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Sphingosine 1-Phosphate Induces Platelet/Endothelial Cell Adhesion Molecule-1 Tyrosine Phosphorylation in Bovine Aortic Endothelial Cells through a PP2-Inhibitable Mechanism

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ABSTRACT: Sphingosine-1-phosphate (S1P) is a low-molecular-weight phospholipid derivative released by activated platelets. S1P transduces signals through a family of G protein-coupled receptors to modulate various physiological behaviors of endothelial cells. Platelet/endothelial cell adhesion molecule-1 (PECAM-1; CD31) is a 130-kDa protein expressed on the surfaces of leukocytes, platelets, and endothelial cells. Upon PECAM-1 activation, its cytoplasmic tyrosine residues become phosphorylated and bind with SH2 domain-containing proteins, thus leading to the downstream functions mediated by PECAM-1. In the present study, we found that S1P induced PECAM-1 tyrosine phosphorylation and SHP-2 association in bovine aortic endothelial cells (BAECs) by immunoprecipitation and western blotting. The pretreatment of BAECs with a series of chemical inhibitors to determine the signaling pathway showed that the PECAM-1 phosphorylation was inhibited by PP2, indicating the participation of Src family kinases. These results demonstrated that S1P induced PECAM-1 tyrosine phosphorylation in BAECs through mediation of Src family kinases, and this may regulate the physiological behaviors of endothelial cells.

KEY WORDS: S1P, PECAM-1, SHP-2, Src family kinases.

INTRODUCTION

Sphingosine 1-phosphate (S1P) is a naturally occurring phospholipid derivative that regulates various important biological functions, such as inflammation, wound healing, and vascular maintenance (Gumina et al., 1996; Lee et al., 2000; Yatomi, 2006). The most prominent source of serum S1P is from platelets (Yatomi et al., 1995, 2001). Upon platelet activation, S1P is released to the bloodstream and activates target cells by binding with its specific receptors. To date, five S1P receptors, $S1P_{1\text{-}5}$, have been identified in mammalian cells (Sanchez and Hla, 2004). Activation of S1P receptors and downstream G proteins results in several intracellular signals, such as increases in inositol phosphates and intracellular calcium concentrations, inhibition of adenylyl cylase, and activation of protein kinase C (Spiegel and Milstien, 2002; Anliker and Chun, 2004). These signals trigger various cellular effects including cell proliferation (Zhang et al., 1991), cell migration (Wang et al., 1999; Lee et al., 2000), endothelial permeability (Garcia et al., 2001; Schaphorst et al., 2003), expression of adhesion molecules (Lee et al., 2004), and cytoskeleton reorganization (Miura et al., 2000).

Platelet/endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kDa member of the immunoglobulin (Ig) gene superfamily expressed on the surfaces of leukocytes and platelets, and by the lateral junctions of endothelial cells (Newman et al., 1990). Endothelial PECAM-1 has been demonstrated to play important roles in junction formation and the control of endothelial permeability (Ayalon et al., 1994; Mamdouh et al., 2003). Upon specific stimulation, tyrosine residues in the PECAM-1 cytoplasmic domain can be phosphorylated and serve as binding sites for SH2 domain-containing proteins. SH2-containing protein phosphatase-2 (SHP-2), phospholipase C, and PI3 kinase have been indicated to bind with tyrosine-phosphorylated PECAM-1

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(Jackson et al., 1997; Masuda et al., 1997; Pellegatta et al., 1998; Pumphrey et al., 1999). However, the participation of a tyrosine kinase is necessary for PECAM-1 phosphorylation. Evidence obtained from co-precipitation, *in vitro* kinase assays, and overexpression studies supports the involvement of Src family kinases in PECAM-1 tyrosine phosphorylation (Lu et al., 1997; Edmead et al., 1999). With different stimulatory conditions, the Csk family and Fer have also been recognized as responsible kinases (Cao et al., 1998; Kogata et al., 2003).

Recent results suggested that PECAM-1 associates with sphingosine kinase 1, the kinase responsible for generating intracellular S1P (Fukuda et al., 2004). In addition, it was suggested that S1P-regulated wound-healing migration was dependent on PECAM-1 (Gratzinger et al., 2003). However, the relationship between S1P and PECAM-1 is still unclear. In the present study, we demonstrate that S1P induced PECAM-1 tyrosine phosphorylation and SHP-2 association in bovine aortic endothelial cells (BAECs). The use of chemical inhibitors suggests that the effect of S1P on PECAM-1 is mediated by Src family kinases. These results indicated that S1P might regulate endothelial behaviors through the phosphorylation of PECAM-1.

MATERIALS AND METHODS

Reagents

S1P, sodium orthovanadate, β-glycerophosphate, phenylmethlysulfonyl fluoride, leupeptin, and pertussis toxin (PTx) were purchased from Sigma (St. Louis, MO). Antibodies against phosphotyrosine, PECAM-1, and SHP-2, and protein A/G beads were obtained from Santa Cruz Biotech (Santa Cruz, CA). The chemical inhibitors, Y27632 dihydrochloride, U73122, PD98059, and wortmannin were purchased from Tocris Cookson (Ellisville, MO). PP2 was purchased from Calbiochem (San Diego, CA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) was obtained from Hyclone Laboratories (Logan, UT). Penicillin-streptomycin and trypsin-EDTA were purchased from Invitrogen (Grand Island, NY). Calphostin C was kindly obtained from Dr. Jiun-Hong Chen (Department of Life Science, Taipei, Taiwan).

Cell culture

Bovine aortic endothelial cells (BAECs) were isolated and characterized as endothelial cells by the presence of the von Willebrand factor and the uptake of DiI-labeled acetylated low-density lipoprotein. Cells were routinely cultured in DMEM containing 10% FBS, 1.5 mM glutamine, 100 IU/mL penicillin, and 50 ng/mL streptomycin. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

S1P treatments

S1P was dissolved in methanol/chloroform (9:1) to make a 1 mM stock solution. Confluent BAECs were washed with phosphate-buffered saline (PBS) and starved in serum-free DMEM for 16 h. S1P was then diluted to 1 μM in DMEM, and 0.005% fatty acid-free BSA was used as the carrier for S1P. In chemical inhibition, BAECs were treated with 1 μM S1P at 37°C for 3 min.

During the experiment, a hyperosmotic 0.3 M sucrose buffer was used as the positive control for PECAM-1 tyrosine phosphorylation (Harada, et al., 1995), and the normal goat IgG antibody was substituted for the PECAM-1 antibody during immunoprecipitation to serve as the negative control.

Cell lysate preparation

BAECs kept in 100-mm culture dishes were washed with cold PBS twice after S1P treatment and lysed with 800 μL of RIPA buffer (50 mM Tris (pH 8.0), 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% sodium dodecylsulfate (SDS) in the presence of phosphatase and protease inhibitors (2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mM β-glycerophosphate, and 10 μg/mL leupeptin). Lysates were collected with scrapers and centrifuged at 14,000 rpm for 10 min at 4°C. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA).

Immunoprecipitation and Western blotting

Total protein lysates, at 350 μg, were incubated with 2 μg of anti-PECAM-1 antibody or normal goat IgG (Santa Cruz, CA) on a rotating mixer at 4°C overnight. Mixtures were then rotated with 20 μL of protein A/G-agarose beads for 2 h at 4°C. The beads were centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was removed, and the pellet was washed three times in ice-cold RIPA buffer with protease inhibitors. After removing the excess buffer, the protein-antibody complexes were boiled with 25 μL of 2x SDS reducing sample buffer at 95°C and electrophoresed on a 10% polyacrylamide gel (SDS-PAGE). Proteins were transferred to PVDF membranes and blocked with 5% BSA in TBSt (10

mM Tris, 150 mM sodium chloride, and 0.1% Tween-20; pH 7.4) for 2 h at room temperature. Anti-phosphotyrosine and HRP-conjugated secondary antibodies diluted to 1:1000 and 1:4000 in 1% BSA in TBSt were incubated with membranes at room temperature for 1.5 h, followed by three washes using TBSt. The chemiluminescent substrate was prepared according to the manufacturer's instructions and incubated with the blots for 3 min. Luminescent signals were detected by exposing film to the blots in a dark room. After detection, membranes were stripped, blocked, and reprobed with the PECAM-1 and SHP-2 antibodies.

Chemical inhibition

Chemical inhibitors for protein kinase C (calphostin C, 200 nM), phospholipase C (U73122, 2 μM), MEK/ERK (PD98059, 50 μM), phosphatidylinositol 3-kinase (wortmannin, 500 nM), ROCK (Y27632, 10 μM), and Src family kinases (PP2, 10 μM) were used to determine the signaling pathways involved in S1P-induced PECAM-1 tyrosine phosphorylation. Inhibitors were prepared in serum-free DMEM and used to pretreat starved BAECs for 1 h before S1P treatments.

RESULTS

S1P induced PECAM-1 tyrosine phosphorylation and SHP-2 association in BAECs

Confluent BAECs that had been treated with 1 μM S1P for different time periods were immunoprecipitated with the PECAM-1 antibody and immunoblotted for phosphotyrosine and PECAM-1. PECAM-1 tyrosine phosphorylation increased after S1P treatment for 3 min and decreased after 5 min (Fig. 1A, top panel). The same blot was stripped and reprobed with the anti-PECAM-1 antibody (Fig. 1A, bottom panel) to show even loading of the samples. The experiments were repeated four times, and the numerical analysis is shown in figure 1B.

SHP-2 has been demonstrated to associate with tyrosine-phosphorylated PECAM-1. Therefore, we determined whether the tyrosine phosphorylation induced by S1P treatment also enhanced the association of SHP-2. The same blots used for detecting phosphotyrosine of precipitated PECAM-1 were reprobed with SHP-2. The results showed that the amount of SHP-2 associated with PECAM-1 increased after S1P treatment for 3 min and decreased after 5 min, consistent with PECAM-1 tyrosine phosphorylation (Fig. 1A, center panel). The experiments were repeated three times, and similar results were obtained. These results indicated that S1P induced PECAM-1 tyrosine phosphorylation and SHP-2 association in BAECs.

S1P induced PECAM-1 tyrosine phosphorylation through a PP2-inhibitable mechanism

To further investigate the pathways involved in S1P-induced PECAM-1 tyrosine phosphorylation, different chemical blockers of S1P signaling pathways with known effective concentrations were used. Confluent BAECs pretreated with 200 nM calphostin C (a PKC inhibitor), 50 μM PD98059 (an MEK inhibitor), 2 μM U73122 (a PLC inhibitor), 10 μM Y27632 (a ROCK inhibitor), and 500 nM wortmannin (a PI3K inhibitor) did not prevent PECAM-1 tyrosine phosphorylation after 1 μM S1P treatment (Fig. 2A). However, pretreatment with 10 μM PP2, a selective inhibitor of Src family kinases, significantly inhibited this S1P effect (Fig. 2B). The experiments were repeated three times, and similar results were obtained. Numerical analysis of PP2 inhibition is shown in figure 2C. These results indicate the involvement of Src family kinases in S1P-induced PECAM-1 tyrosine phosphorylation.

DISCUSSION

S1P and PECAM-1 are both multifunctional modulators of cell physiology. In this report, we first describe the effect of S1P on PECAM-1 tyrosine phosphorylation in bovine aortic endothelial cells, which provides a direct functional linkage between these two molecules.

PECAM-1 tyrosine phosphorylation is induced by a variety of stimuli. In platelets, stimuli such as thrombin, collagen binding, platelet aggregation, and PECAM-1 cross-linking have been demonstrated to induce PECAM-1 phosphorylation (Cicmil et al., 2000; Newman and Newman, 2003). In endothelial cells, collagen, integrin-mediated signaling, fibronectin adhesion, and homophilic binding with recombinant PECAM-1 are able to induce PECAM-1 phosphorylation (Lu et al., 1996; Bird et al., 1999). In addition, physiological mechanical forces (e.g., shear stresses) and osmotic forces also cause increased phosphorylation (Osawa et al., 2002). However, the influence of extracellular ligands on endothelial PECAM-1 has rarely been reported. A previous report suggested that angiopoietin-1 treatment decreases PECAM-1 phosphorylation and lowers the permeability of endothelial cells (Gamble et al., 2000). However, S1P was shown to enhance endothelial barrier integrity (Garcia et al., 2001; Schaphorst et al., 2003). The differences in actions

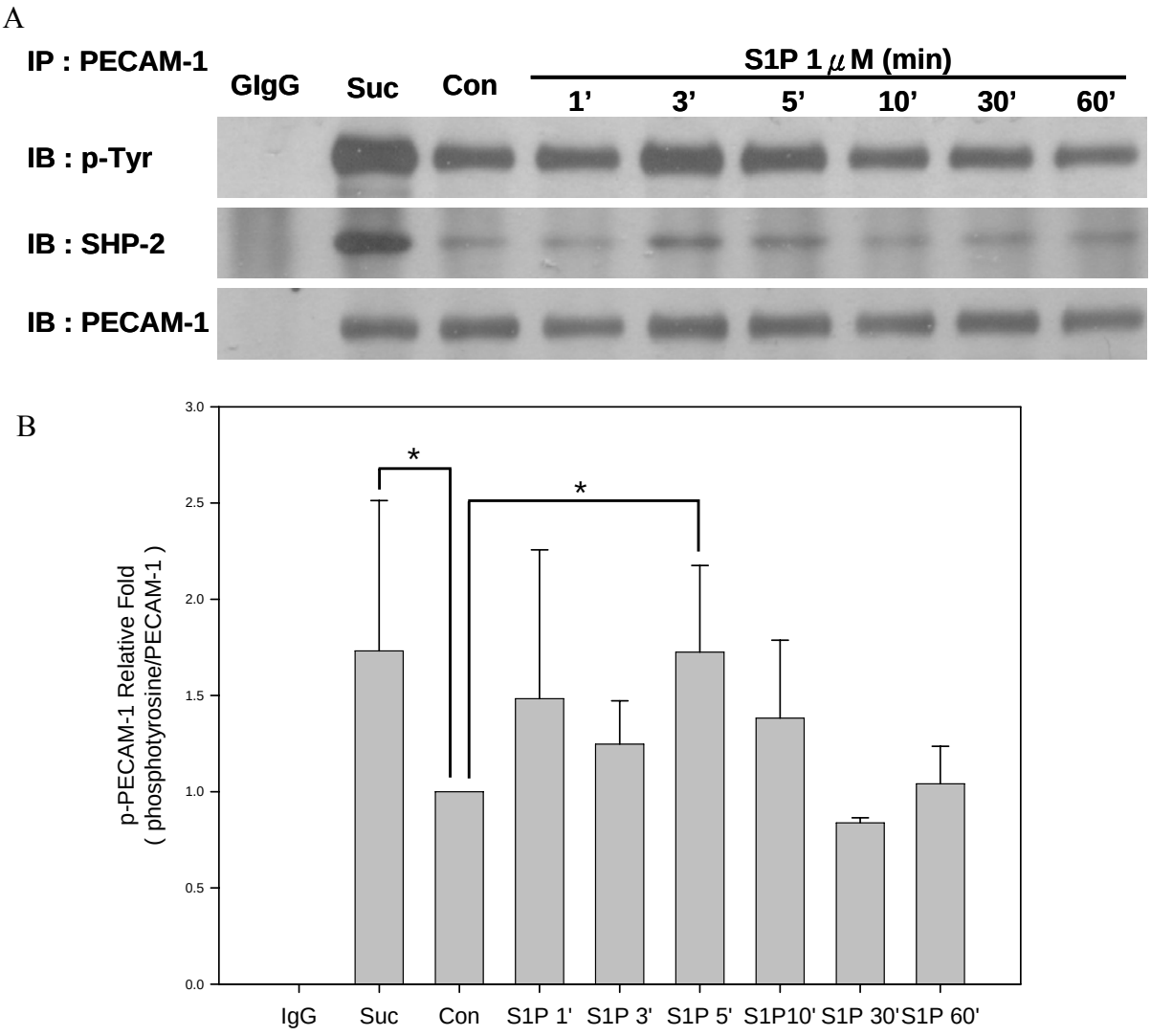

Fig. 1. S1P induced PECAM-1 tyrosine phosphorylation and SHP-2 association in BAECs. A: After S1P treatment, BAECs were lysed and immunoprecipitated with anti-PECAM-1 antibody to detect the level of PECAM-1 tyrosine phosphorylation and SHP-2 association. Compared to the medium-treated control (Con), the amount of tyrosine phosphorylated-PECAM-1 increased within 5 min and declined to control levels after 10 min of S1P treatment. The association of SHP-2 with precipitated PECAM-1 also increased within 5 min and decreased to control levels after 10 min, consistent with the phosphorylation of PECAM-1. Normal goat immunoglobulin G (GIgG) was used as the negative control for the immunoprecipitation, and 0.3 M sucrose in PBS (Suc) served as the positive control for PECAM-1 phosphorylation. Results of one of four independent experiments are shown. B: Quantified results of phosphorylated PECAM-1 of four independent experiments are presented as the mean ± SD. (* $p<0.05$, compared with control)

between these two molecules reflect the complexity in cell signaling networks and physiological systems in endothelial cells. Several investigations have concluded that different tyrosine kinases are responsible for PECAM-1 phosphorylation by different stimuli in various cell types (Ilan and Madri, 2003; Newman and Newman, 2003). Our finding that PP2 efficiently inhibited the S1P-induced PECAM-1 tyrosine phosphorylation suggests that Src family kinases participate in the phospohrylation of PECAM-1. This is consistent with previous findings that members of the Src family are the most frequently utilized tyrosine kinases for PECAM-1 phosphorylation (Ilan and Madri, 2003; Newman and Newman, 2003).

SHP-2 is one of the most frequently mentioned SH2 domain-containing proteins associated with phosphorylated PECAM-1. Binding with PECAM-1 brings SHP-2 closer to its potential substrates, β-catenin and focal adhesion kinase (FAK) (Ilan and Madri, 2003). Activated SHP-2 can dephosphorylate β-catenin and FAK, resulting in stabilization of

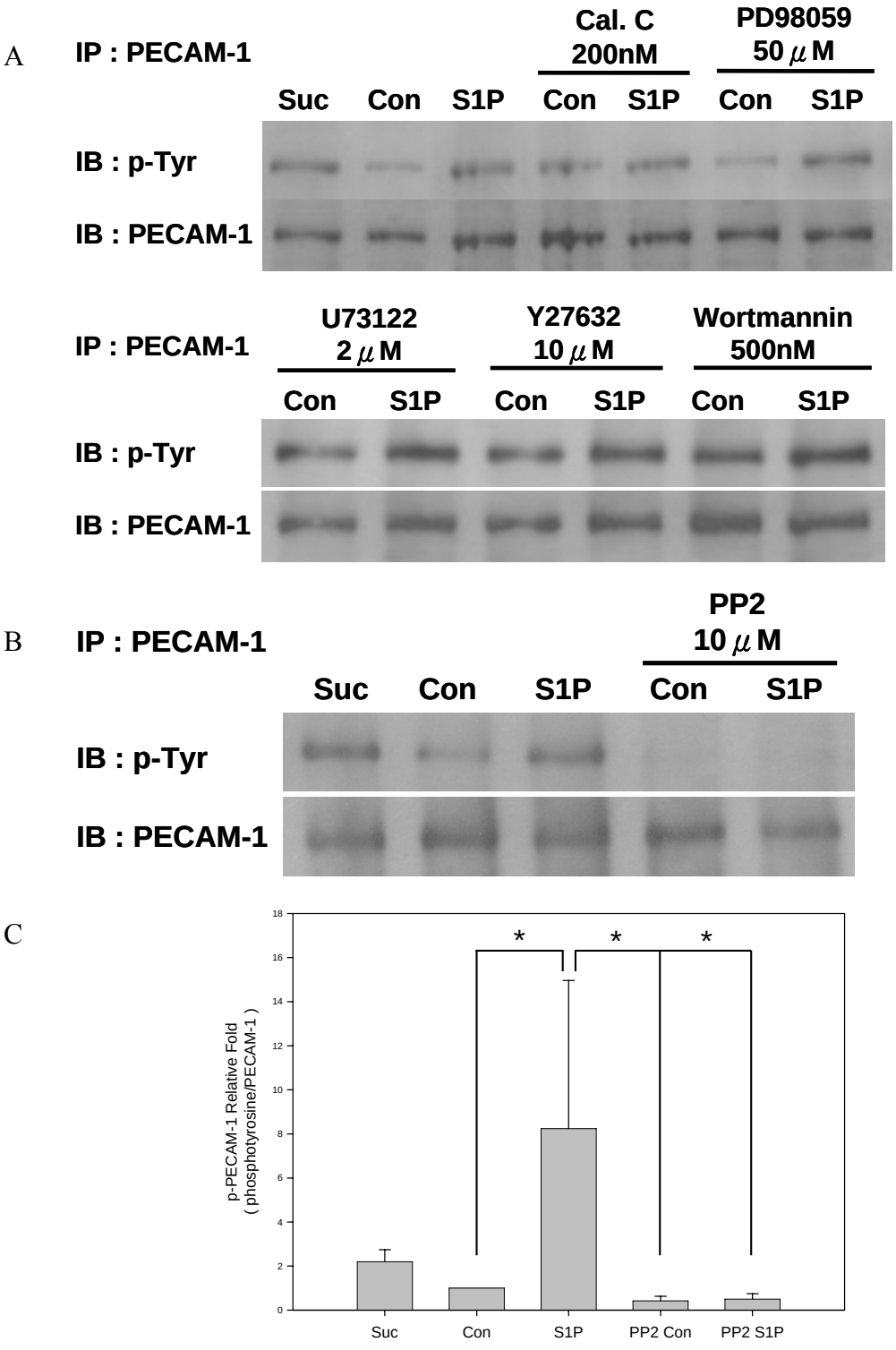

Fig. 2. S1P-induced PECAM-1 tyrosine phosphorylation was inhibited by PP2, a selective Src family kinase inhibitor. Confluent bovine aortic endothelial cells (BAECs) were pretreated with various chemical inhibitors before S1P treatment. A: Calphostin C (a PKC inhibitor), PD98059 (a MEK inhibitor), U73122 (a PLC inhibitor), Y27632 (a ROCK inhibitor), wortmannin (a PI_3K inhibitor), or PDTC (an NFκB inhibitor) pretreatment did not prevent S1P-induced tyrosine phosphorylation. B: Pretreatment of BAECS with 10 μM PP2 (a selective Src family kinase blocker), significantly inhibited S1P-induced PECAM-1 phosphorylation. C: Quantified results of three independent experiments of PP2 inhibition to PECAM-1 phosphorylation are presented as the mean ± SD. (* $p<0.05$, compared with control)

adherens junctions and focal adhesions, and decreased cell motility. S1P, by causing an increase in PECAM-1 phosphorylation, the association and activation of SHP-2, the dephosphorylation of β-catenin and FAK, and the stabilization of cell-cell and cell-matrix adhesions, may strengthen the monolayer endothelial barrier integrity (Garcia et al., 2001; Schaphorst et al., 2003).

Studies of the roles of PECAM-1 tyrosine phosphorylation in wound healing have generated opposite conclusions by different research groups. Gratzinger et al. found that tyrosine-mutated transfectants resulted in faster wound healing and cell migration (Gratzinger et al., 2003), while O'Brien et al. suggested that tyrosine-mutated clones migrated more slowly than wild-type (O'Brien et al., 2004). This contradiction may be due to differences in the cell systems and experimental procedures used. Further investigations of PECAM-1 phosphorylation are necessary to understand the mechanisms of wound healing induced by S1P.

In conclusion, we demonstrated the effect of S1P in inducing PECAM-1 phosphorylation and SHP-2 association through a PP2-inhibitable mechanism. These findings reveal a new route by which S1P modulates endothelial functions through the involvement of PECAM-1 tyrosine phosphorylation.

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S1P 引發牛內皮細胞表面之第一型血小板/內皮細胞附著因子磷酸化機制之研究

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摘　　要

Sphingosine 1-phosphate (S1P)是一種低分子量的多功能性水解磷酸脂，主要經由被活化的血小板釋放到血液中。S1P 和細胞表面特殊的 G 型蛋白耦合受器結合後，會引發下游一連串的細胞訊息傳遞及並影響細胞的基因表現與生理活動，包括：細胞增生、移行、傷口癒合，血管新生及免疫反應的過程。第一型血小板/內皮細胞附著因子(Platelet/endothelial cell adhesion molecule-1; PECAM-1)是一種大量表現在血小板及內皮細胞上的細胞黏著因子。其膜內結構上的酪氨酸在磷酸化後會和具有 SH2 結構之蛋白質結合並活化多種下游之細胞生理活動。在本實驗中，我們使用免疫沉澱與西方墨點法證明了 S1P 的處理會引發牛主動脈內皮細胞上 PECAM-1 酪氨酸的磷酸化以及後續 SHP-2 的結合。使用化學抑制劑所進行的前處理顯示了此 PECAM-1 磷酸化的現象會被 PP2 所抑制，顯示 Src family kinases 在 S1P 所引發的 PECAM-1 酪氨酸磷酸化的過程中扮演了仲介者的角色。釐清此機制的細節將有助於未來在內皮細胞生理功能調控過程的研究。

關鍵詞：S1P、第一型血小板/內皮細胞附著因子、SHP-2、Src family kinases。

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