Platelet-derived growth factor-BB (PDGF-BB) is a potent mitogen and chemotactant for vascular smooth muscle cells (VSMC). To understand its mitogenic and chemotactic signaling events, we studied the role of cytosolic phospholipase A2 (cPLA2) and the Jak/STAT pathway. PDGF-BB induced the expression and activity of cPLA2 in a time-dependent manner in VSMC. Arachidonyl trifluoromethyl ketone, a potent and specific inhibitor of cPLA2, significantly reduced PDGF-BB-induced arachidonic acid release and DNA synthesis. PDGF-BB stimulated tyrosine phosphorylation of Jak-2 in a time-dependent manner. In addition, PDGF-BB activated STAT-3 as determined by its tyrosine phosphorylation, DNA-binding activity, and reporter gene expression, and these responses were suppressed by AG490, a selective inhibitor of Jak-2. AG490 and a dominant-negative mutant of STAT-3 also attenuated PDGF-BB-induced expression of cPLA2, arachidonic acid release, and DNA synthesis in VSMC. Together, these results suggest that induction of expression of cPLA2 and arachidonic acid release are involved in VSMC growth in response to PDGF-BB and that these events are mediated by Jak-2-dependent STAT-3 activation.

Increased vascular smooth muscle cell (VSMC) growth is one of the major components in the thickening of the arterial wall in the pathogenesis of atherosclerosis and restenosis (1). A variety of molecules (including peptide growth factors, hormones, eicosanoids, and oxidants) that are generated at the site of arterial injury and/or inflammation can influence the growth and migration of VSMC (1–6). Indeed, increased levels of growth factors such as platelet-derived growth factor, cytokines, hormones, and oxidants (12–16). Upon release, it is either metabolized via the cyclooxygenase, lipoxynase, or cytochrome P450 monooxygenase pathway, producing prostaglandins, hydroperoxyeicosatetraenoic acids, or epoxyeicosatrienoic acids, respectively, or is reincorporated into membrane phospholipids via esterification involving arachidonoyl-CoA synthase and arachidonoyl lysophospholipid transferase (12, 17). Arachidonic acid and its oxygenative metabolites, known as eicosanoids, are involved in the regulation of a variety of biological processes, including vascular tone (17, 18). In addition, these lipid molecules have been reported to mediate intracellular signaling events in response to a number of stimuli (19–24). A large body of data also suggest that arachidonic acid and its eicosanoid metabolites play an important role in cell survival and growth (25–29). Among the members of the large phospholipase A2 family characterized thus far, cytosolic phospholipase A2 (cPLA2) appears to be the major source of eicosanoid production in response to some agonists in certain cell types (30–32).

Janus-activated kinases (Jak) are a group of non-receptor tyrosine kinases that, via phosphorylation, modulate the activities of a group of transcription factors, viz. signal transducers and activators of transcription (STAT) (33, 34). STAT proteins have been reported to be involved in the regulation of cell growth and differentiation (35–38). To understand the molecular events of platelet-derived growth factor-BB (PDGF-BB)-induced growth and survival in VSMC, we have studied the role of cPLA2 and the Jak/STAT pathway. Here, we report for the first time that PDGF-BB induces the expression of cPLA2 in VSMC in a sustained manner and that this response requires Jak-2-dependent activation of STAT-3. In addition, we show that Jak-2/STAT-3-dependent induction of expression of cPLA2 is required for PDGF-BB-induced arachidonic acid release and growth in VSMC.

MATERIALS AND METHODS

Reagents—Aprotinin, dithiothreitol, phenylmethylsulfonyl fluoride, sodium orthovanadate, sodium deoxycholate, leupeptin, and HEPES were purchased from Sigma. AG490 was obtained from Calbiochem. Arachidonic acid (AA) and a cPLA2 assay kit were bought from Cayman.
Chemical Co., Inc. (Ann Arbor, MI). Anti-cPLA2 (2832) antibodies and phospho-specific anti-STAT-3 (9131S) antibodies were procured from Cell Signaling Technology (Beverly, MA). Phospho-specific anti-Jak-2 (44-426Z) antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Jak-2 (sc-294) and anti-STAT-3 (sc-482) antibodies and consensus oligonucleotides for STAT-3 (5'-GATCCTTCTGGGAATTCCTAGATC-3') (sc-2571) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). T4 polynucleotide kinase was purchased from Invitrogen. [3H]AA (98 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol), and [3H]thymidine (20 Ci/mmol) were obtained from PerkinElmer Life Sciences.

**Cell Culture**—VSMC were isolated from the thoracic aortas of 200–300-g male Sprague-Dawley rats by enzymatic dissociation as described earlier (39). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 95% air and 5% CO2 atmosphere. Cells were growth-arrested by incubation in DMEM containing 0.1% calf serum and 0.2 mM dithiothreitol, 4.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM MgCl2, 0.1% SDS, 100 µg/ml leupeptin, and 1 mM sodium orthovanadate) and washed once with cold 5% trichloroacetic acid and once with cold 70% ethanol. After labeling, cells were washed with cold phosphate-buffered saline, trypsinized, and collected by centrifugation. The cell pellet was suspended in cold 10% (v/v) trichloroacetic acid and vortexed vigorously to lyse cells. After standing on ice for 20 min, the cell lysate mixture was passed through a Whatman GF/C glass-fiber filter. The filter was washed once with cold 5% trichloroacetic acid and once with cold 70% (v/v) ethanol. The filter was dried and placed in a liquid scintillation vial containing the scintillant fluid, and the radioactivity was measured in a Beckman LS 5000TA liquid scintillation counter.

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts were prepared from treated or untreated VSMC as described previously (39). The protein content of the nuclear extracts was determined using a Micro BCA™ protein assay reagent kit (Pierce). Protein-DNA complexes were formed by incubating 5 µg of nuclear protein in a total volume of 20 µl consisting of 15 mM HEPES (pH 7.9), 3 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonil fluoride, 1 mM dithiothreitol, 4.5 µg of bovine serum albumin, 2 µg of poly(dI-dC), 15% glycerol, and 100,000 cpm of [32P]-labeled oligonucleotide probe for 30 min on ice. In some experiments, nuclear extracts were preincubated with anti-STAT-3 antibodies for 3 h prior to protein-DNA binding assay. The protein-DNA complexes were resolved by electrophoresis on a 4% polyacrylamide gel using 1× Tris/glycine/EDTA buffer (25 mM Tris-HCl (pH 8.5), 200 mM glycine, and 0.1 mM EDTA). Double-stranded oligonucleotides were labeled with [γ-32P]ATP using the T4 polynucleotide kinase kit (Invitrogen) following the supplier’s protocol.

**Western Blot Analysis**—After appropriate treatments, VSMC were rinsed with cold phosphate-buffered saline and frozen immediately in liquid nitrogen. Cells were lysed by thawing in 250 µl of lysis buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml phenylmethylsulfonil fluoride, 100 µg/ml soybean trypsin, 100 µg/ml leupeptin, and 1 mM sodium orthovanadate) and scraped into 1.5-ml Eppendorf tubes. After standing on ice for 20 min, the cell lysates were cleared by centrifugation at 12,000 rpm for 20 min at 4 °C. Cell lysates containing an equal amount of protein were resolved by electrophoresis on 1% SDS and 10% polyacrylamide gels.
FIG. 3. A, PDGF-BB stimulates tyrosine phosphorylation of Jak-2 and STAT-3 in a time-dependent manner in VSMC. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) for the indicated times, and cell extracts were prepared. An equal amount of protein (40 μg) from the control and each treatment was analyzed by Western blotting for phospho-Jak-2 (pJak-2) and phospho-STAT-3 (pSTAT-3) using their phospho-specific antibodies. As a loading control, the blot was reprobed with anti-STAT-3 antibodies.

B, PDGF-BB-stimulated tyrosine phosphorylation of STAT-3 is sensitive to inhibition by the Jak-2 inhibitor AG490. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of AG490 (25 μM) for 30 min, and cell extracts were prepared. An equal amount of protein (40 μg) from the control and each treatment was analyzed by Western blotting for phospho-STAT-3 using its phospho-specific antibodies. As a loading control, the blots were reprobed with anti-STAT-3 antibodies. In the case of PDGFR tyrosine phosphorylation analysis, an equal amount of protein from the control and each treatment was immunoprecipitated with anti-PDGFR antibodies, and the resulting immunocomplexes were analyzed by Western blotting using antibody PY20.

FIG. 4. AG490, a potent inhibitor of Jak-2, reduces PDGF-BB-induced translocation of tyrosine-phosphorylated STAT-3 from the cytoplasm to the nucleus. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of AG490 (25 μM) for 30 min, and cytoplasmic and nuclear extracts were prepared. An equal amount of protein (40 μg) from the cytoplasmic and nuclear extracts of the control and PDGF-BB-treated cells was analyzed by Western blotting for phospho-STAT-3 (pSTAT-3) using its phospho-specific antibodies. As a loading control, the blot was reprobed with anti-STAT-3 antibodies.

The proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond, Amersham Biosciences). After blocking in 10 mM Tris-HCl (pH 8.0) containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) nonfat dry milk, the membrane was treated with appropriate primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected using a chemiluminescence reagent kit (Amersham Biosciences).
RESULTS AND DISCUSSION

To understand the mitogenic signaling events of PDGF-BB in VSMC, we have studied the role of cPLA2. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) for various times, and cell extracts were prepared. Equal amounts of protein from control and PDGF-BB-treated cells were analyzed by Western blotting for cPLA2 using its specific antibodies. PDGF-BB induced cPLA2 expression in a time-dependent manner, with 2- and 3-fold increases at 8 and 16 h of treatment, respectively (Fig. 1A). The increase in cPLA2 expression induced by PDGF-BB also resulted in an increase in its activity as measured by hydrolysis of arachidonoyl phosphatidylcholine using a commercially available kit (Fig. 1B). Earlier studies from other laboratories have reported that cPLA2 plays a role in AA release and growth in response to some agonists (15, 40, 41). To test the role of cPLA2 in receptor tyrosine kinase agonist-induced AA release and growth, we studied the effect of arachidonoyl trifluoromethyl ketone (AACOCF3), a specific inhibitor of cPLA2 (42), on PDGF-BB-induced AA release and growth. Quiescent VSMC that were prelabeled with [3H]AA were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of AACOCF3 (10 μM) for 1 h, and [3H]AA release was measured. PDGF-BB stimulated [3H]AA release by ~6-fold, and this effect was significantly inhibited by AACOCF3 (Fig. 2A). To understand the role of cPLA2 in PDGF-BB-induced growth, quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of AACOCF3 (10 μM) for 24 h, and growth was measured by pulse labeling cells with 1 μCi/ml [3H]thymidine for the last 12 h of the 24-h incubation period and determining the trichloroacetic acid-precipitable counts/min. PDGF-BB stimulated [3H]thymidine incorporation by 9-fold, and this response was completely inhibited by AACOCF3 (Fig. 2B). AACOCF3 alone had no toxic effects in VSMC, at least for 72 h as determined by trypan blue dye exclusion assay.

To understand the signaling events underlying PDGF-BB-induced expression of cPLA2 and AA release, we studied the role of the Jak/STAT pathway. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) for various times, and cell extracts were prepared. An equal amount of protein from the control and each treatment was analyzed by Western blotting for tyrosine phosphorylation of both Jak-2 and STAT-3 (Fig. 3A). Jak proteins phosphorylate STAT proteins at tyrosine residues and activate them, although other mechanisms were also reported to be involved in the activation of these transcription factors (43, 44). To determine whether PDGF-BB-stimulated STAT-3 tyrosine phosphorylation is mediated by Jak-2, we tested the effect of AG490, a potent and specific inhibitor of Jak-2 (45). AG490 (25 μM) significantly inhibited PDGF-BB-stimulated tyrosine phosphorylation of STAT-3 (Fig. 3B). The inhibition of PDGF-BB-stimulated tyrosine phosphorylation of STAT-3 by AG490 was not due to its toxic effects in VSMC, as this compound did not
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Fig. 7. AG490 and a dominant-negative mutant of STAT-3 inhibit PDGF-BB-induced [³H]AA release and DNA synthesis in VSMC. A, VSMC that were prelabeled with [³H]AA were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of AG490 (25 μM) and/or forced expression of the dominant-negative STAT-3 mutant for 1 h, and [³H]AA release was measured. B, quiescent VSMC were transfected with and without PDGF-BB (20 ng/ml) in the presence and absence of AG490 (25 μM) and/or forced expression of the dominant-negative STAT-3 mutant for 24 h, and DNA synthesis was measured by pulse labeling cells with 1 μCi/ml [³H]thymidine for the last 12 h of the 24-h treatment period. *, p < 0.01 versus control; **, p < 0.01 versus PDGF-BB treatment alone. C, VSMC were transfected with and without the dominant-negative STAT-3 mutant plasmid (pFS3DM), quiesced, and treated with and without PDGF-BB (20 ng/ml) for 2 h, and

...affect the viability of these cells over a period of 72 h as measured by trypan blue dye exclusion assay. Because earlier studies have reported that PDGF-BB phosphorylates STAT-3 independent of Jak-2 and involving the PDGFR, we also tested whether AG490 affects PDGFR tyrosine phosphorylation. AG490 had no effect on tyrosine phosphorylation of PDGFR induced by PDGF-BB, a finding that suggests that the inhibitory effect of AG490 on STAT-3 tyrosine phosphorylation is specific.

Upon tyrosine phosphorylation, STAT proteins undergo either homo- or heterodimerization and translocate to the nucleus, where they bind (in this case, STAT-3) to their consensus DNA-binding sequence present in the promoter regions of genes and induce transcription (33, 46). To test whether STAT-3, upon its tyrosine phosphorylation induced by PDGF-BB, translocates to the nucleus, quiescent cells were treated with and without PDGF-BB (20 ng/ml) for 30 min, and the cytoplasmic and nuclear extracts were prepared. An equal amount of protein from the cytoplasmic and nuclear extracts of control and PDGF-BB-treated cells was analyzed by Western blotting for the levels of tyrosine-phosphorylated STAT-3. Tyrosine-phosphorylated STAT-3 levels were detected only in the nuclear fraction of PDGF-BB-treated cells, and AG490 reduced these levels (Fig. 4). To determine whether translocation of tyrosine-phosphorylated STAT-3 correlates with increased transcription activation, STAT-3 DNA-binding activity was measured. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) for 2 h, and nuclear extracts were prepared. An equal amount of nuclear protein from the control and each treatment was analyzed by EMSA for STAT-3 DNA-binding activity using ³²P-labeled STAT-3 consensus oligonucleotide as a probe. PDGF-BB increased STAT-3 DNA-binding activity by 6-fold, and it was inhibited by AG490 (Fig. 5A). Preincubation of nuclear extracts with anti-STAT-3 antibodies also significantly reduced PDGF-BB-induced protein-DNA complex formation (Fig. 5A). This result suggests that PDGF-BB-induced protein-DNA complexes contain STAT-3 either as homo- or heterodimers with other members of the STAT transcription factor family. To confirm that increased STAT-3 DNA-binding activity leads to increased transactivation activity, cells were transiently transfected with a STAT-3-dependent reporter plasmid (pSIE-CAT), quiesced, and treated with and without PDGF-BB (20 ng/ml) for 4 h, and cell extracts were prepared. Cell extracts normalized for protein were assayed for CAT activity. PDGF-BB induced STAT-3-dependent CAT activity by 4-fold, and AG490 substantially inhibited this response (Fig. 5E). To understand whether the Jak/STAT pathway plays a role in PDGF-BB-induced expression of cPLA₂, we next studied the effect of AG490 on this event. AG490 completely inhibited PDGF-BB-induced cPLA₂ expression (Fig. 6A). Consistent with this observation, AG490 also inhibited PDGF-BB-induced cPLA₂ activity (Fig. 6B). To obtain additional evidence on the role of the Jak/STAT pathway in PDGF-BB-induced cPLA₂ expression, we tested the effect of a dominant-negative STAT-3 mutant, FS3DM (38). As shown in Fig. 6C, forced expression of FS3DM blocked PDGF-BB-induced expression of cPLA₂. Next, we examined whether the inhibition of cPLA₂ expression by AG490 and the dominant-negative STAT-3 mutant leads to decreased AA release. VSMC that were prelabeled with [³H]AA and quiesced were treated with and without PDGF-BB (20 ng/ml) in the presence or absence of nuclear extracts were prepared. Five micrograms of nuclear protein from the control and each treatment were incubated with 100,000 cpm of ³²P-labeled STAT-3 consensus oligonucleotide probe, and the protein-DNA complexes were separated by EMSA.
AG490 and/or forced expression of the dominant-negative STAT-3 mutant for 1 h, and [3H]AA release was measured. Both AG490 and the dominant-negative STAT-3 mutant substantially reduced PDGF-BB-induced [3H]AA release (Fig. 7A). To investigate whether the Jak/STAT-dependent induction of expression of cPLA2 and AA release are required for PDGF-BB-induced VSMC growth, the effects of AG490 and the dominant-negative STAT-3 mutant on PDGF-BB-stimulated DNA synthesis were studied. As shown in Fig. 7B, PDGF-BB-induced DNA synthesis was significantly reduced by both AG490 and FS3DM. To validate the effects of FS3DM on PDGF-BB-induced cPLA2 expression and DNA synthesis, its effect on endogenous STAT-3 DNA-binding activity was tested. Forced expression of FS3DM substantially reduced PDGF-BB-induced endogenous STAT-3-DNA complexes in VSMC (Fig. 7C).

The important findings of this study are as follows. 1) PDGF-BB, a receptor tyrosine kinase agonist and a potent VSMC mitogen, induced the expression of cPLA2 in a sustained manner in VSMC. 2) PDGF-BB-induced expression of cPLA2 also resulted in an increase in cPLA2 activity. 3) ACOCCF, a selective inhibitor of cPLA2, attenuated PDGF-BB-induced AA release and growth in VSMC. 4) Jak-2-dependent STAT-3 activation mediated PDGF-BB-induced cPLA2 expression and growth in VSMC. A number of PLA2 enzymes, particularly group V secretory PLA2 and group IV cPLA2 enzymes, are involved in agonist-induced AA release (31, 32, 47, 48). Interestingly, cross-activation between secretory PLA2 and cPLA2 enzymes has been observed in acute and delayed production of eicosanoids in many cell types, including human neutrophils and murine macrophages and mast cells (30, 47, 48). Acute activation of cPLA2 in response to a number of agonists that are coupled to Ca2+ mobilization, particularly G protein-coupled receptor agonists, cytokines, and phorbol esters, is mediated by the mitogen-activated protein kinase cascade and/or protein kinase C (49, 50). In this study, we have shown for the first time that PDGF-BB stimulated sustained cPLA2 activity via induction of its expression. Furthermore, the sustained expression and activity of cPLA2 appeared to be mediated by and involved in Jak/STAT-dependent PDGF-BB-induced growth in VSMC. This conclusion is supported by the findings that AG490, a selective inhibitor of Jak-2, and a dominant-negative mutant of STAT-3 substantially reduced the expression and activity of cPLA2 and DNA synthesis induced by PDGF-BB. Studies from other laboratories also indicate that cPLA2 plays a role in serum-induced growth in human coronary artery smooth muscle cells (40). A potential role for the Jak/STAT pathway in the regulation of cell growth, differentiation, and survival activities in many cell types, including hematopoietic cells, has been demonstrated (35–38, 51). Based on these findings and the present observations, it is likely that cPLA2 is one of the effector molecules that are involved in Jak/STAT signaling leading to induction of growth in VSMC by PDGF-BB. Some STAT transcription factors such as STAT-1 have also been reported to be involved in the induction of expression of cell cycle inhibitory molecules such as p21WAF1/CIP1 and pro-apoptotic enzymes such as caspase-1 and thereby in apoptosis (52, 53). In this regard, it is noteworthy that cPLA2-dependent AA release mediates oxidant-induced apoptosis in some cell types (54). In view of these findings, it can be speculated that cPLA2 is distal in the path of Jak/STAT signaling to cell proliferation and/or apoptosis. Future studies are required to test whether the responsiveness of cPLA2 to various agonists of cell growth and apoptosis is dependent on activation of different members of the STAT transcription factor family.

In summary, we have reported for the first time that the receptor tyrosine kinase agonist PDGF-BB induces the expression of cPLA2 in a manner that is dependent on activation of the Jak/STAT pathway. In addition, we have shown that Jak/STAT-dependent induction of expression of cPLA2 and AA release are involved in PDGF-BB-induced growth in VSMC.

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Cytosolic Phospholipase A2 Is an Effector of Jak/STAT Signaling and Is Involved in Platelet-derived Growth Factor BB-induced Growth in Vascular Smooth Muscle Cells

Chandrahasa R. Yellaturu and Gadiparthi N. Rao

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