Role of Janus Kinase/Signal Transducer and Activator of Transcription and Mitogen-activated Protein Kinase Cascades in Angiotensin II- and Platelet-derived Growth Factor-induced Vascular Smooth Muscle Cell Proliferation*

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In vascular smooth muscle cells, the induction of early growth response genes involves the Janus kinase (JAK)/signal transducer and activators of transcription (STAT) and the Ras/Raf-1/mitogen-activated protein kinase (MAPK) cascades. In the present study, we found that electroporation of antibodies against MEK1 or ERK1 abolished vascular smooth muscle cell proliferation in response to either platelet-derived growth factor or angiotensin II. However, anti-STAT1 or -STAT3 antibody electroporation abolished proliferative responses only to angiotensin II and not to platelet-derived growth factor. AG-490, a specific inhibitor of the JAK2 tyrosine kinase, prevented proliferation of vascular smooth muscle cells, complex formation between JAK2 and Raf-1, the tyrosine phosphorylation of Raf-1, and the activation of ERK1 in response to either angiotensin II or platelet-derived growth factor. However, AG-490 had no effect on angiotensin II- or platelet-derived growth factor-induced Ras/Raf-1 complex formation. Our results indicate that: 1) STAT proteins play an essential role in angiotensin II-induced vascular smooth muscle cell proliferation; 2) JAK2 plays an essential role in the tyrosine phosphorylation of Raf-1; and 3) convergent mitogenic signaling cascades involving the cytosolic kinases JAK2, MEK1, and ERK1 mediate vascular smooth muscle cell proliferation in response to both growth factor and G protein-coupled receptors.

Previous work by our laboratory (1–6) on cultured rat aortic vascular smooth muscle cells (VSMC)† and phenotypically similar glomerular mesangial cells has shown that protein tyrosine phosphorylation plays a critical role in angiotensin II (Ang II)-mediated intracellular signaling cascades. This is true despite the fact that G protein-coupled receptors in general and the Ang II AT1 receptor in particular possess no intrinsic tyrosine kinase activity. It is also now recognized that Ang II can act not only as a vasoactive peptide but also as a growth factor. In particular, Ang II has been shown to stimulate proliferative and hypertrophic growth in VSMC, glomerular mesangial cells, cardiac fibroblasts, and myocytes via AT1 receptor binding (4, 7–9). Like classic growth factors (e.g. platelet-derived growth factor [PDGF] and epidermal growth factor) and some cytokines (e.g. interferons and interleukins) (4, 8–10), Ang II is also capable of stimulating a rapid increase in the mRNA levels of c-fos, an early growth response gene implicated in VSMC proliferation (4, 7, 8). However, the Ang II-stimulated intracellular signaling cascades responsible for c-fos induction and therefore proliferation in VSMC have not been well defined.

One candidate mitogenic signaling cascade involves the activation of the small GTP-binding protein, Ras, which is traditionally mediated via classic growth factor receptors (4). Ras activation promotes the formation of a membrane-bound complex with Raf-1 (a serine/threonine protein kinase). Subsequent tyrosine phosphorylation of Raf-1 leads to its activation and the sequential stimulation of several cytoplasmic protein kinases, collectively known as the mitogen-activated protein kinase (MAPK) pathway. This phosphorylation cascade in turn activates a set of regulatory elements leading to the stimulation of early response genes and cellular growth (4). Our laboratory (5) has previously shown that as with classic growth factors, Ang II-induced protein tyrosine phosphorylation promotes the activation of p21Vos in VSMC.

A second mitogenic cascade that is activated by many cytokine receptors (e.g. interferons and interleukins) involves the JAK (Janus kinase) family of cytoplasmic tyrosine kinases (11, 12). JAK-mediated tyrosine phosphorylation of STAT (signal transducers and activators of transcription) family members promotes the translocation of these transcription factors to the nucleus, where they bind to specific DNA motifs and induce c-fos gene transcription (11–14). In VSMC, our laboratory (6) has previously shown that Ang II stimulates the tyrosine phosphorylation of JAK isoforms (JAK2 and TYK2), the tyrosine kinase activity of JAK2, and the tyrosine phosphorylation of transducer and activator of transcription; Temed, N,N,N’-tetramethylethylenediamine; DMEM, Dulbecco’s modified Eagle’s medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt.

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† The abbreviations used are: VSMC, vascular smooth muscle cells; Ang II, angiotensin II; PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; JAK, Janus kinase; STAT, signal...
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STAT isoforms (STAT1, STAT2, and STAT3). Finally, Ang II induces the formation of a complex between JAK2 and the AT1 receptor itself.

Our present study examines the role of JAK/STAT and Ras/Raf-1/MAPK signaling cascades in the cellular proliferation mediated by activation of G protein-coupled AT1 receptor and classic growth factor receptors (e.g. PDGF). Ang II plays a crucial role in the regulation of systemic arterial blood pressure, cardiovascular and renal growth, and sodium homeostasis (7). Importantly, angiotensin-converting enzyme inhibitors have become a mainstay in the treatment of hypertension, congestive heart failure, cardiac hypertrophy, myocardial infarction, and chronic renal failure (4). Better definition of Ang II-mediated mitogenic signaling provides the potential for additional specific therapeutic interventions.

MATERIALS AND METHODS

Reagents—Tween 20, acrylamide, SDS, N,N'-methylenebisacrylamide, Temed, and nitrocellulose membranes were purchased from BioRad Laboratories. PDGF-BB, molecular weight standards, immunoprecipitin, protein A- and G-agarose, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, and all medium additives were obtained from Life Technologies, Inc. Anti-phosphotyrosine (PY20), -Raf-1, -Ras, -PDGF-β receptor, -JAK2, -STAT1, -STAT3, -MEK1, and -ERK1 antibodies were obtained from Santa Cruz Biotechnology, Inc. or Transduction Laboratories. AG-490 was purchased from Calbiochem. Phospho-specific MAPK antibody for detection of catalytically activated ERK1 and ERK2 was obtained from New England Biolabs, Inc. (15). The enhanced chemiluminescence kit was obtained from Amersham Corp. Angiotensin II, goat anti-mouse IgG, and all other chemicals were purchased from Sigma.

Cell Culture—Rat aortic VSMC were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 10 mg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 5% CO2 enriched, humidified atmosphere as we have previously described (1, 2, 5, 6). Cells from passages 5 and 6 were routinely subcultured 1:5 or 1:10 at 7-day intervals, and the medium was changed at 2–3-day intervals.

Cell Proliferation Assay and Coulter Counting—Proliferation was measured using the Cell Titer 96® AQueous nonradioactive cell proliferation assay (Promega, Inc., Madison, WI) (16). This assay is based on the cellular conversion of the colorimetric reagent, MTS (3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt), into soluble formazan by dehydrogenase enzymes found only in metabolically active, proliferating cells. MTS in Dulbecco's phosphate-buffered saline (pH 6.0) was mixed with the electron-coupling reagent, phenazine methosulfate. The absorbance of formazan, measured at 490 nm using a 96-well enzyme-linked immunosorbent assay plate reader interfaced with a personal computer (model 3550, Bio-Rad), is directly proportional to the number of living cells in culture. To confirm the accuracy of our MTS proliferation assay, the actual increase in cell number was also directly assessed with a Coulter counter (model ZM, Coulter Corp., Hialeah, FL).

VSMC were grown in a 75-mm2 flask to confluence and detached with trypsin-EDTA (0.05% trypsin, 0.53 mol/liter EDTA; Life Technologies Inc.). 20,000 cells were plated into 96-well plates and allowed to settle for 4 h in DMEM supplemented with 10% fetal bovine serum. Prior to experiments, cells were then growth-arrested in serum-deprived DMEM for 24 h (time 0). Cells were then stimulated with 10–7 mol/liter Ang II (Sigma) or 0.33 mmol/liter PDGF (Life Technologies Inc.). After timed ligand exposure, the phenazine methosulfate/MTS mix was added to each well (final volume, 20 μl/100 μl medium) and then incubated for an additional 60 min in 5% CO2 at 37 °C. A 10% SDS solution was then added to stop the reaction, and the absorbance of formazan was measured at 490 nm.

[3H]Thymidine Incorporation—VSMC were plated in 96-well plates and maintained in DMEM supplemented with 10% fetal bovine serum as described for the cell proliferation assay above. 24 and 48 h after ligand exposure, cells were pulsed with 1 μCi/ml [3H]thymidine (New England Nuclear, Boston, MA) and then harvested into trichloroacetic acid-precipitable material. Cells were washed with phosphate-buffered saline, incubated in 10% trichloroacetic acid at 4 °C, dissolved at room temperature in 1 mol/liter, and dried on filter paper. The paper was washed three times with phosphate-buffered saline, and then the samples were placed in scintillation liquid and counted on a scintillation counter (Beckman Inc., Palo Alto, CA). Data were plotted as the number of cpm/well. Each experimental data point represents duplicate wells from at least four different experiments.

Electroporation Procedure—Cells were plated in 96-well plates and growth-arrested in serum-deprived DMEM for 24 h prior to experiments. As described previously (1, 5), VSMC were electroporated in 96-well plates using a Multi-Coaxial electrode (model P/N 747, BTX Inc. San Diego, CA) was performed in Ca2⁺- and Mg2⁺-free Hanks' balanced salt solution (pH 7.4, 5 mmol/liter KCl, 0.3 mmol/liter KH2PO4, 138 mmol/liter NaCl, 4 mol/liter NaHCO3, and 0.3 mmol/liter NaHPO4) containing antibodies at a final concentration of 10 mg/ml. Following electroporation, cells were incubated for an additional 30 min at 37 °C (5% CO2), washed once with serum-free DMEM, and then left in serum-free DMEM prior to the experiments.

Immunoprecipitation and Western Blotting—VSMC were stimulated with Ang II or PDGF for timed periods. The immunoprecipitation and Western blotting was performed as described previously (1–3, 5, 6). To immunoprecipitate proteins we used the following antibodies: anti-Raf-1 (2 μg/ml), anti-Ras (4 μg/ml), or anti-phosphotyrosine (PY20 clone, 10 μg/ml lysate). The recovered immunoprecipitated proteins were transferred to a nitrocellulose membrane and blotted with anti-JAK2, anti-Raf-1, or anti-PDGF-β receptor or phospho-specific MAPK

FIG. 1. Effect of anti-STAT, -ERK1, or -MEK1 antibody electroporation on Ang II- and PDGF-induced VSMC cell proliferation. A, VSMC were exposed to serum-free DMEM only ( ), or serum-free DMEM supplemented with Ang II (10–7 mol/liter; ) or PDGF (0.33 mmol/liter; ) for timed periods prior to measuring cell proliferation. B, VSMC were electroporated with 10 mg/ml rabbit anti-MEK1 (closed symbols) or -ERK1 (open symbols) prior to exposure to Ang II (circles) or PDGF (squares). C, VSMC were electroporated with anti-STAT1 (open symbols) or -STAT3 (closed symbols) prior to exposure to Ang II (circles) or PDGF (squares). Cell proliferation is expressed as absorption of formazan at 490 nm. Data represent the means ± S.D. for at least four experiments (each in duplicate).
significant. (Jandel Scientific, San Rafael, CA). Probability, 0.05 was considered significant.

RESULTS

Cellular proliferation, determined by the MTS assay (see "Materials and Methods"), was measured in VSMC after timed exposures to \(10^{-7}\) mol/liter Ang II or 0.33 mmol/liter PDGF. Both PDGF and Ang II significantly stimulated proliferation within 24 h when compared with cells that had not been exposed to either growth factor or G protein-coupled receptor ligands (Fig. 1A). PDGF-induced proliferation exceeded Ang II-induced proliferative responses.

MEK1 and ERK1 Are Required for Both Ang II- and PDGF-induced VSMC Proliferation—Physiologic cell growth and differentiation mediated by the Ras/Raf-1/MAPK cascade involves the activation of the serine/threonine MAPK kinase, MEK1, and the serine/threonine MAPK, ERK1 (4). Other investigators have recently demonstrated that both Ang II and PDGF are capable of activating ERK1 in VSMC (4). To evaluate the potential role of the MAPK cascade in VSMC proliferation, antibodies against MEK1 and ERK1 were electroporated into VSMC prior to exposure to Ang II or PDGF. VSMC proliferation in response to Ang II or PDGF was abolished in the presence of anti-MEK1 or -ERK1 antibodies (Fig. 1B). In serum-free negative controls or electroporation experiments with pooled rabbit IgG or sham-absorbed anti-MEK1 or -ERK1 antibodies, no inhibition of Ang II- or PDGF-induced VSMC proliferation was observed (data not shown). In VSMC electroporated with mock antibody (anti-IgG), DNA synthesis measured as \([\text{H}]\text{thymidine incorporation}\) increased significantly within 24 h of Ang II or PDGF exposure (Fig. 2A). Also consistent with our proliferation results (Fig. 1A), \([\text{H}]\text{thymidine incorporation}\) was greater after PDGF than Ang II exposure. We then tested the role of MAPK cascade components in VSMC DNA-synthesis. Indeed, the electroporation of anti-MEK1 or -ERK1 antibodies abolished DNA synthesis in response to either Ang II or PDGF (Fig. 2A).

These results suggested that VSMC proliferation and DNA synthesis, in response to both G protein-receptor coupled (i.e. Ang II) and growth factor (i.e. PDGF) receptor ligands, involve the MAPK cascade and are dependent on the activation of MEK1 and ERK1.

STAT1 and STAT3 Are Required for Ang II- but Not PDGF-induced VSMC Proliferation—Previous work by our laboratory (6) has shown that the cytosolic tyrosine kinase, JAK2, plays a critical role in Ang II-mediated signaling events, including the activation of STAT proteins. In the present study, we found that Ang II-induced proliferation was virtually abolished by the electroporation of anti-STAT1 or anti-STAT3 (Fig. 1C). In contrast, there was no statistical difference between PDGF-in-
Therefore, we investigated the role of JAK2 tyrosine kinase in timed exposure to Ang II (10^{-7} mol/liter; lower band). Top, VSMC lysates were immunoprecipitated with an anti-JAK2 antibody and then probed with anti-phosphotyrosine antibody. Representative bands corresponding to the molecular mass of JAK2 (135 kDa) are shown from lysates from cells with (right) or without (left) 10 μM AG-490 pretreatment for 16 h prior to timed exposure to Ang II (10^{-7} mol/liter; upper band) or PDGF (0.33 mmol/liter; lower band). Bottom, VSMC were exposed to serum-free DMEM only (circles) or serum-free DMEM supplemented with the specific JAK2 inhibitor, AG-490 (10 μM) (triangles), for 16 h prior to timed exposure to Ang II (10^{-7} mol/liter) (open symbols) or PDGF (0.33 mmol/liter) (closed symbols). Bands were quantitated by densitometry using a La Cie scanner interfaced with a personal computer. Each band was scanned in two dimensions, and the density was corrected for the background present in the lane. Data represent corrected densities for each time point and are expressed as arbitrary units plotted against time of Ang II or PDGF exposure (mean ± S.E.; n = 3). Results were similar if the addition of antibodies was reversed (i.e. immunoprecipitated with anti-phosphotyrosine antibody and probed with anti-JAK2 antibody) (data not shown).

duced proliferative responses observed in normal VSMC (Fig. 1A) compared with VSMC electroporated with anti-STAT1 or -STAT3 antibodies (Fig. 1C). The latter observation suggested that blockage of Ang II-induced proliferation was not simply a toxic effect of the electroporated antibodies. In electroporation experiments with sham-absorbed anti-STAT1 or -STAT3 antibodies, no inhibitory effect on Ang II- or PDGF-induced VSMC proliferation was observed (data not shown).

Similarly, Ang II-induced [3H]thymidine incorporation was completely prevented in cells electroporated with either anti-STAT1 or -STAT3 antibodies (Fig. 2B). The latter observation suggested that blockage of Ang II-induced proliferation was not simply a toxic effect of the electroporated antibodies. In electroporation experiments with sham-absorbed anti-STAT1 or -STAT3 antibodies, no inhibitory effect on Ang II- or PDGF-induced VSMC proliferation was observed (data not shown).

JAK2 Tyrosine Kinase Activity Is Essential for Both Ang II- and PDGF-induced VSMC Proliferation—Our laboratory (6) has previously shown that in VSMC Ang II induces the rapid tyrosine phosphorylation of and activation of the cytoplasmic tyrosine kinase JAK2. JAK2 activation, in turn, promotes the phosphorylation of STAT1 and STAT3 tyrosine residues. Our above results suggested that STAT1 and STAT3 are necessary and specific for VSMC proliferative responses linked to the G protein-coupled AT_{1} receptor but not the PDGF-β receptor. Therefore, we investigated the role of JAK2 tyrosine kinase in VSMC proliferation. We were unsuccessful in blocking Ang II- or PDGF-induced VSMC proliferation, DNA synthesis, or autotyrosine phosphorylation of JAK2 with the electroporation of commercially available anti-JAK2 polyclonal antibodies (data not shown). Because not all antibodies block or neutralize the biologic activities of the respective antigens, we investigated the effect of AG-490, a specific JAK2 inhibitor (17, 18). AG-490 belongs to the tyrphostin family of tyrosine kinase inhibitors, and these inhibitors inhibit protein tyrosine kinases by binding to the substrate binding site (19). Pretreatment of VSMC with 10 μM AG-490 did indeed block Ang II-induced VSMC proliferation (Fig. 3A), DNA synthesis (Fig. 3B), and the tyrosine phosphorylation of JAK2 (Fig. 4). AG-490 also blocked PDGF-induced VSMC proliferation (Fig. 3A), DNA synthesis (Fig. 3B), and JAK2 tyrosine phosphorylation (Fig. 4). We found that 16 h of pretreatment with AG-490 produced maximal inhibition of Ang II- and PDGF-induced JAK2 tyrosine phosphorylation events while still allowing recovery of VSMC proliferative responses when the AG-490 was removed from the bath. Because PDGF-induced VSMC proliferation and DNA synthesis required JAK2 activity but were unaffected by anti-STAT1 or -STAT3 antibody electroporation, we examined the possibility that JAK2-dependent proliferative responses were mediated through an alternative mitogenic pathway other than the JAK/STAT cascade.

Evidence from several groups suggests that JAK2 forms a membrane complex with Ras/Raf-1 and is required for Raf-1 activation in several different nonvascular mammalian cell types (20–22). We found that JAK2 inhibition with AG-490 pretreatment blocked both Ang II- and PDGF-induced complex formation between JAK2 and Raf-1 (Fig. 5) and the tyrosine phosphorylation of Raf-1 (Fig. 6). We then examined the effect of JAK2 inhibition on Ang II- and PDGF-mediated stimulation of ERK tyrosine phosphorylation. VSMC lysates were probed with a phosho-specific MAPK antibody that recognizes only the catalytically activated forms (phosphorylated on tyrosine
Several groups have previously shown that two mitogenic cascades, the JAK/STAT and Ras/Raf-1/MAPK, are stimulated by the AT1 receptor in VSMC (4, 6, 14, 23, 24). Both cascades link the binding of ligands to cell surface receptors, with intracellular signaling elements that promote nuclear translocation events resulting in cellular growth (4). Our laboratory (6) has shown that Ang II stimulates the tyrosine phosphorylation and activation of JAK2 and subsequently the tyrosine phosphorylation of STAT isoforms in VSMC. Bhat et al. (23, 24) have also demonstrated in cultured neonatal fibroblasts that Ang II induces STAT protein phosphorylation, translocation of STAT proteins into the nucleus, and initiation of early response gene transcription. Our laboratory (5) has shown that in VSMC Ang II stimulates the proto-oncogene, p21ras, and transcription. Our laboratory (5) has shown that in VSMC Ang II stimulates the proto-oncogene, p21ras, and transcription. Our laboratory (5) has shown that in VSMC Ang II stimulates the proto-oncogene, p21ras, and transcription. Our laboratory (5) has shown that in VSMC Ang II stimulates the proto-oncogene, p21ras, and transcription. Our laboratory (5) has shown that in VSMC Ang II stimulates the proto-oncogene, p21ras, and transcription.

***DISCUSSION***

Several groups have previously shown that two mitogenic cascades, the JAK/STAT and Ras/Raf-1/MAPK, are stimulated by the AT1 receptor in VSMC (4, 6, 14, 23, 24). Both cascades link the binding of ligands to cell surface receptors, with intracellular signaling elements that promote nuclear translocation events resulting in cellular growth (4). Our laboratory (6) has shown that Ang II stimulates the tyrosine phosphorylation and activation of JAK2 and subsequently the tyrosine phosphorylation of STAT isoforms in VSMC. Bhat et al. (23, 24) have also demonstrated in cultured neonatal fibroblasts that Ang II induces STAT protein phosphorylation, translocation of STAT proteins into the nucleus, and initiation of early response gene transcription. Our laboratory (5) has shown that in VSMC Ang II stimulates the proto-oncogene, p21ras, and transcription. Activated Ras then forms a membrane-bound complex with Raf-1 (a serine/threonine protein kinase), leading to the activation of Raf-1 by tyrosine phosphorylation (4). Raf-1 then phosphorylates and activates MEK1, which in turn leads to the activation of ERK1.

Because G protein-coupled receptors lack intrinsic tyrosine kinase activity, the activation of these mitogenic signaling cascades requires the recruitment of cytosolic tyrosine kinases, such as pp60 \( \text{src} \) or MEK1, and RAF2. Previous work by our laboratory (5) has shown that blocking of pp60 \( \text{src} \) with sorafenib and sorafenib antibodies prevented Ang II-induced formation of the Ras/Raf-1 membrane complex in VSMC. Our present study shows that inhibition of JAK2 with AG-490 and inhibition of MEK1 with antibody electroporation prevents VSMC proliferation and DNA synthesis in response to Ang II. Together, these observations indicate that the G protein-coupled AT1 receptor stimulates major mitogenic signaling pathways in VSMC via tyrosine phosphorylation and, in particular, the JAK/STAT and the Raf/Ras/RAF-1/MAPK cascades.

In contrast to the G protein-coupled AT1 receptor, classic growth factor receptors that possess intrinsic tyrosine kinase activity (e.g., PDGF and epidermal growth factor receptors) are thought not to require cytosolic tyrosine kinases to mediate downstream proliferative signaling events (7, 8). Consistent with this premise, our laboratory (2, 5) has previously demon-
intracellular signaling pathways involved in VSMC proliferation. Our present and past studies (1–6) suggest that Ang II-induced VSMC proliferation requires protein tyrosine phosphorylation and growth factor receptors provides a convergent signaling element for these two diverse mitogenic cascades in VSMC. Our present and past studies (1–6) suggest that Ang II-induced VSMC proliferation requires protein tyrosine phosphorylation via JAK2, MEK1, and pp60c-src, which in turn are necessary for the activation of several diverse mitogenic factors, specifically STAT proteins, Raf-1, ERK1, and phospholipase C-γ1. More importantly, the inhibition of these individual signaling molecules prevents VSMC proliferation. Current clinical therapeutic interventions for the prevention or regression of maladaptive cardiovascular growth (e.g., hypertension, congestive heart failure, cardiac hypertrophy, atherosclerosis, angioplasty injury) include the inhibition of G protein-coupled AT1 receptors (e.g., AT1 receptor antagonist and losartan) or their respective ligand (e.g., angiotensin-converting enzyme inhibitors) (4, 7, 8, 25). Our better understanding of the two mitogenic signaling pathways investigated in the present study presents potential new and specific targets for future therapeutic interventions in various cardiovascular diseases associated with VSMC proliferation.

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Fig. 8. Effects of JAK2 inhibition on the autophosphorylation of the PDGF-β receptor by PDGF. Top, VSMC lysates were immunoprecipitated with an anti-phosphotyrosine antibody and then probed with anti-PDGFB-β receptor antibody. Representative bands corresponding to the molecular mass of PDGF-β receptor (180 kDa) are shown from lysates from cells with (right) or without (left) 10 μM AG-490 pretreatment for 16 h prior to timed exposures to PDGF (0.33 mmol/liter). Bottom, VSMC were exposed to serum-free DMEM and then probed with anti-PDGF-β receptor antibody. Representative bands to corresponding to the molecular mass of PDGF-β receptor antibody. (data not shown).

Fig. 9. Effect of JAK2 inhibition on the Ang II- and PDGF-induced Ras/Raf-1 complex formation. VSMC lysates were immunoprecipitated with an anti-Ras antibody and then probed with anti-Raf-1 antibody. Representative experiments show Ras/Raf-1 complex formation in lysates from cells with (right) or without (left) 10 μM AG-490 pretreatment for 16 h prior to timed exposures to 10−7 mol/liter Ang II (n = 3) or 0.33 mmol/liter PDGF (n = 3). The Raf-1 protein standard is shown on the far left.

JAK-induced tyrosine phosphorylation of STAT1 or STAT3 transcription factors. Several groups have shown that growth hormone-, interferon-, and interleukin-induced activation of early growth response genes (e.g. c-myc, c-fos, and c-jun), cell proliferation, Ras/JAK2/Raf-1 complex formation, and Raf-1 kinase activity are dependent on JAK2 in several nonvascular mammalian cell types (20–22). Indeed, in the present study we find that both Ang II- and PDGF-induced JAK2/Raf-1 complex formation, Raf-1 tyrosine phosphorylation, and ERRK1 and ERRK2 kinase activity are dependent on JAK2 activity. Therefore, our data provide a key molecular link between the mitogenic JAK/STAT and Ras/Raf-1/MAPK cascades in VSMC.

In summary, our present study emphasizes the important role played by the JAK/STAT and Ras/Raf-1/MAPK cascades in mediating VSMC proliferation in response to both G protein-coupled AT1 receptors and classic growth factor receptors. We have shown that JAK2 activation by both G protein-coupled and growth factor receptors provides a convergent signaling element for these two diverse mitogenic cascades in VSMC. Our present and past studies (1–6) suggest that Ang II-induced VSMC proliferation requires protein tyrosine phosphorylation via JAK2, MEK1, and pp60c-src, which in turn are necessary for the activation of several diverse mitogenic factors, specifically STAT proteins, Raf-1, ERK1, and phospholipase C-γ1. More importantly, the inhibition of these individual signaling molecules prevents VSMC proliferation. Current clinical therapeutic interventions for the prevention or regression of maladaptive cardiovascular growth (e.g. hypertension, congestive heart failure, cardiac hypertrophy, atherosclerosis, angioplasty injury) include the inhibition of G protein-coupled AT1 receptors (e.g. AT1 receptor antagonist and losartan) or their respective ligand (e.g. angiotensin-converting enzyme inhibitors) (4, 7, 8, 25). Our better understanding of the two mitogenic signaling pathways investigated in the present study presents potential new and specific targets for future therapeutic interventions in various cardiovascular diseases associated with VSMC proliferation.

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