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VEGF stimulation of endothelial cell PAF synthesis is mediated by group V 14 kDa secretory phospholipase A_2

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1 Vascular endothelial growth factor (VEGF) is a potent inducer of inflammation, and we have shown that this latter effect is mediated through endothelial cell (EC) PAF synthesis. Since the phospholipid remodelling pathway enzymes (CoA-independent transacylase, CoA-IT; phospholipase A_2, PLA_2; and lyso-PAF acetyltransferase, lyso-PAF-AT) may participate in PAF synthesis, we assessed their contribution to VEGF-induced PAF synthesis in bovine aortic EC (BAEC) and human umbilical vein EC (HUVEC).

2 VEGF enhanced BAEC and HUVEC PAF synthesis by up to 28 and 4 fold above basal levels respectively.

3 A pretreatment with a CoA-IT and lyso-PAF-AT inhibitor (Sanguinarin; 500 nM) blocked VEGF-induced PAF synthesis by 95%, a specific CoA-IT inhibitor (SKF45905; 10–50 μM) was without effect, confirming the crucial role of the PLA_2 and lyso-PAF-AT.

4 Treatment with secreted PLA_2 ($sPLA_2$) inhibitors which have been shown to inhibit both groups IIA and V $sPLA_2$ (SB203347; 10 μM and LY311727; 100 μM) blocked EC PAF synthesis by up to 90%, whereas selective inhibition of group IIA $sPLA_2$ (LY311727; 1 μM) had no significant effect.

5 RT–PCR and Western blot analyses demonstrated the presence of group V $sPLA_2$ whereas group IIA $sPLA_2$ was undetected in EC.

6 Treatment with cytosolic and calcium-independent PLA_2 inhibitors (Arachidonyl trifluoromethyl ketone, Bromoenol lactone, Methyl arachydonyl fluorophosphate, up to 50 μM) did not prevent but rather potentiated the VEGF effect on EC PAF synthesis.

7 These results provide evidence that with VEGF activation of EC cells, the group V $sPLA_2$ provides substrate for EC PAF formation.

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Keywords: VEGF; PAF; PLA_2; inflammation; angiogenesis

Abbreviations: $AACOCF_3$, arachidonyl trifluoromethyl ketone; BAEC, bovine aortic endothelial cells; BEL, bromoenol lactone; CoA-IT, coenzyme A-independent transacylase; DMEM, Dulbecco's modified Eagle's medium; EC, endothelial cells; HBSS, Hank's balanced salt solution; HPLC, high performance liquid chromatography; HUVEC, human umbilical vein endothelial cells; $iPLA_2$, calcium-independent phospholipase A_2; lyso-PAF-AT, lyso-PAF acetyltransferase; MAFP, methyl arachidonyl fluorophosphate; PAF, platelet-activating factor; PGI_2, prostacyclin; RT–PCR, reverse transcriptase-polymerase chain reaction; $sPLA_2$, secreted phospholipase A_2; VEGF, vascular endothelial growth factor

Introduction

Over the past 20 years, strong evidence has confirmed that endothelial cells (EC) represent a metabolically active tissue rather than a simple barrier between blood and interstitial fluid. The pluripotent ability of these cells allows them to respond to a wide range of stimuli known to play a critical role not only in the balance of vascular tone and permeability, but also in the pathogenesis of certain diseases such as tumor growth, diabetic retinopathy as well as psoriasis (Folkman, 1991; Folkman & Klagsbrun, 1997). Aberrant angiogenesis, characterized by uncontrolled growth of new capillaries from pre-existing blood vessels, has been found to be a crucial component of these pathologies (Folkman & Klagsbrun, 1997).

Several reports suggest that inflammation regularly and perhaps invariably precedes and/or accompanies angiogenesis (Jackson *et al*., 1997; 1998). Blood vessels in and around tumours display increased vascular permeability (Dvorak *et al*., 1988) and inflammatory monocytes/macrophages can be found at sites where angiogenesis is occurring in an abnormal environment (Sunderkotter *et al*., 1994). One likely candidate for the regulation of pathological angiogenesis is the vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) (Ferrara, 1995). This is suggested in part by observations that high levels of VEGF are produced by various types of tumours (Kondo *et al*., 1994), and that tumour growth is attenuated *in vivo* by anti-VEGF antibodies (Kim *et al*., 1993). Although many growth factors such as VEGF can induce endothelial cell (EC) migration and proliferation in culture, VEGF is the only one capable of enhancing vascular permeability (Connolly *et al*., 1989;

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Folkman & Klagsbrun, 1997), and it is likely that its angiogenic properties are mediated in part by its ability to modulate fluid and protein extravasation. We have recently reported that VEGF-induced protein extravasation *in vivo* was abolished by a pretreatment with a selective platelet-activating factor (PAF) receptor antagonist, suggesting that VEGF effect on vascular permeability was mediated through PAF synthesis (Sirois & Edelman, 1997). This hypothesis was supported by the rapid induction of PAF synthesis in cultured bovine aortic endothelial cells (BAEC) treated with VEGF. In addition, we showed that VEGF effect on BAEC migration, proliferation and PAF synthesis was dependent on the activation of the receptor encoded by the Flk-1/KDR gene (Bernatchez *et al*., 1999). However, the intracellular events related to VEGF-induced PAF production remain unknown.

Two synthetic pathways for PAF synthesis have been described. One route, *de novo* synthesis, is thought to produce constitutively a small amount of PAF in tissues, which allows efficient homeostasis between the bloodstream and interstitial fluid (Venable *et al*., 1993). The dominant inflammatory mechanism of PAF biosynthesis in EC is thought to occur through a two step remodelling pathway where the acyl moiety of alkylacylglycerophosphorylcholine, a membrane-associated phospholipid, is initially removed by either the direct action of a phospholipase A_2 (PLA_2) or by a CoA-independent transacylase (CoA-IT) to form lyso-PAF. The final step, the acetylation of the lyso-PAF, is catalyzed by the acetylCoA:-lyso-PAF acetyltransferase (lyso-PAF-AT) leading to PAF synthesis (Bussolino & Camussi, 1995; Snyder *et al*., 1996).

Several inflammatory mediators were shown to directly induce the activation of the remodelling route, leading to very-early, early and delayed PAF synthesis (Bussolino & Camussi, 1995). However, there are no data regarding the capacity of growth factors to activate this pathway. Consequently, we sought to determine whether the elevation of PAF synthesis elicited by VEGF might be related to an increased activity of the remodelling pathway.

Methods

Drugs

VEGF (human recombinant vascular endothelial growth factor, 165 amino acid peptide) was purchased from PeproTech (Rocky Hill, NJ, U.S.A.). Arachidonoyl trifluoromethyl ketone ($AACOCF_3$) and Scalaradial were purchased from Calbiochem (La Jolla, CA, U.S.A.), Sanguinarin was purchased from Sigma (St-Louis, MO, U.S.A.), and Methyl arachydonyl fluorophosphate (MAFP) and Bromoenol lactone (BEL) were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). SB203347 and SKF45905 were donated by Dr James D. Winkler (SmithKline Beecham Pharmaceuticals; King of Prussia, PA, U.S.A.). LY311727 was kindly provided by Dr Jerome Fleisch (Lilly Research Laboratories, Indianapolis, IN, U.S.A.).

Cell culture

BAEC and human umbilical vein endothelial cells (HUVEC) were isolated from freshly harvested aorta or umbilical cords respectively, and cultured and characterized as described previously (Sirois & Edelman, 1997; Bernatchez *et al*., 1999). BAEC and HUVEC were not passaged for more than 10 and five passages respectively.

Measurement of PAF synthesis

PAF production by BAEC and HUVEC was measured by incorporation of ^{3}H-acetate into lyso-PAF (Sirois & Edelman, 1997; Bernatchez *et al*., 1999). Confluent BAEC or HUVEC (6-well tissue culture plate) were rinsed with HBSS (Hank's balanced salt solution)/HEPES (10 mM; pH 7.4). Cells were then stimulated for 15 min in 1 ml of HBSS-HEPES (10 mM, pH 7.4) + $CaCl_2$ (0 – 10 mM) + ^{3}H-acetate (25 μCi) plus the appropriate concentration of VEGF. Inhibitors were added 5 to 30 min prior to the addition of VEGF (1 nM). The reaction was stopped by addition of acidified methanol (50 mM acetic acid), the wells were scraped and added to chloroform (2.5 ml) and 0.1 M sodium acetate (1 ml) mixture. Culture plates were washed twice with 1 ml of methanol, added to the chloroform mixture, shaken vigorously and centrifuged for 2 min at 1700 r.p.m. The upper phase was discarded and the chloroform phase was washed twice with 2 ml of the organic phase of a HBSS-HEPES (10 mM)-methanol-chloroform-sodium acetate (0.1 M) solution (1 : 2.5 : 3.75 : 1). Isolated lipids were evaporated under a stream of N_2 gas, resuspended in 175 μl of mobile phase solvent (water-chloroform-methanol 5 : 40 : 55) and purified by a silica-based normal-phase HPLC column (4.5 × 250 mm, 5 μm silica particle size; Varian, Harbour City, CA, U.S.A.) and eluted with the mobile phase solvent at a 0.5 ml min^{-1} flow rate. Fractions corresponding to ^{3}H-alkyl-PAF were quantified by counting radioactivity with a β-counter. The authenticity of synthesized ^{3}H-alkyl-PAF was confirmed by the similar HPLC elution pattern as standard ^{3}H-alkyl-PAF (New England Nuclear, MA, U.S.A.), and by its ability to induce platelet aggregation as standard alkyl-PAF (Avanti Polar Lipids, Alabaster, AL, U.S.A.) (Sirois & Edelman, 1997).

Western blot analysis of secreted phospholipases A_2 expression

Confluent BAEC and HUVEC (100 mm tissue culture plate) were rinsed with HBSS-HEPES (10 mM; pH 7.4). Cells were then stimulated for 15 min in 3 ml of HBSS-HEPES (10 mM, pH 7.4) + $CaCl_2$ (10 mM) + acetate (6 nM) with or without VEGF (1 nM). The supernatants and the cells were collected. Total proteins were isolated by the addition of 500 μl of lysis buffer with PMSF 1 mM (Sigma), leupeptin 10 μg ml^{-1} (Sigma), aprotinin 30 μg ml^{-1} (Sigma) and $NaVO_3$ 1 mM (Sigma). Plates were scraped using a plastic policeman and the protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). Total protein (40 μg) was separated by a 10% tricine SDS – PAGE gel (Novex) and transblotted onto a Immobilon-P PVDF membrane (Millipore, Bedford, MA, U.S.A.). Membranes were blocked in immunoblot buffer (mM:Tris-HCl 10 (pH 8.0), EDTA 1, NaCl 150, 0.1% Triton X-100) with 5% bovine serum albumin and 5% nonfat dry milk for 1 h at room temperature with gentle agitation. Membranes were then incubated for 1 h in Immunoblot buffer containing 1%

Bovine Serum Albumin and 1% nonfat dry milk with sPLA$_2$ (human synovial) polyclonal antiserum (dilution 1 : 4000, Cayman Chemical) which recognizes both group IIA and group V PLA$_2$. Membranes were washed three times with Immunoblot buffer and incubated with biotinylated goat anti-rabbit IgG antibodies (dilution 1 : 10,000, Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min. Membranes were washed three times with Immunoblot buffer and incubated with horseradish peroxidase streptavidin (dilution 1 : 10,000, Vector Laboratories) for 30 min. Membranes were washed with immunoblot buffer and horseradish peroxidase was revealed by chemiluminesence (ECL kit, Amersham). Human synovial fluid group IIA sPLA$_2$ (Cayman Chemicals) and P388D1 macrophage cell lysate were used as positive controls. Kaleidoscope molecular weight marker proteins were used as standards for SDS–PAGE.

Reverse transcriptase-polymerase chain reaction analysis of group IIA and V sPLA$_2$ gene expression

Total RNA from BAEC and HUVEC was prepared using the Trizol reagent system (Life Technologies, Grand Island, NY, U.S.A.). Total RNA from human brain, heart and intestine was from Clonetech (Palo Alto, CA, U.S.A.). Single strand cDNA was synthesized in a reaction that contained 2 μg of total RNA with 0.5 μg random hexamers (Amersham Pharmacia, Uppsala, Sweden) and 200 units M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.) at 37°C for 1 h. 0.3 μg of cDNA was then utilized for PCR using Taq DNA polymerase (Promega). Reaction conditions were as follows: denature at 94°C for 40 s, anneal at 50°C for 1 min, extend at 72°C for 1 min for 30 cycles. The primers used to amplify group IIA sPLA$_2$ were designed using the human sequence: 5′-CTT ACC ATG AAG ACC CTC CTA CTG TTG GCA-3′ and 5′-GAG GGG ACT CAG CAA CGA GGG GTG CT-3′. Primers for group V sPLA$_2$ were from highly conserved regions of the human and mouse sequences: 5′-GGC TTC TAC GGC TGT TAC TG-3′ and 5′-GTA GAC GAG CTT CCG GTC AC-3′.

Statistical analysis

Data are mean ± s.e.mean. Statistical comparisons were made by analysis of variance followed by an unpaired Student's *t*-test. Data were considered significantly different if values of $P<0.05$ were observed.

Results

Induction of endothelial cell PAF synthesis by VEGF is [Ca^{2+}]-dependent

We recently reported that VEGF dose-dependently induced the synthesis of PAF in BAEC (Sirois & Edelman, 1997; Bernatchez *et al*., 1999). However, these experiments were performed exclusively in the presence of high extracellular Ca^{2+} concentration (10 mM). In the present study we assessed the contribution of extracellular Ca^{2+} for the induction of EC PAF production mediated by VEGF on BAEC. In the presence of 10 mM $CaCl_2$, VEGF (1 nM) induced an increase in PAF synthesis which was considered maximal (100%) as compared to control buffer (0%) (Figure 1). However, when BAEC were incubated in the absence of extracellular $CaCl_2$ (0 mM) or $CaCl_2$ (0 mM) + EDTA (1 mM), VEGF failed to significantly increase the basal production of PAF by EC. We then sought to determine the Ca^{2+} concentration required by the BAEC to synthesize PAF in response to VEGF. Although an increase of $CaCl_2$ to 1 and 10 μM concentrations did not modulate the basal PAF production, the application of $CaCl_2$ to 100 μM and 1 mM induced a significant increase of PAF synthesis, which represented 1.2 and 67% of the maximal amount of PAF synthesized by BAEC when stimulated with VEGF (1 nM) in the presence of 10 mM $CaCl_2$. As a result, the subsequent experiments were performed in the presence of 10 mM $CaCl_2$.

The effect of VEGF on EC PAF synthesis is attenuated by remodelling pathway and group V sPLA$_2$ inhibitors

Since growing evidence indicates that the remodelling route is activated during inflammation and other hypersensitivity responses, we used a range of specific inhibitors of the aforementioned pathway to determine its possible involvement in VEGF-induced PAF production in EC from two different sources, namely BAEC and HUVEC (Figure 2). First, VEGF (1 nM) increased by 28 and 4 fold the basal production of PAF by BAEC and HUVEC as compared to PBS control buffer ($P<0.001$), respectively (Figure 3A,B). The addition of a potent inhibitor of both the lyso-PAF-AT and the CoA-IT activities (Sanguinarin; 500 nM) (Snyder *et al*., 1996) blocked the amount of PAF synthesized by BAEC and HUVEC by 95 and 97%, respectively, in response to VEGF treatment (1 nM) (Figure 3A,B). In contrast, a pretreatment with a selective CoA-IT inhibitor (SKF45905; 10–50 μM; $IC_{50}=6$ μM) (Winkler *et al*., 1996a) did not

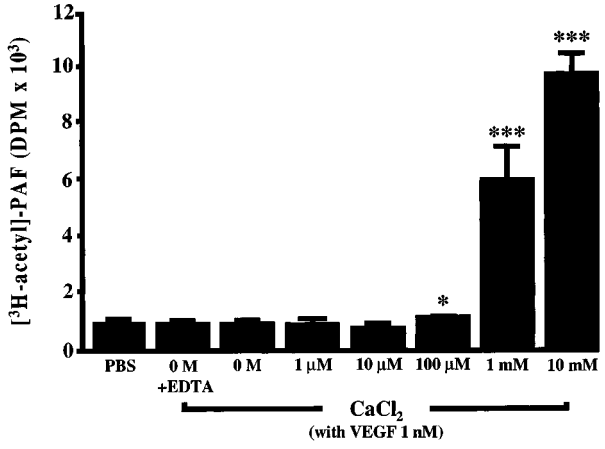

Figure 1 PAF biosynthesis induced by VEGF is Ca^{2+}-dependent. Confluent BAEC (6-well tissue culture plate) were incubated with ^{3}H-acetate and stimulated with VEGF (1 nM) for 15 min with various concentrations of $CaCl_2$ (0–10 mM). The radioactive polar lipids samples were extracted by the Bligh and Dyer procedure. The samples were injected into a 4.6 × 250 mm Varian Si-5 column and eluted with a mobile phase (H_2O:$CHCl_3$:MeOH; 5 : 40 : 55; 0.5 ml min^{-1}). Fractions were collected every min after injection and radioactivity was determined with a β-counter. The values are means of at least six experiments. *$P<0.05$ and ***$P<0.001$ as compared to control buffer (PBS) as determined by analysis of variance followed by an unpaired Student's *t*-test.

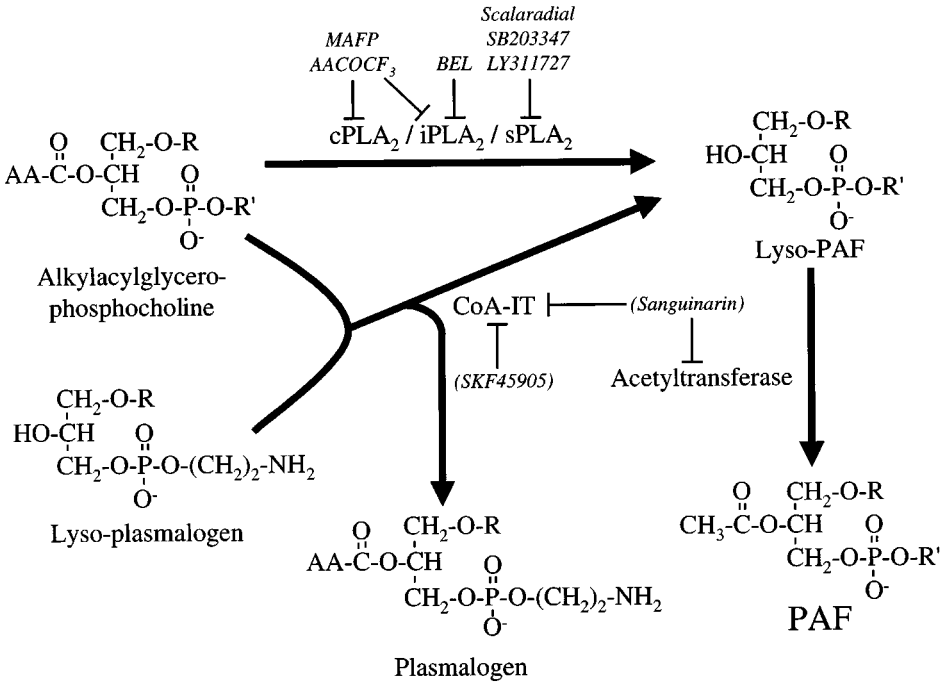

Figure 2 Biosynthesis of lyso-PAF and PAF *via* the remodelling pathway. The initial hydrolysis of the acyl moiety of alkylacylglycerophosphocholine to form lyso-PAF and arachidonic acid (AA) can be catalyzed by the action of a direct phospholipase A_2 (PLA_2) or a CoA-independent transacylase (CoA-IT). Lyso-plasmalogen and other lyso-glycerophospholipids can act as the acyl acceptor in the CoA-independent transacylase type of reaction. The lyso-PAF is then converted to PAF by the acetyl-CoA:lyso-PAF acetyltransferase. Above, are the names of selective inhibitors used to identify the enzymes involved in VEGF-mediated PAF synthesis upon remodelling pathway activation. $R=(CH_2)_n\text{-}CH_3$ where $n=15$ to 17 and $R'=(CH_2)_2N^+(CH_3)_3$.

attenuate PAF synthesis (Figure 3A,B), suggesting that CoA-IT is not involved in EC PAF synthesis.

Considering that PLA_2 were also shown to be capable of mediating the production of lyso-PAF, we used a variety of PLA_2 inhibitors. First, a pretreatment with a competitive inhibitor of the group IV 85 kDa cytosolic PLA_2 ($cPLA_2$) and group VI Ca^{2+}-independent PLA_2 ($iPLA_2$) ($AACOCF_3$; 10–50 μM; $IC_{50}=100$ nM for human recombinant $cPLA_2$ and 15 μM for semi-purified $iPLA_2$) (Ackermann *et al*., 1995) did not reduce VEGF-induced EC PAF synthesis. In contrast, a potent irreversible inhibitor of both the $cPLA_2$ and $iPLA_2$ (MAFP; 10 μM; $IC_{50}=600$ nM for recombinant $cPLA_2$ and 500 nM for $iPLA_2$) (Leslie, 1997; Balsinde *et al*., 1997; Fujishima *et al*., 1999) increased VEGF effect on BAEC and HUVEC PAF synthesis by 126 and 531%, respectively (Figure 3A,B). Interestingly, a specific inhibitor of the group VI $iPLA_2$ (BEL; 10 μM; $IC_{50}=60$ nM) (Ackermann *et al*., 1995) also potentiated VEGF effect, elevating PAF biosynthesis by 113 and 129% in BAEC and HUVEC respectively (Figure 3A,B). These results clearly show that both $cPLA_2$ and $iPLA_2$ activity are not directly involved in EC PAF synthesis upon VEGF stimulation as their inhibition did not prevent PAF synthesis. In contrast, a broad-range 14 kDa $sPLA_2$ inhibitor (Scalaradial; 10 μM) (de Carvalho & Jacobs, 1991; Marshall *et al*., 1994; 1995) which has been shown to block several $sPLA_2$s attenuated by 87 and 73% the synthesis of PAF mediated by VEGF in BAEC and HUVEC respectively, thereby suggesting that this biological response is initiated by a $sPLA_2$. Furthermore, the use of a specific group IIA and V $sPLA_2$ inhibitor (SB203347; 10 μM; $IC_{50}=500$ nM) (Marshall *et al*., 1995) blocked by 90% VEGF effect on both EC types (Figure 3A,B). Interestingly, the use of a structurally designed $sPLA_2$ inhibitor (LY311727) at a concentration known to block specifically group IIA $sPLA_2$ (1 μM; $IC_{50}=<1$ μM for group IIA $sPLA_2$) (Murakami *et al*., 1998) did not have any effect as well on PAF synthesis induced by VEGF, whereas it attenuated by 62 and 81% the synthesis of PAF in BAEC and HUVEC, respectively, when used at a concentration reported to block both groups IIA and V $sPLA_2$ (100 μM; $IC_{50}=>50$ μM for group V $sPLA_2$) (Figure 3A,B) (Murakami *et al*., 1998).

BAEC and HUVEC express group V but not group IIA $sPLA_2$

Considering that the addition of SB203347 (10 μM) and LY311727 (100 μM) attenuated PAF biosynthesis, we attempted to determine if BAEC and HUVEC express either group IIA and/or group V $sPLA_2$. Confluent cells were stimulated with VEGF (1 nM) for 15 min, the supernatants and the cells were collected, the proteins were separated by SDS–PAGE and a Western blot analysis was performed with the use of an anti-human $sPLA_2$ antibody which recognizes both group IIA and V $sPLA_2$. Human synovial $sPLA_2$ (group IIA $sPLA_2$ positive control) and P388D1 (group V $sPLA_2$ positive control) (Balboa *et al*., 1996) presented a different migration pattern from each other (Figure 4). Interestingly, the application of 40 μg of BAEC and HUVEC protein extracts that were stimulated with

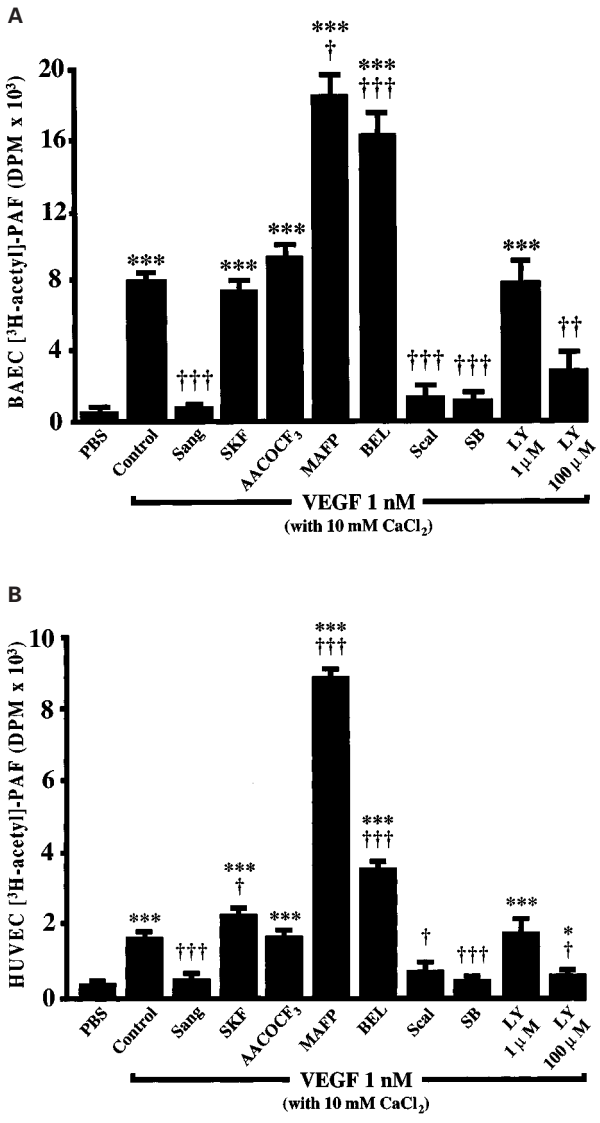

Figure 3 Effect of the remodelling pathway inhibitors on VEGF-induced PAF synthesis. (A) Confluent BAEC (6-well tissue culture plate) were pretreated 5 or 30 min with the remodelling pathway inhibitors Sanguinarine (Sang; 500 μM), SKF45905 (SKF; up to 50 μM), $AACOCF_3$ ($AACOCF_3$; 50 μM), MAFP (MAFP; 10 μM), BEL (BEL; 10 μM), Scalaradial (Scal; 10 μM), SB203347 (SB; 10 μM) and LY311727 (LY; up to 100 μM) stimulated with VEGF (1 nM) + ^{3}H-acetate + 10 mM $CaCl_2$, and the lipids were extracted and purified as described in Figure 1. The values are means of at least four experiments. (B) Confluent HUVEC were treated as described for BAEC. $^*P<0.05$ and $^{***}P<0.001$ as compared to control buffer (PBS), and $\dagger P<0.05$, $\dagger\dagger P<0.01$, $\dagger\dagger\dagger P<0.001$ as compared to VEGF (1 nM) as determined by analysis of variance followed by an unpaired Student's *t*-test.

VEGF revealed that both EC types express group V $sPLA_2$, whereas group IIA $sPLA_2$ expression was not detected (Figure 4). In another experiment, we have observed a constitutive endogenous expression of group V $sPLA_2$ in non-treated BAEC and HUVEC (data not shown). This enzyme remained cell-associated as we did not detect the presence of group V or group IIA $sPLA_2$ in the supernatant of BAEC and HUVEC treated or not with VEGF (1 nM) for 15 min (data not shown).

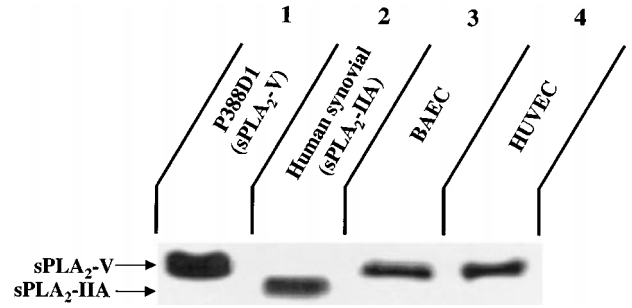

Figure 4 Expression of group IIA and V $sPLA_2$ by BAEC and HUVEC. Confluent BAEC and HUVEC (100 mm tissue culture plate) were rinsed and stimulated with VEGF (1 nM) for 15 min. Cells were scraped and total proteins were isolated. Forty μg of crude proteins were separated by a 10% SDS–PAGE and transblotted onto a PVDF membrane. Proteins were detected by a $sPLA_2$ (human synovial) polyclonal antiserum which recognizes both group IIA and group V $sPLA_2$. Lane 1: P388D1 macrophage cell lysate (group V $sPLA_2$ positive control). Lane 2: Human synovial fluid $sPLA_2$ (group IIA $sPLA_2$ positive control). Lane 3: BAEC lysate. Lane 4: HUVEC lysate.

HUVEC express group V but not group IIA $sPLA_2$ mRNA

Since the data presented in Figure 4 demonstrate the presence of group V $sPLA_2$ and the absence of group IIA $sPLA_2$ in both BAEC and HUVEC, we then sought to confirm by RT–PCR if these two EC types synthesize constitutively group IIA and V $sPLA_2$ mRNA. This approach allows a far more specific and sensitive analysis of the group IIA and V $sPLA_2$ gene expression. The present RT–PCR experiments confirmed the presence of group IIA $sPLA_2$ mRNA in human intestine RNA extracts (positive control), its absence in human brain RNA extracts (negative control) (450 base-pairs; Figure 5A) and the presence of group V $sPLA_2$ in human brain and heart RNA extracts (positive controls) (225 base-pairs; Figure 5B). Interestingly, group IIA $sPLA_2$ was not detected in both stimulated and non-stimulated BAEC and HUVEC (Figure 5A), whereas group V $sPLA_2$ was detected in stimulated and non-stimulated HUVEC but not in BAEC (Figure 5B). As the bovine group V $sPLA_2$ cDNA sequence is still unknown, we may hypothesize that this enzyme has not been detected in BAEC by RT–PCR because of possible mismatches between the human and mouse oligonucleotides used in these experiments, and the bovine group V $sPLA_2$ cDNA sequences.

Discussion

Vascular endothelial growth factor, unlike any other growth factors studied to date, is capable of inducing protein extravasation and it is likely that its angiogenic properties are mediated in large part through the induction of plasma protein leakage (Dvorak *et al*., 1988; Connolly *et al*., 1989). In the present study, we report that VEGF induces PAF synthesis by EC through the action of $sPLA_2$, most likely group V $sPLA_2$. These findings not only shed light on the intracellular events initiated by VEGF on the vascular endothelium, but also may enable rational interventions to regulate vascular diseases such as inflammation and possibly uncontrolled angiogenesis attributable to VEGF overexpression.

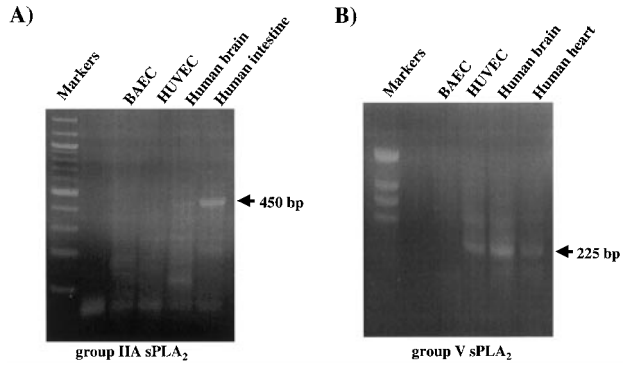

Figure 5 Expression of group IIA and V $sPLA_2$ mRNA by BAEC and HUVEC. Confluent BAEC and HUVEC were lysed, total RNA was extracted and used for RT–PCR. The sizes of the expected amplified fragments are 450 base-pairs for group IIA $sPLA_2$ (A) and 225 base-pairs for group V $sPLA_2$ (B). Human brain and intestine mRNA were used as negative and positive controls respectively, for group IIA $sPLA_2$ RT–PCR (A). Human brain and human heart mRNA were used as positive controls for group V $sPLA_2$ RT–PCR (B).

VEGF increased EC PAF synthesis in vitro

In a previous report we showed for the first time the effect of VEGF on mediating PAF synthesis in BAEC (Sirois & Edelman, 1997). More recently, others have confirmed the capacity of VEGF to elicit PAF synthesis in HUVEC (Montrucchio *et al*., 2000). It is of interest to note that BAEC are far more potent than HUVEC to synthesize PAF in response to VEGF. This observation is supported by previous studies that have shown that venous endothelial cells produce far less PAF as compared to arterial cells (Whatley *et al*., 1988). Thus, this difference of response to VEGF is most likely not attributable to differences in VEGF activity on BAEC and HUVEC, but rather to differences between the capacity of the enzymes to synthesize PAF in these two EC types.

VEGF stimulated the remodelling pathway through the action of group V $sPLA_2$

Micromolar (μM) calcium levels are known to be a crucial cofactor for a wide range of intracellular events (Chakravarthy *et al*., 1999). However, our results showed that an extracellular millimolar (mM) Ca^{2+} concentration is required to fully elicit PAF synthesis. These results are in accordance with previous studies that showed that maximal BAEC PAF synthesis is directly dependent on millimolar extracellular Ca^{2+} concentration (Whatley *et al*., 1989). These authors furthermore demonstrated that this important Ca^{2+} requirement is attributable to the PLA_2-mediated conversion of membrane phospholipids into lyso-PAF induced by various pro-inflammatory stimuli. Interestingly, the Ca^{2+}-concentration dependence of enzymatic activity is one of the major defining characteristics for 14 kDa $sPLA_2$s, since they were reported to bind millimolar Ca^{2+} within a highly conserved calcium binding loop in order to stabilize the transition state of the phospholipid substrate (Dennis, 1994; 1997; Tishfield, 1997). Hence, our results demonstrate that biosynthesis of PAF in BAEC through Flk-1/KDR tyrosine kinase receptor activation by VEGF is at least in part similar to the one observed following activation of G protein-coupled receptors and raise the possibility that $sPLA_2$ may participate in mediating VEGF-induced EC PAF synthesis.

Sanguinarin has previously been shown to have a high degree of selectivity towards the inhibition of both the lyso-PAF-AT and CoA-IT activities (Snyder *et al*., 1996). Therefore, this inhibitor should be a valuable tool to assess the possible involvement of the remodelling pathway in numerous cellular events. Pretreatment of BAEC and HUVEC with Sanguinarin almost completely abolished the synthesis of PAF induced by VEGF, suggesting that VEGF is capable of inducing the activation of the remodelling pathway. To the best of our knowledge, these data are the first to show that a growth factor can mediate such activity, and also confirm that the remodelling route is influenced by proinflammatory stimuli.

A possible key player in the synthesis of PAF through the remodelling pathway is the CoA-IT. This enzyme transfers an *sn*-2 acyl group from a diacylglycerophosphatide to a lyso-phospholipid in the presence or absence of Coenzyme A (Snyder *et al*., 1992; Winkler *et al*., 1996a). However, a pretreatment with a specific CoA-IT inhibitor (SKF45905) (Winkler *et al*., 1996a) did not attenuate PAF synthesis in both EC types. This observation is in accordance with previous reports indicating that EC contain far less CoA-IT activity than inflammatory cells such as neutrophils and monocytes (Winkler & Chilton, 1995; Winkler *et al*., 1996b). Taken together, these observations demonstrate that the CoA-IT is unlikely to take part in EC lyso-PAF synthesis mediated by VEGF and suggest that lyso-PAF synthesis is initiated through the activation of a $sPLA_2$. This hypothesis is relevant since others have shown that VEGF could promote the synthesis of prostaglandins in EC, which is recognized as a marker of PLA_2 activity (Murohara *et al*., 1998).

Phospholipases A_2 consist of a growing superfamily of enzymes, also capable of hydrolyzing membrane phospholipids with the concomitant production of lyso-phospholipids (Dennis, 1994; 1997; Tishfield, 1997). Though some of these enzymes are very unlikely to play a role in VEGF-induced PAF synthesis by BAEC, others may be directly involved in EC PAF synthesis. Since our results support that Ca^{2+} is a crucial cofactor for VEGF-induced PAF production by EC, likely candidates for mediating such activity are the groups IIA and V $sPLA_2$. Nonetheless, the possible effects of other recently described $sPLA_2$ (groups IID, IIE, IIF and X) cannot be ruled out (Six & Dennis, 2000), despite the fact that their expression have not been reported in EC yet.

Hence, we used different inhibitors of the groups IIA and V $sPLA_2$. A pretreatment with a non-specific 14 kDa $sPLA_2$ inhibitor (Scalaradial) previously reported to block several $sPLA_2$s including group IIA and most probably group V $sPLA_2$ (de Carvalho & Jacobs, 1991; Marshall *et al*., 1994; 1995) blocked almost completely EC PAF synthesis. Moreover, an inhibitor of both groups IIA and V $sPLA_2$ (SB203347) (Marshall *et al*., 1995) which possess a 40 fold specificity for groups IIA and V $sPLA_2$ over group IV 85 kDa $cPLA_2$ abrogated almost completely VEGF-induced EC PAF synthesis. In contrast, treatment with LY311727 (1 μM), another well-described $sPLA_2$ inhibitor which at such

concentration blocks group IIA sPLA$_2$ activity, did not significantly affect VEGF induction of PAF synthesis in both BAEC in HUVEC. However, when used at a concentration reported to block both groups IIA and V sPLA$_2$ (100 μM) (Murakami *et al*., 1998), LY311727 inhibited significantly VEGF-induced PAF synthesis. Taken together, these results demonstrate that group V sPLA$_2$ is responsible for the synthesis of lyso-PAF induced by VEGF. Interestingly, several pieces of evidence support such hypothesis. Others have reported that group V sPLA$_2$ can catalyze more efficiently the hydrolysis of phospholipid bilayers than group IIA sPLA$_2$, liberating arachidonic acid and lyso-phospholipids, while group IIA sPLA$_2$ appears to have a relatively low affinity for membrane phospholipids (Han *et al*., 1998).

Group IV cPLA$_2$ and group VI iPLA$_2$ are two cell-associated phospholipases both capable of influencing cellular fatty acid metabolism by increasing free arachidonic acid levels (Balsinde & Dennis, 1996; Leslie, 1997). Thus, these two enzymes could possibly be linked to the synthesis of lyso-PAF from membrane phospholipids initiated by VEGF. A specific inhibitor of both the group IV cPLA$_2$ and VI iPLA$_2$ (AACOCF$_3$) (Ackermann *et al*., 1995, McNicol & Nick-olaychuk, 1995; Leslie, 1997; Fujishima *et al*., 1999) did not significantly modulate PAF synthesis induced by VEGF. In contrast, an irreversible inhibitor of both the cPLA$_2$ and iPLA$_2$ with a greater effect on iPLA$_2$ than cPLA$_2$ (MAFP) (Leslie, 1997; Balsinde & Dennis, 1997; Fujishima *et al*., 1999) greatly potentiated VEGF effect. Moreover, a specific iPLA$_2$ inhibitor (BEL) also increased the synthesis of PAF induced by VEGF in BAEC and HUVEC. Thus, this clearly demonstrates that cPLA$_2$ and iPLA$_2$ are not directly involved in EC lyso-PAF biosynthesis induced by VEGF, and that iPLA$_2$ inhibition by either BEL or MAFP potentiates VEGF effect on EC PAF synthesis. This furthermore suggests that group VI iPLA$_2$ may hydrolyze similar membrane phospholipids to the ones required to mediate EC PAF synthesis, and as a result may act in a competition fashion with the enzyme involved in PAF synthesis. Hence, by blocking the iPLA$_2$, a pool of membrane phospholipids may be exclusively hydrolyzed by the group V sPLA$_2$ leading to increased amount of lyso-PAF synthesized. This hypothesis is furthermore supported by the fact that these two enzymes do not demonstrate substrate selectivity, unlike cPLA$_2$ which prefers arachidonic acid-containing phospholipids. Finally, these data indicate as well that cPLA$_2$ is unlikely to take part in EC PAF production and evidence for this is starting to emerge. Indeed, several reports indicate that cPLA$_2$ is not involved in monocyte or neutrophil PAF biosynthesis but rather in prostanoid production (Leslie, 1997; Marshall *et al*., 1997; Winkler *et al*., 1997). As a result, this enzyme is most probably not implicated in EC PAF synthesis. In addition, this enzyme is active at low micromolar Ca^{2+}, and we showed that at such concentrations VEGF did not induce the synthesis of PAF.

It is interesting to note that previous studies have shown that cPLA$_2$ activity is essential for the pro-inflammatory activity of group V sPLA$_2$ (Balsinde *et al*., 1998; Cho, 2000). However, these studies investigate the implication of these two enzymes to prostaglandin synthesis. In contrast, we show that PAF synthesis mediated by group V sPLA$_2$ is independent of cPLA$_2$ activity. This is in agreement with a previous report that has also shown that PAF formation is dependent of sPLA$_2$ and independent of cPLA$_2$ (Marshall *et al*., 1999), one might hypothesize that cPLA$_2$ activity is required for prostanoid synthesis, whereas cPLA$_2$ appears not to be implicated in PAF synthesis. This hypothesis is furthermore supported by a recent report in which it has been shown that group V sPLA$_2$ alone could mediate lyso-PAF synthesis (Murakami *et al*., 2001).

In order to support the hypothesis that group V sPLA$_2$ mediates BAEC PAF synthesis, we then sought to determine if BAEC and HUVEC express a significant amount of group IIA and/or V sPLA$_2$ enzymes. Western blot analysis indicated that both BAEC and HUVEC either stimulated or not with VEGF indeed express group V sPLA$_2$ and not group IIA sPLA$_2$, thereby confirming the possible involvement of group V sPLA$_2$ in PAF synthesis. Finally, group V sPLA$_2$ remained cell-associated and was not detected in the supernatant of BAEC and HUVEC challenged or not with VEGF. We confirmed also by RT–PCR the presence of group V sPLA$_2$ and the absence of group IIA sPLA$_2$ at least in HUVEC. Since our data support a direct role for group V sPLA$_2$ in VEGF-induced PAF synthesis in BAEC and that Western blot analysis clearly shows that BAEC express group V sPLA$_2$, one might hypothesize that the discrepancy observed between Western blot and RT–PCR analyses for BAEC expression of group V sPLA$_2$ may be rationalized by the fact that the oligonucleotides used for RT–PCR were synthesized according to the human group V sPLA$_2$ cDNA sequences, since the bovine gene has not yet been sequenced. As a result, mismatches between bovine group V sPLA$_2$ mRNA and the RT–PCR oligomers used might have been present. Nonetheless, our RT–PCR experiments support the crucial role of group V sPLA$_2$ in HUVEC, and completely dissipates any possible ambiguity regarding a role of group IIA sPLA$_2$ in HUVEC PAF synthesis since this enzyme was not detected in HUVEC by Northern and Western blot analyses. Interestingly, others have shown that group IIA sPLA$_2$ expression is inducible by pro-inflammatory agents (Murakami *et al*., 1997). Moreover, the fact that HUVEC do not express group IIA sPLA$_2$ and that the blockade of this enzyme by LY311727 (1 μM) did not reduce PAF synthesis confirms that the inhibitory effect of the two specific inhibitors of groups IIA and V sPLA$_2$, namely SB203347 and LY311727 (100 μM), on PAF synthesis induced by VEGF is not through the inhibition of group IIA sPLA$_2$ but rather through the inhibition of group V sPLA$_2$.

VEGF and group V sPLA$_2$

Though group V sPLA$_2$ has been cloned only recently (Chen and Dennis, 1998), a growing body of evidence has confirmed that this novel enzyme 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 P388D$_1$ (Balboa *et al*., 1996), as well as in eicosanoid formation in mast cells (Reddy *et al*., 1997). Moreover, group V rather that group IIA as previously believed, is the primary 14 kDa PLA$_2$ synthesized by P388D$_1$ cells (Balboa *et al*., 1996). In the present study, we demonstrate that this newly characterized enzyme is likely responsible for VEGF-induced PAF synthesis, that it is expressed in EC from both bovine aortas and human umbilical veins, and that it remains associated with the cells

that were challenged with VEGF. This is in agreement with a recent report that has shown that group V sPLA$_2$ was cell associated and identified intracellularly (Bingham *et al*., 1999).

Previous investigations have proposed that an increase in microvascular permeability is a crucial step in angiogenesis associated with tumours and wounds (Connolly *et al*., 1989; Folkman & Klagsbrun, 1997). According to this hypothesis, a major function of VEGF is probably the induction of plasma protein leakage. We have shown earlier that VEGF-induced protein extravasation was abolished by a PAF receptor antagonist *in vivo* (Sirois & Edelman, 1997). Since this study supports the idea that group V sPLA$_2$ is responsible for the PAF synthesis mediated by VEGF and that diverse studies suggest that PAF acts as a pro-angiogenic factor (Camussi *et al*., 1995; Bussolino & Camussi, 1995; Montrucchio *et al*., 2000), it would be of interest to investigate if the inhibition of this enzyme may interfere with VEGF inflammatory effect *in vivo*, as well as with its angiogenic potential.

In conclusion, our data showed that the production of PAF elicited by VEGF on BAEC and HUVEC is mediated by the activation of the remodelling pathway, and more specifically through the action of the group V sPLA$_2$ and lyso-PAF-AT. As the inflammatory reaction mediated by VEGF might be an essential key factor of angiogenesis, the inhibition of PAF synthesis mediated by VEGF might prevent the deleterious inflammation and angiogenesis imputable to VEGF overexpression *in vivo*.

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