A Potential Role for Extracellular Signal-regulated Kinases in Prostaglandin F2α-induced Protein Synthesis in Smooth Muscle Cells*

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To understand the mechanisms of prostaglandin F2α (PGF2α)-induced protein synthesis in vascular smooth muscle cells (VSMC), we have studied its effect on two major signal transduction pathways: mitogen-activated protein kinases and phosphatidylinositol 3-kinase (PI3-kinase) and their downstream targets ribosomal protein S6 kinase (p70S6k) and eukaryotic initiation factor 4E (eIF4E) and its regulator 4E-BP1. PGF2α-induced protein synthesis is phosphorylated by mitogen-activated protein kinases (e.g. p38 MAPK, JNK, and ERK2) and PI3-kinase-dependent. Phosphorylation of eIF4E and 4E-BP1 was found to be sensitive to inhibition by both wortmannin and rapamycin. PI3-kinase activation, which is required for optimal eIF4E phosphorylation, is blocked by wortmannin. The phosphorylation of eIF4E and 4E-BP1 is also prevented by rapamycin, indicating that PI3-kinase activation is essential for the phosphorylation of eIF4E and 4E-BP1. These findings demonstrate that 1) PI3-kinase-dependent and independent mechanisms appear to be involved in PGF2α-induced activation of ERK2; 2) PGF2α-induced eIF4E phosphorylation is dependent on both ERK and PI3-kinase-dependent rapamycin-sensitive mechanisms; and 3) ERK-dependent eIF4E phosphorylation, but not PI3-kinase-dependent p70S6k activation correlates with PGF2α-induced global protein synthesis and bFGF-2 expression in VSMC.

Translational control plays an important role in regulation of gene expression (1–4). Several kinase cascades are implicated in the regulation of protein synthesis (5–7). One of the events associated with protein synthesis is the phosphorylation of ribosomal protein S6 (8). The serine/threonine kinase, p70S6k, phosphorylates ribosomal protein S6 (8, 9). Studies have reported that phosphatidylinositol 3-kinase (PI3-kinase)1 plays a role in the activation of p70S6k in response to a variety of mitogens (10–12). Another event that is critical in the regulation of protein synthesis is the binding of mRNA to ribosomes, which is facilitated by a translation initiation complex, eIF4F (1–4). eIF4F consists of three polypeptides as follows: eIF4A, an RNA helicase; eIF4E, the cap-binding protein; and eIF4G, a bridging protein for eIF4A and eIF4E (7, 13). The binding of eIF4F to mRNA is catalyzed by its smallest subunit eIF4E via interaction with the cap structure present in the 5′ of the eukaryotic mRNAs (1, 3). eIF4F complex is formed in combination with another initiation factor, eIF4B, thought to unwind the mRNA secondary structure thereby rendering it capable of binding to ribosomes (3). Because of its lowest abundance and its critical role in mRNA binding to ribosomes, eIF4E is considered to be a regulator of protein synthesis (1, 3). In fact, eIF4E activity has been reported to be regulated by several kinase cascades including protein kinase C and Mnk1, a downstream target of MAPKs (14–16). In addition, eIF4E activity is negatively regulated by its binding proteins, 4E-BP1 and -2 (17–20).

Vascular smooth muscle cell (VSMC) growth exhibits characteristic features of several proliferative cardiovascular diseases such as atherosclerosis and restenosis (21). Both receptor tyrosine kinase and G-protein-coupled receptor (GPCR) agonists can induce VSMC growth (22–26). Of interest, receptor tyrosine kinase agonists such as platelet-derived growth factor and fibroblast growth factor (FGF) cause a hyperplastic effect in these cells, whereas G protein-coupled receptor agonists such as angiotensin II (ang II), endothelin, and PGF2α often induce a hypertrophic effect (21–26). All three of the latter agonists mediate their effects via Gα-coupled receptors whose activation is characterized by increases in phospholipase C activity and intracellular Ca2+ mobilization (27–30). The hypertrophic effect of ang II has been reported to be mediated by p70S6k and ERKs (31, 32). Although PGF2α, a cyclooxygenase metabolite of arachidonic acid that is produced in a variety of

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‡ The abbreviations used are: PI3-kinase, phosphatidylinositol 3-kinase; bFGF-2, basic fibroblast growth factor-2; eIF4E, eukaryotic translation initiation factor 4E; 4E-BP1, eIF4E binding protein 1; ERK2, extracellular signal-regulated kinase 2; JNK1, Jun N-terminal kinase 1; MAPKs, mitogen-activated protein kinases; PGF2α, prostaglandin F2α; VSMC, vascular smooth muscle cells; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; MOPS, 4-morpholinepropanesulfonic acid; GPCR, G protein-coupled receptor; ang II, angiotensin II; mTOR, mammalian target of rapamycin.
cells in response to various stimuli including oxidant stress, has been shown to be a potent hypertrophic agonist for cardiac myocytes and VSMC, the mechanisms underlying this effect are largely unclear. The purpose of the present study is to investigate the signaling events that are evoked in response to PGF$_{2\alpha}$, leading to increased protein synthesis in VSMC. We found that PGF$_{2\alpha}$ activates ERK2 and JNK1 groups of MAPKs, P13-kinase, and p70S6k and induces phosphorylation of the translation regulators eIF4E and 4E-BP1 in VSMC. We also show a correlation between PGF$_{2\alpha}$-induced ERK activations, eIF4E and 4E-BP1 phosphorylation, global protein synthesis, and bFGF-2 expression. In addition, the present findings suggest that ERK-dependent and P13-kinase-dependent rapamycin-sensitive mechanisms modulate PGF$_{2\alpha}$-induced eIF4E and 4E-BP1 phosphorylation in VSMC.

**EXPERIMENTAL PROCEDURES**

**Materials**—Aprotinin, ATP, bovine myelin basic protein, dibutyryl cyclic AMP (cAMP), EGTA, β-glycerophosphate, leupeptin, phenylmethylsulfonyl fluoride (PMSF), phosphatidylinositol, sodium deoxycholate, sodium fluoride, sodium orthovanadate, and sodium pyrophosphate were obtained from Sigma. Prostaglandin F$_2\alpha$ (PGF$_2\alpha$), was from Cayman Chemical Co. (Ann Arbor, MI). Silica gel 60A thin layer chromatography plates and PS1 phosphocellulose filter paper were purchased from Whatman. Anti-ERK2 (sc-154), anti-JNK1 (sc-474), anti-p70S6k (sc-330), and anti-PGF-2 (sc-79) rabbit polyclonal antibodies and GST-HSV-1 ICP44 recombinant protein were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-P13-kinase rabbit polyclonal antibody (06-195) was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-phospho-p38 MAPK antibodies and PD 098059 were provided by New England Biolabs (Beverly, MA). Rapamycin and wortmannin were from Calbiochem. [3H]Thymidine (70 Ci/mmol), [35S]Methionine (1000 Ci/mmol), [3H]Prothorophosphatic acid (8500 Ci/mmol), [γ-32P]ATP (8000 Ci/mmol), and [methyl-3H]-thymidine (70 Ci/mmol) were obtained from NEN Life Science Products.

**Cell Culture**—VSMC were isolated from the thoracic aorta of 200-300 g male Sprague-Dawley rats by enzymatic dissociation as described earlier (33). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 95% air, 5% CO$_2$ atmosphere. Cells were growth-arrested by incubating in Dulbecco’s modified Eagle’s medium containing 0.1% (v/v) fetal bovine serum, followed by 1% (v/v) ethanol for an additional hour. The beads were washed three times with lysis buffer, three times with wash buffer (100 mM Tris-Cl, pH 7.6, 500 mM lithium chloride, 0.1% Triton X-100 and 1 mM diethiothreitol), and there with kinase buffer (12.5 mM Mops, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl$_2$, 0.5 mM EGTA, 0.5 mM Na$_2$VO$_4$, and 0.5 mM Na$_3$VO$_4$). The ERK2 activity present in the immunoprecipitates was determined by resuspension in 30 μl of kinase buffer containing 5 μg of myelin basic protein, 20 μM ATP, and 1 μCi of [γ-32P]ATP and incubation at 30 °C for 20 min. For JNK1 assay, incubation with the kinase buffer was the same as that for ERK2 assay except that 1 μg of GST-HSV-1 ICP44 (sc-79) was used instead of myelin basic protein (34). The reactions were stopped by adding 20 μl of 4× Laemmli sample buffer and heating the samples at 95 °C for 5 min. The samples were analyzed by electrophoresis on 0.1% SDS-12% polyacrylamide gels. The dried gel was exposed to X-Omat AR x-ray film with an intensifying screen for 1 to 4 h at −80 °C and developed. For determination of p38 MAPK activity, an equal amount of protein from control and test samples was analyzed by Western blotting for phosphorylated p38 MAPK using its phospohispecific antibodies.

**PI3-Kinase Assay**—After appropriate treatments, cells were lysed in 1 ml of lysis buffer (20 mM Heps, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM diethiothreitol, 1 mM Na$_2$VO$_4$, 1% Triton X-100, 10% glycerol, 2 μM leupeptin, 10 units/ml aprotinin, and 400 μM PMSF) for 10 min on ice. The cell lysates were cleared by centrifugation at 12,000 rpm for 15 min at 4 °C. Five hundred micromolars of protein from control and each treatment was immunoprecipitated with 5 μl of anti-PI3-kinase antibodies for 2 h at 4 °C, followed by incubation with 40 μl of 50% (w/v) protein A-Sepharose beads for an additional hour. The immunoprecipitates were washed three times with lysis buffer, three times with wash buffer, and three times with TNF buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 1 mM EDTA, and 10 μM Na$_3$VO$_4$). The kinase activity was measured by resuspending the immunoprecipitates in 30 μl of TNF buffer and incubating with 10 μl of 2 mg/ml phosphatidylinositol, 10 μl of 100 mM MgCl$_2$, 2 μl of 100 mM ATP, and 20 μCl of [γ-32P]ATP for 10 min at 22 °C. The reaction was terminated by addition of 20 μl of 5× HCl and 200 μl of chloroform:methanol (1:1) mix. The aqueous and organic phases were separated by centrifugation at 2000 rpm for 10 min. The organic phase containing the phosphoinositides was spotted onto silica gel 60A TLC plate coated with 1% potassium oxalate and developed in a solvent system consisting of chloroform:methanol:water:ammonium hydroxide (90:70:14.6:5.4). The TLC plate was exposed to X-Omat AR x-ray film for 4–6 h at −80 °C and developed.

**Western Blot Analysis**—After appropriate treatments, medium was aspirated, and cells were rinsed with cold PBS and lysed in 500 μl of lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EGTA, 80 mM β-glycerophosphate, 50 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate (Na$_3$VO$_4$)), was added to the frozen monolayers, thawed on ice for 15 min, and scraped into 1.5-ml Eppendorf tubes. The cell lysates were subjected to centrifugation at 12,000 rpm for 20 min at 4 °C. Protein content of the supernatants was determined using Bradford reagent from Bio-Rad. Cell lysates containing equal amounts of protein from each condition were incubated with 5 μl of eIF4E or 4E-BP1 polyclonal antibodies (35, 36) for 2 h with gentle rocking. Forty microliters of 10% (v/v) protein A-Sepharose beads were added, and incubation was continued for another 2 h. The beads were collected by centrifugation at 4000 rpm for 2 min at 4 °C and washed five times with cold lysis buffer and once with cold PBS. The beads were heated in 40 μl of Laemmli sample buffer at 95 °C for 5 min. The proteins were resolved by electrophoresis on 0.1% SDS-10% polyacrylamide gel. The gel was dried and exposed to X-Omat AR x-ray film with an intensifying screen for 2–4 h at −80 °C and developed.

**Protein and DNA Synthesis**—Growth-arrested VSMC were treated with the indicated concentrations of PGF$_{2\alpha}$ for the indicated times or were left untreated. Protein and DNA synthesis were measured by labeling cells with 1 μCi/ml [3H]thymidine and 1 μCi/ml [35S]methylene, respectively. After labeling, cells were washed with cold PBS, trypsinized, and collected by centrifugation. The cell pellet was suspended in cold 10% (v/v) trichloroacetic acid and vortexed vigorously to lyse the cells. After standing on ice for 20 min, the mixture was passed through a glass fiber filter (GF/C, Whatman). The filter was washed once with 5% cold trichloroacetic acid and once with cold 70% (v/v) ethanol. The filter was dried, placed in a liquid scintillation vial containing the mixture, and the radioactivity was measured in a liquid scintillation counter (LS 3801, Beckman).

All the experiments were repeated at least three times with similar
PGF₂α Induces Global Protein Synthesis via ERK Activation

FIG. 1. Effect of PGF₂α on protein and DNA synthesis in VSMC. Growth-arrested VSMC were treated with and without various concentrations of PGF₂α for 24 h (A) or with 1 μM PGF₂α, for different times (B) and protein synthesis was measured by [³⁵S]methionine incorporation. Cells were labeled with 1 μCi/ml [³⁵S]methionine for the last 12 h of the 24-h incubation period in the dose-response experiment and continuously in the time course experiment. The control values shown in B were that of VSMC labeled for 24 h in the absence of PGF₂α. No significant differences were observed between this and earlier time point controls. To determine DNA synthesis, growth-arrested VSMC were treated with and without 1 μM PGF₂α, or 10% (v/v) calf serum for 24 h and labeled with 1 μCi/ml [³⁵H]thymidine for the last 12 h of the 24-h incubation period (C). * p < 0.01 versus control.

RESULTS

To test the hypertrophic effect of PGF₂α in VSMC, we first studied the dose-response effect of PGF₂α on protein synthesis. Growth-arrested VSMC were treated with and without various concentrations of PGF₂α for 24 h, and protein synthesis was determined by [³⁵S]methionine incorporation. As shown in Fig. 1A, PGF₂α induced protein synthesis in a dose-dependent manner with a maximum effect (2-fold) at 1 μM. Increase in protein synthesis by PGF₂α occurred in a time-dependent manner as well (Fig. 1B). Earlier studies have reported that PGF₂α causes mitogenesis in some cell types (30). To find whether PGF₂α-induced increase in protein synthesis is due to stimulation of cell division, we tested its effect on DNA synthesis. PGF₂α had no significant effect on VSMC DNA synthesis as determined by [³⁵H]thymidine incorporation (Fig. 1C). Serum (10% v/v), which served as a positive control, stimulated VSMC DNA synthesis by 2-fold as compared with control. These results clearly indicate that PGF₂α induces protein synthesis without an effect on DNA synthesis in VSMC.

To understand the signal transduction pathways of PGF₂α-induced protein synthesis, we first studied the role of MAPKs. Growth-arrested VSMC were treated with and without 1 μM PGF₂α for various times or with different concentrations of PGF₂α, for 10 min, and the MAPK activities were determined. ERK2 and JNK1 were measured by immunocomplex kinase assay using myelin basic protein and GST-c-Jun-(1–79) as substrates, respectively, whereas p38 MAPK activation was determined by Western blotting using its phosphospecific antibodies. PGF₂α activated ERK2 and JNK1 in a time- and dose-dependent manner (Fig. 2, A–D). Activation of ERK2 by PGF₂α was maximum (7-fold) at 10 min, and thereafter it declined gradually reaching almost basal levels by 60 min (Fig. 2A). PGF₂α also activated JNK1 with a peak activity (4-fold) at 10 min, which then persisted at least for 60 min (Fig. 2B). Dose-response study showed that PGF₂α at 1 μM concentration was found to be more potent in the activation of ERK2 (Fig. 2C), whereas higher concentrations appear to be more effective in the activation of JNK1 (Fig. 2D). PGF₂α, however, had no significant effect on the activation of p38 MAPK in VSMC (Fig. 2E). Ang II (100 nM), which served as a positive control, activated p38 MAPK 3-fold as compared with untreated cells. For comparative purposes, the PGF₂α was used at 1 μM concentration.

A role for PI3-kinase was implicated both in receptor tyrosine kinase and GPCR agonist-induced activation of MAPKs (37–39). To find whether PGF₂α activates PI3-kinase in VSMC, growth-arrested cells were treated with and without 1 μM PGF₂α for various times, and the PI3-kinase activity was measured. PGF₂α induced PI3-kinase activity in a time-dependent manner in VSMC with a peak activity (6-fold) at 10 min (Fig. 3). PI3-kinase activity, however, had no significant effect on protein synthesis. Growth-arrested VSMC with various concentrations of wortmannin or 50 ng/ml rapamycin, and ERK2 activity was measured. Wortmannin inhibited the PGF₂α-induced ERK2 activity in a concentration-dependent manner with maximum effect (>50%) at 1 μM (Fig. 4A). Rapamycin also partially (35%)...
PGF$_{2\alpha}$ activates ERK2 and JNK1 groups of MAPKs in a time- and dose-dependent manner in VSMC. Growth-arrested VSMC were treated with and without 1 µM PGF$_{2\alpha}$ for the indicated times or with different concentrations of PGF$_{2\alpha}$ for 10 min, and cell lysates were prepared. Equal amount of protein from each condition was immunoprecipitated with anti-ERK2 or anti-JNK1 antibodies, and the kinase activities in the immunocomplexes were measured by immunocomplex kinase assay using myelin basic protein as a substrate for ERK2 activity and GST-c-Jun-(1-79) as a substrate for JNK1 activity. The reaction mixtures were separated by electrophoresis on SDS-polyacrylamide gel and subjected to autoradiography. For determination of p38 MAPK activation, equal amount of protein from control and various times of PGF$_{2\alpha}$-treated VSMC were analyzed by Western blotting using its phosphospecific antibodies. Ang II (100 nM) was used as a positive control. MBP, myelin basic protein.

FIG. 2. PGF$_{2\alpha}$ activates ERK2 and JNK1 groups of MAPKs in a time- and dose-dependent manner in VSMC. Upper panel, growth-arrested VSMC were treated with and without 1 µM PGF$_{2\alpha}$ for the indicated times, and cell lysates were prepared. Equal amount of protein from each condition was immunoprecipitated with anti-ERK2 or anti-JNK1 antibodies, and the kinase activities in the immunocomplexes were measured by immunocomplex kinase assay using myelin basic protein as a substrate for ERK2 activity and GST-c-Jun-(1-79) as a substrate for JNK1 activity. The reaction mixtures were separated by electrophoresis on SDS-polyacrylamide gel and subjected to autoradiography. For determination of p38 MAPK activation, equal amount of protein from control and various times of PGF$_{2\alpha}$-treated VSMC were analyzed by Western blotting using its phosphospecific antibodies. Ang II (100 nM) was used as a positive control. MBP, myelin basic protein.

FIG. 3. PGF$_{2\alpha}$ activates PI3-kinase in a time-dependent manner in VSMC. Upper panel, growth-arrested VSMC were treated with and without 1 µM PGF$_{2\alpha}$ for the indicated times, and cell lysates were prepared. Equal amount of protein from control and each treatment was immunoprecipitated with anti-PI3-kinase antibodies followed by SDS-PAGE (Fig. 5, upper panel). As expected, rapamycin (50 ng/ml) for 5 min, and the PI3-kinase activity was measured as described above. PIP, phosphatidylinositol 1,4,5-trisphosphate; ORI, origin.

Ribosomal protein S6 kinase, p70S6k, was reported to play an important role in protein synthesis initiation process (2, 3, 4, 7). To examine the effect of wortmannin (1 µM) and rapamycin (50 ng/ml) on the activation of JNK1 by PGF$_{2\alpha}$. As shown in Fig. 4B, wortmannin had no effect on PGF$_{2\alpha}$-induced JNK1 activity. Rapamycin also did not affect the JNK1 activation by PGF$_{2\alpha}$ (data not shown).

Ribosomal protein S6 kinase, p70S6k, was reported to play an important role in protein synthesis initiation process (8, 9, 43, 44). We, therefore, wanted to examine the effect of PGF$_{2\alpha}$ on p70S6k. Growth-arrested VSMC were treated with and without 1 µM PGF$_{2\alpha}$ for various times, and p70S6k activity was determined. PGF$_{2\alpha}$ activated p70S6k in a time-dependent manner as determined by the slower mobility of the phosphorylated p70S6k as compared with its nonphosphorylated form on SDS-PAGE (Fig. 5, upper panel). As expected, rapamycin (50 ng/ml), a potent and specific inhibitor of p70S6k (43, 44), completely blocked the p70S6k activity by PGF$_{2\alpha}$. To find the possible upstream kinases that are involved in PGF$_{2\alpha}$-induced p70S6k activation, we next studied the effect of wortmannin (1 µM) and rapamycin (50 ng/ml), the potent and specific inhibitors of the PI3-kinase (40, 41) and ERKs (42), respectively, on PGF$_{2\alpha}$-induced p70S6k activation. Wortmannin completely inhibited the PGF$_{2\alpha}$-induced p70S6k activation (Fig. 5, middle panel). PD 098059 also significantly inhibited the PGF$_{2\alpha}$-induced p70S6k activation (Fig. 5, lower panel).

A large body of evidence indicates that eIF4E plays a determinant role in protein synthesis (8, 9, 43, 44). When we examined the effect of PGF$_{2\alpha}$ on eIF4E phosphorylation. Growth-arrested VSMC were treated with and without 1 µM PGF$_{2\alpha}$ for various times, and the phosphorylation state of eIF4E was determined by immunoprecipitation using its specific antibodies followed by SDS-PAGE (35). PGF$_{2\alpha}$ induced phosphorylation of eIF4E in a time-dependent manner with a maximum effect (5-fold) at 20 min, which persisted at least for 60 min (Fig. 6, upper panel). To test the role of PI3-kinase, ERKs and rapamycin-sensitive targets in PGF$_{2\alpha}$ activation of eIF4E, we next studied the effect of wortmannin (1 µM), PD 098059 (50 µM), and rapamycin (50 ng/ml) on eIF4E phosphorylation. As shown in Fig. 6, lower panel, all three inhibitors, respectively, on eIF4E phosphorylation induced by PGF$_{2\alpha}$. A large body of evidence indicates that eIF4E plays a determinant role in protein synthesis (8, 9, 43, 44). When we examined the effect of PGF$_{2\alpha}$ on eIF4E phosphorylation. Growth-arrested VSMC were treated with and without 1 µM PGF$_{2\alpha}$ for various times, and the phosphorylation state of eIF4E was determined by immunoprecipitation using its specific antibodies followed by SDS-PAGE (35). PGF$_{2\alpha}$ induced phosphorylation of eIF4E in a time-dependent manner with a maximum effect (5-fold) at 20 min, which persisted at least for 60 min (Fig. 6, upper panel). To test the role of PI3-kinase, ERKs and rapamycin-sensitive targets in PGF$_{2\alpha}$ activation of eIF4E, we next studied the effect of wortmannin (1 µM), PD 098059 (50 µM), and rapamycin (50 ng/ml) on eIF4E phosphorylation. As shown in Fig. 6, lower panel, all three inhibitors, respectively, on eIF4E phosphorylation induced by PGF$_{2\alpha}$. All three
drugs blocked the PGF$_{2\alpha}$-induced eIF4E phosphorylation by 60–70% (Fig. 6, lower panel). PD 098059 and rapamycin also inhibited the basal eIF4E phosphorylation.

The activity of eIF4E was reported to be regulated by its binding proteins 4E-BP1 and 4E-BP2 (17–20). In addition, several studies have reported that wortmannin-dependent rapamycin-sensitive RAFT1/FRAP/mTOR phosphorylates 4E-BP1 (45–47). Upon phosphorylation, 4E-BP1 dissociates from eIF4E allowing the latter to be phosphorylated and activated. To find whether PGF$_{2\alpha}$ induces phosphorylation of 4E-BP1, and if so, the role of ERKs, PI3-kinase, and RAFT1/FRAP/mTOR in this phenomenon, growth-arrested and $[^{32}P]$orthophosphoric acid-labeled VSMC were treated with and without PGF$_{2\alpha}$ (1 mM) in the presence and absence of wortmannin (1 mM), PD 098059 (50 mM), or rapamycin (50 ng/ml) for 20 min, and the phosphorylation state of 4E-BP1 was measured by immunoprecipitation using its specific antibodies (36). PGF$_{2\alpha}$ induced 4E-BP1 phosphorylation 4-fold, and this effect was sensitive to inhibition by all three compounds (Fig. 7).

To relate the above signaling events to PGF$_{2\alpha}$-induced protein synthesis, we next studied the effect of wortmannin, rapamycin, and PD 098059 on PGF$_{2\alpha}$-induced activation of MAPKs. Growth-arrested VSMC were treated with and without 1 mM PGF$_{2\alpha}$ in the presence and absence of wortmannin (100 nM or 1 mM), rapamycin (50 ng/ml), or PD 098059 (50 mM) for 10 min, and the ERK2 and JNK1 activities were measured as described in Fig. 1. MBP, myelin basic protein.

To relate the above signaling events to PGF$_{2\alpha}$-induced protein synthesis, we next studied the effect of wortmannin, rapamycin, and PD 098059 on PGF$_{2\alpha}$-induced activation of MAPKs. Growth-arrested VSMC were treated with and without 1 mM PGF$_{2\alpha}$ in the presence and absence of wortmannin (100 nM or 1 mM), rapamycin (50 ng/ml), or PD 098059 (50 mM) for 10 min, and the ERK2 and JNK1 activities were measured as described in Fig. 1. MBP, myelin basic protein.

find whether the inhibition of PGF$_{2\alpha}$-induced protein synthesis by cAMP correlates with decreased ERKs activities, we further determined the effect of cAMP on PGF$_{2\alpha}$ activation of ERK2. As shown in Fig. 9, cAMP also inhibited the PGF$_{2\alpha}$-induced ERK2 activation. In order to find the role of the above mechanisms of PGF$_{2\alpha}$-induced global protein synthesis in the modulation of a specific protein, whose expression may be induced by this eicosanoid, we first studied its effect on the expression of bFGF-2. To study the possible mechanisms underlying PGF$_{2\alpha}$-induced bFGF-2 expression, we determined the effect of wortmannin (1 mM), PD 098059 (50 mM), and rapamycin (50 ng/ml) on PGF$_{2\alpha}$-induced bFGF-2 expression. Whereas wortmannin inhibited the PGF$_{2\alpha}$-induced bFGF-2 expression only partially (30%), PD 098059 completely blocked the effect. Rapamycin had no significant effect on PGF$_{2\alpha}$-induced bFGF-2 expression (Fig. 10, lower panel).

**DISCUSSION**

The novel findings of the present study are as follows: 1) PGF$_{2\alpha}$, an eicosanoid and a G protein-coupled receptor agonist,
activates several early response serine/threonine kinases such as MAPKs, PI3-kinase, and p70 S6k and induces eIF4E and 4E-BP1 phosphorylation, global protein synthesis, and bFGF-2 expression in growth-arrested VSMC; 2) PGF2α-induced global protein synthesis and bFGF-2 expression to a major extent require activation of ERKs but not PI3-kinase or p70S6k; 3) PGF2α activates ERK2 in a manner that is dependent and independent of PI3-kinase; and 4) although multiple signaling pathways seem to be involved in PGF2α-induced eIF4E phosphorylation, a correlation was observed only between ERK-dependent eIF4E phosphorylation and global protein synthesis by PGF2α. Duckworth and Cantley (37) have reported that both PI3-kinase-dependent and -independent mechanisms play a role in the activation of ERKs in Swiss 3T3 cells in response to platelet-derived growth factor, a receptor tyrosine kinase agonist. In addition, Seva et al. (39) have shown that gastrin, a GPCR agonist, activates ERKs via two signal transduction pathways in AR4–2J cells, of which one is sensitive to inhibition by PI3-kinase inhibitors. Our findings suggest at least two redundant pathways for activation of ERKs in VSMC by

FIG. 6. PGF2α induces phosphorylation of eIF4E in VSMC. Growth-arrested and [32P]orthophosphoric acid-labeled VSMC were treated with and without 1 µM PGF2α for various times or for 20 min in the presence and absence of the indicated inhibitors (wortmannin, 1 µM; PD 098059, 50 µM; rapamycin, 50 ng/ml), and cell lysates were prepared. Equal amount of protein from control and each treatment was immunoprecipitated with anti-eIF4E antibodies, and the immunocomplexes were separated by electrophoresis on SDS-polyacrylamide gel. The phosphorylated eIF4E was visualized by autoradiography.

FIG. 7. PGF2α induces phosphorylation of 4E-BP1 in VSMC. Growth-arrested and [32P]orthophosphoric acid-labeled VSMC were treated with and without 1 µM PGF2α in the presence and absence of the indicated inhibitors (wortmannin, 1 µM; PD 098059, 50 µM; rapamycin, 50 ng/ml) for 20 min, and cell lysates were prepared. Equal amount of protein from control and each treatment was immunoprecipitated with anti-4E-BP1 antibodies, and the immunocomplexes were separated by electrophoresis on SDS-polyacrylamide gel. The phosphorylated 4E-BP1 was visualized by autoradiography.

FIG. 8. PGF2α-induced global protein synthesis requires ERKs activities. Growth-arrested VSMC were treated with and without 1 µM PGF2α for 24 h in the presence and absence of the indicated inhibitors (wortmannin, 1 µM; PD 098059, 50 µM; rapamycin, 50 ng/ml; cAMP, 1 mM), and protein synthesis was measured by pulse-labeling the cells for the last 12 h of the 24-h incubation period with 1 µCi/ml [35S]methionine. *, p < 0.01 versus control; **, p < 0.01 versus PGF2α alone.

FIG. 9. Effect of cAMP on PGF2α-induced activation of ERK2. Growth-arrested VSMC were treated with and without 1 µM PGF2α in the presence and absence of 1 mM cAMP for 10 min, and the ERK2 activity was measured as described in the legend to Fig. 1. MBP, myelin basic protein.
PGF$_{2\alpha}$, of which one is sensitive to PI3-kinase inhibitors. Numerous studies have reported a requirement for PI3-kinase activity in p70$^{S6K}$ activation by various stimulants in several cell types (10–12, 40). The present finding that wortmannin, a potent inhibitor of PI3-kinase, blocks the PGF$_{2\alpha}$-induced p70$^{S6K}$ activation is consistent with the above reports. Since wortmannin blocked the PGF$_{2\alpha}$-induced p70$^{S6K}$ activation completely and ERK2 activation partially, it is likely that PI3-kinase lies upstream to and signals several kinase cascades leading to gene expression. Although wortmannin inhibited the PGF$_{2\alpha}$-induced ERK2 activity by 50%, it had no significant effect on PGF$_{2\alpha}$-induced global protein synthesis, which was sensitive to inhibition by PD 098059, a MEK1 inhibitor, suggesting that PI3-kinase-independent ERK2 activation is more important than PI3-kinase-dependent rapamycin-sensitive mechanisms in the induction of global protein synthesis by PGF$_{2\alpha}$. This view can be further supported by the finding that rapamycin, although it inhibits ERK2 activity partially, fails to block PGF$_{2\alpha}$-induced global protein synthesis. These findings further suggest complexity in the coupling of ERKs to various signaling kinase cascades targeting modulation of different effector molecules (25, 37, 38).

The present findings suggest that ERKs play an important role in the phosphorylation of eIF4E and 4E-BP1, the regulators of protein synthesis, by PGF$_{2\alpha}$. Our results also indicate a role for PI3-kinase-dependent rapamycin-sensitive mechanisms in PGF$_{2\alpha}$-induced phosphorylation of eIF4E and 4E-BP1 as these events were inhibited by wortmannin and rapamycin. Indeed, insulin-induced eIF4E and PHAS-1 (4E-BP1) phosphorylation was also reported to be inhibited by wortmannin and rapamycin in myeloid progenitor cells (50). However, the PI3-kinase-dependent rapamycin-sensitive eIF4E and 4E-BP1 phosphorylation may also be mediated by ERKs as the PI3-kinase inhibitor, wortmannin, and the RAFT1/FRAP/mTOR inhibitor, rapamycin, significantly reduced ERKs activities induced by PGF$_{2\alpha}$. ERKs may play a role downstream to mTOR in the signaling pathway of PI3-kinase-dependent rapamycin-sensitive eIF4E and 4E-BP1 phosphorylation. Based on the sensitivity of eIF4E and 4E-BP1 phosphorylation to inhibition by various kinase inhibitors, it is tempting to speculate a role for multiple signal transduction pathways in the modulation of activity of these important regulators of protein synthesis. Furthermore, different groups of MAPKs may be involved in the activation of eIF4E by different stimulants (51). To cite an example in support of this notion, Morley and McKendrick (52) have demonstrated that serum-induced eIF4E phosphorylation was blocked by the ERK MAPK pathway inhibitor PD 098059, whereas anisomycin-induced eIF4E phosphorylation was attenuated by the p38 MAPK inhibitor SB 203580. It is noteworthy that while PD 098059, wortmannin, and rapamycin decreased PGF$_{2\alpha}$-induced phosphorylation of eIF4E and 4E-BP1 by more than 60%, only PD 098059 significantly inhibited the protein synthesis stimulated by PGF$_{2\alpha}$. These findings suggest that enhanced phosphorylation of eIF4E and 4E-BP1 alone may not be sufficient for global protein synthesis induced by PGF$_{2\alpha}$. From these findings, it is also conceivable that coordinate regulation of both transcriptional and translational events may be required for increased protein synthesis by PGF$_{2\alpha}$. Indeed, this view further supports the role of ERKs in PGF$_{2\alpha}$-induced protein synthesis as these kinases modulate the activities of both translation regulators such as eIF4E and its binding proteins 4E-BP1/2 and transcriptional factors such as AP-1 (7, 53). Additional evidence in support of a role for transcriptional mechanisms in the induction of global protein synthesis comes from the recent findings that calcineurin, a calcium-dependent phosphatase, plays a role in cardiac hypertrophy (54). Calcineurin modulates the activity of several transcription factors including nuclear factor of activated T cells (55). Although further studies are required to test whether calcineurin-nuclear factor of activated T cells plays a role in PGF$_{2\alpha}$-induced protein synthesis, these findings together with those of Sussman et al. (54) and Molkentin et al. (55) clearly suggest a role for both transcriptional and translational events in induced global protein synthesis.

PI3-kinase was reportedly shown to play a role in the activation of p70$^{S6K}$ in a manner that is sensitive to inhibition by rapamycin (10–12). A similar mechanism appears to be operative in the activation of p70$^{S6K}$ by PGF$_{2\alpha}$ as this event is blocked by both wortmannin and rapamycin, the PI3-kinase, and mTOR inhibitors, respectively. Rapamycin, complexed with its receptor FKB12, binds to mTOR (mammalian target of rapamycin) protein and inhibits its function (56). mTOR plays a role in the activation of p70$^{S6K}$ (47, 57). In addition, it was reported that mTOR regulates the activities of p70$^{S6K}$ and 4E-BP1 in a parallel manner (57). Although our results demonstrate the ability of PGF$_{2\alpha}$ in the activation of both of these signaling pathways in VSMC, their role, in particular of p70$^{S6K}$ in PGF$_{2\alpha}$-induced hypertrophy is obscure as the inhibitors of these pathways failed to significantly reduce the protein synthesis induced by this agonist. However, ang II, a peptide hormone and a G protein-coupled receptor agonist, was reported to stimulate global protein synthesis via a mechanism involving p70$^{S6K}$ activation in VSMC (31). It was also reported that ang II induces 4E-BP1 phosphorylation via a MAPK-independent mechanism (58). In addition, we have previously shown that ang II induces eIF4E phosphorylation and PKC plays a role in this event (59). Based on these observations as well as the present findings, it is likely that different signaling mechanisms are involved in the induction of protein synthesis by different G protein-coupled receptor agonists in VSMC. PGF$_{2\alpha}$ activation of the p70$^{S6K}$ pathway may be linked to the regulation of a subset of genes whose identity has not yet been addressed.
The present study demonstrates that PGF$_{2\alpha}$, an arachidonic acid metabolite and a G protein-coupled receptor agonist, induces protein synthesis via activation of ERKs in VSMC. These findings also provide the first evidence for the role of PI3-kinase-dependent and -independent ERK MAPK pathways in the phosphorylation of eIF4E induced by PGF$_{2\alpha}$ in VSMC. In addition, these results indicate a major role for PI3-kinase-dependent ERK-mediated eIF4E phosphorylation in PGF$_{2\alpha}$-induced global protein synthesis and bFGF-2 expression in VSMC.

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