observations suggest that LPA and S1P may play important roles in endothelial cell invasion by regulating the expression of MMP-2.

**Keywords**: LPA; S1P; endothelial cell

**Introduction**

Bioactive lysophospholipids (LPLs), including lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), are membrane-derived lipid mediators, which are generated from phospholipid precursors of membranes and secreted by platelets [1, 2], macrophages, epithelial cells [3-5], and some cancer cells [6-8]. These lysophospholipids regulate migration, proliferation, and survival of endothelial cells [9-11]. LPA and S1P bind multiple G protein-coupled receptors of the endothelial differentiation gene (Edg) family [9, 12]. To date, nine members of the family have been discovered in mammalian cells: LPA1 (Edg-2), LPA2 (Edg-4), LPA3 (Edg-7), and LPA4 receptors for LPA [13, 14], and S1P1 (Edg-1), S1P2 (Edg-5), S1P3 (Edg-3), S1P4 (Edg-6), and S1P5 (Edg-8) are receptors for S1P [9, 15]. Activation of LPL receptors results in a wide spectrum of intracellular events, such as increases in inositol phosphates and intracellular calcium [9], inhibition of adenylyl cyclase [16], and activation of kinases such as protein kinase C (PKC) and many other signaling pathways [17-19].

The major sources of LPA and S1P are activated platelets, injured cells, and cells stimulated by growth factors, suggesting potential roles in inflammation, wound healing, and tumor formation [6]. The concentration of S1P is estimated to be 200 nM in plasma serum [20], and that of LPA is approximately 1-10 μM in human serum with variable reports of plasma concentrations from undetectable to 2 nM [21]. The growth-promoting activity of LPA has been observed in numerous cell lines. The ability of serum to induce mitosis appears to be mediated, in large part, by the lipids LPA and S1P [22].

Endothelial cells are normally quiescent and form a tight monolayer by interacting with the underlying extracellular matrix and with surrounding endothelial cells [23]. Interactions between endothelial cells and extracellular matrix proteins are important determinants of endothelial cell migration and signaling. In addition, endothelial cell migration is important during vascular development and angiogenesis. Both LPA and S1P stimulate endothelial cell migration [22, 24, 25]. Matrix metalloproteinases (MMPs) have been reported to play critical roles in this process [26-28].

MMPs are a family of enzymes that degrade components of the extracellular matrix (ECM). They are grouped by their substrate preferences and also domain structures: collagenases (MMP-1, MMP-8, and MMP-13) degrade fibrillar collagen; gelatinases (MMP-2 and MMP-9) are potent in non-fibrillar and denatured collagen degradation; stromelysins (MMP-3, MMP-10, and MMP-11) prefer proteoglycans and glycoproteins as substrates; and membrane-type MMPs (MT1-, MT2-, MT3-, MT4-, and MT5-MMP) contain a C-terminal transmembrane domain directing cell surface localization [26, 29, 30].

The 72-kDa gelatinase A (MMP-2) is the most widely distributed of all the MMPs and is expressed constitutively by a number of cells, including endothelial cells. It participates in the breakdown of collagen type IV, a major component of subendothelial basement membranes [29]. Therefore, MMP-2 production by endothelial or surrounding cells may be vital for the formation of new functional blood vessels, in either the early process of degradation or the later reconstruction of the underlying basement membrane. Paradoxically, at low levels, the specific physiological MMP-2 inhibitor, tissue inhibitor of metalloproteinases-2 (TIMP-2), promotes this activation by forming a membrane complex with MT1-MMP, anchoring the pro-MMP-2 to the cell surface [31-33]. A recent study also showed that both TIMP-2 and TIMP-3 enhance the activation of pro-MMP-2 by MT3-MMP [34]. It has been
shown that MMP-2 and TIMP-2 are required for normal development of zebrafish embryos [35, 36], indicating that MMP-2 and TIMP-2 expressions are essential during embryogenesis.

Activities of MMPs are tightly regulated at several levels including gene expression and secretion of proenzymes that require activation by other MMPs or serine proteinases [37]. Once MMPs are activated, they are subjected to inhibition by TIMPs, which determine the net enzymatic activity in the extracellular space [26]. A balance between MMPs and TIMPs is involved in the morphogenesis of normal tissue as well as in mechanisms of tumor invasion and metastasis [37-40].

Aberrant expression of MMPs has been noted in cancer, angiogenesis [41], arthritis, inflammation, emphysema, multiple sclerosis [42], and chronic wounds [6, 8]. In this study, we hypothesized that LPA and S1P may affect endothelial cell invasion through regulating MMP-2 expression. We present evidence that LPA and S1P enhance MMP-2 expression in human endothelial cell hybridoma EA.hy926 cells in concentration- and time-dependent manners. By using chemical inhibitors, we show that LPA and S1P enhance MMP-2 through pathways dependent on MEK/ERK-, NF-κB-, and calcium influx-mediated signaling. Furthermore, we used a modified Boyden chamber to investigate the invasive activity of EA.hy926 cells, and found that the invasive activity was enhanced by LPL treatments. This enhancement was likely due to an increase in MMP-2 expression in EA.hy926 cells since the enhancement was prevented by pretreatment with the synthetic MMP inhibitor, GM6001. These results imply that LPL’s enhancement of endothelial cell invasion is likely mediated through induction of MMP-2 expression.

**Results and discussion**

**LPA and S1P enhance MMP-2 mRNA levels in human endothelial cells.**

We first determined the Edg receptor expression profile of EAhy926 cells by RT-PCR (Fig. 1). Our results indicated that these cells express S1P1, S1P5, LPA1, and LPA3 receptors, and therefore can be used as a good model to study LPL’s effects in human endothelial cells.

LPLs have multiple effects in many different cell types, such as promoting cell migration, which requires protease. Therefore, we were interested in determining if LPLs regulate MMP-2 expression in endothelial cells. We first investigated the effects of LPLs on MMP-2 mRNA levels in human umbilical vein endothelial cells (HUVECs). HUVECs were treated with 1 μM of LPA and S1P for 4 h. RNAs from treated cells were isolated and subjected to RT-PCR using specific primer sets for human MMP-2 and β-actin. MMP-2 mRNA levels were elevated after the treatments, especially after treatment with LPA (Fig. 2A, left panel). Expression patterns of β-actin, which was used as the loading control, did not significantly differ in either LPA- or S1P-treated samples (Fig. 2A, right panel). To further confirm that the observed bands were MMP-2 mRNA, PCR products from agarose gels were cloned and sequenced. Sequencing results verified the bands are human MMP-2. We then checked if the same effects could be detected in EAhy926 cells. Cells were treated with different concentrations of LPA and S1P. RNA from treated EAhy926 cells was harvested and subjected to RT-PCR using specific primer sets for human MMP-2 or GAPDH. The expression patterns of GAPDH, which was used as the loading control, did not significantly differ in either LPA- or S1P-treated samples. LPA and S1P also upregulated MMP-2 mRNA expression in EAhy926 cells in concentration-dependent manners (Fig. 2B). The MMP-2 mRNA level increased at 0.1 μM, and the expression peaked at 1 μM of S1P treatment and around 0.5-1 μM of LPA. Therefore, HUVECs and EAhy926 cells have similar effects after LPL treatment. HUVECs can only undergo 5 passages. On the other hand, EAhy926 cells can passage through up to 20 generations and have been shown to express S1P1, S1P5, LPA1, and LPA3 receptors (Fig. 1). Therefore, we chose EAhy926 cells as a suitable model for the following experiments.

In the dose-response experiments, we
showed that the enhancement of MMP-2 mRNA expression by both LPA and S1P in EAhy926 cells was concentration-dependent. Therefore, we intended to investigate whether the effects of LPA and S1P on MMP-2 mRNA levels in EAhy926 cells were time-dependent. EAhy926 cells were incubated with LPA (1 μM) and S1P (1 μM) for different time intervals as indicated, and RNA was subjected to RT-PCR using specific primer sets for human MMP-2 or GAPDH. Enhanced MMP-2 mRNA in EAhy926 cells was first observed as early as 1 h after treatments were initiated, peaked at 4 h, and then declined thereafter. We show that LPA and S1P upregulated MMP-2 mRNA expression in EAhy926 cells in a time-dependent manner (Fig. 2C).

LPA and S1P enhance MMP-2 protein levels in human endothelial cells.

Since the mRNA levels were increased by LPLs, we further investigated if the elevated mRNA levels of MMP-2 were also correlated with protein expression levels. Cells were treated with different concentrations of LPA and S1P, and total protein was collected and detected by Western blot analysis. After a 4-h treatment, LPA and S1P both enhanced MMP-2 protein expression in concentration-dependent manners (Fig. 3A). Human β-actin Western blotting was used to show that the amount of sample loading did not significantly differ between control and treated samples. Consistent with the RT-PCR results, the enhancement effects of LPA and S1P on MMP-2 expression in EAhy926 cells peaked at 1 μM. For LPA treatment, MMP-2 protein expression appeared to peak at 0.5 μM and dropped at 5 μM; while for S1P, the peak was around 0.5–1 μM, then it dropped at 10 μM. Similar results were also observed in HUVECs (data not shown).

In the dose-response experiments, we showed that enhancement of MMP-2 protein levels by both LPA and S1P in EAhy926 cells was concentration-dependent. Therefore, we wanted to test if the effects of LPA and S1P on MMP-2 protein expression were time-dependent. EAhy926 cells were incubated with LPA (1 μM) and S1P (1 μM) for different time durations as indicated, and total protein was subjected to Western blotting using an anti-MMP-2 antibody. A human β-actin antibody was used to show that the amount of sample loading did not significantly differ between control and treated samples. These enhanced MMP-2 protein levels in EAhy926 cells were first observed as early as 1 h after treatment was initiated, peaked at around 4 h for S1P treatment and 4–8 h for LPA treatment, and declined thereafter. With overnight treatment, MMP-2 expression remained high in S1P-treated but returned to basal levels in LPA-treated samples. Therefore, we have shown that LPA and S1P upregulate MMP-2 protein expression in EAhy926 cells in a time-dependent manner (Fig. 3B).

LPA and S1P enhance MMP-2 enzymatic activity in human endothelial cells.

Since protein levels were increased by LPLs, we further investigated if the elevated protein levels of MMP-2 were also correlated with enzymatic activity. We next investigated the effects in both HUVECs and EAhy926 cells. Cells were treated with different concentrations of LPA and S1P, and conditioned medium were collected and resolved on a substrate gel (containing 0.1% gelatin). After staining, increased gelatinolytic activity was detected, and we found that LPLs induced MMP-2 activity in a concentration-dependent manner both in HUVECs (Fig. 4A) and EAhy926 cells (Fig. 4B). These results were consistent with the results of mRNA levels. HUVECs and EAhy926 cells showed similar results after LPL treatment in these assays. MMP-2 enzymatic activity first increased at 0.5 μM, and the activities increased with the concentration. The effect of S1P was more significant than that of LPA in regulating MMP-2 enzymatic activity. These results indicated that LPA and S1P induce endothelial cell proteolytic activity, which might stimulate the invasive capacity of endothelial cells. Incubation of the gel with EDTA significantly inhibited the enzymatic activity of the gelatinase, indicating that the responsible gelatinase was a metalloproteinase (data not shown).

In the dose-response experiments, we
show that the enhancement of MMP-2 enzymatic activity by both LPA and S1P in EAhy926 cells was concentration-dependent. We further investigated whether the effects of LPA and S1P on MMP-2 enzymatic activity were time-dependent or due to starvation. Cells were simultaneously subjected to LPL treatments or serum-free medium for different time durations. The conditioned media, including LPL-treated and control media, were collected and assayed on a substrate gel (containing 0.1% gelatin). LPLs induced MMP-2 activity in a time-dependent manner (Fig. 4C). The enhancement effects of LPA and S1P were first observed at 2 h post-ligand treatment. Although control medium showed a slight increase in MMP-2 activity, LPL treatment generated a more-significant induction of MMP-2 enzymatic activity. Therefore, we subtracted the control value to quantify the increased multiples of MMP-2 activity stimulated by LPLs. It should be noted that in addition to starvation before treatment, cells in the overnight treatment were incubated in a serum-free condition for 48 h. Such a stressful situation may have caused a dramatic increase in cell secretion in general. That might be the reason why the conditioned medium collected in the overnight treatment showed significantly increased MMP-2 gelatinolytic activity.

**LPLs effects on MMP-2 mRNA expression are involved in MEK/ERK-, NF-κB-, and calcium influx-dependent signaling pathways**

Since LPA and S1P are ligands for Edg receptors, and at least 4 different Edg receptors are expressed on EAhy926 cells (Fig. 1), we further investigated if the effects of LPLs on MMP-2 expression are mediated through these receptors. To investigate the signaling pathway involved in the induction of MMP-2 by LPA and S1P, we used several chemical inhibitors known to impede certain signaling pathways. Cells were pretreated with pertussis toxin (a Gi blocker), PD98059 (a MEK blocker), U73122 (a PLC blocker), PDTC (an NF-κB blocker), PP2 (an Src family kinase blocker), and SKF96365 (a receptor-mediated calcium influx blocker) for 1 h and then were treated with control, 1 μM LPA, and 1 μM S1P for 4 h. After the RT reaction, we used real-time PCR to detect modulation of MMP-2 mRNA levels by these inhibitors as enhanced by LPLs. Pretreatment using chemical inhibitors for 1 h had no significant effect on the GAPDH loading control. However, the stimulatory effects of 1 μM LPA and S1P on MMP-2 mRNA expression in EAhy926 cells were significantly suppressed by PD98059, PDTC, and SKF96365. On the other hand, pretreatment with U73122, LY 294002, PP2, and pertussis toxin had no effect on either control or LPL-enhanced MMP-2 expression (Fig. 6). These results suggested that the enhancement effects of LPLs on MMP-2 mRNA expression are MEK-, NF-κB-, and calcium influx-dependent but independent of the function of Gi, PLC, PI3K, and Src family kinases.

**LPLs effects on MMP-2 protein expression are involved in MEK/ERK-, NF-κB-, and calcium influx-dependent signaling pathways**

Since the enhancement effects on mRNA levels by LPLs were blocked by the above mentioned chemical inhibitors, we further investigated if these inhibitors also affected MMP-2 protein expression induced by LPLs. Cells were pretreated with the inhibitors mentioned above for 1 h, followed by control and 1 μM of LPA and S1P treatments for 4 h. Human β-actin Western blotting was used to indicate that sample loadings were even. Consistent with the mRNA results, we found that LPL induction of MMP-2 protein expression was blocked by PDTC, PD98059 and SKF96365 but not by pertussis toxin. These results suggested that the enhancement effects of LPLs on MMP-2 protein expression are MEK-, NF-κB-, and calcium influx-dependent but independent of the function of Gi (Fig. 7).

**Enhancement of MMP-2 gelatinolytic activity by LPL treatments is partially suppressed by chemical inhibitors.**

Since the enhancement effects on mRNA and protein levels by LPLs were blocked by certain chemical inhibitors, we further investigated if the effects of these inhibitors
were also correlated with enzymatic activity. Cells were pretreated with the inhibitors mentioned above for 1 h, followed by control and 1 μM of LPA and S1P treatments for 4 h. Conditioned media were collected and resolved on a substrate gel. We found that LPL induction of MMP-2 activity was significantly blocked by SKF96365, but partially suppressed by PDTC and PD98059, implying that calcium mobilization, MEK/ERK, and NF-κB signaling were involved (Fig. 8). These results are consistent with the above-mentioned mRNA and protein results.

LPA and S1P induce MMP-dependent endothelial cell invasion.

We further investigated if enhancements of MMP by LPLs were responsible for the cell invasive activity. We tested the hypothesis that LPL treatments induce endothelial cell invasion by an in vitro Boyden chamber invasion assay. No significant cell invasion was observed under control conditions, while dramatic enhancement was seen in the presence of LPLs. The enhancement effects occurred in a concentration-dependent manner (Fig. 9A). The maximal effects were about 4- and 5-fold increases by S1P and LPA treatments, respectively.

To further demonstrate that LPL-induced EAhy926 cell invasion was due to the induction of MMPs, we used GM6001, a chemically synthesized MMP inhibitor in the invasion experiments. We found that LPL-induced EAhy926 cell invasion was significantly inhibited by GM6001 (Fig. 9B). The amounts of invading cells were also monitored by microscopy (Fig. 9C). Together, these results suggested that the LPL-induced endothelial cell invasive capacity is MMP-dependent.

GM6001 inhibited basal or LPL induced-endothelial cell adhesion to gelatin

Since endothelial cell invasion of gelatin gel was enhanced by LPLs through up-regulating MMP-2 enzymatic activity, we further investigated if these enhancement effects were due to increase of cell adhesion. We tested the hypothesis by an in vitro adhesion assay. Treatments with 1 μM LPA or S1P had limited effects on EAhy926 cell adhesion to gelatin gel. However, significant suppression of cell adhesion was observed in both control- and treated EAhy926 cells co-incubated with 10 μM GM6001 (Fig. 10). These results indicated that LPA and S1P strongly enhanced endothelial cell invasion but had limited effects on endothelial cell adhesion. On the other hand, GM6001, a broad MMP inhibitor, showed strong suppression effects on both endothelial cell invasion and adhesion. These results indicated that MMP-2 participates in both endothelial cell invasion of and adhesion to gelatin gel. In addition, the inhibitory effects of GM6001 on endothelial cell adhesion might also be responsible for the suppression of endothelial cell invasion induced by LPLs.

計畫成果自評

本計畫之研究成果與計畫提出之目的大致相符，均為研究水解磷酸脂之於內皮細胞之生理作用。研究成果應可使我們對水解磷酸脂之生炎機制有進一步之了解，並進而對其拮抗劑之藥理作用建立一偵測系統。本實驗成果已發表於美國生理學會2005年之年會。另一方面，本研究之成果已投稿至Endocrinology期刊並獲得接受，將於不久之將來獲得刊登於Endocrinology期刊上。

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### Fig. 9

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### Fig. 10

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Control | LPA | S1P | LPA + GM6001 | S1P + GM6001

- GM6001
- +GM6001

A

B

C

Fig. 9

Fig. 10