Phosphorylation of STAT-3 in Response to Basic Fibroblast Growth Factor Occurs through a Mechanism Involving Platelet-activating Factor, JAK-2, and Src in Human Umbilical Vein Endothelial Cells

EVIDENCE FOR A DUAL KINASE MECHANISM

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Platelet-activating factor (PAF) is a potent proinflammatory phospholipid with multiple pathological and physiological effects. We have shown that basic fibroblast growth factor (bFGF) supplementation induces rapid proliferation of human umbilical vein endothelial cells (HUVEC), which is reduced upon removal of bFGF or by bFGF immunoneutralization. The PAF receptor antagonist LAU-8080 inhibited bFGF-stimulated HUVEC proliferation, indicating the involvement of PAF in the bFGF-mediated signaling of HUVEC. Although FGF receptor phosphorylation was not affected by LAU-8080, the bFGF-mediated prolonged phosphorylation, and activation of Erk-1 and -2 were attenuated. Phosphorylation of STAT-3 was observed in the presence of PAF or bFGF, which was attenuated by PAFR antagonists. PAF-induced STAT-3 phosphorylation observed in HUVEC pretreated with either Src inhibitor PP1 or JAK-2 inhibitor AG-490 indicated (i) immediate (1 min) phosphorylation of STAT-3 is dependent on Src, (ii) JAK-2-dependent STAT-3 phosphorylation occurs after the delayed (30 min) PAF exposure, and (iii) prolonged (60 min) STAT-3 phosphorylation may be either through Src and/or JAK-2. Attenuation of the STAT-3 phosphorylation by the PAFR antagonists indicated signaling through the PAF receptor. Taken together, these findings suggest the production of PAF is important for bFGF-mediated signaling and that a dual kinase mechanism is involved in the PAF-mediated signal transduction cascade.

PAF1 (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is an ether phospholipid second messenger that mediates a number of biological responses, including inflammatory and immune responses, shock, embryogenesis, and cell differentiation (for review, see Ref. 1). PAF is also a potent mediator of pathological angiogenesis associated with tumor expansion and metastasis (2, 3). Hepatocyte growth factor, tumor necrosis factor-α, and thrombopoietin have been shown to induce angiogenesis through a mechanism involving PAF (4, 5). Many cells produce PAF, including monocytes, endothelial cells, neutrophils, and lymphocytes, and these cell types can themselves become targets of PAF bioactions (6). PAF acts through a specific G-protein-linked receptor containing seven α-helical domains that span the plasma membrane (7) and has been localized to the plasma membrane (8) and a large endosomal compartment on human umbilical vein endothelial cells (HUVEC) (9). PAF also up-regulates the expression of its own receptor in several cell types including human alveolar macrophages (10) and rat epithelial cells (11), thus potentially providing a positive feedback loop for PAF action.

Of the 20 members of the FGF family of growth factors, only acidic FGF and basic FGF (bFGF) have been shown to regulate proliferation and migration of capillary endothelial cells (for review, see Refs. 12 and 13). Although bFGF does not contain a traditional signal sequence, it is now clear that it is secreted via a tightly regulated non-conventional secretory pathway and is localized in the basement membrane and extracellular matrix of numerous tissues (14). bFGF binds to its high affinity receptor (FGFR-1 and FGFR-2) and induces their dimerization and activation of protein-tyrosine kinase activity and autophosphorylation. The activation of the receptor induces the Ras-independent cascade (via activation of phospholipase Cγ) (15) and the Ras-dependent cascade (initiated via Grb-2, Sos, and Ras) (16), resulting in the activation of Raf. The Ras-dependent pathway results in the phosphorylation of mitogen-activated protein kinase kinase (MEK) and subsequent phosphorylation of MAP kinase (Erk) (17). Activation of Erk causes downstream activation of phospholipase A2. Using cloned guinea pig receptor stably expressed in Chinese hamster ovary cells, Honda et al. (18) have shown that PAF potentially activates MAP kinase and both the 42- and 44-kDa Erk-1 and Erk-2 and that PAF receptor couples to both pertussis toxin-sensitive and -insensitive G proteins in Chinese hamster ovary cells. Like the Ras-dependent cascade, the Ras-independent cascade also results in the activation of phospholipase A2, which is responsible for the hydrolysis of 1-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine to lyso-PAF and arachidonic acid. Lyso-PAF is converted to PAF by an acetyltransferase.

It has been demonstrated that PAF receptor activation was facilitated through both the associated G-protein and through...
an unknown tyrosine kinase (19). Although the study did not identify the tyrosine kinase, the data were consistent with a Janus kinase or Src-family kinase as possible suspects. The Janus kinase/signaling transducers and activators of transcription (JAK/STAT) pathway is one of the major mechanisms by which cytokine receptors transduce intracellular signals. To date, four mammalian JAKs (JAK-1, JAK-2, JAK-3, and TYK-2) and seven STATs have been identified and characterized (20). Both JAKs and STATs become phosphorylated on their tyrosine residues followed by homo- or heterodimerization, nuclear translocation, and finally transcriptional activation of specific genes (21). Thrombopoietin induced a rapid phosphorylation of STAT-5B (a target of JAK-1, JAK-2, JAK-3, and TYK-2), which was inhibited by the PAF receptor antagonist WEB-2170 (22). In a recent study, Lukashova et al. (23), using monocytic cell lines U937 and MonoMac-1, show that G-protein-independent activation of TYK-2 occurred after activation of the PAF receptor with PAF, which was followed by a time-dependent activation and tyrosine phosphorylation of signal transducers and activators of transcription (STAT-1, STAT-2, STAT-3, and STAT-5) (23). Dhar and Shukla (24) introduced anti-γ-Src antibody into rabbit platelets and found that PAF-stimulated inositol phosphate production and aggregation were significantly reduced. Furthermore, PAF caused phosphorylation of both Src and phospholipase Cγ in rabbit platelets and A431 cells, suggesting direct phosphorylation of phospholipase Cγ by Src.

In the present report we show that bFGF stimulates HUVEC proliferation, which is antagonized by the PAF receptor (PAFR) antagonist LAU-8080. The PAFR antagonists do not affect activation of the FGF receptor by bFGF, but the downstream activation of Erk by bFGF is partially dependent on PAF. Antagonism of the bFGF-stimulated STAT-3 phosphorylation by PAFR antagonists suggests the importance of STAT-3 in PAF-mediated signaling. Also, prolonged activation of STAT-3 by bFGF may be through the production of PAF and activation of PAFR. JAK-2 and Src are both involved in bFGF-mediated HUVEC proliferation and are also phosphorylated upon exposure of HUVEC to bFGF. PAFR antagonists attenuate bFGF-stimulated JAK-2 phosphorylation but have no effect on the phosphorylation of Src by bFGF. Finally, Src is involved in the PAF-stimulated immediate phosphorylation of STAT-3, whereas JAK-2 is pivotal in the delayed STAT-3 phosphorylation. The prolonged phosphorylation of STAT-3 may be either through Src and/or JAK-2.

**EXPERIMENTAL PROCEDURES**

**Reagents—** PAF was obtained from Sigma-Aldrich, PAF receptor antagonists CV-3988 and BN-52021 (Ginkgolide B) were obtained from Biomol Research Laboratories Inc., Plymouth Meeting, PA, and LAU-8080 was synthesized by Prof. Julio Alvarez-Builla at the Universidad de Alcalá, Madrid, Spain. Antibodies used were monoclonal mouse anti-FGF-receptor (Ab-1) and monoclonal mouse anti-bFGF (Ab-3) (OncoGenex Research Products, Boston, MA), monoclonal mouse anti-phosphotyrosine (Cell Signaling Technology, Beverly, MA), rabbit antisera to JAK-2 and rabbit polyclonal anti-human TYK-2 (Upstate Biotechnology, Lake Placid, NY), monoclonal mouse anti-phospho-JAK-2 (Sigma-Aldrich), rabbit polyclonal anti-STAT-3, rabbit polyclonal anti-phospho-STAT-3, horseradish peroxidase-conjugated goat anti-mouse, and goat anti-rabbit antibodies (Cell Signaling Technology). Phosphorylated Erk-1 and -2 were detected using rabbit polyclonal anti-phospho-p44/42 MAP kinase, and MAP kinase activity was detected using the nonradioactive p44/42 MAP kinase assay kit (Cell Signaling Technology, Beverly, MA). PAFR antagonists were from Biomol Research Laboratories, Logan, UT. Aortic smooth muscle cells (ASMC) were a kind gift from Dr. Emel Songu-Mize and were maintained on M199 medium (Sigma-Aldrich).

**Immunoprecipitation of FGFRI and Erk Activity—** HUVEC were incubated with bFGF and/or 10 μM LAU-8080 for 10 min at 37°C. Cells were then lysed with the cell lysis buffer, and cell lysates were incubated with anti-FGF receptor Ab-1 antibody at 4°C overnight and precipitated by incubation with 100 μg of protein G-agarose for 2 h at 4°C. After washing 3 times with lysis buffer, complexes were dissolved in 1× loading buffer, separated by 7.5% SDS-PAGE, blotted onto a nitrocellulose membrane, incubated with anti-phosphotyrosine antibody for 1 h at room temperature, and detected using the chemiluminescent detection kit (Pierce). Phosphorylated Erk-1 and -2 were detected using the antibodies on a 12% SDS-PAGE (40 μg protein/lane), blotted onto a nitrocellulose membrane, incubated with anti-phospho-p44/42 Erk antibody, and chemiluminescent detection. Erk activity in HUVEC treated with bFGF and/or 10 μM LAU-8080 for different time points was detected using the nonradioactive p44/42 Erk assay kit. Briefly, 200 μg of cell lysate protein was incubated with immobilized phospho-p44/42 Erk antibody overnight at 4°C. For the positive control, 2 ng of active Erk was added to control cell lysate and treated subsequently like all the other samples. The samples were centrifuged, and the pellets were washed twice with lysis buffer followed by kinase buffer. Pellets were then suspended in kinase buffer and supplemented with 200 μM ATP and 2 μg of Elk-1 fusion protein. After incubation for 30 min at 30°C, the reaction was terminated with 3× SDS sample buffer. Samples were boiled for 5 min, separated on a 12% SDS-PAGE, blotted onto nitrocellulose membranes, incubated with phospho-Erk-1 antibody, and detected by chemiluminescence.

**Western Blot Analysis—** The growth factor-starved HUVEC were exposed to either 10-7 M PAF, bFGF, 10 μM LAU-8080, 10 μM BN-52021, or 3 μM CV-3988 for various time points. To inhibit the Src or JAK-2 pathways, overnight starved cells were pretreated for 30 min with either 170 nM PP1 or 50 μM AG-490. After the completion of each time point, cells were lysed in the cell lysis buffer containing 0.1% w/v bromphenol blue, and samples were boiled for 5 min and separated on a 7.5% SDS-PAGE (40 μg total protein/lane). The separated proteins were then transferred onto nitrocellulose membranes, blocked with Tris-buffered saline containing 5% nonfat dry milk for 1 h at room temperature, and incubated with the antibodies against the phosphorylated forms of the Erk kinases or phospho-Src antibodies. Membranes were stripped by incubation in 1× Tris-HCl (pH 6.8), 10% SDS, and 10 mM 2-mercaptoethanol for 30 min at 55°C. After washing, membranes were blocked with blocking
factors were added to the basal medium individually or in combination at 37 °C. Proliferation was measured by the incorporation of [3H]thymidine after 144 h. No loss of proliferation was seen when LAU-8080 was combined with IGF-1 and EGF. Only a slight loss of proliferation was observed with VEGF. There was a significant loss of proliferation in medium containing bFGF, indicating that PAF is involved in bFGF-mediated proliferation of HUVEC (p < 0.05, Student’s t test). Panel B, a mixture of growth factors was added to each well such that only one growth factor was omitted from the medium and the other three remained, with or without LAU-8080. A loss of proliferation is observed in every instance where bFGF remains in the medium but is lost upon removal of bFGF. Panel C, basal medium was pretreated individually with bFGF, monoclonal anti-bFGF Ab-3 antibody, LAU-8080, boiled Ab-3, or with combinations of the above for 4 h at 37 °C. Growth factor-starved HUVEC were then added and analyzed by [3H]thymidine incorporation. Panel D, minimum essential medium with Earle’s salts supplemented with 10% FBS or M199 medium was pretreated for 4 h with bFGF, 10 μM LAU-8080, 10 μM BN-52021, or 3 μM CV-3988 individually or in combination at 37 °C. WI-38 or ASMC were then transferred, respectively, and proliferation was measured by [3H]thymidine incorporation after 144 h.

buffer and reprobed with antibodies against the non-phosphorylated proteins, respectively, and developed as described above.

RESULTS

PAF Is Involved in the bFGF-mediated HUVEC Proliferation—PAF receptor antagonist LAU-8080 was used to elucidate the involvement of PAF in HUVEC proliferation mediated by the bFGF-signaling pathway. The basal level of HUVEC growth was achieved by an overnight incubation of HUVEC in basal medium. After establishing the basal growth rates, bFGF, VEGF, EGF, and IGF-1 were added individually to HUVEC maintained on EBM-2 supplemented with 2% FBS with or without 10 μM LAU-8080. As seen in Fig. 1A, a small but significant reduction in HUVEC growth was observed when LAU-8080 was included in the medium containing VEGF. However, the addition of LAU-8080 to the medium containing bFGF resulted in a much larger growth reduction of HUVEC. In a subsequent experiment, a mixture of each of the above growth factors was added whereby only one growth factor was removed from the medium, and the other three remained with or without the presence of LAU-8080. Growth reduction in the presence of LAU-8080 was observed when VEGF, EGF, and IGF were removed from the medium, whereas the growth of HUVEC was not reduced by LAU-8080 when bFGF was removed from the medium (Fig. 1B). These observations suggest that the involvement of PAF in the bFGF-mediated signaling pathway is affected by the PAFR antagonist LAU-8080.

Attenuation of the bFGF-stimulated HUVEC proliferation was observed after bFGF immunoneutralization using monoclonal anti-bFGF Ab-3 antibody (Fig. 1C), suggesting that the bFGF-mediated signaling pathway stimulates HUVEC proliferation. The addition of monoclonal antibody Ab-3 alone or the boiled antibody (used as a negative control) had no effect on HUVEC proliferation. Attenuation in HUVEC proliferation was observed when LAU-8080 was added to HUVEC along with bFGF. LAU-8080 alone had no effect on HUVEC proliferation as compared with the basal level. Attenuation of HUVEC proliferation by the PAF receptor antagonist suggests the involvement of PAF at some point in the bFGF-mediated signaling of HUVEC. As seen in Fig. 1D, bFGF also stimulated the proliferation of the lung fibroblasts (WI-38) and ASMC, which was attenuated by the PAF receptor antagonists LAU-8080, BN-52021, and CV-3988. This observation further suggests that the growth promoting effect of bFGF is blocked by the PAF receptor antagonists not only in HUVEC but also in other cell types.

Phosphorylation of FGFR and Erk—Binding of bFGF to the FGFR leads to its immediate activation and phosphorylation at tyrosine residues. To elucidate the effect of PAF receptor antagonists LAU-8080 on the level of FGFR phosphorylation, HUVEC were exposed to a short (10 min) pulse of bFGF with or without 10 μM LAU-8080. Antibodies specific for phosphotyrosine revealed the immunoprecipitated FGFR. As shown in Fig. 2, there is no significant difference in the level of tyrosine phosphorylation or activity of Erk-1 and -2 were observed until 15 min.

We hypothesized that activation of FGFR by bFGF would lead to the early phosphorylation and activation of Erk, whereas prolonged stimulation of Erk would be through downstream production of PAF, antagonized by LAU-8080. To test this hypothesis, HUVEC were starved of growth factors overnight (EBM supplemented with 0.2% FBS), after which they were exposed to bFGF with or without 10 μM LAU-8080, and the levels of phosphorylation (Fig. 3A) and activity (Fig. 3B) of Erk-1 and -2 were determined. As seen in Fig 3C, no phosphorylation or activity of Erk-1 and -2 were observed until 15 min.
in independent experiments is shown. Phospho-Erk-1, phospho-Erk-2, and phospho-Elk-1 levels obtained after 

Fig. 4. PAF antagonism inhibits phosphorylation of STAT-3. Growth factor-starved HUVEC were exposed to endothelial growth medium containing bFGF, VEGF, EGF, and IGF-1 for various time points with or without the PAFR antagonists (10 μM LAU-8080, 10 μM BN-52021, or 3 μM CV-3988). Cell lysates obtained after each time point were separated on a 7.5% SDS-PAGE and Western-blotted. Panel A, phosphorylated STAT-3 levels were obtained after incubation with phospho-STAT-3 antibody and chemiluminescent detection. The proteins levels were verified by stripping and reblotting with STAT-3 antibody. Panel B, levels of phospho-STAT-3 obtained after densitometric analysis and represented as percentage of control against time (control being set at 100%). The results of one of the three independent experiments are shown.

JAK-2 and Src Are Involved in the Growth-promoting Effects of bFGF on HUVEC—JAK-2 inhibitor AG-490 and Src inhibitor PP1 were used to elucidate the involvement of JAK-2 and Src in bFGF-mediated HUVEC proliferation. After establishing basal growth rates, HUVEC were exposed to bFGF, AG-490, or PP1 individually or in combination. As seen in Fig. 6, bFGF-stimulated HUVEC proliferation was attenuated by

STAT-3 phosphorylation in HUVEC stimulated with EGM-2 obtained after each time point

Fig. 3. PAFR antagonist LAU-8080 attenuated prolonged Erk-1 and Erk-2 phosphorylation and activity. Panel A, HUVEC were exposed to bFGF and/or LAU-8080 for 1–60 min. The cell lysates obtained were separated on a 12% SDS-PAGE and blotted onto a nitrocellulose membrane. Phosphorylated Erk-1 and Erk-2 were revealed by incubation with phospho-p44/42 Erk antibody and chemiluminescent detection. Panel B, Erk activity in the cells treated with bFGF and/or LAU-8080 for different time points was revealed by incubating the immunoprecipitated Erk with 200 μM ATP and Elk-1 fusion protein for 30 min at 30 °C. The kinase reaction was terminated with SDS sample buffer, separated on a 12% SDS-PAGE, incubated with phospho-Elk-1 antibody, and detected by chemiluminescence. Panel C, phosphorylated Erk-1, phospho-Erk-2, and phospho-Elk-1 levels obtained after densitometric analysis. A representative result of one of the three independent experiments is shown.

After 15 min, phosphorylation and the activity of Erk-1 and -2 were observed, which peaked 30 min after treatment with bFGF and continued until 60 min after bFGF exposure. LAU-8080 attenuated the prolonged phosphorylation and activity of Erk-1 and -2 after 15 min in the cells treated with bFGF, demonstrating that prolonged activation of Erk by bFGF involves PAF.

Involvement of STAT-3—After an overnight incubation in EBM supplemented with 0.2% FBS, HUVEC were treated with 10 μM LAU-8080, 10 μM BN-52021, or 3 μM CV-3988 in the presence of EGM-2 (containing bFGF, VEGF, IGF-1, EGF, 2% FBS). STAT-3 phosphorylation in HUVEC stimulated with EGM-2 was investigated over time (Fig. 4A). As seen in Fig. 4B, there was a significant reduction in the level of STAT-3 phosphorylation in the presence of each of the PAF receptor antagonists (LAU-8080, BN-52021, CV-3988) as compared with vehicle-treated cells (Control). Because STAT-3 is phosphorylated by a number of cytokines and growth factors, inhibition of phosphorylation in the presence of the PAFR antagonists suggested that STAT-3 is one of the components involved in PAF-mediated signaling.

activation of STAT-3 by bFGF and PAF—Of the various members of the Janus kinase family of protein kinase, we have determined that TYK-2 and JAK-2 are expressed in HUVEC, whereas JAK-1 and JAK-3 are not (data not shown). TYK-2 is not phosphorylated even after stimulation of HUVEC with PAF or bFGF at all the time points (data not shown). JAK-2 is constitutively expressed in HUVEC regardless of the addition of PAF or bFGF (data not shown). STAT-1 and STAT-3 are expressed in HUVEC, but STAT-5a, STAT-5b, and STAT-6 are not (data not shown). HUVEC were exposed to bFGF after an overnight exposure to basal medium devoid of growth factors. As seen in Fig. 5, a slight increase in phosphorylation of STAT-3 was observed after 1 min followed by a rapid increase in STAT-3 phosphorylation after 15 min of bFGF exposure. This increased STAT-3 phosphorylation is maintained after prolonged exposure to bFGF alone through the 30- and 60-min time points. The PAF receptor antagonists LAU-8080, BN-52021, and CV-3988 antagonize the bFGF-stimulated STAT-3 phosphorylation at all time points. Phosphorylation of STAT-3 in the presence of PAF is not observed until 15 min of exposure, which peaks at the 30-min time point. All PAF antagonists antagonize this delayed phosphorylation of STAT-3 by PAF, suggesting that PAF causes the phosphorylation of STAT-3 through PAFR-associated kinases that are blocked by the PAFR antagonists. Exposure of HUVEC to bFGF and PAF alone results in maximal phosphorylation of STAT-3 after 30 min, suggesting that bFGF would induce the prolonged activation of STAT-3 through the production of PAF and activation of PAF receptor.
both AG-490 and PP1. Attenuation of bFGF-mediated proliferation was greater in the presence of AG-490 than PP1 and was comparable with that of AG-490 alone (without supplementation with bFGF). PP1 alone attenuated HUVEC proliferation, but this effect was to some extent overcome by stimulation of HUVEC by bFGF. These observations suggest that although signaling through JAK-2 is critical for HUVEC proliferation, both JAK-2 and Src are involved in bFGF-mediated HUVEC growth.

**Effect of bFGF on the Phosphorylation of JAK-2 and Src**—Growth factor-starved HUVEC were exposed to bFGF with or without the PAFR antagonists, and the level of JAK-2 and Src phosphorylation was investigated at different time intervals. Phospho-JAK-2 and phospho-Src levels are shown in Fig. 7A. As seen in Fig. 7C, phosphorylation of JAK-2 was observed after 1 min of bFGF exposure and was maintained through the 30-min time point and decreased slightly after 60 min of bFGF stimulation. The PAFR antagonists LAU-8080, BN-52021, and CV-3988 attenuated this bFGF-stimulated JAK-2 phosphorylation at all time points, suggesting that the PAF-PAFR pathway is involved in the bFGF-mediated JAK-2 phosphorylation in HUVEC. Levels of phospho-Src and Src are shown in Fig. 7B. bFGF alone did not induce phosphorylation of Src until 15 min.

**Fig. 5.** bFGF and PAF phosphorylate STAT-3 through a mechanism involving the PAF receptor. Panel A, HUVEC, incubated overnight in basal medium, were exposed to bFGF or 10^{-7} M PAF individually or in combination with 10 \mu M LAU-8080, 10 \mu M BN-52021, or 3 \mu M CV-3988 for different time points. The cell lysates obtained after each time point were separated by 7.5% SDS-PAGE, blotted onto nitrocellulose membrane, incubated with phospho-STAT-3 antibody, and revealed by chemiluminescence. The membranes were stripped and reprobed with STAT-3 antibody to verify the protein levels in each lane. Panel B, phospho-STAT-3 levels were obtained by densitometric analysis and represented as a percentage of control against time (control being set at 100%). Shown is a representative of three independent experiments.
and was followed by a rapid phosphorylation at the 30-min time point (Fig. 7D). The PAFR antagonists did not antagonize this bFGF-stimulated phosphorylation of Src, suggesting that Src is also a component of the bFGF-signaling pathway, and its stimulation by bFGF is independent of the PAF-PAFR pathway.

STAT-3 Phosphorylation via JAK-2 and Src—To further elucidate the involvement of JAK-2 and Src in the phosphorylation of STAT-3, HUVEC were pretreated for 30 min in the presence of the JAK-2 inhibitor AG-490 and/or Src inhibitor PP1 to obtain the baseline phosphorylation of STAT-3. After this pretreatment, HUVEC were stimulated with PAF with or without the PAFR antagonists for different time intervals. Control cells were exposed to PP1 and AG-490 for the duration of the experiment. As seen in Fig. 8B, JAK-2 was not phosphorylated in the presence of AG-490 but was unaffected by PP1. Phosphorylation of JAK-2 occurred in PP1-pretreated HUVEC cells after 15 min of PAF stimulation (Fig. 8E), reached maximal levels after 30 min, and dropped to the baseline level after 60 min of PAF exposure. PAF receptor antagonists LAU-8080, BN-52021, and CV-3988 attenuated JAK-2 phosphorylation below the baseline level at all time points. Phosphorylation of Src was observed in the AG-490-pretreated HUVEC cells but was inhibited in the presence of PP1 (Fig. 8C). Rapid phosphorylation of Src occurred after 1 min of PAF stimulation in AG-490-pretreated cells (Fig. 8F). Src phosphorylation decreases with time, reaching nadir at 30 min, and was rephosphorylated after 60 min of PAF exposure. Attenuation of the Src phosphorylation was observed at all time points in the presence of the PAF receptor antagonists LAU-8080, BN-52021, and CV-3988. Phospho-STAT-3 and STAT-3 levels are shown in Fig. 8A. When JAK-2 was inhibited by AG-490, STAT-3 was rapidly phosphorylated at 1 min after exposure to PAF (Fig. 8D). The level of STAT-3 phosphorylation then decreased with time through the 30-min time point. Phosphorylation of STAT-3 was again observed after prolonged exposure to PAF (60 min). However, as seen in Fig. 8D, when Src was inhibited by PP1, profound phosphorylation of STAT-3 was observed only after 30 min of PAF stimulation and was maintained through 60 min. STAT-3 phosphorylation was attenuated at all time points by the PAF receptor inhibitors, suggesting the involvement of the PAF receptor.

The above observations taken together suggest that the immediate (1 min) PAF-mediated phosphorylation of STAT-3 is independent of JAK-2 and may be dependent on Src, since phosphorylation of Src as well as STAT-3 are observed to be maximal at this time point in the AG-490-pretreated cells. PP1 pretreatment caused an attenuation of both Src as well as STAT-3 phosphorylation after 1 min of PAF exposure, further supporting the immediate Src-dependent phosphorylation of STAT-3. Maximal phosphorylation of STAT-3 occurred at 30 min following stimulation by PAF, corresponding to maximal

Fig. 8. PAF phosphorylates STAT-3 through JAK-2 and Src. HUVEC, starved of growth factors overnight, were pretreated for 30 min with 50 μM AG-490 or 170 nM PP1 and exposed to 10−7 M PAF individually or in combination with 10 μM LAU-8080, 10 μM BN-52021, or 3 μM CV-3988 for various time points. Cell lysates were separated on a 7.5% SDS-PAGE, Western-blotted, and incubated with phospho-JAK-2 antibody (panel A), phospho-JAK-2 antibody (Panel B), or phospho-Src antibody (panel C). Phosphorylated proteins were revealed by chemiluminescence. The same membranes were stripped and rebotted with respective protein antibodies to verify the proteins levels. Control represents cells were exposed to 50 μM AG-490 or 170 nM PP1 alone. Panel D, phospho-STAT-3 levels are represented as percentage of control after densitometric analysis. Panel E, levels of phospho-JAK-2 in PP1 pretreated cells, represented as percentage of control. Panel F, phospho-Src levels as percentage of control in AG-490-pretreated cells. (the control was set at 100%). Results of an individual experiment are shown that are representative of three separate experiments.
JAK-2 phosphorylation in the PP1-pretreated cells. The presence of the JAK-2 inhibitor AG-490 caused complete attenuation of both JAK-2 and STAT-3 phosphorylation at this time point, suggesting that at 30 min, STAT-3 phosphorylation is dependent upon JAK-2. The prolonged (60 min) phosphorylation of STAT-3 observed in both AG-490- and PP1-pretreated cells suggests that the signaling of the PAF receptor upon binding PAF may be through both JAK-2 and/or Src. Src may be activated through the G-protein or through protein kinase A (PKA). Activation of Src by the PAF receptor leads to differential activation of both JAK-2 and Src. Src can be activated through the G-protein or through protein kinase A (PKA). Activation of Src by the PAF receptor leads to differential activation of both JAK-2 and Src. Src may be activated through the G-protein or through protein kinase A (PKA). Activation of Src by the PAF receptor leads to differential activation of both JAK-2 and Src.

**Discussion**

PAF is now considered as a major lipid second messenger in the regulation of a number of biological responses including tumor expansion and angiogenesis (25, 26). PAF induces endothelial cell proliferation (27) and migration through the basement membrane by inducing the production of plasminogen activator protein and DNA synthesis (28, 29). There are data to suggest that the production of PAF may result through the binding of many different pro-angiogenic protein growth factors to their tyrosine receptors. Therefore, the production of PAF may be an important signaling event for many growth factors (30). Although PAF is known to be involved in a number of biological functions, the mechanisms involving PAF-mediated action have yet to be identified. Results presented in this report indicate the possible PAF-mediated signal transduction mechanism involved in the stimulation of HUVEC.

Members of the FGF family of growth factors are potent inducers of angiogenesis. Cellular responses mediated by FGFs include cell migration, proliferation, and differentiation (31). The FGF family consists of at least 20 factors, which are 30–70% identical in their primary amino acid sequences, of which FGF-1 (acidic FGF) and FGF-2 (bFGF) are the most extensively studied (13). The cellular effects of FGFs are mediated via receptor tyrosine kinases (14) and the low affinity FGF receptors consisting of heparan sulfate proteoglycans on the cell surface (32), resulting in the dimerization and autophosphorylation of the FGF receptors (see Fig. 9A). We found that stimulation of HUVEC with bFGF resulted in their rapid proliferation as compared with VEGF, IGF-1, or EGF, whereas bFGF deprivation significantly reduced their growth. Furthermore, attenuation of bFGF-stimulated HUVEC growth was observed in the presence of the PAF receptor antagonist LAU-8080, whereas there was no appreciable difference in growth when bFGF was removed from the medium containing LAU-8080 or when bFGF was immunonutralized as compared with the cells treated with LAU-8080 alone. This led us to the conclusion that bFGF is a key growth factor responsible for the proliferation of HUVECs. PAF is involved in the bFGF signal transduction pathway. This phenomenon was also observed in lung fibroblasts (WI-38) and ASMC, since the PAF receptor antagonists LAU-8080, BN-52021, and CV-3988 attenuated the growth-promoting effect of bFGF in these cells. The level of tyrosine phosphorylation of the FGFR in HUVEC remained the same in the presence of LAU-8080, suggesting that PAF might be acting at a downstream level in the bFGF signal transduction pathway (Fig. 9A).

The activation of the FGFR induces the Ras-independent and Ras-dependent signal transduction cascades. The Ras-independent cascade is induced through the activation of phospholipase Cγ (15), which results in the activation of phosphatidylinositol turnover, protein kinase C, and Raf (reviewed by Shukla in Ref. 33). The release of inositol triphosphate results in the mobilization of Ca^{2+}, activation of phospholipase A_2, hydrolysis of alkylarachidonoylphosphatidylcholine to arachidonic acid and lyso-PAF (34–36). The Ras-dependent pathway is initiated through the binding of adaptor proteins (Grb2, Sos) and the activation of Ras and Raf (16). Thus, the activation of Raf represents a convergence of the Ras-independent and Ras-dependent pathways (see Fig. 9A). The resulting phosphorylation of MAP kinase kinase (Mek) and subsequent phosphorylation of MAP kinase (Erk) results in another convergence of the Ras-independent and Ras-dependent pathways.

Interestingly Erk is a candidate for upstream regulators of PAF-induced mitogenesis in lymphocytes (37) and differentiation of neuronal cells (38). PAF reportedly causes phosphorylation of the 42-kDa Erk-2 in human neutrophils (39) and sheep platelets (40). Stimulation of B cell lines with PAF induced tyrosine phosphorylation of a protein identified as MAP-2 kinase (41). PAF has been demonstrated to activate Mek and Erk in corneal epithelial cells (42). More recently, PAF has been shown to stimulate Erk activation in primary hippocampal neurons in a process that can be blocked by PAFR antagonists.
(43). Our results indicate that although there is no immediate stimulation of Erk-1 and -2 upon bFGF exposure to HUVEC, maximum stimulation was observed after 30 min of exposure, which continued for 60 min, suggesting that treatment of HUVEC with bFGF induced a delayed activation of Erk through a Ras-dependent pathway. Attenuation of Erk phosphorylation and activity by the PAF receptor antagonist LAU-8080 suggested that PAF was being produced after stimulation of HUVEC with bFGF and was influencing the activity of Erk. Given that the Ras-dependent and Ras-independent pathways induced by bFGF could lead to the production of PAF after hydrolysis by phospholipase A<sub>2</sub>, it is not surprising that PAFR antagonism affected bFGF-induced Erk phosphorylation.

PAF-signaling mechanisms are complex and are not clearly understood (Fig. 9B). The PAF receptor has been cloned and sequenced (44). As with the cytokine receptors, PAF receptors have no intrinsic catalytic activity. Previous data indicate that it belongs to the super family of G-protein-coupled receptors, suggesting that PAF might elicit its effects through G-protein mediation (45, 46). One of the major signaling pathways activated by the ligand-coupled receptors is the JAK/STAT pathway. Studies using interferons and growth hormones indicate that specific Janus kinases may be preferentially activated depending on the type of the receptor that is being activated (47, 48). The activated Janus kinase phosphorylates the tyrosine residue of a novel group of transcription factors named STAT (49). Reddy et al. (50) and Darnell (49) observe that the nature of the STATs that are activated depend on the cell line that is used in the study rather than the nature of the JAK activated by the ligand/receptor interaction. Recently, the peptide hormone bradykinin has been shown to activate TYK-2 after binding to its receptor, resulting in the tyrosine phosphorylation of STAT-3 in the bovine aortic endothelial cells (51). As mentioned earlier, PAF receptor antagonist WEB-2170 inhibited the thrombopoietin-induced phosphorylation of STAT-5B (22). More recently, PAF receptor has been shown to activate TYK-2 upon binding to PAF and, later, to activate various STAT proteins, bypassing the G-protein in the monocytic cell lines U937 and MonoMac-1 (23).

Our studies have demonstrated that simultaneous exposure of HUVEC to VEGF, bFGF, EGF, and IGF-1 induced phosphorylation of STAT-3 through a mechanism involving PAF, since the PAF receptor antagonists LAU-8080, BN-52021, and CV3988 all reduced STAT-3 phosphorylation. Stimulation of delayed and prolonged STAT-3 phosphorylation by bFGF alone, and its antagonism by the PAF receptor inhibitors further implicates the involvement of PAF in the bFGF-signaling cascade. PAF itself caused a delayed phosphorylation of STAT-3 that peaked after 30 min of exposure. Given that the PAFR has no intrinsic kinase activity, this delayed action of PAF demonstrates the involvement of another kinase responsible for STAT-3 phosphorylation. Although PAF itself maximally stimulated STAT-3 phosphorylation after 30 min of exposure, bFGF alone was able to prolong the phosphorylation of STAT-3 for 60 min, suggesting that bFGF induced the production of PAF and activation of PAF receptor to cause the prolonged STAT-3 phosphorylation.

Activation of FGF receptor by ligand binding has been implicated in regulation of Src kinase activity (52). Klint et al. (53) observe that Immortomice brain endothelial cells (a capillary endothelial cell line) showed an inhibition of FGF-2 induced tube formation when treated with the Src family inhibitor PP1. However, FGF-2-induced cell survival was not affected upon treatment with PP1, and there was only a modest stimulatory effect on Src activity by FGF-2 in these cells. Ligation of the bFGF receptor failed to induce JAK activity in AIDS-derived Kaposi’s sarcoma cells (54). In contrast to the above observations, our results indicate that inhibition of JAK-2 or Src attenuates bFGF-stimulated HUVEC proliferation, thus demonstrating the importance of these proteins in the growth of HUVEC mediated by bFGF. Stimulation of cells with bFGF could not overcome the growth attenuation by the JAK-2 inhibitor, suggesting that signaling through JAK-2 was indispensable for cell survival. bFGF induced the phosphorylation of JAK-2, which was attenuated by the PAFR antagonists, implicating the involvement of PAF-PAFR pathway in bFGF-stimulated JAK-2 phosphorylation. bFGF also stimulated the phosphorylation of Src in a time-dependent manner. However, the PAFR antagonists did not antagonize the Src phosphorylation at any time point, demonstrating that Src is a member of the bFGF-signaling pathway, and its phosphorylation induced by bFGF is independent of the PAF-PAFR pathway.

STAT-3 is a target of phosphorylation by both Janus kinases (22) and p60<sup>Src</sup>. We hypothesized that in HUVEC, both JAK and p60<sup>Src</sup> are activated by PAF binding, suggesting the involvement of a dual kinase regulatory mechanism for the PAF-mediated signal transduction pathway (Fig. 9B). We have used a specific JAK-2 inhibitor AG-490 and Src inhibitor PP1 to elucidate the phosphorylation of STAT-3 through both of these pathways. Our results demonstrate that inhibition of the Src pathway by PP1 does not affect the phosphorylation of JAK-2, which peaks at 30 min upon exposure to PAF. Inhibition of JAK-2 by AG-490 allows phosphorylation of Src to occur after 1 min of PAF stimulation, after which it decreases with time to 30 min but is rephosphorylated after 60 min in the presence of PAF. Attenuation of JAK-2 and Src phosphorylation by the PAF receptor antagonists suggests that their activation by PAF is specific to the PAF receptor.

When STAT-3 phosphorylation was observed in HUVEC pretreated with AG-490, we found that PAF induced a significant amount of STAT-3 phosphorylation at 1 and 60 min of exposure, whereas phosphorylation of STAT-3 was drastically attenuated at 30 min of PAF stimulation. This pattern of STAT-3 phosphorylation is similar to that of Src phosphorylation observed in cells pretreated similarly, suggesting that the immediate (1 min) and prolonged (60 min) STAT-3 phosphorylation is dependent on Src activation by PAF through its specific PAF receptor. However, this STAT-3 phosphorylation profile is drastically different from that observed when HUVEC were exposed to PAF alone without any pretreatment of cells with inhibitors. In the absence of any inhibitor, both JAK-2 and Src are active and aid in transferring the signal to activate STAT-3 after the stimulation of cells with PAF.

Furthermore, STAT-3 phosphorylation peaked at 30 min of PAF exposure when Src was inhibited by PP1. This delayed (30 min) activation of STAT-3 by PAF is specifically mediated through JAK-2, which itself gets maximally phosphorylated at this time point upon binding of PAF to its receptor. The attenuation of STAT-3 phosphorylation at all time points by the PAF receptor antagonists in both PP1- and AG-490-pretreated cells suggests the involvement of the PAF receptor in STAT-3 phosphorylation through PAF. Prolonged (60 min) phosphorylation of STAT-3 in PP1-pretreated cells suggests that JAK-2 may be involved in STAT-3 activation along with Src as noted previously. Thus, there appears to be a loss of dependence on either JAK-2 or Src in the prolonged PAF-mediated STAT-3 phosphorylation in HUVEC.

Studies using Src-transformed NIH-3T3 cells showed that v-Src can bind to STAT-3 and phosphorylate it in vitro (55). Chaturvedi et al. (56) observe that although STAT-5 required interaction with JAK-2 to mediate its phosphorylation, STAT-3 phosphorylation could be independent of JAK activation but in
turn be dependent on interaction with c-Src for its activation. Phosphorylation of Src and phospholipase Ca by exposure to PAF has been shown in rabbit platelets and A431 cells (24). In these studies, PAF-stimulated inositol production and the aggregation of cells was significantly reduced. Given our current data, it is unclear if activation of Src occurs through the coupled G-protein or through activation of protein kinase A.

In conclusion, we propose a dual kinase activation of STAT-3 upon binding of PAF to its PAF receptor in HUVEC. After the binding of PAF to its G-protein-associated seven-transmembrane domain receptor, a Src-family kinase is immediately activated, which further phosphorylates STAT-3. Continued stimulation of the PAF receptor by PAF results in the phosphorylation and activation of associated JAK-2. Activated JAK-2 then phosphorylates STAT-3 protein, with concomitant dephosphorylation of JAK-2. After STAT-3 activation, phosphorylation and activation of associated JAK-2. Activated JAK-2 might activate Ras, leading to increased invasion, migration, and proliferation of HUVEC. Activation of the Src-family kinase by the PAF receptor might activate Ras, leading to the activation of Erk, which in turn might activate phospholipase A2, resulting in the production of lysophosphatidylcholine which then activates the PAF receptor and, thus, activation of STAT-3 protein.
Phosphorylation of STAT-3 in Response to Basic Fibroblast Growth Factor Occurs through a Mechanism Involving Platelet-activating Factor, JAK-2, and Src in Human Umbilical Vein Endothelial Cells: EVIDENCE FOR A DUAL KINASE MECHANISM

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