Activation of Platelet-activating Factor Receptor-coupled \(\alpha_q\) Leads to Stimulation of Src and Focal Adhesion Kinase via Two Separate Pathways in Human Umbilical Vein Endothelial Cells*

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Platelet-activating factor (PAF), a phospholipid second messenger, has diverse physiological functions, including responses in differentiated endothelial cells to external stimuli. We used human umbilical vein endothelial cells (HUVECs) as a model system. We show that PAF activated pertussis toxin-insensitive \(\alpha_q\) protein upon binding to its seven transmembrane receptor. Elevated cAMP levels were observed via activation of adenylate cyclase, which activated protein kinase A (PKA) and was attenuated by a PAF receptor antagonist, blocking downstream activity. Phosphorylation of Src by PAF required \(\alpha_q\) protein and adenylate cyclase activation; there was an absolute requirement of PKA for PAF-induced Src phosphorylation. Immediate (1 min) PAF-induced STAT-3 phosphorylation required the activation of \(\alpha_q\) protein, adenylate cyclase, and PKA, and was independent of these intermediates at delayed (30 min) and prolonged (60 min) PAF exposure. PAF activated PLC\(\beta_3\) through its \(\alpha_q\) protein-coupled receptor, whereas activation of phospholipase C\(\gamma_1\) (PLC\(\gamma_1\)) by PAF was independent of \(G\) proteins but required the involvement of Src at prolonged PAF exposure (60 min). We demonstrate for the first time in vascular endothelial cells: (i) the involvement of signaling intermediates in the PAF-PAF receptor system in the induction of TIMP2 and MT1-MMP expression, resulting in the coordinated proteolytic activation of MMP2, and (ii) a receptor-mediated signal transduction cascade for the tyrosine phosphorylation of FAK by PAF. PAF exposure induced binding of p130\(^{càs}\), Src, SHC, and paxillin to FAK. Clearly, PAF-mediated signaling in differentiated endothelial cells is critical to endothelial cell functions, including cell migration and proteolytic activation of MMP2.

1-O-Alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF), a mediator of homotypic and heterotypic cell-to-cell communication known to activate platelets, neutrophils, monocytes and lymphocytes, is assuming an increasing relevance as a major lipid second messenger (reviewed in Ref. 1). A wide variety of PAF bioactions have been elucidated, including platelet activation, embryogenesis, cell differentiation, and shock, inflammatory, and immune responses (2). PAF is so potent that it can always elicit significant biological responses at nanomolar concentrations in vitro and in vivo (3). Many cells and organs have been shown to produce PAF, which can themselves become targets of PAF bioactions (2). PAF induces endothelial cell migration, which depends on a chemotactic rather than a chemokinetic effect, and promotes in vivo angiogenesis, thus acting as a mediator of vascularization for tumor growth and metastasis (4, 5).

PAF acts through its specific \(G\) protein-coupled receptor, found to be localized to the plasmalemma and a large endosomal compartment in human umbilical vein endothelial cells (HUVECs) (6). The PAF receptor contains seven \(\alpha\)-helical domains that span the plasma membrane and relates the binding of PAF to an intracellular signal through the coupled \(G\) protein (7). Depending on the cell types, multiple \(G\) proteins interact with the PAF receptor resulting in a myriad of distinct signaling pathways. In leukocytes, chemotactic responses to PAF use the pertussis toxin-resistant \(G\) proteins (8), whereas in eosinophils, PAF signals through both pertussis toxin-sensitive and -resistant \(G\) proteins (9). Activation of the p85 MAPK by PAF in Chinese hamster ovary (CHO) cells occurs through the pertussis toxin-insensitive \(\alpha_q\) protein, whereas the activation of extracellular signal-regulated kinases 1 and 2 upon PAF stimulation in these cells signals through the \(\alpha\) subunit of pertussis toxin-sensitive \(G_q\) but not \(G_i\) protein (10, 11).

The activation of adenylate cyclase and \(\alpha\)-dependent protein kinase (protein kinase A (PKA)) by the PAF-PAF receptor system regulates different effector functions depending on the cell type. Stimulation of adenylate cyclase leads to increased levels of cAMP in mesangial cells upon binding of PAF to its receptor, whereas in CHO cells, PAF stimulation antagonized adenylate cyclase activity, leading to decreased levels of cAMP (12, 13). The PAF-induced activation of PKA leads to stimulation of the acrosome reaction in human spermatozoon, and causes generation of superoxide anions and degradation in eosinophils (14, 15).

One consequence of PAF receptor activation is the stimulation of specific isoenzymes of phospholipase C (PLC) depending on cell type and stimulation conditions.

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1 The abbreviations used are: PAF, 1-O-alkyl-sn-glycero-3-phosphocholine or platelet-activating factor; HUVEC, human umbilical vein endothelial cell; STAT, signal transducers and activators of transcription; JAK, Janus kinase; EGK, endothelial growth medium; EBM, endothelial basal medium; FBS, fetal bovine serum; PKA, protein kinase A; PLC, phospholipase C; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; MMP2, matrix metalloproteinase 2 (gelatinase-A); MT1-MMP, membrane type 1 matrix metalloproteinase; PTX, pertussis toxin; TIMP2, tissue inhibitor of metalloproteinase 2; CHO, Chinese hamster ovary; STAT, signal transducers and activators of transcription.
on the cell type. Of the three classes of PLC, namely PLCβ, PLCγ, and PLCδ, PAF stimulates the phosphorylation and activation of only PLCβ and PLCγ (16). Signaling to PLCβ isoforms occurs through either Goζ protein (17) or through the Gq protein using the βγ subunit (18). There appears to be a requirement for the phosphorylation of a tyrosine residue on PLCγ to enable its activation (19). In a B lymphoblastoid cell line, PAF induces an immediate phosphorylation of the tyrosine residue on PLCγ1 leading to its activation (20). Introduction of anti-Src antibodies into rat aortic smooth muscle cells attenuated tyrosine phosphorylation of PLCγ1 upon stimulation of the G protein-coupled angiotensin II receptor (21). Involvement of Src in the PAF-dependent PLCγ activation has also been demonstrated in platelets (22).

Among the various tyrosine kinases, focal adhesion kinase (FAK) is involved in the regulation of cellular motility, adhesion, and cytoskeletal assembly (23). FAK has a molecular structure that is distinct from other identified tyrosine kinases. The catalytic domain is flanked on one side by the N terminus, which interacts with integrins and growth factor receptors (23), and on the other side by the C terminus, which has binding sites for SH2 and SH3 domains that link FAK to the activation of various downstream signaling pathways (24). The unique terminal focal adhesion targeting sequence contains proline-rich sequences that serve as binding sites for paxillin, an adapter protein (25), and the structural protein, talin (26). In rat cerebral cortex and hippocampus, binding of PAF to its receptor has been shown to stimulate a rapid tyrosine phosphorylation of FAK (27, 28). Thus, a variety of different signaling proteins directly associate with FAK, and this combination of proteins affects the involvement of FAK in diverse signaling pathways.

Degradation of the matrix surrounding the interstitial space, occurs through the stringent regulation of matrix metalloproteinases (MMP), including membrane type 1 MMP (MT1-MMP) and MMP2 (29), and the action of tissue inhibitors of metalloproteinases, such as TIMP2 (30), leading to cellular invasion. PAF has been shown to stimulate the expression and activity of MMPs in corneal epithelial cells (31). In neuroblastoma clones isolated from the human LaN1 neuroblastoma cell line, PAF exposure reduced the expression MMPs and activation of MMP2, resulting in inhibition of invasiveness through Matrigel by these cells (32). We have recently demonstrated that the stimulation of quiescent HUVECs with PAF induces increases in mRNA levels for TIMP2 and MT1-MMP (55). PAF does not increase mRNA levels or protein levels of MMP2 but instead results in proteolytic activation of MT1-MMP (55). PAF does not increase mRNA levels or protein levels of MMP2 through the coordinated activation of the extracellular membrane-bound heterotrimeric TIMP2-pro-MMP2-MT1-MMP complex. In this complex, TIMP2 binds to pro-MMP2, which is bound to the extracellular matrix. TIMP2 then associates with a membrane-bound MT1-MMP molecule, which positions the pro-MMP2 so that a second, membrane-bound MT1-MMP can cleave the pro-domain from pro-MMP2, releasing active MMP2 into the extracellular milieu.

In the present report, we show that activation of the PAF receptor induces a specific receptor-associated pertussis toxin-insensitive Goζ protein in HUVECs. Stimulation of adenylate cyclase by PAF, resulting in increased levels of cAMP, induces the activation of PKA in HUVECs, which requires the activation of Goζ protein coupled to the PAF receptor. Phosphorylation of Src by PAF is attenuated by the PAF receptor antagonist LAU-8080 as well as upon blocking the activation of Goζ protein and adenylate cyclase; indeed, there is an absolute requirement for PKA activation in the PAF-PAF receptor signaling cascade of HUVECs with PAF suggesting the importance of Src in the PAF-mediated activation of PLCγ1. The Goζ-protein-coupled PAF-PAF receptor-signaling cascade also phosphorylates FAK in a time-dependent manner, and its activation is independent of the adenylate cyclase/PKA axis in HUVECs. Stimulation of the tyrosine phosphorylation of FAK by PAF-PAF receptor signaling cascade induces the binding of Src, p130Cas, SHC, and paxillin to FAK in HUVECs. Finally, PAF induces a time-dependent increase in expression of TIMP2 and MT1-MMP, which are dependent upon the activity of Goζ and Src, but has no effect on the level of pro-MMP2 in growth factor-starved HUVECs. However, the PAF-mediated increased TIMP2 and MT1-MMP levels, result in the proteolytic activation of pro-MMP2 to active MMP2 in the growth medium.

EXPERIMENTAL PROCEDURES

Reagents—PAF was obtained from Sigma-Aldrich. PAF receptor antagonist LAU-8080 was synthesized by Prof. Julio Alvares-Builla at the Universidad de Alcala, Madrid, Spain, whereas CV-3988 and EN-52021 were obtained from BIOMOL Research Laboratories Inc., Plymouth Meeting, PA. Antibodies used were rabbit polyclonal anti-STAT-3 (#9131), rabbit polyclonal anti-phospho-STAT-3 (#9132), rabbit polyclonal anti-PLCβ3 (#2482), rabbit polyclonal anti-phospho-PLCβ3 (#2481), rabbit polyclonal anti-PLCγ1 (#2822), rabbit polyclonal anti-phospho-PLCγ1 (#2821), and horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies (Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-Src (#07-335), rabbit polyclonal antibody anti-phospho-Src (#07-020), rabbit polyclonal anti-phospho-FK357 (#07-014), rabbit polyclonal anti-p130Cas (#06-500), rabbit polyclonal anti-SHOC (#03-003) antibodies (Upstate Biotechnology, Lake Placid, NY), mouse monoclonal anti-paxillin antibody (#6HO0492) (BIO-SOURCE, Camarillo, CA), rabbit polyclonal anti-rabbit FAK/C-20 (#sc-558), rabbit polyclonal PLCβ1 (#sc-205), rabbit polyclonal anti-PLCγ2 (#sc-206), rabbit polyclonal anti-PLCβ4 (#sc-404), and rabbit polyclonal anti-PLCγ2 (#sc-401) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-TIMP2 (#MAB13435), rabbit polyclonal anti-MMP2 (#MAB13435), and mouse monoclonal anti-β-tubulin (#MAB380) antibodies (Chemicon International, Temecula, CA). Pertussis toxin, GP Antagonist-2, GP Antagonist-2A, suramin, adenylate cyclase inhibitor SQ-25538, PKA inhibitor H-89, and Src family inhibitor PP1 were from BIOMOL Research Laboratories, whereas the JAK-2 inhibitor Tyrphostin AG-490 (T-9142) (Tyrphostin B42) was from LC Laboratories, Beverly, MA. Detection of cAMP was done using a Direct cAMP enzyme immunoassay kit from Sigma-Aldrich (St. Louis, MO), and PKA activity was detected using the PKA assay kit from Upstate Biotechnology. PBS, endothelial growth medium (EGM-2), and endothelial basal medium (EBM-2) were from Cambrex (Walkersville, MD).

Cell Culture—HUVECs were grown to sub-confluent levels in EGM-2 obtained from Cambrex, Inc. plated onto 6-well culture plates (1 × 10⁶ cells/well). HUVECs were then washed once with EBM-2 and starved of growth factors by switching them to EBM-2 supplemented with 0.2% PBS (Cambrex) for 4 h. Cells were again washed once with EBM-2 and then exposed to various experimental conditions for different time points. At the end of each time point, cells were washed with ice-cold phosphate-buffered saline (Invitrogen), lysed in cell lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 2 mM Na3VO4, 50 mM NaF, 70 mM 2-mercaptoethanol, 1% w/v Triton X-100, 2% w/v
Briefly, HUVECs were treated for different time points with various Gly (Kemptide), using the PKA assay kit (Upstate Biotechnology). The reaction was stopped with the stop solution and read at 405 nm.

The three independent experiments are shown.

**RESULTS**

**PAF Induces Activation of Pertussis Toxin-insensitive Goα Protein—**HUVECs were starved of growth factors overnight (EBM supplemented with 0.2% FBS), after which they were exposed to 1 μM pertussis toxin (overnight) or, for 30 min with 60 μM suramin, 25 μM GP Antagonist-2 (pertussis toxin-sensitive Goα and Goq protein inhibitor), or 10 μM GP Antagonist-2A (pertussis toxin-insensitive Goα protein inhibitor), or overnight with 1 μM/ml pertussis toxin (PTX) and exposed to 10^{-7} M PAF for various time points. Cell lysates were obtained after each time point and separated using 7.5% SDS-PAGE and Western blotted. A, phosphorylated Src levels were obtained after incubation with anti-phospho-Src antibody and chemiluminescence detection. The protein levels were verified by stripping and detection with anti-Src antibody. B, levels of phospho-Src obtained after densitometric analysis and represented as percentage of control against time (control being set at 100%). Typical results of one of the three independent experiments are shown.
The inhibition observed with GP Antagonist-2A treatment exceeded the inhibition observed with the broad spectrum G protein inhibitor suramin. 

**Adenylate Cyclase Is Activated by PAF through PAF Receptor and Goq.**—We hypothesized that adenylate cyclase is induced in HUVECs following PAF receptor stimulation and activation of a specific G protein. To test this hypothesis, HUVECs were starved overnight by exposing them to basal medium devoid of growth factors and then exposed for various time points to PAF with or without PAF receptor antagonists LAU-8080, BN-52021, or CV-3988. Adenylate cyclase activity was analyzed by measuring the amount of cAMP produced in each condition. As seen in Fig. 2A, cAMP levels were maximal at 10 min of PAF exposure. The PAF-stimulated cAMP production was attenuated at each time point by the PAF receptor antagonists alone or in combination with PAF, suggesting that PAF stimulates the activity of adenylate cyclase through the PAF receptor in HUVECs.

Furthermore, growth factor-starved HUVECs were exposed to suramin, SQ-22536 (adenylate cyclase inhibitor), or pertussis toxin-sensitive or -insensitive G protein inhibitors for 30 min prior to PAF exposure over time Fig. 2B. Basal levels of cAMP were observed upon inhibition of adenylate cyclase activity by SQ-22536 with or without PAF in HUVECs. Pertussis toxin or GP Antagonist-2, which inhibit the pertussis toxin-sensitive Goq or Goq proteins, did not antagonize PAF-stimulated cAMP production. Decreased levels of cAMP were observed in the presence of the general G protein inhibitor suramin, which was not altered in the presence of PAF. Significant attenuation of cAMP levels were observed at the 10-min time point when the pertussis toxin-insensitive Goq protein was specifically blocked by GP Antagonist-2A, and PAF stimulation could not alter this effect, suggesting that PAF activates adenylate cyclase through the involvement of PAF receptor and its associated Goq protein.

**PAK Is Activated via the PAF-PAF Receptor-signaling Cascade.**—As seen in Fig. 3A, maximal stimulation of PAK activity by PAF occurred following 10 min of PAF exposure in growth factor-starved HUVECs; this effect was antagonized by each of PAF receptor antagonists at all time points, further supporting the involvement of PAF in PAK activation. Inhibition of PAK activity by H-89 (a specific PAK inhibitor) could not be recovered by the addition of PAF at any time point (Fig. 3B). Although PAF-stimulated PAK activity was unaffected upon blocking the pertussis toxin-sensitive G proteins, attenuation of PAF-induced activation of PAK was observed upon inhibiting Goq protein or adenylate cyclase activity by GP Antagonist-2A or SQ-22536, respectively (Fig. 3B). These observations suggest that PAK is a component of the PAF signal transduction cascade and is activated by PAF through the PAF receptor, its associated Goq protein, and adenylate cyclase.

**Goq Adenylate Cyclase, and PAK Are Involved in PAF-mediated Signaling to Src and STAT-3.**—HUVECs, incubated overnight in basal medium, were pretreated for 30 min with: A, PAF receptor antagonists (10 μM LAU-8080, 10 μM BN-52021, or 3 μM CV-3988); or B, GPA-2 (25 μM), GPA-2A (10 μM), suramin (60 μM), PTX (1 μg/ml, overnight), or SQ-22536 (SQ, 50 μM, adenylate cyclase inhibitor), and then exposed to PAF (10⁻⁷ M) for various time points. Cells were lysed using 0.1 M HCl after each time point. cAMP levels in the cell lysates were estimated using the Direct cAMP enzyme immunoassay kit (Sigma-Aldrich) to assess adenylate cyclase activity and are represented as percentage of control against time (control being set at 100%). Typical results of one of the three independent experiments are shown.
Activation of Src and FAK by PAF

As seen in Fig. 4B, Src was maximally phosphorylated at 1 and 60 min of PAF exposure, which was antagonized at all time points by the PAF receptor antagonist LAU-8080. A time-dependent decrease in PAF-induced phosphorylation of Src was observed when the Goq protein and adenylate cyclase were inhibited, whereas Src phosphorylation by PAF was reduced to nadir upon inhibition of PKA activity in HUVECs. These observations suggest that signaling to Src through the PAF-PAF receptor pathway requires the involvement of the PAF receptor-coupled Goq protein and activation of adenylate cyclase, whereas there is an absolute requirement of PKA activation for PAF-induced Src phosphorylation.

In a subsequent experiment, phosphorylation of STAT-3 was analyzed under similar conditions (Fig. 5A). Although PAF-induced STAT-3 phosphorylation was antagonized at all time points by LAU-8080 suggesting the signaling to STAT-3 was through the activation of PAF-receptor by PAF, only the immediate (1 min) PAF-stimulated phosphorylation of STAT-3 was attenuated by blocking Goq protein, adenylate cyclase, and PKA (Fig. 5B). Continuous PAF exposure recovered this initial attenuation of STAT-3 phosphorylation in a time-dependent manner up to 30 min, suggesting that the immediate phosphorylation of STAT-3 signals through the PAF-PAF receptor pathway involving Goq protein, adenylate cyclase, and PKA, whereas the delayed (30 min) and prolonged (60 min) STAT-3 phosphorylation by PAF is through the PAF-PAF receptor mechanism that is independent of these signaling intermediates in HUVECs. Our previous data suggests that STAT-3 is phosphorylated by JAK-2 at these later time points (33).

**PAF Phosphorylates PLCβ and PLCγ**—Among the various isoforms of PLCβ and PLCγ, we observed that PLCβ1, PLCβ2, PLCβ3, PLCγ1, and PLCγ2 were expressed in HUVECs, whereas PLCβ4 was not (data not shown). PAF induced phosphorylation of PLCβ3 and PLCγ1 but had no effect on the other PLC isoforms (data not shown). To elucidate the involvement of the PAF-signaling mechanism in the phosphorylation of PLCβ3, growth factor-starved HUVECs were pretreated for 30 min in the presence of LAU-8080, pertussis toxin, GP Antagonist-2A, and Src inhibitor PP1 (Fig. 6A). After this pretreatment, HUVECs were stimulated with PAF for various time intervals. Phospho-PLCβ3 and PLCβ3 levels are shown in Fig. 6A. As seen in Fig. 6B, PAF alone induced an immediate (1 min) phosphorylation of PLCβ3, which reached a maximum at 15 min of PAF exposure and returned to basal levels at prolonged (60 min) PAF stimulation. Inhibition of G proteins by pertussis toxin or Src by PP1 had no effect on PAF-induced PLCβ3 phosphorylation, suggesting that PAF does not signal to PLCβ3 through the pertussis toxin-sensitive G proteins or through Src. However, LAU-8080 and GP Antagonist-2A antagonized phosphorylation of PLCβ3 by PAF at all time points.
FIG. 6. PAF induces phosphorylation of PLCβ3 and PLCγ1. HUVECs, incubated overnight in basal medium, were pretreated with LAU-8080 (10 μM), GPA-2A (10 μM), or PP1 (170 nM) for 30 min, or overnight with PTX (1 μg/ml), followed by 10^{-7} M PAF exposure for various time intervals. After each time point, cells were lysed and the lysate was separated using 7.5% SDS-PAGE and Western blotting. The phosphorylated proteins were revealed by incubating with anti-phospho-PLCβ3 antibody (A) or anti-phospho-PLCγ1 antibody (C) followed by chemiluminescence detection. The membranes were stripped and subsequently detected with the respective anti-protein antibodies to verify protein levels. B, densitometric analysis of phospho-PLCβ3 levels, represented as percentage of control. D, phospho-PLCγ1 levels, represented as percentage of control against time after densitometric analysis (control being set at 100%). Typical results of one of the three independent experiments are shown.
suggesting that PAF induces PLCβ3 phosphorylation by stimulating its receptor and activating pertussis toxin-independent Gαq protein.

Phosphorylation and activation of PLCγ1 were investigated under similar conditions (Fig. 6C). As seen in Fig. 6D, immediate (1 min) phosphorylation of PLCγ1 by PAF reached maximal level at 15 min, dropping to baseline at 30 min of PAF exposure, followed by rephosphorylation at 60 min of PAF stimulation. PAF receptor antagonist LAU-8080 attenuated PLCγ1 phosphorylation below the baseline at all time points. Exposure of HUVECs to PP1 did not alter PAF-induced PLCγ1 phosphorylation up to 30 min. However, attenuation of PLCγ1 phosphorylation by PP1 was observed at 45-min PAF stimulation, which continued until 60 min of PAF exposure, suggesting that prolonged stimulation of PAF receptor by PAF involves signaling through Src to activate PLCγ1. Exposure of HUVECs with pertussis toxin or GP Antagonist-2A prior to stimulation with PAF did not change the pattern of PAF-induced PLCγ1 phosphorylation at all time points, suggesting that PAF receptor-associated pertussis toxin-sensitive and -insensitive Gα proteins are not required for the activation of PLCγ1 upon binding of PAF to its receptor.

Phosphorylation of FAK and Its Binding to Various Proteins Is Mediated by PAF—We hypothesized that activation of FAK involves the PAF-FAK receptor-signaling mechanism in HUVECs and that the pathway of PAF-induced FAK activation is different from that involved in activation of Src by PAF. To test this hypothesis, HUVECs, grown overnight in serum-depleted basal medium, were preincubated with LAU-8080, GP Antagonist-2A, SQ-22536, or H-89 for 30 min followed by PAF exposure for various time points (Fig. 7A). Rapid phosphorylation of FAK occurred at 1 min of PAF exposure, which decreased to basal level at 10 min, followed by a time-dependent rephosphorylation up to 60 min of PAF stimulation (Fig. 7B). Neither inhibition of adenylate cyclase by SQ-22536, nor PKA by H-89 could alter the PAF-dependent phosphorylation of FAK at all time intervals. This suggests that both adenylate cyclase and PKA, which are important for activation of Src in the PAF-signaling cascade, are not required for activation of FAK by PAF. Attenuation of PAF-induced FAK phosphorylation was observed at all time points in the presence of LAU-8080 and GP Antagonist-2A, suggesting that PAF receptor and its associated Gαq protein are activated upon binding of PAF, leading to activation and phosphorylation of FAK in a time-dependent manner.

To elucidate the proteins that bind to FAK upon stimulation of HUVECs with PAF, serum-starved HUVECs were preincubated with LAU-8080 and GP Antagonist-2A for 30 min and then exposed to PAF for 10 min. FAK was immunoprecipitated from the lysate and various antibodies specific to the proteins that bind to FAK were used to analyze the PAF-induced FAK-binding proteins. PAF induced the binding of p130Cas, Src, SHC, and paxillin to FAK, which was decreased in the presence of the PAF receptor antagonist LAU-8080 and upon blocking the Gαq protein with GP Antagonist-2A, suggesting that activation of the PAF receptor and Gαq protein by PAF is required for the binding of these proteins to FAK in HUVECs (data not shown).

PAF Induces TIMP2 and MT1-MMP Expression, Leading to Activation of MMP2—To elucidate the involvement of the PAF signaling cascade in the induction of expression and activation of matrix metalloproteinases, growth factor-starved HUVECs were preincubated for 30 min with LAU-8080, GP Antagonist-2A, AG-490 (JAK-2 inhibitor), and PP1 (Src inhibitor) followed by exposure to PAF for various time points. After each time point, the cells were collected and either lysed or processed for preparation of the membrane fraction, whereas the medium was collected and concentrated. TIMP2 expression in cellular lysates (Fig. 8A) and concentrated growth medium (Fig. 8B) was maximally induced by PAF at the 8-h time point (Fig. 8, B and D). PAF-induced expression of TIMP2 was attenuated in the lysate as well as growth medium upon inhibition of the PAF receptor, Gαq, JAK-2, and Src at this time point, suggesting the involvement of PAF-signaling mechanism in TIMP2 expression.

Protein expression and proteolytic activation of MT1-MMP was induced in the lysate (Fig. 8E) and membrane fraction of HUVECs (Fig. 8G) after 8 h of PAF stimulation (Fig. 8, F and H), which rapidly increased through 16–24 h of PAF exposure. Inhibition of the PAF receptor, its associated Gαq protein, JAK-2, and Src by specific inhibitors reduced the expression and activation of MT1-MMP at all time points, suggesting that the PAF-PAF receptor signaling is involved in MT1-MMP expression and activation.

PAF signaling cascade had no affect on the level of pro-MMP2 in the cellular lysate or concentrated growth medium (Fig. 8, K and D), confirming our recent data that MMP2 mRNA levels are not affected by PAF (55). However, as expected, after 8 h of PAF stimulation, an elevated level of active MMP2 was observed in the medium, which increased rapidly at 16 and 24 h of PAF exposure (Fig. 8J). Again, attenuation of PAF-induced MMP2 activation by LAU-8080, GPA-2A, AG-490, and
The PAF receptor pathway induces proteolytic activation of MMP2 by coordinated expression of MT1-MMP and TIMP2. Stimulation of HUVECs by PAF induces increased expression of TIMP2 by 8 h as observed in both cellular lysates (A and B) and in concentrated growth medium (C and D). Inhibition of the PAF receptor with LAU-8080, the Gαq protein with GPA-2A, JAK-2, with AG-490, or Src with PP1 all completely abrogated the increased expression observed with PAF alone. Likewise, MT1-MMP, which proteolytically activates pro-MMP2 to active MMP2 in conjunction with TIMP2 on the extracellular surface of the cellular membrane, was induced by PAF as observed in cellular lysates (E and F) and in membrane fractions (G and H). Again, each of the inhibitors tested reduced expression of MT1-MMP. The coordinated induction of TIMP2 and MT1-MMP by the PAF signaling cascade resulted in proteolytic activation of pro-MMP2 to active MMP2 in the growth medium (I and J). As expected, neither MMP2 nor changes in pro-MMP2 levels were observed intracellularly from cellular lysates (K).
PP1 at all time points suggests that the PAF-signaling mechanism is involved in the proteolytic activation of MMP2 in the medium.

DISCUSSION

PAF is one of the most potent phospholipid agonist that transmits outside-in signals to intracellular transduction systems and effector mechanisms in a variety of cell types. PAF has been shown to be important in inflammatory and immune responses, as well as in vascular functions (35). Many types of cells, including macrophages, platelets, basophils, neutrophils, eosinophils, and endothelial cells produce PAF (36, 37). The PAF signaling system is a point of convergence at which injurious stimuli can trigger and amplify both acute inflammatory and thrombotic cascades, amplify these cascades when acting with other mediators, and mediate molecular and cellular interactions (cross-talk) between inflammation and thrombosis (reviewed in Ref. 38). Many studies involving exogenous administration of PAF or its endogenous generation in experimental animals indicate that it has signaling roles in vitro and in vivo (39). PAF has been shown in earlier studies to be a fluid-phase mediator that could activate platelets and various leukocytes in suspension (36). PAF, in concert with polypeptide mediators, may have an autocrine action on tumor cells by promoting their motility and proliferation, and paracrine action on endothelial cells by stimulating the development of new vessels (40). We have previously demonstrated that binding of basic fibroblast growth factor to its receptor, results in PAF production and the activation of the PAF receptor-signaling mechanism in HUVECs (33). In the same study we also demonstrated the involvement of a PAF-induced dual kinase mechanism for the activation of STAT-3, involving Src and JAK-2. Results presented in this report indicate the signaling components involved in the stimulation of multiple pathways upon activation of PAF receptor by PAF in HUVECs.

The intracellular actions of PAF are mediated through the PAF receptor that is expressed on the surface of a variety of cell types (recently reviewed in Ref. 41). The PAF receptor is associated with heterotrimeric guanine nucleotide-binding proteins, which in part are responsible for relaying the binding of PAF to an intracellular signal (42). Haribabu et al. (8) have reported that, although G proteins involved in chemotactic factor response are often pertussis toxin-sensitive, chemotactic responses to PAF seem to be extremely pertussis toxin-resistant. The PAF receptor can interact with multiple G proteins, resulting in the simultaneous activation of distinct signaling pathways depending on the cell type (9). Our results indicate that, in HUVECs, stimulation of the PAF receptor by PAF leads to the downstream activation of Src in a time-dependent biphasic fashion. Attenuation of PAF-induced Src phosphorylation was observed when the pertussis toxin-insensitive Gαq protein was specifically blocked, suggesting the involvement of Gαq protein in PAF-mediated signaling to Src (Fig. 9). Stimulation of Src phosphorylation by PAF was also attenuated by pretreatment of HUVECs with the broad spectrum G protein inhibitor suramin, but this was less than that observed upon blocking Gαq protein. Neither pretreatment with pertussis toxin nor GP Antagonist-2, which blocks the pertussis toxin-sensitive Gαq and Gαi proteins, could attenuate Src phosphorylation by PAF, further confirming that PAF signals through Gαq protein to Src in HUVECs. The biphasic nature of Src phosphorylation may occur as a result of immediate (1 min) stimulation of Src following PAF receptor activation, followed by prolonged (60 min) activation via feed forward activation of PLA2 and further production of PAF, with subsequent stimulation of the PAF receptor (Fig. 9).

Stimulation of cAMP has been demonstrated when cultured mesangial cells were exposed to PAF (12). However, in CHO cells expressing wild type or mutant PAF receptor, PAF exposure led to the inhibition of forskolin-induced cAMP suggesting the inhibition of adenylate cyclases in these cells (13). In neutrophils, the basal levels of cAMP were unaltered upon stimulation with PAF suggesting that adenylate cyclase is not activated when these cells are exposed to PAF (43). Our studies demonstrated that exposure of HUVECs to PAF maximally increases the intracellular cAMP levels at 10 min of PAF stimulation. Attenuation of PAF-induced cAMP at all time points by PAF receptor antagonists and adenylate cyclase inhibition suggests that PAF activates adenylate cyclase through the PAF receptor. Involvement of G proteins in the activation of adenylate cyclase by PAF was observed when the broad spectrum inhibitor suramin decreased the cAMP levels at 10 min of exposure, which could not be restored by PAF stimulation.
None of the pertussis toxin-sensitive G proteins was involved in PAF signaling to adenylate cyclase, because the cAMP levels were unaltered upon exposure of HUVECs to either pertussis toxin or GP Antagonist-2. However, specific blocking of Gαq protein by GP Antagonist-2A significantly attenuated the levels of cAMP, which could not be restored by PAF, suggesting that Gαq protein is involved in the PAF-PAF receptor signaling to activate adenylate cyclase in HUVECs. Furthermore, our results demonstrate that PAF-dependent PKA activation is maximal at 10 min of PAF exposure, which is antagonized by PAF receptor antagonists suggesting the involvement of the PAF-PAF receptor signaling mechanism involved in activation of PKA. Blocking of pertussis toxin-insensitive G proteins did not alter the effect of PAF on activation of PKA. However, inhibition of pertussis toxin-insensitive Gαq protein and adenylate cyclase, respectively, significantly attenuated PKA activation by PAF, suggesting the involvement of Gαq protein and adenylate cyclase in the PAF-dependent intracellular signaling cascade leading to the activation of PKA in HUVECs.

The activation of Src by the cAMP/PAK cascade has been shown to regulate cellular functions in a number of cell lines. Schmitt and Stork (44) have shown that cAMP antagonizes growth factor-dependent activation of cell growth via PKA and Src in fibroblasts. In our previous study, we showed that PAF can independently lead to phosphorylation of Src through the PAF receptor in HUVECs (33). At that time, we were not able to determine if Src phosphorylation by PAF was due to its direct interaction with the PAF receptor or indirectly through the activation of intermediate signaling molecules. Our present study demonstrates that PAF signals to Src by activation of the PAF receptor and its associated Gαq protein, because Src phosphorylation by PAF was significantly attenuated by the PAF receptor antagonist LAU-8080 and GP Antagonist-2A, which specifically blocks the pertussis toxin-independent Gαq protein. Furthermore, phosphorylation of Src by PAF was reduced in a time-dependent manner upon inhibition of adenylate cyclase activity, whereas attenuation of PKA activity reduced the PAF-induced Src phosphorylation to nadir. We demonstrate for the first time that both adenylate cyclase and PKA are involved in the activation of Src by PAF through the Gαq protein-coupled PAF receptor in HUVECs.

The presence of a dual kinase mechanism in HUVECs, involving JAK-2 and Src, for the activation of STAT-3 upon binding of PAF to its PAF receptor has been demonstrated by us (33). The PAF receptor antagonist LAU-8080 attenuated the immediate (1 min) phosphorylation of STAT-3 observed in this report upon stimulation of HUVECs with PAF. Furthermore, PAF-induced STAT-3 phosphorylation was also attenuated at 1 min of PAF exposure upon specifically blocking Gαq protein, adenylate cyclase, and PKA, suggesting that the immediate phosphorylation of STAT-3 by PAF involves activation of its specific receptor, Gαq protein, adenylate cyclase, and PKA. Because Src is also activated by the same mechanism observed earlier, our study suggests that PAF binds to its receptor and induces an immediate signal-transduction cascade involving the sequential activation of Gαq protein, adenylate cyclase, PKA, and Src, finally leading to the activation of STAT-3. Continuous exposure of HUVECs by PAF induces high levels of STAT-3 phosphorylation that are antagonized by the PAF receptor antagonist LAU-8080 at all time points and are unaffected by any other antagonist, suggesting that the delayed (30 min) and prolonged (60 min) phosphorylation of STAT-3 through the PAF-PAF receptor signaling mechanism is independent of these signaling intermediates. Because we had demonstrated earlier that JAK-2 is involved in the delayed (30 min) activation of STAT-3 and that the prolonged (60 min) PAF-induced STAT-3 phosphorylation depends on both JAK-2 and Src (33), our present study confirms these observations and also explains the independence from the requirement of any signaling intermediates for phosphorylation of STAT-3 by PAF at these time points.

Three classes of phospholipase C (PLC) have been observed, PLCβ, PLCγ, and PLCδ, of which each occurs in several isoforms (16). All four PLCβ isoforms are activated to varying extent by Gαq protein (17), whereas PLCβ2 and PLCβ3 are also activated by the βγ subunit of the Gi family of G proteins (18). Tyrosine phosphorylation of PLCγ1 is required for its activation, but the mechanism by which PLCδ8 is activated is unknown (19). We sought to determine whether the PLCβ isoforms are activated by PAF in HUVECs; only PLCβ3 and PLCγ1 were phosphorylated by PAF stimulation. The initial PAF-induced PLCβ3 phosphorylation reached maximal levels at 15 min and returned to baseline at 30 min of PAF exposure. Blocking the PAF receptor and Gαq protein by LAU-8080 and GP Antagonist-2A, respectively, antagonized the PAF-induced PLCβ3 phosphorylation at all time points, suggesting that PLCβ3 is activated by PAF through its specific receptor and associated Gαq protein. Exposure of HUVECs to pertussis toxin or PP1, which is a specific inhibitor of Src, did not have any effect on the PLCβ3 phosphorylation induced by PAF, suggesting that pertussis toxin-sensitive G proteins or Src are not involved in the activation of PLCβ3 by PAF. Our observations are in accordance to those of Ali et al. (45), who have shown that PAF uses the pertussis toxin-insensitive G protein to activate PLCβ3 in RBL-2H3 cells.

Although most G protein-coupled receptors activate PLCβ, stimulation of G protein-coupled angiotensin II receptors in rat aortic smooth muscle cells caused tyrosine phosphorylation of PLCγ1, which is eliminated by the introduction of anti-pp60Src antibodies in these cells (21). Our study demonstrates that the initial phosphorylation of PLCγ1 upon stimulation of HUVECs with PAF was maximal at 15 min, after which it decreases with time to 30 min but is re-phosphorylated after 60 min in the presence of PAF. Attenuation of PLCγ1 phosphorylation by the PAF receptor antagonist suggests that the activation of PLCγ1 by PAF is specific to the PAF receptor. Signaling of PAF to PLCγ1 through the PAF receptor does not involve the Gαs proteins, because blocking the Gαs proteins with either pertussis toxin or GP Antagonist-2A did not alter the effect of PAF on PLCγ1 phosphorylation. Whether PAF signaling to PLCγ1 is through the activation of Gβγ subunits, remains to be determined. Our observation suggests that Src-dependent phosphorylation of PLCγ1 in PAF stimulated HUVECs is a downstream event in the PAF-PAF receptor signaling cascade, because attenuation of PAF-stimulated PLCγ1 phosphorylation was observed after 30-min exposure to PP1, a specific inhibitor of Src.

Multiple stimuli, acting on distinct cell surface receptors, including growth factors, neuropetides, and lysophosphatidic acid, stimulate tyrosine phosphorylation of FAK (46–48). FAK has been implicated in a number of biological processes, including controlling the rate of cell spreading, cell migration, and generating an antiapoptotic signal in response to cell adhesion (49, 50). Of the six tyrosine residues within the FAK that have been identified as sites of phosphorylation, the major site of autophosphorylation is tyrosine 397 (51, 52), which lies just upstream of the kinase domain and is a docking site for the SH2 domains of a number of proteins, including the Src family of tyrosine kinases (51). Our results have demonstrated that PAF stimulates an immediate (1 min) phosphorylation of FAK on tyrosine 397, which reaches baseline at 10 min, followed by re-phosphorylation in a time-dependent manner at 50 min of PAF exposure in HUVECs. The PAF-induced phosphorylation...
of FAK signals through the PAF receptor coupled to its associated Goα protein and is independent of the involvement of adenylate cyclase or PKA. As expected, phosphorylation and activation of FAK by PAF induces the binding of a number of proteins to FAK, including Src, p130Cas, SHC, and paxillin. The binding of these proteins to FAK is decreased in the presence of PAF receptor antagonists and upon blocking of the Goα protein. Because these proteins lead to binding of adaptor proteins to the complex, which stimulate a variety of different signaling pathways involved in the regulation of intracellular events, it is tempting to speculate that PAF may be a key molecule that is responsible for a major regulatory pathway in HUVECs.

Matrix metalloproteinases (MMPs) and the associated tissue inhibitors of metalloproteinases (TIMPs) are major components in the process of cellular invasion (30). PAF has been shown to activate gene expression of TIMP1 and -2 and lead to overexpression of MMP9 in corneal epithelial cells, thus creating an imbalance leading to corneal ulcers (53). In human uterine cervical fibroblasts, PAF accelerates collagenolysis by inducing imbalance leading to corneal ulcers (53). In human uterine fibroblasts, PAF accelerates collagenolysis by inducing the activation of TIMP2 expression and activation of MT1-MMP by PAF. Because these proteins lead to binding of adaptor proteins to the complex, which stimulate a variety of different signaling pathways involved in the regulation of intracellular events, it is tempting to speculate that PAF may be a key molecule that is responsible for a major regulatory pathway in HUVECs.

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Activation of Platelet-activating Factor Receptor-coupled Goq Leads to Stimulation of Src and Focal Adhesion Kinase via Two Separate Pathways in Human Umbilical Vein Endothelial Cells

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