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Enhancement of Human Endothelial Cell Adhesion to Type I Collagen by Lysophosphatidic Acid (LPA) and Sphingosine-1-Phosphate (S1P)

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ABSTRACT: The diverse cellular effects of lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are transduced by two structurally homologous subfamilies of G protein-coupled receptors, which are encoded by endothelial differentiation genes (Edg Rs). Human umbilical cord vein endothelial cells (HUVECs) express Edg Rs for LPA (Edg2) and S1P (Edg1 and 3), which transduce signals for migration of HUVECs through micropore filters coated with type I collagen. Since activation of integrins is essential for optimal migration of endothelial cells, we now examine the capacity of LPA and S1P to augment integrin mediation of endothelial cell binding to type I collagen. Lysophospholipid enhancement of HUVEC adhesion to type I collagen is detectable within 20 minutes. Enhancement of adhesion by both LPA and S1P is significant at 50 nM and optimal at 5 μM. Pertussis toxin (PTx), a specific inhibitor of Gi, and C3 exotoxin, a specific inhibitor of Rho, both suppress LPA and S1P enhancement of HUVEC adhesion. In contrast, PD98059, which blocks MAP kinase kinase (MEK), and wortmannin, which inhibits phosphatidylinositol 3-kinase (PI3K), had no effect on LPA- or S1P-enhancement of HUVEC adhesion. Neutralizing monoclonal antibodies specific for α2 and β1 integrin chains, concomitantly decrease LPA and S1P enhancement of HUVEC adhesion to type I collagen. LPA and S1P thus promote type I collagen-dependent adhesion and migration of HUVECs by recruiting α2 and β1 integrin through both Gi and Rho pathways. Integrin α2/β1 therefore appears to be critical on the effects of LPA and S1P on endothelial cell physiology.

KEY WORDS: LPA, S1P, Adhesion, Endothelial cell, Integrin.

INTRODUCTION

LPA and S1P are potent lipid growth factors with diverse biological activities (Moolenaar, 1995; Spiegel, 1998; Goetzl and An, 1998; Spiegel *et al.*, 1998; Graler and Goetzl, 2002; Mills and Moolenaar, 2003; Ozaki *et al*., 2003). Concentrations of LPA and S1P reach micromolar levels in serum, and in some circumstances in plasma, and account for some cellular growth effects of serum (Eichholtz *et al*., 1993; Tokumura, 1995; Yatomi *et al*., 1997). The most prominent sources of LPA and S1P are activated platelets, injured cells, macrophages stimulated by cytokines and protein growth factors, and ovarian cancer cells, suggesting potential roles in vascular, inflammatory and tissue healing events (Yatomi *et al*., 1997; Goetzl and An, 1998; Shen *et al.*, 1998; Valet *et al.*, 1998; Xu *et al.*, 1998; Watsky *et al.*, 2000). Recent evidences suggested that autotoxin, a previously identified exo-enzyme, might be responsible for the generation of these two phospholipids (Umezu-Goto *et al.*, 2002; Clair *et al.*, 2003; Ferry *et al.*, 2003).

The cellular effects of LPA and S1P are mediated by two sub-families of G protein-coupled receptors (GPCRs) encoded by endothelial differentiation genes (Edg Rs) (An *et al.*, 1998; Chun *et al.*, 1999; Goetzl *et al.*, 1999; Hla *et al.*, 1999; Moolenaar, 1999; Spiegel and Milstien, 2000). Human Edg1 (S1P1), Edg3 (S1P3), Edg5 (S1P2), Edg6 (S1P4) and Edg8 (S1P5) transduce signals from S1P. Human Edg2 (LPA1), Edg4 (LPA2) and Edg7

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(LPA3) transduce signals from LPA. G protein-linked intracellular signaling pathways activated by cloned Edg receptors, which have been characterized in heterologous expression systems, include ras-dependent activation of Erk 1/2, increases in $[Ca^{++}]_i$ and recruitment of rho GTPase and its downstream targets (Ye *et al.*, 2002; Hla, 2003; Meacci *et al.*, 2003; Ozaki *et al.*, 2003).

Endothelial cells form the inner lining of blood vessels and participate in important physiological processes including materials exchange, coagulation and wound healing. Several pathological phenotypes, including arteriosclerosis, inflammation and cancer, are associated with excessive activation or abnormalities of endothelial cells (Folkman and Shing, 1992; Siess, 2002). Endothelial cells express at least three types of Edg receptors, including Edg1, Edg2 and Edg3 (Lee *et al.*, 2000a). LPA and S1P regulate several endothelial functions through Edg Rs, including proliferation, migration and secretion of proteases (Lee *et al.*, 1999a; Wang *et al.*, 1999a; Lee *et al.*, 2000a; Panetti *et al.*, 2001). Several reports have also suggested that these lipids might also play a role in the regulation of angiogenesis and blood vessel integrity (Lee *et al.*, 1999a; Lee *et al.*, 1999b; Liu *et al.*, 2000).

Cell migration is a complicated process, which requires precise regulation of the processes of engagement of cells with matrix proteins (for review, see Sheetz *et al.*, 1998). The interactions between cells and matrix protein substrates are dependent on the expression and function of integrins. Endothelial cells express multiple integrin subunits, including α1, α2, α3, α4, α5, αV, β1 and β3, which constitute several complete integrins and interact with diverse extracellular matrix proteins (Short *et al.*, 1998). Upon binding to their substrates, activated integrins generate *outside-in* signals to induce tyrosine phosphorylation of focal adhesion kinase and regulate the assembly of focal adhesion complexes and associated stress fibers (Huttenlocher *et al.*, 1995).

I hypothesized that LPA and S1P stimulate endothelial cell adhesion to extra-cellular matrix proteins and concomitantly enhance adherence-dependent functions by activating distinct integrins. I now show evidence that LPA and S1P promote HUVEC adherence to type I collagen by activating integrin α2 and β1 through a Gi/o- and Rho-dependent mechanism, and consequently augment migration through type I collagen.

MATERIALS AND METHODS

Reagents

Sphingosine 1-phosphate (S1P) and PD98059 were obtained from Biomol (Plymouth, PA, USA). 1-Oleoyl-lysophosphatidic acid (LPA), 1-β-D-galatosylsphingosine (psychosine, PSY), lysophosphatidylserine (LPS), wortmannin, fatty acid-free bovine serum albumin (FAF-BSA), and crystal violet were purchased from Sigma (St. Louis, MO, USA). Pertussis toxin (PTx) was from CalBiochem (La Jolla, CA, USA). C3 exoenzyme was purchased from List Biological Laboratory (Campbell, CA, USA). Blocking antibodies for integrins were purchased from Chemicon (Temacula, CA, USA). Fetal bovine serum (FBS), trypsin and media were obtained from UCSF Cell Culture Facilities.

Human endothelial cell culture

Human umbilical cord vein endothelial cells (HUVEC) were purchased from Clonetics/Biowhittaker (Walkersville, MD, USA) and cultured in endothelial cell growth medium (EGM, Clonetics/Biowhittaker) supplemented with 10% FBS and passaged weekly. Only HUVEC passage numbers 2 through 6 were used in these experiments.

Quantification of HUVEC adhesion

Adhesion of HUVEC to type I collagen was determined by using CytoMatrix Cell Adhesion Strips from Chemicon (Temecula, CA, USA) according to the manufacturer's instructions. Briefly, the dehydrated extra-cellular matrix proteins coated on culture wells were re-hydrated by adding 100 μl of PBS for 30 minutes to generate a simulated matrix environment for cells to adhere. Single cell suspensions of HUVEC were prepared by 1 minute trypsinization, washing and resuspension in phosphate buffered saline (PBS) at 10^5 HUVEC/100 μl volume, plating of triplicate 100 μl aliquates onto re-hydrated strips with 100 μl of PBS alone or different concentrations of lipids. The strips were incubated at 37°C with 5% CO_2 for 30 minutes, and the unattached cells were removed by gentle washing of the strips two to three times with PBS. The adherent cells were stained with 0.2% crystal violet in 10% ethanol and the HUVEC-associated crystal violet was extracted into solubilization buffer (a 50/50 mixture of 0.1 M NaH_2PO_4, pH 4.5 and 50% of ethanol). Absorbance at 595 nm in a 96-well plate reader (Molecular Devices, CA, USA) was used to calculate the number of adherent HUVEC.

Pharmacological inhibition of HUVEC adhesion

HUVEC cultured in 10 cm plates were treated with buffer alone, 15 ng/ml of PTx or 300 ng/ml of C3 toxin for 16 h, 10 μM of PD98059 for 45 minutes or 10 nM of wortmannin for 1 h. The treated cells were trypsinized and cell suspensions were prepared. HUVEC adhesion then was determined by the standard assay.

Anti-integrin antibody blocking of HUVEC adhesion

Suspensions of HUVEC were treated with 1 μg/ml of the anti-integrin α1, α2, α3, α4, α5, αV, β3, α2β1 (Chemicon; Temecula, CA, USA) and β1 (clone A2B2, kindly provided by Dr. Caroline Damsky at UCSF) for 1 h. Adhesion then was determined by the standard assay.

Statistical analysis

All data were statistically analyzed by one-way ANOVA followed by Fisher's protected least significant difference (StatView, Abacus Concept, Berkeley, CA, USA). A p value of < 0.05 was considered statistically significant.

RESULTS

LPA and S1P stimulate endothelial cell adhesion to type I collagen in a ligand-specific and concentration-dependent manner

Endothelial cells adhere to multiple extracellular matrix proteins, including fibronectin, vitronectin, laminin and collagen. As HUVEC expressed Edg1, Edg2 and Edg3 receptors, as detected by RT-PCR (Lee, 2000a), they are expected to respond to LPA and S1P. LPA and S1P both enhanced HUVEC adhesion to vitronectin, laminin, type I, and type IV collagen, (Fig. 1). I focused on type I collagen, which is widely accessible to endothelial cells in damaged tissue, since LPA and S1P enhancement of HUVEC adhesion was greatest for this matrix protein (Fig. 1) and LPA and S1P also stimulate migration on type I collagen (Wang *et al.*, 1999b; Lee *et al.*, 2000a). LPA and S1P enhanced HUVEC adherence to type I collagen in a similar concentration-dependent manner (Fig. 2A). HUVECs are more sensitive to S1P than LPA at lower concentrations, which is consistent with our previous observation that S1P is a more potent stimulus than LPA of endothelial cell migration (Lee *et al.*, 2000a). In order

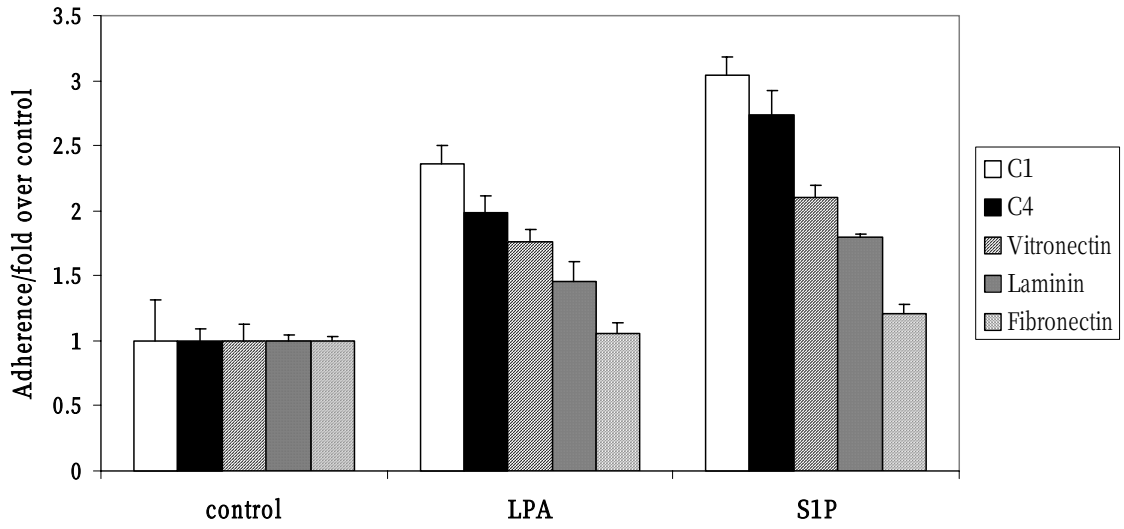

Fig. 1. LPA and S1P enhancement of HUVEC adherence to diverse extracellular matrix proteins. Suspensions of HUVEC were added to 96-well plates coated with vitronectin, laminin, fibronectin, and type I (C1) and type IV (C4) collagen in the presence or absence of 5 μM of LPA or S1P. After 30 minutes, attached cells were stained with crystal violet and dissolved for quantification of OD595 nm in a plate reader. Data are presented as mean ± SE (n = 3).

to confirm the specificity of LPA and S1P increases in endothelial cell adhesion, I showed that neither 10 μM LPS (a structural analog of LPA), PSY (structural analog of S1P) nor the diluted lipid solvent (10% chloroform; 90% methanol) have any effect on HUVEC adhesion to type I collagen (Fig. 2B).

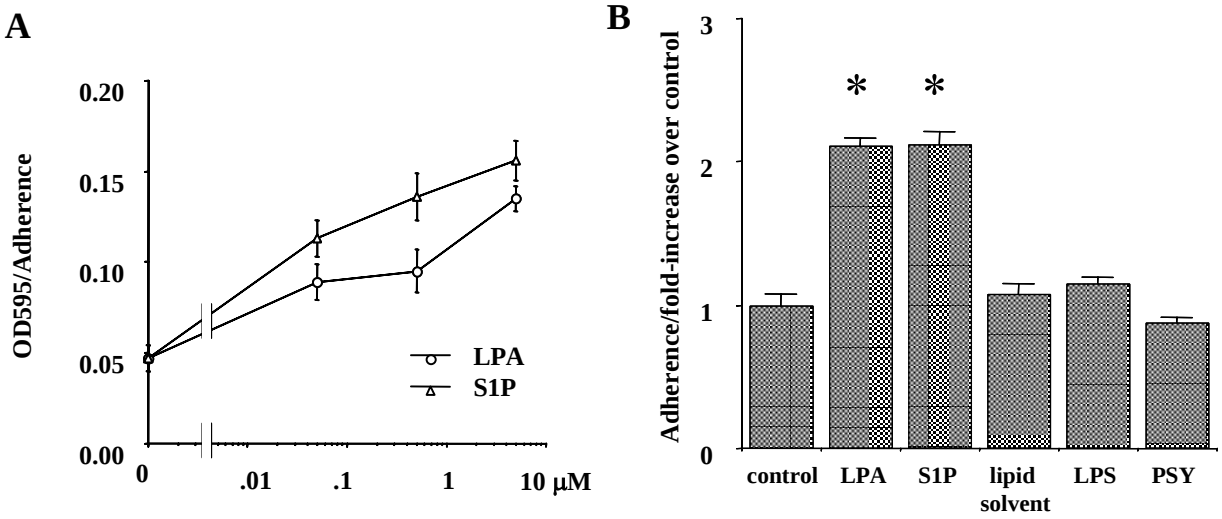

Fig. 2. Characteristics of LPA and S1P enhancement of HUVEC adherence to type I collagen. A: Concentration-dependence of enhancement of HUVEC adhesion. HUVECs in single cell suspensions were added to type I collagen-coated 96-well plates in the presence or absence of LPLs as concentrations indicated. After 30 minutes, attached cells were stained with crystal violet and dissolved for quantification of OD595 nm in a plate reader. B: Ligand specificity of LPA and S1P enhancement of HUVEC adherence. HUVEC adherence to type I collagen was quantified in medium (control), 5 μM of LPA (LPA), 5 μM of S1P (S1P), 5 μM of lysophosphatidylserine (LPS), 5 μM of psychosine (PSY) and 1:4 (vol/vol) of chloroform and methanol (lipid solvent) by the standard adhesion assay. All data are presented as mean ± SE (n = 3).

LPA and S1P effect on endothelial cell adherence is mediated through Gi- and Rho-dependent pathways and is independent of MEK and PI3K.

LPA and S1P binding to Edg-1, -2 and -3, activate multiple G proteins and transduce diverse downstream signals. To identify the specific signaling mechanisms utilized by LPA and S1P to enhance HUVEC adherence to type I collagen, relevant pharmacological inhibitors were applied at optimal concentrations. At 15 ng/ml, PTx, inhibitor of Gi/o-dependent signaling suppresses HUVEC adherence, activated by 5 μM LPA and S1P, up to means of 59% and 76%, respectively. At this concentration, PTx had little or no effect on the basal adhesion of HUVEC. At 300 ng/ml, C3 exotoxin, inhibitor for Rho, prevents HUVEC adherence activated by 5 μM LPA and S1P, up to means of 27% and 53%, respectively. The same concentration of C3 exotoxin had no significant effect on HUVEC basal adhesion to type I collagen (Fig. 3A). In contrast, 10 μM PD98059, an inhibitor of MEK, and 10 nM wortmannin, an inhibitor of PI3K, had no significant effect on LPL-stimulated HUVEC adherence to the substrate (Figs. 3B and 3C).

LPA and S1P enhancement of endothelial cell adhesion to type I collagen is mediated through activation of integrin α2β1

Since HUVEC adhesion is critically dependent on activation of integrins, we delineated which integrins were recruited by LPA and S1P in the course of HUVEC adhesion to type I collagen. HUVECs pre-treated with neutralizing antibodies against human integrin α2 or β1 showed markedly diminished adhesion responses to LPA and S1P, while antibodies against integrin α1, α3, α4, α5, α6, αv, β3 have little or no effect on HUVEC adhesion to type I collagen (Figs. 4A and 4B). Furthermore, functional blocking antibodies against VLA2, a complex of integrin α2β1, also blocked the effect of LPA- and S1P-stimulated HUVEC adhesion to type I collagen (Fig. 4C).

DISCUSSION

Through binding to Edg receptors on both vascular smooth muscle cells and endothelial cells, LPA and S1P have been shown to participate in processes such as angiogenesis (Lee *et al.*, 1999a; Lee *et al.*, 2000b), development of vascular integrity (Liu *et al.*, 2000), and pathological angiogenesis of diverse diseases (Xu *et al.*, 1998; Hu *et al.*, 2001). In addition, several reports using both *in vitro* (Lee *et al.*, 2000a) and *in vivo* (Goetzl *et al.*, 2002; Rother *et al.*, 2003; Watsky *et al.*, 2000) systems suggested that LPA and S1P are factors that facilitate wound healing. Activated platelets in the wounded tissues release LPA and S1P, which induce the damaged endothelium to migrate, proliferate, and thus enhance the wound healing process. My lab and others have previously shown that lysophospholipids are regulators of endothelial cell migration (Lee *et al.*, 1999b; Wang *et al.*, 1999a; Lee *et al.*, 2000a; Panetti *et al.*, 2000; Paik *et al.*, 2001). In this report, I investigated the mechanisms of LPLs on endothelial cell migration. Cell migration requires the delicate regulation of attachment and dissociation between cell and matrix (Huttonlocher *et al.*, 1995; Sheetz *et al.*, 1998). LPA and S1P have been shown to regulate cell adhesion to ECM both positively and negatively (Wang *et al.*, 1999b; Panetti *et al.*, 2001). In this study, I demonstrated that both LPA and S1P induce endothelial cell attachment to substrate proteins, which is consistent with their roles in enhancing endothelial cell migration.

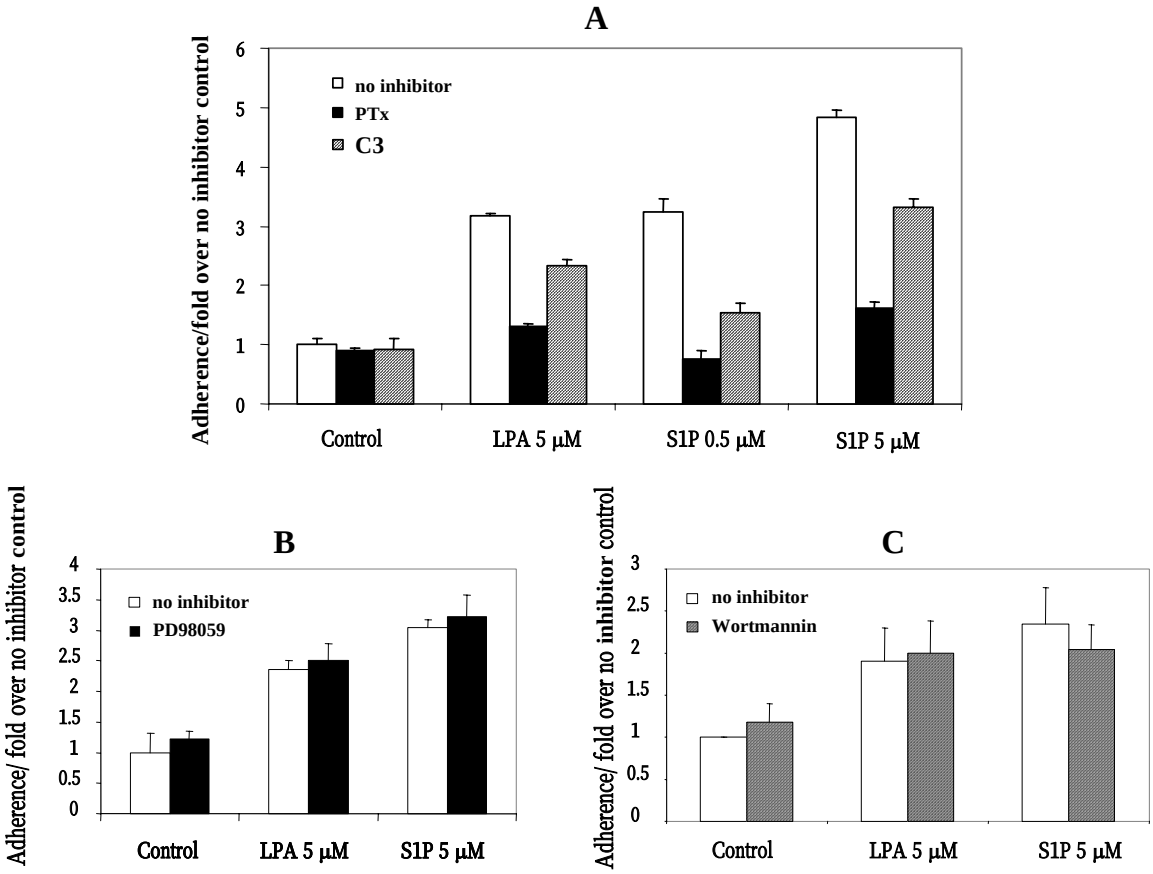

Fig. 3. Biochemical prerequisites for LPA and S1P enhancement of HUVEC adherence. A: Dependence of LPA and S1P enhancement of HUVEC adherence on Gi/o protein and Rho. Sub-confluent cultures of HUVECs were treated with 15 ng/ml of PTx or 300 ng/ml of C3 exotoxin (C3) for 16 hours. Pretreated cells were trypsinized, and single cell suspensions were used in adhesion assays in the absence or presence of LPLs. B: Lack of dependence of LPA and S1P enhancement of HUVEC adherence on MEK. Sub-confluent cultures of HUVECs were treated with 10 μM PD98059 for 45 minutes, trypsinized into single cell suspensions and subjected to adhesion assay as above. C: Lack of dependence of LPA and S1P enhancement of HUVEC adherence on PI3K. Sub-confluent cultures of HUVECs were treated with 10 nM wortmannin for 1 h, trypsinized into single cell suspensions and subjected to adhesion assay as above. All data are presented as mean ± SE (n = 3).

The interaction between cell and ECM is dependent on the action of different type of adhesion molecules. Integrins have been shown to be the most important among these molecules. Endothelial cells express multiple integrins including α1, α2, α3, α4, α5, αv, β1, and β3 (Short *et al.*, 1998). Using blocking antibodies, I demonstrated that LPA and S1P enhance endothelial cell adhesion to type I collagen, and this is mediated through α2β1 integrins. α2β1, also termed VLA2, has been shown to interact with type I collagen in different cell types. VEGF, a peptide growth factor that regulates endothelial cell functions, also up-regulates α2β1 expression in endothelial cells (Senger *et al.*, 1997), suggesting important roles of this integrins in endothelial cell function. Furthermore, recent report showed S1P effect on GD25 fibroblast contraction and endothelial cell tube formation in collagen gel are mediated through the action of α2β1 (Cooke *et al.*, 2000; Bayless and Davis, 2003), is consistent with our results. These results suggested that α2β1 might play important roles in mediating lysophospholipids effects on endothelial cells.

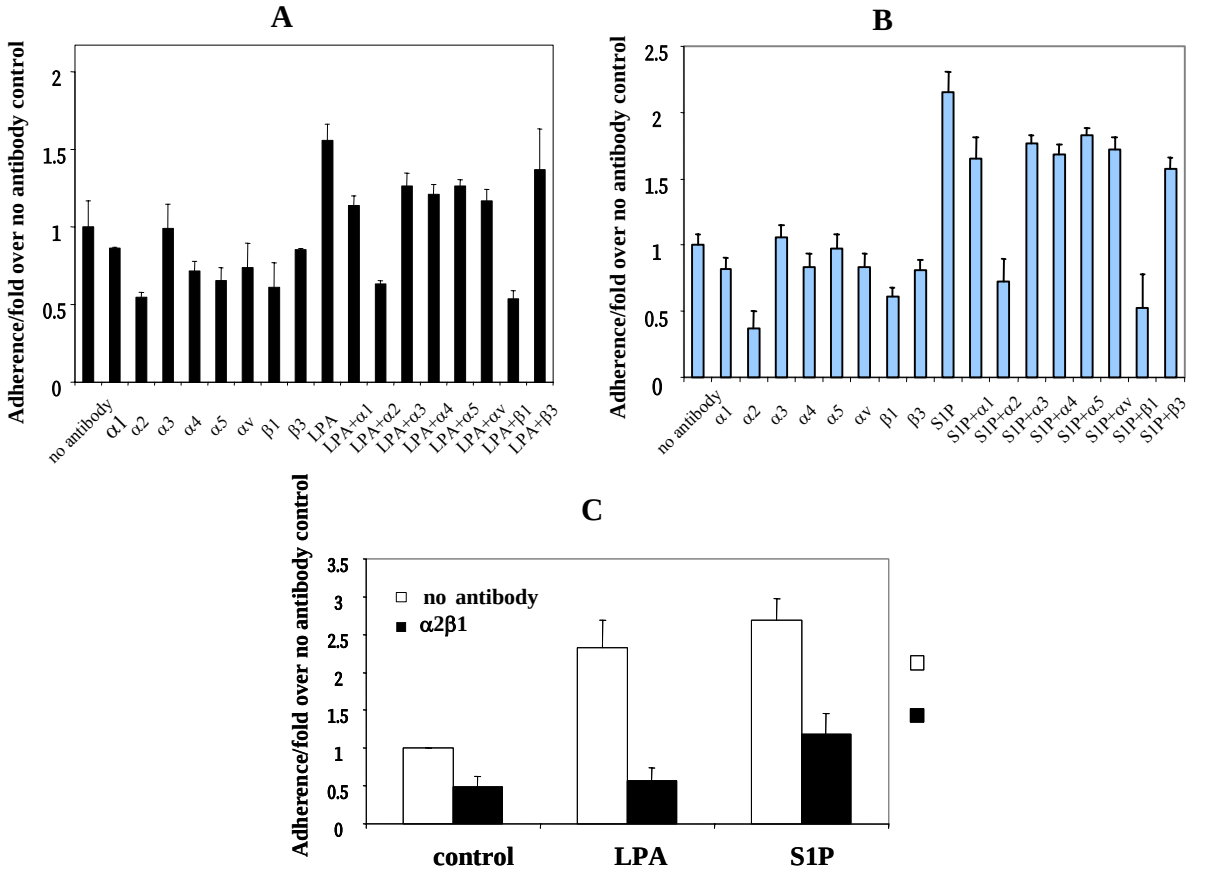

Fig. 4. Suppression of LPA and S1P enhancement of HUVEC adherence by neutralizing anti-integrin antibodies. Single cell suspensions of HUVECs were incubated with 1 μg/ml of neutralizing integrin antibodies against α1, α2, α3, α4, α5, αv, β1 and β3 integrins for 30 minutes. (A and B); or against integrin VLA2 (C) for 1 hour. The antibody-treated cells were used in adhesion assays without or with 5 μM of LPA (A and C) or S1P (B and C). Data are presented as mean ± SE (n = 3).

From this report, I found that the activation of integrin by LPA and SIP occurred in the matter of minutes. Judging from the kinetics of the activation, LPLs might regulate affinity of integrins by changing the conformation of these molecules. This activation could occur in two non-mutual-exclusive ways. First, lateral association of integrins with other membrane proteins can enhance their affinity of integrin. For example, cross-linking of CD98 enhances β1-integrin-dependent cell adhesion (Fenczik *et al.*, 1997). Second, the extracellular conformation status of integrin can be regulated by signals originated from its cytoplasmic tail; and this is referred to as inside-out signaling (Ginsberg *et al.*, 1992). For example, αIIβ3 integrin is activated by platelet agonists binding to G-protein-coupled receptors by this mechanism (Shattil *et al.*, 1998). Inactivation of Rho by C3 exoenzyme blocks α4β1-dependent adhesion of chemoattractant-stimulated lymphocytes to VCAM-1 and αLβ2-dependent adhesion of neutrophils to fibrinogen (Laudanna *et al.*, 1996). Since LPLs effects on endothelial cell adhesion is blocked by C3, suggests that an inside-out signaling mechanism is involved. It is unclear how "activating" signals transduce to the ligand-binding region of α2β1. However, the mechanisms of the interaction of Rho and the cytoplasmic tail of integrin β1 leading to the activation of α2β1 are currently under investigation.

LPA and S1P bind to Edg receptors and activate multiple G proteins including Gi, Gq, and G12/13 (An *et al.*, 1998). These activated G proteins will turn on downstream signaling events including MAPK phosphorylation, Ca^{++} mobilization and cyto-skeleton reorganization (Chun *et al.*, 1999; Hla *et al.*, 1999; Moolenaar, 1999; Spiegel and Milstien, 2000). I tested the effects of chemical inhibitors of these processes to identify the signaling mechanisms involved in endothelial cell adhesion induced by LPA and S1P. PTx has been used as a specific inhibitor for Gi dependent signaling pathways, while C3 exotoxin has been used to suppress the activity of Rho in multiple cell types. Here I reported that the effects of LPA and S1P on endothelial cell adhesion can be inhibited by 15 ng/ml of PTx and 300 ng/ml of C3 exotoxin treatments. This is consistent with the observation that LPA and S1P enhance endothelial cell migration and can be blocked by these treatments (Lee *et al.*, 1999b; Wang *et al.*, 1999a; Lee *et al.*, 2000a; Paik *et al.*, 2001). On the other hand, PD98059, a specific inhibitor for MEK, and wortmannin, an inhibitor for PI3K, had no significant effect on LPA and S1P induced endothelial cell adhesion, suggesting that the effects of LPA and S1P on endothelial cell adhesion to type I collagen are mostly Gi- and Rho- dependent while MEK and PI3K did not play an important role in these processes. These observations are consistent with the fact that LPA and S1P activate G proteins coupled to Edg receptors. Due to reagent limitation, I was unable to determine that whcich specific Edg receptor(s) is/are responsible for mediating effects for LPA and S1P on HUVEC adherence to matrix proteins. The report by Paik showed that introducing phosphothioate oligonucleotides of anti-sense Edg1 and Edg3 suppressed S1P effect on endothelial cell migration suggests that these receptors might be involved (Paik *et al.*, 2001).

It has been shown that signaling processes activated by growth factors may be modulated by the interaction between integrins and the substrate environment (Aplin *et al.*, 1999; Juliano *et al.*, 2001). The effect of LPLs on endothelial cell adhesion to matrix proteins might modulate the proliferation signals generated by both growth factors and by LPLs themselves. This suggests that the substrate environment encountered by wounded endothelial cells might provide additional stimuli other than that of wound healing factors alone. The multiple effects of LPLs on endothelial cell including proliferation, migration and enhancement of interaction with substrate proteins indicate that LPLs generate a plethora of signaling inputs during wound healing.

In this report, I conclude that both LPA and S1P enhance endothelial cell adhesion to substrate proteins by activating integrin $\alpha 2\beta 1$ through Gi/o- and Rho- dependent mechanisms. These results provide a potential mechanism for LPL-induced endothelial cell migration. The mechanisms of integrin $\alpha 2\beta 1$ activation are currently under investigation in my laboratory.

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水解磷酸脂促進內皮細胞與第一類纖維蛋白之附著

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摘　　要

水解磷酸脂 LPA 與 S1P 之複雜生理功能，是經由與兩類結構相似之接受器結合後所達成。人類臍帶靜脈內皮細胞表達至少三種水解磷酸脂接受器，活化後會造成細胞於第一類纖維蛋白環境中之移行。由於 integrin 蛋白接受器之活化與細胞移行有相當關聯，此研究目的即在於偵測水解磷酸脂是否會影響該蛋白質之活性。研究結果顯示水解磷酸脂的確會於 20 分鐘內造成內皮細胞與第一類纖維蛋白之附著且於 50 nM 之處理濃度即可見到顯著效果，而於 5 μM 之處理濃度達到最高效果。以訊息傳遞之化學抑制物質處理後發現水解磷酸脂之作用是經由 Gi 以及 Rho 蛋白來達成其作用。而使用 integrin 蛋白接受器活性抑制抗體處理後之結果發現，α2 與 β1 integrin 實際參與了水解磷酸脂增強內皮細胞與第一類纖維蛋白之附著之作用。實驗結論推斷水解磷酸脂應可活化 α2 與 β1 integrin，且於內皮細胞生理現象之調控可能扮演重要角色。

關鍵詞：水解磷酸脂、附著、內皮細胞、第一類纖維蛋白。

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