Journal of Cellular Biochemistry 90:719–731 (2003)

Sphingosine 1-Phosphate Effect on Endothelial Cell PAF Synthesis: Role in Cellular Migration

Pascal N. Bernatchez, François Tremblay, Simon Rollin, Paul-Eduard Neagoe, and Martin G. Sirois*

Montreal Heart Institute and Department of Pharmacology, University of Montreal, Montreal, Quebec, Canada

Abstract Sphingosine 1-phosphate (S1P) and vascular endothelial growth factor (VEGF) are two inflammatory mediators capable of promoting endothelial cell (EC) migration and angiogenesis. As VEGF inflammatory effect is mediated by the synthesis of endothelial platelet-activating factor (PAF) which is also contributing to VEGF chemotactic activity, we wanted to assess if S1P can trigger PAF synthesis in EC and if S1P-induced migration is PAF-dependent. Treatment of bovine aortic EC (BAEC) with S1P (10^{-10}–10^{-6} M) increased dose- and time-dependently the synthesis of PAF by up to 3.3-fold above the basal level, with a maximal amount of PAF detected at 20 min post-stimulation. This biological response was attenuated by inhibiting p38 mitogen-activated protein kinase (MAPK), cytosolic or secreted phospholipase A_2 ($cPLA_2$, $sPLA_2$) activity, suggesting that p38 MAPK activation by S1P promotes the conversion of membrane phospholipids into PAF through the combined activation of $cPLA_2$ and $sPLA_2$. Interestingly, pretreatment of BAEC with extracellular PAF receptor antagonists (BN52021, 10^{-5} M and CV3988, 10^{-6} M) reduced by up to 42% the cellular migration induced by S1P (10^{-6} M). These data demonstrate the capacity of S1P to induce PAF synthesis, which contributes in part to S1P chemotactic activity. J. Cell. Biochem. 90: 719–731, 2003.

Key words: endothelial cells; inflammatory mediators; MAP kinases; phospholipases

Sphingosine 1-phosphate (S1P) is a biologically active sphingolipid secreted mainly by platelets at clotting sites and also by several cell types in response to various extracellular stimuli [Olivera and Spiegel, 1993; Yatomi et al., 1995; Pyne et al., 1996]. Its precursor, sphingomyelin, is a membrane phospholipid that can be converted into S1P through the cascade activation of the sphingomyelinase, ceramidase, and sphingosine kinase enzymes [Hannun and Bell, 1989; Olivera et al., 1998]. So far, five members of the endothelial differentiation gene (*EDG*) family have been identified as S1P receptors in a wide range of cell lines. EDG-1/S1P1, the first member of this receptor family to be cloned, was identified as an inducible transcript expressed during endothelial cell (EC) differentiation in vitro [Hla and Maciag, 1990]. The other EDG isoforms that bind S1P as a high affinity ligand are EDG-3/S1P3, EDG-5/S1P2, EDG-6/S1P4, and EDG-8/S1P5 [Spiegel and Milstein, 2003].

Once secreted, S1P is metabolically stable in plasma, remains bound to albumin [Yatomi et al., 1995] and is involved in a variety of physiological processes, including thrombosis and hemostasis [English et al., 2001]. However, recent works have shown that S1P is capable of inducing angiogenesis which can lead to the pathogenesis of vascular diseases characterized by the growth of new blood vessels, such as atherosclerosis and tumor growth [Folkman, 1991; Lee et al., 1999]. On the other hand, angiogenesis is a highly desirable event in settings where revascularization is sought, for instance in infarcted myocardium. Several angiogenic factors, such as vascular endothelial growth factor (VEGF), have been identified and investigated allowing the design of promising pro- or anti-angiogenic therapies [Folkman and Klagsburn, 1987]. As S1P is suspected to induce both therapeutic and pathologic angiogenesis, a better comprehension of the mechanisms by which

Grant sponsor: Canadian Institutes of Health Research (CIHR); Grant number: MOP-43919; Grant sponsor: Heart and Stroke Foundation of Québec (to MGS).

*Correspondence to: Dr. Martin G. Sirois, PhD, Montreal Heart Institute, 5000, Belanger Street, Montreal, Que., Canada H1T 1C8. E-mail: mgsirois@icm.umontreal.ca

Received 14 July 2003; Accepted 14 August 2003

DOI 10.1002/jcb.10686

S1P mediates its angiogenic activity is of great interest.

Compelling data have shown that angiogenesis is often initiated through a local increase in vascular permeability closely followed by the migration and proliferation of EC [Senger et al., 1983; Connolly et al., 1989; Dvorak et al., 1995]. Interestingly, others have shown that S1P is likely to take part in the induction of inflammation in vivo [Ammit et al., 2001] and that it is a potent regulator of EC survival, migration, and proliferation, which are all directly linked to blood vessel growth and assembly [Lee et al., 1999; Boguslawski et al., 2000]. Though these recent works have extensively described the mitogenic and chemotactic effect of S1P on EC, the mechanisms by which S1P may promote inflammation are unknown.

We have recently shown that VEGF-driven inflammation is mediated through the synthesis by EC of platelet-activating factor (PAF), a potent inflammatory mediator also capable of promoting EC migration [Sirois and Edelman, 1997; Bernatchez et al., 1999]. Furthermore, we have reported that VEGF catalyzes the conversion of EC membrane phospholipids into PAF through group V secreted phospholipase A_2 (sPLA$_2$-V) and lyso-PAF acetyltransferase (lyso-PAF-AT) [Bernatchez et al., 2001a], and that the latter enzymes are regulated by p42/44 and p38 mitogen-activated protein kinases (MAPK), and phosphatidylinositol 3-kinase (PI3K) [Bernatchez et al., 2001b]. As S1P was shown to activate p42/44, p38 MAPK, and PI3K in EC [Lee et al., 1999; Morales-Ruiz et al., 2001], we tested the hypothesis that stimulation of EC with S1P triggers PAF synthesis and that S1P-induced EC migration is PAF-dependent.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) or J.T. Baker (Philipsburg, NJ) unless stated otherwise. Kinase inhibitors were purchased from Calbiochem (San Diego, CA), whereas PLA$_2$ inhibitors were purchased from Cayman Chemicals (Ann Arbour, MI). S1P, BN 52021, and CV3988 were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). LAU 8080 (formerly known as BN 50730) was kindly provided by Dr. Nicolas G. Bazan (LSU Health Sciences Center, New Orleans, LA). SB203347 was kindly provided by Dr. James D. Winkley (SmithKline Beecham Pharmaceuticals, King of Prussia, PA).

Cell Culture

Bovine aortic EC (BAEC) were isolated from freshly harvested aortas, cultured in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Burlington, Canada) containing 5% fetal bovine serum (FBS; Hyclone Lab., Logan, UT) and antibiotics. BAEC were characterized by their cobblestone monolayer morphology, Factor VIII immunohistochemistry and by diiodoindocarbocyanide acetylated LDL uptake as described previously [Sirois and Edelman, 1997; Bernatchez et al., 1999].

Measurement of PAF Synthesis

PAF production by BAEC was measured by incorpration of ^{3}H-acetate into lyso-PAF as described previously [Sirois and Edelman, 1997; Bernatchez et al., 1999]. Briefly, confluent BAEC (6-well tissue culture plate) were rinsed with Hank's balanced salt solution (HBSS) + HEPES (10^{-2} M; pH 7.4) and stimulated for various periods of time in 1 ml of HBSS–HEPES (10^{-2} M, pH 7.4) + $CaCl_2$ (10^{-2} M) + ^{3}H-acetate (2.5×10^{-5} Ci) (New England Nuclear, Boston, MA) + PBS with bovine serum albumin (BSA) or S1P (10^{-10}–10^{-6} M). The reaction was stopped by the addition of acidified methanol, polar lipids were isolated by the Bligh and Dyer method [Bligh and Dyer, 1959], evaporated under a stream of N_2 gas and purified by a silica-based normal-phase HPLC column. Fractions corresponding to ^{3}H-PAF were quantified by counting radioactivity with a β-counter. The authenticity of synthesized ^{3}H-PAF was confirmed by the similar HPLC elution pattern as standard ^{3}H-PAF (New England Nuclear), and by its ability to induce platelet aggregation as standard PAF (Avanti Polar Lipids, Alabaster, AL) [Sirois and Edelman, 1997].

Chemotaxic Assays

Cell migration was evaluated using a microchamber apparatus (Neuroprobe, Cabin John, MD). Subconfluent BAEC (100 mm tissue culture plate) were trypsinized using trypsin–EDTA and resuspended in DMEM containing 5% FBS. Cells were centrifuged for 1 min at 2,000 rpm and resuspended at a concentration of 1×10^6 cells/ml in DMEM + 1% FBS. Fifty microliters of this solution was added in the upper chamber of the system, whereas the lower

chamber was filled with DMEM + 1% FBS + S1P (10^{-10}–10^{-6} M). The two sections of the system were assembled and separated by a polycarbonate filter (5 μm pores) coated with a gelatin solution (1.5 mg/ml). Five hours post-incubation at 37°C, the apparatus was dismantled, the non-migrated cells were scraped with a plastic policeman and the migrated cells were stained by using Quick-Diff solutions (Shandon Lipshaw, Pittsburg, PA). The filter was mounted on a glass slide using immersion oil and migrated cells were counted using a microscope.

Western Blot Analyses of $sPLA_2$, $cPLA_2$ $iPLA_2$, and PAFR Expression

Confluent BAEC (100 mm tissue culture plate) were rinsed with DMEM, lysed with PMSF 10^{-3} M, 0.15 U/ml aprotinin, 10 μg/ml leupeptin, and Na_2VO_3 10^{-3} M. Plates were scraped by using a plastic policeman and total proteins (50 μg) were separated by a SDS–PAGE gel and transblotted onto a Immobilon-P PVDF membrane (Millipore, Bedford, MA). Membranes were blocked with 5% BSA for 1 h at room temperature with gentle agitation. Membranes were then incubated for 1 h in TTBS containing 3% BSA and either a monoclonal anti-$cPLA_2$ antiserum (dilution 1:500), a monoclonal anti-group V $sPLA_2$ antiserum (dilution 1:200) which does not cross-react with group IIA $sPLA_2$, a monoclonal anti-group IIA $sPLA_2$ antiserum (dilution 1:200) which does not cross-react with group V $sPLA_2$, a polyclonal anti-$iPLA_2$ (dilution 1:500) or a goat polyclonal anti-PAFR antiserum (dilution 1:500) (all from Cayman Chemicals). Membranes were washed three times with TTBS and incubated with horseradish peroxidase-coupled secondary antibodies (dilution 1:1,000 to 2,500; Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h. Membranes were washed three times with TTBS and horseradish peroxidase was revealed by chemiluminesence (ECL kit, Amersham, Baie D'urfé, Canada). Kaleidoscope molecular weight marker proteins (Bio-Rad, Hercules, CA) were used as standards for SDS–PAGE.

Western Blot Analyses of p42/44 MAPK, p38 MAPK, and Akt Phosphorylation

Confluent BAEC (100 mm tissue culture plate) were serum-starved for 12 h in DMEM and then stimulated with S1P (10^{-6} M) for various periods of time with or without inhibitors as described above. Cell lysing, SDS–PAGE, and immunoblotting were performed as described previously [Bernatchez et al., 2001b] by using anti-phospho-p42/44 MAPK, anti-phospho-p38 MAPK, or anti-phospho-Akt rabbit polyclonal antiserum (New England Biolabs, Beverley, MA). Kaleidoscope prestained standards (Bio-Rad) were used for SDS–PAGE.

Immunofluorescence Analysis of PAFR Expression and Distribution

BAEC were cultured on glass coverslips (18 mm) in 12-well culture plates. At 50% confluence, cells were fixed in 3% paraformaldehyde (pH 7.3) for 15 min at room temperature and washed with PBS or with PBS-Triton X-100 (0.5%) solution (permeabilization) for 10 min and incubated overnight with 10% normal donkey serum (NDS) at 40°C. The cells were rinsed with PBS, incubated with goat polyclonal anti-PAFR antibodies (1:200 in 1% NDS) at room temperature for 1 h, incubated with donkey anti-goat Alexa 488 antibodies (1:500 in 1% NDS) (Molecular Probes, Eugene, OR) at room temperature for 1 h, then washed with PBS. Glass coverslips were mounted using a 1,4-diazabicyclo-2-2-2-octane (DABCO)/glycerol (1:1) solution. Finally, cells were observed on a Zeiss Axiovert 100 M microscope adapted with a LSM 510 confocal system. Images were captured with the LSM 510 software and analyzed with Adobe Photoshop.

RESULTS

Effect of S1P on EC PAF Synthesis

As several data support the hypothesis that S1P may promote EC PAF synthesis, confluent BAEC were challenged with S1P at a concentration known to induce significant biological activities, and PAF synthesis was quantified by the incorporation of [^{3}H]-acetate into lyso-PAF. Stimulation of confluent BAEC with S1P (10^{-6} M) for 5, 10, 15, 20, or 30 min induced a significant time-dependent increase in PAF synthesis (Fig. 1A), raising the basal radioactivity counts from 175 ± 48 dpm (time 0) to $1{,}540 \pm 166$ dpm (20 min; $P < 0.001$), which represents a 3.1-fold increase as compared to a similar PBS stimulation.

In order to determine the optimal concentration of S1P for the stimulation of PAF synthesis in BAEC, we challenged the cells for 20 min with various concentrations of S1P ranging from

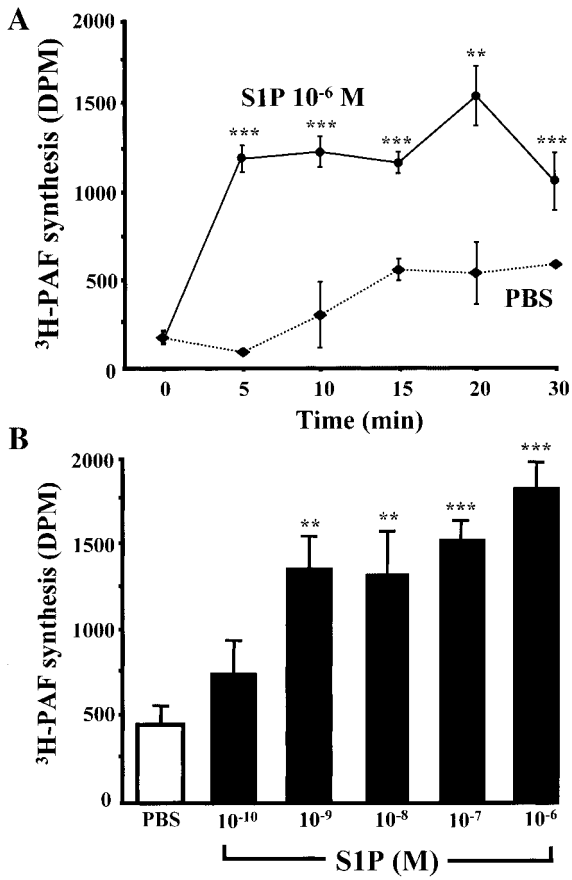

Fig. 1. Sphingosine 1-phosphate (S1P) effect on endothelial cell (EC) platelet-activating factor (PAF) synthesis. S1P effect on PAF synthesis is time-dependent (**A**), and concentration-dependent (**B**). Confluent bovine aortic EC (BAEC) were incubated with [^{3}H]-acetate and were stimulated with S1P 10^{-6} M or with vehicle (PBS) for 5–30 min (A), or with various concentrations of SIP for 20 min (B). The radioactive polar lipid samples were extracted by the Bligh and Dyer procedure and purified by HPLC. Fractions were collected every minute after injection, and radioactivity was determined with a β-counter. The values are means of at least four experiments in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to vehicle (PBS) with similar treatment time as determined by analysis of variance followed by an unpaired Student's *t*-test.

10^{-10} to 10^{-6} M. We observed that stimulation of BAEC with S1P (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) induced PAF synthesis in a dose-dependent manner, with maximal increases of 0.7-, 2.1-, 2.0-, 2.4-, and 3.3-fold, respectively, as compared to PBS-treated cells (Fig. 1B).

Effect of MAPK and PI3K Inhibitors on S1P-Induced PAF Synthesis

We recently reported that VEGF-induced PAF synthesis by BAEC is regulated by the activation of p42/44 MAPK, p38 MAPK, and PI3K pathways [Bernatchez et al., 2001b]. Having the knowledge that S1P activates these three intracellular pathways, we sought to investigate their contribution to S1P-induced PAF synthesis by pretreating BAEC with a range of selective inhibitors. This approach allowed the use of native non-transfected EC which possess the intracellular pathways found in normal vascular endothelium. Pretreatment of BAEC with a MAPK kinase inhibitor (PD98059; 10^{-5} M; $IC_{50} = 10^{-6}$ M) [Pang et al., 1995] did not block but rather potentiated S1P-induced PAF synthesis by 95% (Fig. 2A). In contrast, inhibition of p38 MAPK (SB203580;

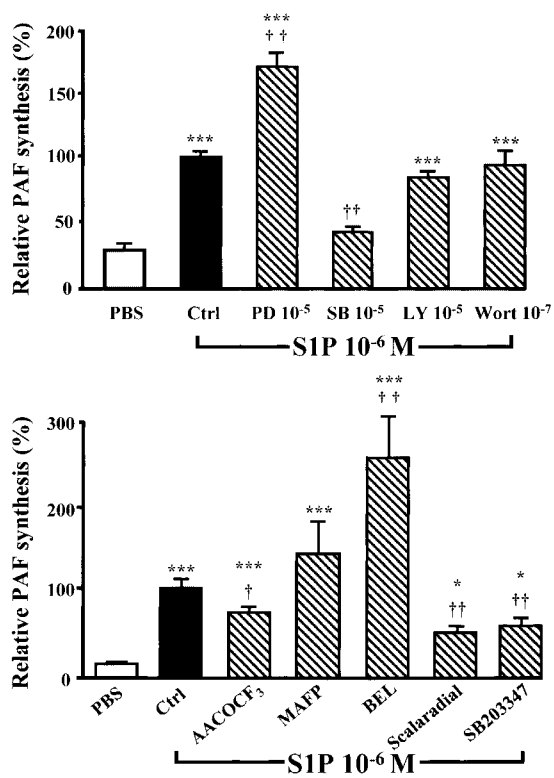

Fig. 2. Effect of kinase and phospholipase A_2 (PLA_2) inhibitors on S1P-induced PAF synthesis. **A**: Effect of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) inhibitors on PAF synthesis. Confluent BAEC were pretreated with MAPKK inhibitor PD98059 (PD, 10^{-5} M), p38 MAPK inhibitor SB203580 (SB, 10^{-5} M), PI3K inhibitors LY294002 (LY, 10^{-5} M), and Wortmannin (Wort, 10^{-7} M), and stimulated for 20 min with either S1P 10^{-6} M or vehicle alone (PBS). **B**: Effect of PLA_2 inhibitors on PAF synthesis. Confluent BAEC were pretreated with $cPLA_2$ inhibitor $AACOCF_3$ (10^{-5} M), $cPLA_2$ and $iPLA_2$ inhibitor MAFP (10^{-5} M), $sPLA_2$ inhibitor Scalaradial (10^{-5} M), groups IIA and V $sPLA_2$ inhibitor SB203347 (10^{-5} M) and stimulated for 20 min with S1P 10^{-6} M or vehicle (PBS). The values are means of at least four experiments in duplicate. $^{*}P < 0.05$; *** $P < 0.001$ as compared to vehicle (PBS) and $^{\dagger}P < 0.05$; $^{\ddagger}P < 0.01$ as compared with S1P 10^{-6} M determined by analysis of variance followed by an unpaired Student's *t*-test.

10^{-5} M; $IC_{50} = 6 \times 10^{-7}$ M) [Cuenda et al., 1995] almost completely blocked S1P-mediated PAF synthesis. Finally, pretreatment with two structurally unrelated inhibitors of PI3K (LY294002; 10^{-5} M and Wortmannin; 10^{-7} M; $IC_{50} = 1.4 \times 10^{-6}$ and 5×10^{-9}, respectively) [Bonser et al., 1991; Vlahos et al., 1995] did not significantly modulate S1P-induced PAF synthesis.

Effect of PLA_2 Inhibitors on S1P-Induced PAF Synthesis

Considering that EC can synthesize the precursor of PAF, lyso-PAF, through the activation of PLA_2, we pretreated BAEC with different PLA_2 inhibitors to characterize the intracellular pathways by which S1P triggers PAF synthesis. Pretreatment of BAEC with a $cPLA_2$ inhibitor ($AACOCF_3$; 10^{-5} M; $IC_{50} = 10^{-7}$ M for human recombinant $cPLA_2$ and 15 mM for semi-purified $iPLA_2$) [Ackermann et al., 1995] reduced significantly S1P-mediated PAF synthesis by 28% ($P < 0.05$) (Fig. 2B). Pretreatment with methyl arachidonyl fluorophosphate (MAFP; 10^{-5} M; $IC_{50} = 6 \times 10^{-7}$ M for recombinant $cPLA_2$ and 500 nM for $iPLA_2$) [Balsinde and Dennis, 1997; Leslie, 1997] which provides an inhibitory activity on cytosolic and calcium-independent phospholipases A_2 ($cPLA_2$ and $iPLA_2$) did not significantly modulate S1P-induced PAF synthesis, whereas the specific inhibition of $iPLA_2$ with bromoenol lactone (BEL; 10^{-5} M; $IC_{50} = 6 \times 10^{-8}$ M) [Ackermann et al., 1995] increased by 1.55-fold the synthesis of PAF synthesis mediated by S1P treatment (Fig. 2B). As $sPLA_2$ can also synthesize lyso-PAF, we used specific inhibitors of $sPLA_2$ activity. Inhibition of $sPLA_2$ activity with Scalaradial (10^{-5} M) [De Carvalho and Jacobs, 1991; Marshall et al., 1995] prevented by up to 62% S1P effect on PAF synthesis. More interestingly, pretreatment of BAEC with a specific inhibitor of groups IIA and V $sPLA_2$ (SB203347; 10^{-5} M $IC_{50} = 5 \times 10^{-7}$ M) [Marshall et al., 1995] attenuated S1P-induced PAF synthesis by up to 60% (Fig. 2B).

S1P Effect on Intracellular Kinase Activation

As p38 but not p42/44 MAPK or PI3K appears to regulate positively S1P-induced PAF synthesis, we sought to determine their activation pattern in BAEC by Western blot analyses. Treatment with S1P (10^{-6} M) promoted a transient time-dependent activation of the p42/44 and p38 MAPK pathways peaking within 10 min post-stimulation (Fig. 3A,B). S1P also induced the activation of the PI3K pathway as revealed by the phosphorylation of Akt, a substrate of PI3K. At 10 min post-stimulation, we observed a marked increase in Akt phosphorylation, followed by a time-dependent decrease (Fig. 3C).

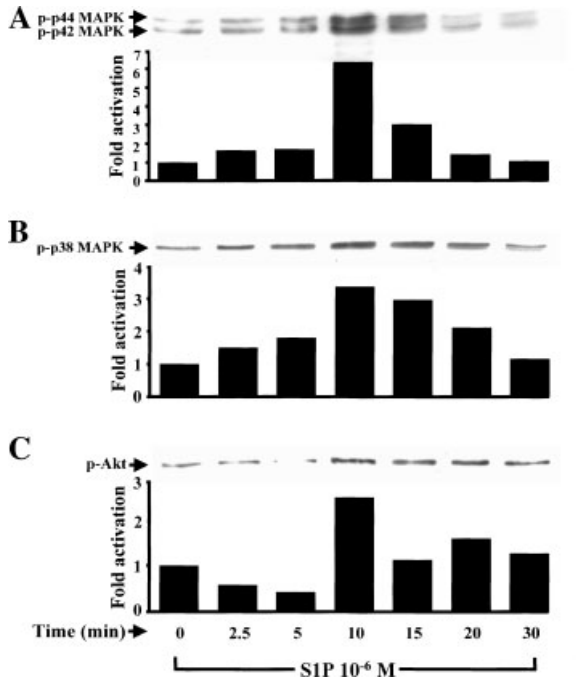

Fig. 3. Effect of S1P on p42/44 MAPK, p38 MAPK, and Akt phosphorylation. S1P effect on p42/44 MAPK (**A**), p38 MAPK (**B**), and Akt phosphorylation (**C**) is time-dependent. Confluent BAEC (100 mm culture plates) were stimulated for 2.5–30 min with S1P 10^{-6} M. Proteins were isolated, separated by SDS–PAGE, transblotted onto a PVDF membrane and probed by using either an anti-phospho-p42/44 MAPK, anti-phospho-p38 MAPK, or anti-phospho-Akt antibodies. The histogram represents the increase in fold activation in function of the time as illustrated on the Western blot.

Effect of MAPK and PI3K Inhibitors on Intracellular Pathways Activation

We next sought to confirm that the inhibitors used in our studies blocked their respective kinases in a selective fashion. Hence, we pretreated BAEC with the inhibitors of p42/44 MAPK kinase, p38 MAPK, and PI3K and investigated their effect on S1P-induced phophorylation of these kinases. As expected, the p42/44 MAPK inhibitor (PD98059, 10^{-5} M) attenuated only p42/44 MAPK activation and the p38 MAPK inhibitor (SB203580, 10^{-5} M) attenuated only p38 MAPK phosphorylation. Finally, the PI3K inhibitors (Wortmannin, 10^{-7} M; LY294002, 10^{-5} M) almost completely

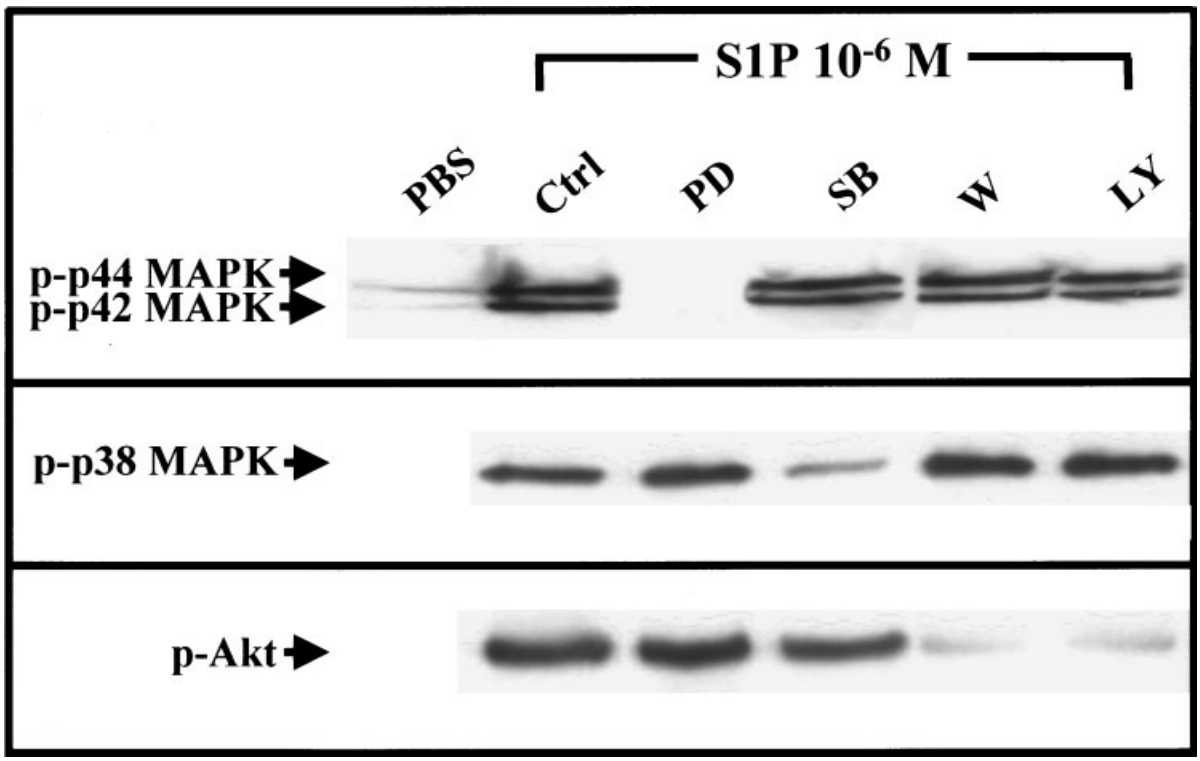

Fig. 4. Effect of kinase inhibitors on p42/44 MAPK, p38 MAPK, and Akt phosphorylation. Confluent BAEC were pretreated with kinase inhibitors as described in Figure 2A. Cells were stimulated with S1P 10^{-6}, lysed and p42/44 MAPK, p38 MAPK, and Akt phosphrylation was determined as described in Figure 3.

blocked Akt phosphorylation induced by S1P treatment (Fig. 4).

Role of PAF in S1P-Induced Migration

As the present data demonstrate that S1P promotes PAF synthesis, and that PAF has a chemotactic activity on ECs, we hypothesized that S1P-induced EC migration could be PAF-dependent. By using a modified Boyden chamber assay, we observed that addition of S1P (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) to the lower compartment of the Boyden chamber increased dose-dependently the migration of BAEC (0.9-, 1.0-, 2.2-, 3.8-, and 6.7-fold, respectively) as compared to PBS-treated cells (Fig. 5A). Pretreatment with a selective intracellular and extracellular PAF receptor antagonist (CV-3988, 10^{-6} M; $IC_{50} = 254$ nM) [Marcheselli et al., 1990; Bazan et al., 1991] reduced by 42% ($P < 0.01$) the migration of BAEC mediated by SIP (10^{-6} M). Similarly, pretreatment with a selective antagonist of extracellular PAF receptor (BN52021; 10^{-5} M; $IC_{50} = 164$ nM) [Marcheselli et al., 1990; Bazan et al., 1991] resulted in a 41% reduction ($P < 0.01$) in S1P-induced BAEC migration, whereas the blockade of the intracellular pool of PAF receptors with LAU8080 (10^{-7} M; $IC_{50} =$ 590 pM) [Marcheselli and Bazan, 1994] had no significant inhibitory effect on S1P-induced BAEC migration (Fig. 5B). The basal migration of BAEC was not affected by pretreatment with these PAF receptor antagonists (Fig. 5B).

Role of Intracellular Pathways in S1P-Induced Migration

We next investigated the contribution of p42/44 MAPK, p38 MAPK, and PI3K pathways on S1P-induced EC migration. Pretreatment with the p42/44 MAPK kinase inhibitor (PD98059; 10^{-5} M) failed to attenuate S1P-induced cell migration, whereas the inhibition of p38MAPK (SB203580; 10^{-5} M) decreased by 42% the S1P (10^{-6} M) chemotactic activity on BAEC. However, inhibition of PI3K with LY294002 (10^{-5} M) or Wortmannin (10^{-7} M) did not prevent S1P chemotactic effect on EC (Fig. 5C).

Expression of Phospholipases A_2 in BAEC

Considering that pretreatment of BAEC with Scalaradial (10^{-5} M), SB203337 (10^{-5} M), or $AACOCF_3$ (10^{-5} M) attenuated S1P-induced PAF synthesis and that a pretreatment with

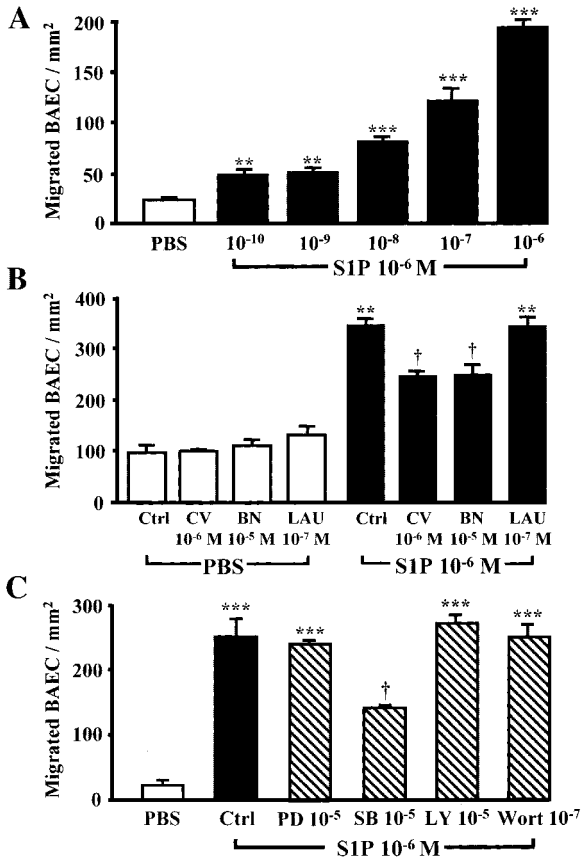

Fig. 5. S1P chemotactic effect on BAEC is PAF and p38 MAPK-dependent. **A**: S1P effect on EC migration is dose-dependent. BAEC were trypsinized and resuspended in DMEM, 1% FBS, and antibiotics; 5×10^4 cells were added in the higher chamber of the modified Boyden chamber apparatus, and the lower chamber was filled with DMEM, 1% FBS with various concentrations of S1P. Five hours post-incubation at 37°C, the migrated cells were stained and counted by using a microscope adapted to a digitized video camera. The values are means of migrating cells/mm^2 from six chambers for each treatment. **B**: S1P chemotactic effect is PAF dependent. BAEC were treated as described above, and PAF receptor antagonists BN52021 (BN, 10^{-5} M), LAU8080 (LAU, 10^{-7} M), CV3988 (CV, 10^{-6} M) were added to the higher chamber prior to S1P stimulation. **C**: S1P chemotactic effect is p38 MAPK dependent. BAEC were treated as described above, p42/44 MAPK inhibitor PD98059 (PD, 10^{-5} M), p38 MAPK inhibitor SB203580 (SB, 10^{-5} M), PI3K inhibitors LY294002 (LY, 10^{-5} M), and Wortmannin (Wort, 10^{-7} M) were added to the higher chamber prior to S1P stimulation. $^{**}P < 0.01$; $^{***}P < 0.001$ as compared with vehicle (PBS), and $^{\dagger}P < 0.05$ as compared to S1P 10^{-6} M as determined by analysis of variance followed by an unpaired Student's *t*-test.

BEL increased PAF synthesis, we sought to confirm that the BAEC are expressing sPLA$_2$-V, cPLA$_2$, and iPLA$_2$. Moreover, as previous studies have confused sPLA$_2$-V and sPLA$_2$-IIA expression and activities, we also sought to determine if BAEC express sPLA$_2$-IIA. Confluent BAEC were lysed, total proteins were isolated and separated by SDS–PAGE. Western blot analyses were performed with monoclonal antibodies recognizing specifically sPLA$_2$-V, sPLA$_2$-IIA, or cPLA$_2$, and with a goat polyclonal antibody recognizing specifically iPLA$_2$. Our data confirm the expression of sPLA$_2$-V, cPLA$_2$, and iPLA$_2$ but not sPLA$_2$-IIA in BAEC (the latter negative result being confirmed by the use of human synovial fluid as positive control) (Fig. 6).

Expression of PAF Receptors in BAEC

As our results suggest a role for PAF and its extracellular receptor in S1P-induced migration of BAEC, we sought to confirm the expression of PAF receptor (PAFR) in BAEC. By Western blot analysis, we detected a 39 kDa band which corresponds to PAFR molecular weight [Ihida et al., 1999] (Fig. 7A). In addition, we performed immunofluorescence analyses of PAFR. By confocal microscopy, we detected in non-permeabilized BAEC the expression of PAFR at the cell membrane surface (Fig. 7B). Cell permeabilization allowed the labeling of intracellular and extracellular PAFR, which resulted in a greater immunofluorescence (Fig. 7D). The specificity of PAFR expression in BAEC was confirmed by replacing the goat polyclonal anti-PAFR antibodies by normal non-specific goat IgG antibodies, which led to no or marginal immunofluorescence in non-permeabilized and permeabilized ECs (Fig. 7C,E).

DISCUSSION

Angiogenesis is a tightly regulated process that is essential to both physiological and pathological situations. Crucial steps in the angiogenic process support an early increase in vascular permeability, closely followed by migration of EC [Connolly et al., 1989; Dvorak et al., 1995; Jackson et al., 1997; Bergers and Benjamin, 2002]. Recent studies have shown that S1P is involved in the induction of angiogenesis in part by promoting EC migration [Lee et al., 1999]. Herein, we demonstrate that S1P is also capable of promoting the synthesis of PAF, a highly potent vascular permeability mediator, in a time- and dose-dependent fashion, and that PAF is involved in the induction of EC migration by S1P. We furthermore demonstrate that S1P-induced PAF synthesis is driven through the activation of p38 MAPK, cPLA$_2$, and sPLA$_2$-V.

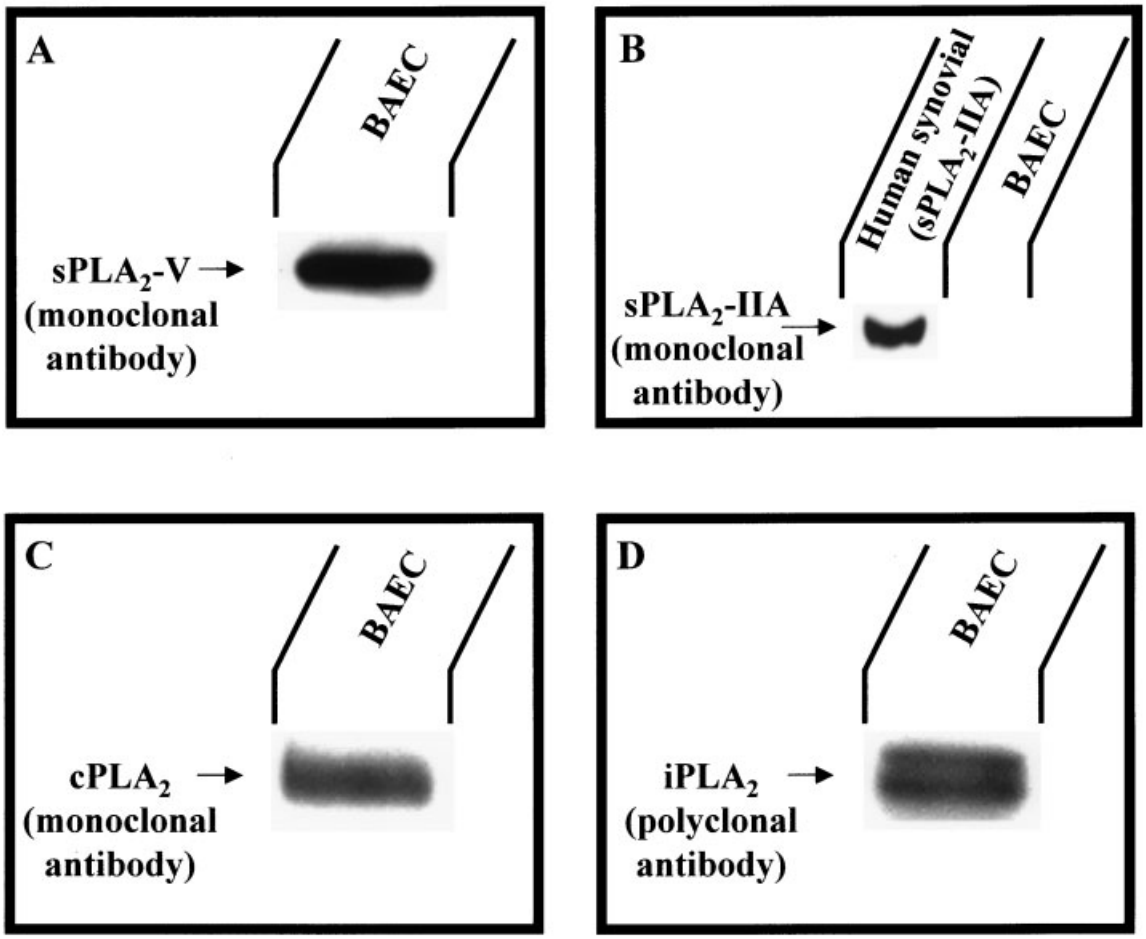

Fig. 6. PLA_2 expression by BAEC. Confluent BAEC were lysed, proteins separated by SDS–PAGE and transblotted onto a PVDF membrane. **A**: Expression of $sPLA_2$-V by BAEC. The membrane was probed by using an anti-$sPLA_2$-V monoclonal antibody that does not cross-react with $sPLA_2$-IIA. **B**: BAEC do not express $sPLA_2$-IIA. The membrane was probed by using an anti-$sPLA_2$-IIA monoclonal antibody that does not cross-react with $sPLA_2$-V. Human synovial proteins were used as a positive control for $sPLA_2$-IIA expression. **C**: Expression of $cPLA_2$ by BAEC. The membrane was probed by using an anti-$cPLA_2$ monoclonal antibody that does not cross-react with any other known PLA_2. **D**: Expression of $iPLA_2$ by BAEC. The membrane was probed by using an anti-$iPLA_2$ polyclonal antibody that does not cross-react with any other known PLA_2.

S1P Is a Rapid Inducer of PAF Synthesis

Resting EC contain low amounts of PAF [Camussi et al., 1983] which can be increased when challenged with appropriate stimulus. PAF synthesis in EC can occur in a very early (0–10 min), early (10–40 min), or delayed (4–8 h) manner, depending of the stimulus used [Camussi et al., 1983; Prescott et al., 1984]. Our data suggest that the induction of PAF synthesis by S1P takes place very early and early after stimulation since marked increases in PAF levels were observed from 5 to 20 min post-stimulation.

We previously reported that other agonists, such as VEGF or ATP, can increase PAF synthesis by up to 30-fold in BAEC [Sirois and Edelman, 1997]. However, the 3.3-fold increase in PAF synthesis observed following S1P treatment suggests that S1P is a more modest inducer of PAF synthesis.

Effect of MAPK, PI3K, and PLA_2 Inhibitors on S1P-Induced PAF Synthesis

Previous studies have shown that early PAF synthesis in EC is mediated through the activation of the enzymatic phospholipid remodeling pathway. These enzymes, PLA_2 and lyso-PAF AT, convert membrane phospholipids into lyso-PAF and lyso-PAF into PAF, respectively [Bussolino and Camussi, 1995]. Since these enzymes are thought to be regulated by phosphorylation [Xing and Insel, 1996; Nixon et al., 1999], we investigated the effect of intracellular

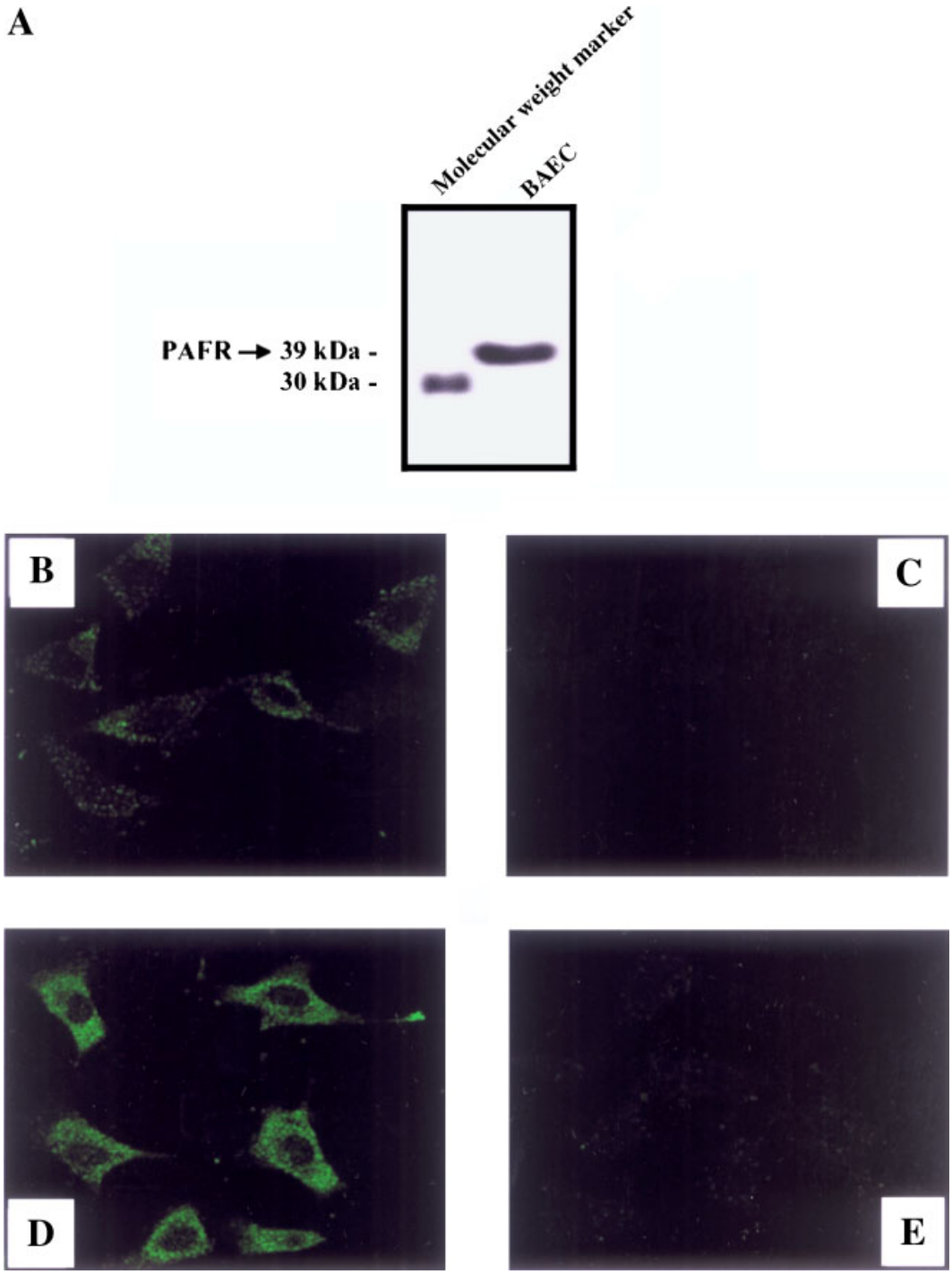

Fig. 7. BAEC express extracellular and intracellular PAFR. **A**: Confluent BAEC were lysed, and Western blot analysis was performed by using an anti-PAFR antibody. **B**–**E**: Immunochemistry analyses of PAFR localization. The extracellular localization of PAFR expression in BAEC was performed by incubating non-permeabilized cells with an anti-PAFR or control goat IgG antibodies (**top left** and **right panels**, respectively). The intracellular and extracellular PAFR distribution was determined in permeabilized BAEC incubated with the same anti-PAFR or control goat IgG antibodies (**lower left** and **right panels**, respectively). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

kinase inhibitors on S1P-induced PAF synthesis. Our data show that inhibition of the p42/44 MAPK pathway with PD98059 potentiates S1P-induced PAF synthesis, suggesting that p42/44 MAPK acts as a negative regulator of PAF synthesis induced by S1P. These observations are in contrast with a previous report of our own in which we show that p42/44 MAPK activation

is crucial to VEGF-induced PAF synthesis [Bernatchez et al., 2001a]. These data suggests that p42/44 MAPK can be both a positive or negative regulator of PAF synthesis, depending on the agonist used. However, the p42/44 MAPK target that is responsible for such variations in the amount of PAF synthesized following stimulation is unknown. Previous studies have shown that p42/44 MAPK can interact with p38 MAPK and $cPLA_2$, two enzymes known to take part in EC PAF synthesis. This shows that PAF synthesis is a complex and tightly regulated biological response that requires a critical activation pattern of several key enzymes.

On the other hand, p38 MAPK activation appears to play a crucial role in S1P-induced PAF synthesis since its inhibition by SB203580 completely blocked PAF synthesis. Others have shown that p38 MAPK, known to be activated by cellular stresses, inflammatory cytokines and growth factors, is capable of directly phosphorylating and activating some PLA_2 isoforms and also lyso-PAF AT [Borsch-Haubold et al., 1998; Martin-Blanco, 2000]. As p38 MAPK activation is also crucial for VEGF-induced PAF synthesis [Bernatchez et al., 2001b], these data illustrate the importance of p38 MAPK to EC PAF synthesis following stimulation with various agonists.

Finally, two structurally unrelated inhibitors of PI3K had no effect on PAF synthesis. To our knowledge, PI3K is not capable of phosphorylating the remodeling pathway enzymes, which rationalizes the lack of effect of PI3K inhibitors on S1P-induced PAF synthesis. Taken together, these data show that p38 MAPK drives S1P-induced PAF synthesis, whereas p42/44 MAPK acts as a negative regulator of PAF synthesis.

We then confirmed that S1P can activate p42/44 MAPK, p38 MAPK, and PI3K by performing Western blot analyses with antibodies that recognize the phosphorylated form of these kinases. Figure 2 reveals that S1P does induce a transient activation of p42/44 MAPK, p38 MAPK, and Akt, a substrate of PI3K. Moreover, pretreatment of BAEC with the afore-mentioned kinase inhibitors prevented the phosphorylation of their respective enzymes in a selective fashion. This supports our findings concerning the effect of p42/44 and p38 MAPK inhibitors on PAF synthesis (Fig. 1) and also confirms the biochemical activity of the PI3K inhibitors used in our studies.

S1P Effect on PAF Synthesis Is Both $cPLA_2$- and $sPLA_2$-Dependent

Phopholipases A_2 consist of a superfamily of enzymes capable of hydrolysing membrane phospholipids with the concomitant production of lyso-phospholipids [Murakami and Kudo, 2002]. $cPLA_2$ and $iPLA_2$ are two cell-associated phospholipases both capable of influencing cellular fatty acid metabolism by increasing free arachidonic acid levels [Balsinde and Dennis, 1997; Leslie, 1997]. Thus, these two enzymes could possibly be linked to the synthesis of lyso-PAF from membrane phospholipids upon stimulation by S1P. Pretreatment with a $cPLA_2$ inhibitor that is also known to interfere slightly with $iPLA_2$ ($AACOCF_3$) [Ackermann et al., 1995; Leslie, 1997] significantly attenuated PAF synthesis induced by S1P by up to 28%, whereas a pretreatment with an irreversible inhibitor of both $cPLA_2$ and $iPLA_2$ with a greater effect on $iPLA_2$ than $cPLA_2$ (MAFP) [Balsinde and Dennis, 1997; Leslie, 1997] had no significant effect. Pretreatment with a specific $iPLA_2$ inhibitor (BEL) potentiated considerably S1P effect on PAF synthesis. Taken together, these data clearly demonstrate that $cPLA_2$ and $iPLA_2$ are both involved in EC lyso-PAF biosynthesis induced by S1P, but at different levels. $cPLA_2$ activity appears to have a positive role in PAF synthesis, likely by mediating lyso-PAF synthesis and downstream PAF production, whereas $iPLA_2$ seems to act in a competitive fashion with the enzymes involved in PAF synthesis. Hence, by blocking $iPLA_2$ without affecting $cPLA_2$ (BEL), a pool of membrane phospholipids may be exclusively hydrolyzed into lyso-PAF, which will result in a greater amount of PAF synthesized in part through $cPLA_2$ activity. Recent reports indicated that $cPLA_2$ might have a minor role in EC PAF synthesis, furthermore, it has been shown that $cPLA_2$ is not involved in monocyte or neutrophil PAF biosynthesis [Leslie, 1997; Marshall et al., 1997; Winkler et al., 1997]. These findings rationalize our observation that $cPLA_2$ inhibition causes only a minor decrease in S1P-induced PAF synthesis, and that the participation of $cPLA_2$ to PAF synthesis might be agonist-selective.

Other possible players in EC PAF synthesis are $sPLA_2$. Hence, we pretreated BAEC with inhibitors of this enzymatic class. Pretreatment with a $sPLA_2$ inhibitor that possesses no selectivity towards the different $sPLA_2$ isoforms

(Scalaradial) greatly attenuated S1P effect on BAEC PAF synthesis, which suggests that $sPLA_2$ play an important role in these events. More importantly, inhibition of both groups IIA and V $sPLA_2$ (SB203347) blocked similarly S1P-induced PAF synthesis. Taken together, these results clearly demonstrate that $cPLA_2$ and groups IIA and/or V $sPLA_2$ mediate S1P-induced PAF synthesis. It remains to be determined how $cPLA_2$, $sPLA_2$-V, and/or $sPLA_2$-IIA are activated following S1P stimulation. One interesting hypothesis is that p38 MAPK might phosphorylate not only lyso-PAF AT but also $cPLA_2$ and $sPLA_2$. Previous studies have shown that $cPLA_2$ can be phosphorylated by p38 MAPK [Borsch-Haubold et al., 1998], although no regulatory mechanisms have been uncovered yet for $sPLA_2$-V.

To confirm the presence of $cPLA_2$, $iPLA_2$, $sPLA_2$-V, and $sPLA_2$-IIA in BAEC, we performed Western blot analyses by using selective antibodies that recognize specifically these proteins with no cross-reactivity. These experiments reveal that BAEC express $cPLA_2$, $iPLA_2$, and $sPLA_2$-V, thereby confirming their involvement in S1P-induced PAF synthesis (Fig. 6). On the other hand, these data show as well that BAEC do not express detectable levels of $sPLA_2$-IIA. Consequently, this demonstrates that the inhibitory effect of SB203347 on PAF synthesis is mediated through the inhibition of $sPLA_2$-V and not $sPLA_2$-IIA.

Though cloned only recently, a growing body of evidence has confirmed that $sPLA_2$-V is constitutively expressed mainly in the cardiovascular system [Chen et al., 1994] and is indeed an active effector in fatty acid metabolism. It was shown to take part in arachidonic acid-mediated signal transduction in macrophage-like cell line P388D1 [Balboa et al., 1996], in eicosanoid formation in mast cells [Reddy et al., 1997] and is directly involved in VEGF-induced PAF synthesis [Bernatchez et al., 2001a]. Moreover, group V rather that group IIA as previously believed, is the primary 14 kDa PLA_2 synthesized by P388D1 cells [Balboa et al., 1996]. Hence, this demonstrates that this newly characterized enzyme is likely responsible for S1P-induced PAF synthesis and that it is expressed in BAEC.

VEGF Chemotactic Effect Is PAF-Dependent

PAF is well-known to be a potent extracellular agonist capable of stimulating a vast array of biological functions such as EC migration and inflammation [Bussolino and Camussi, 1995; Montrucchio et al., 2000]. These actions were long thought to be exclusively mediated through its 39 kDa extracellular receptor [Ihida et al., 1999]. However, recent evidence suggest that PAF may also act as an intracellular messenger through an internal pool of PAFR [Ihida et al., 1999; Honda et al., 2002] which would furthermore emphasize the importance of PAF to EC biology. Hence, as S1P induces both PAF synthesis and migration, we tested the hypothesis that PAF drives S1P-induced EC migration. To do so, we pretreated BAEC with selective PAFR antagonists and investigated their effect on S1P-induced migration. Our data show that S1P induced a robust, time-dependent increase in BAEC migration (Fig. 5A) which can be partially inhibited by a pretreatment with the extracellular PAFR antagonist (BN52021) or the extracellular and intracellular PAFR antagonist (CV3988). In contrast, the blockade of intracellular PAFR (LAU8080) had no such effect. Taken together, these data show that the extracellular pool of PAFR, and not the intracellular pool, participates partially to S1P-induced migration. These data illustrates the capacity of PAF synthesized de novo to promote in an autocrine fashion the activation of its cell-surface receptor. However, it remains to be determined what other intracellular effector, besides PAF, drives S1P-induced migration.

The p38 MAPK pathway is known to take part to EC migration by promoting actin reorganization and stress fiber formation [Rousseau et al., 1997]. Others have shown that it can induce lyso-PAF AT activation [Nixon et al., 1999]. Hence, it is possible that the p38 MAPK pathway may also promote EC migration in a PAF-independent manner. Our data show that p38 MAPK pathway inhibition (SB203580) blocks only partially (42%) S1P-induced migration, which is similar to the inhibitory effect of extracellular PAFR antagonists. These data suggest that the partial inhibitory effect of SB203580 on migration is mediated through the near complete inhibition of S1P-induced PAF synthesis, as observed in Figure 2. As PAF plays only a partial role in BAEC migration, this rationalizes the incomplete inhibition of S1P-induced migration by SB203580. Finally, p42/44 MAPK or PI3K pathway inhibition had no significant effect on BAEC migration.

As PAF and its extracellular PAFR play a role in S1P-induced migration, we sought to confirm that BAEC do express PAFR. The data presented in Figure 7 show that BAEC do express PAFR, as shown by Western blot analysis, and that PAFR are localized both at the membrane and in the cytoplasm.

In conclusion, S1P is believed to take part to several pathological situations characterized by uncontrolled angiogenesis [Lee et al., 1999; Boguslawski et al., 2000; Ammit et al., 2001; English et al., 2001]. Studies have shown that activated platelets adhere to injured tissues, aggregate and release growth substances, including S1P, that are capable of promoting angiogenesis through EC activation. Our data describe how S1P regulates the synthesis of PAF, a highly potent inflammatory mediator, and illustrate its role in S1P-induced EC migration. Hence, it is possible that EC PAF synthesis induced by S1P may play a role in the progression of vascular diseases characterized by local inflammation and angiogenesis such as atherosclerosis.

ACKNOWLEDGMENTS

Dr. Bernatchez and Dr. Sirois are, respectively, recipients of a studentship and a scholarship from the CIHR.

REFERENCES

Ackermann EJ, Conde-Friebos K, Dennis EA. 1995. Inhibition of macrophage $Ca^{(2+)}$-independent phospholipase A_2 by bromoenol lactone and trifluoromethyl ketones. J Biol Chem 270:445–450.

Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, Kane SA, Peters SP, Penn RB, Spiegel S, Panettieri RA, Jr. 2001. Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. FASEB J 15:1212–1224.

Balboa MA, Balsinde J, Winstead MV, Tishfield JA, Dennis EA. 1996. Novel group V phospholipase A_2 is involved in arachidonic acid mobilization in murine P388D1 macrophages. J Biol Chem 271:32381–32384.

Balsinde J, Dennis EA. 1997. Function and inhibition of intracellular calcium-independent phospholipase A_2. J Biol Chem 272:16069–16072.

Bazan NG, Squinto SP, Braquet P, Panetta T, Marcheselli VL. 1991. Platelet-activating factor and polyunsaturated fatty acids in cerebral ischemia or convulsions: Intracellular PAF-binding sites and activation of a Fos/Jun/AP-1 transcriptional signaling system. Lipids 26:1236–1242.

Bergers G, Benjamin LE. 2002. Tumorigenesis and the angiogenic switch. Nat Rev 2:401–410.

Bernatchez P, Soker S, Sirois MG. 1999. VEGF effect on endothelial cell proliferation, migration, and PAF synthesis is mediated through the activation of Flk-1 receptor. J Biol Chem 274:31047–31054.

Bernatchez PN, Winstead MV, Dennis EA, Sirois MG. 2001a. VEGF stimulation of endothelial cell PAF synthesis is mediated by group V 14 kDa secretory phospholipase A_2. Br J Pharmacol 134:197–205.

Bernatchez PN, Allen BG, Gélinas DS, Guillemette G, Sirois MG. 2001b. Regulation of VEGF-induced endothelial cell PAF synthesis: Role of p42/44 MAPK, p38 MAPK, and PI3K. Br J Pharmacol 134:1252–1262.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917.

Boguslawski G, Lyons D, Harvey KA, Kovala T, English D. 2000. Spingosylphophorylcholine induces endothelial cell migration and morphogenesis. Biochem Biophys Res Commun 272:603–609.

Bonser RW, Thompson NT, Randall RW, Tateson JE, Spacey GD, Hodson HF, Garland LG. 1991. Demethoxyviridin and Wortmannin block phospholipase C and D activation in the human neutrophil. Br J Pharmacol 103:1237–1241.

Borsch-Haubold AG, Bartoli F, Asselin J, Dudler T, Kramer RM, Apitz-Castro R, Watson SP, Gelb MH. 1998. Identification of the phosphorylation sites of cytosolic phospholipase A_2 in agonist-stimulated human platelets and HeLa cells. J Biol Chem 273:4449–4458.

Bussolino F, Camussi G. 1995. Platelet-activating factor produced by endothelial cells. Eur J Biochem 229:327–337.

Camussi G, Aglietta M, Malavasi F, Tetta C, Sanavio F, Piacibello W, Bussolino F. 1983. The release of platelet activating factor from human endothelial cells in culture. J Immunol 131:2397–2403.

Chen J, Engle SJ, Seilhamer JJ, Tishfield JA. 1994. Cloning and recombinant expression of a novel human low molecular weight Ca^{2+}-dependent phospholipase A_2. J Biol Chem 269:2365–2368.

Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. 1989. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 84:1470–1478.

Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. 1995. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 364: 229–233.

De Carvalho MS, Jacobs RS. 1991. Two-step inactivation of bee venom phospholipase A_2 by scalaradial. Biochem Pharmacol 42:1621–1626.

Dvorak HF, Brown LF, Detmar M, Dvorak AM. 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 146:1029–1039.

English D, Garcia JGN, Brindley DN. 2001. Platelet-released phospholipids link haemostasis and angiogenesis. Cardiovasc Res 49:588–599.

Folkman J. 1991. What is the evidence that tumors are angiogenesis-dependent. J Natl Cancer Inst 82:4–6.

Folkman J, Klagsburn M. 1987. Angiogenic factors. Science 235:442–447.

Hannun YA, Bell RM. 1989. Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. Science 243:500–507.

Hla T, Maciag T. 1990. An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. J Biol Chem 265:9308–9313.

Honda Z, Ishii S, Shimizu T. 2002. Platelet-activating factor receptor. J Biochem 131:773–779.

Ihida K, Pedescu D, Czekay RP, Palade GE. 1999. Platelet activating factor receptor (PAF-R) is found in a large endosomal compartment in human umbilical vein endothelial cells. J Cell Sci 112:285–289.

Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD. 1997. The codependence of angiogenesis and chronic inflammation. FASEB J 11:457–465.

Lee OH, Kim YM, Lee YM, Moon EJ, Lee DJ, Kim JH, Kim KW, Kwon YG. 1999. Sphingosine 1-phosphate induces angiogenesis: Its angiogenic action and signaling mechanism in human umbilical vein endothelial cells. Biochem Biophys Res Commun 264:743–750.

Leslie CC. 1997. Properties and regulation of cytosolic phospholipase A_2. J Biol Chem 272:16709–16712.

Marcheselli VL, Bazan NG. 1994. Platelet-activating factor is a messager in the electroconvulsive shock-induced transcriptional activation of c-fos and zif-268 in hippocampus. J Neurosci Res 37:54–61.

Marcheselli VL, Rossowska MJ, Domingo MT, Braquet P, Bazan NG. 1990. Distinct platelet-activating factor binding sites in synaptic endings and in intracellular membranes of rat cerebral cortex. J Biol Chem 265:9140–9145.

Marshall LA, Hall RH, Winkler JD, Badger A, Bolognese B, Roshak A, Flamberg PL, Sung CM, Chabot-Fletcher M, Adams JL, Mayer RJ. 1995. SB203347, an inhibitor of 14 kDa phospholipase A_2, alters human neutrophil arachidonic acid release and metabolism and prolongs survival in murine endotoxic choc. J Pharmacol Exp Ther 274: 1254–1262.

Marshall LA, Bolognese B, Winkler JD, Roshak J. 1997. Depletion of human monocyte 85-kDa phospholipase A_2 does not alter leukotriene formation. J Biol Chem 272:759–765.

Martin-Blanco E. 2000. p38 MAPK signaling cascades: Ancient roles and new functions. Bioassays 22:637–645.

Montrucchio G, Alloatti G, Camussi G. 2000. Role of platelet-activating factor in cardiovascular pathophysiology. Phys Rev 80:1669–1699.

Morales-Ruiz M, Lee MJ, Zollner S, Gratton JP, Scotland R, Shiojima I, Walsh K, Hla T, Sessa WC. 2001. Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. J Biol Chem 276: 19672–19677.

Murakami M, Kudo I. 2002. Phospholipase A_2. J Biochem 131:285–292.

Nixon AB, O'Flaherty JT, Salyer JK, Wykle RL. 1999. Acetyl-CoA: 1-*O*-alkyl-2-lyso-sn-glycero-3-phosphocholine acetyltransferase is directly activated by p38 kinase. J Biol Chem 274:5469–5473.

Olivera A, Spiegel S. 1993. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. Nature 365:557–560.

Olivera A, Kohama T, Tu Z, Milstien S, Spiegel S. 1998. Purification and characterization of rat kidney sphingosine kinase. J Biol Chem 273:12576–12583.

Pang L, Sawada T, Decker SJ, Saltiel AR. 1995. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J Biol Chem 270: 13585–13588.

Prescott SM, Zimmerman GA, McIntyre TM. 1984. Human endothelial cells in culture produce platelet-activating factor (1-alkyl-2-acetyl-sn-glycerol-3-phosphocholine) when stimulated with trombin. Proc Natl Acad Sci USA 81:3534–3538.

Pyne S, Chapman J, Steele L, Pyne NJ. 1996. Sphingomyelin-derived lipids differentially regulate the extracellular signal-regulated kinase 2 (ERK-2) and c-Jun N-terminal kinase (JNK) signal cascades in airway smooth muscle. Eur J Biochem 237:819–826.

Reddy ST, Winstead MV, Tishfield JA, Hershman HR. 1997. Analysis of the secretory phospholipase A_2 that mediates prostaglandin production in mast cells. J Biol Chem 272:13591–13596.

Rousseau S, Houle F, Landry J, Huot J. 1997. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Oncogene 15:2169–2177.

Senger DR, Galli SJ, Dvorak AM, Perruzi CA, Harvey VS, Dvorak HF. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985.

Sirois MG, Edelman ER. 1997. VEGF effect on vascular permeability is mediated by the synthesis of platelet-activating factor. Am J Physiol 272:H2746–H2756.

Spiegel S, Milstein S. 2003. Sphingosine 1-phosphate: An enigmatic signaling lipid. Nat Rev 4:397–407.

Vlahos CJ, Matter WF, Brown RF, Traynor-Kaplan AE, Heyworth PG, Prossnitz ER, Ye RD, Marder P, Schelm JA, Rothfuss KJ. 1995. Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol 3-kinase. J Imunol 154:2413–2422.

Winkler JD, Bolognese B, Roshak AK, Sung CM, Marshall LA. 1997. Evidence that the 85 kDa phospholipase A_2 is not linked to CoA-independent transacylase-mediated production of platelet-activating factor in human monocytes. Biochim Biophys Acta 1346:173–184.

Xing M, Insel PA. 1996. Protein kinase C-dependent activation of cytosolic phospholipase A_2 and mitogen-activated protein kinase by alpha 1-adrenergic receptors in Madin–Darby canine kidney cells. J Clin Invest 97:1302–1310.

Yatomi Y, Ohmori T, Rile G, Kazama F, Okamoto H, Sano T, Satoh K, Kume S, Tigyi G, Igarashi Y, Ozaki Y. 1995. Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. Blood 96:3431–3438.