Thrombin induces expression of FGF-2 via activation of PI3K-Akt-Fra-1 signaling axis leading to DNA synthesis and motility in vascular smooth muscle cells

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Thrombin induces expression of FGF-2 via activation of PI3K-Akt-Fra-1 signaling axis leading to DNA synthesis and motility in vascular smooth muscle cells. Am J Physiol Cell Physiol 290: C172–C182, 2006. First published September 7, 2005; doi:10.1152/ajpcell.00284.2005.—To understand the mechanisms by which thrombin induces vascular smooth muscle cell (VSMC) DNA synthesis and motility, we have studied the role of phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR)-S6K1 signaling. Thrombin stimulated the phosphorylation of Akt and S6K1 in VSMC in a sustained manner. Blockade of PI3K-Akt-mTOR-S6K1 signaling by LY-294002, and rapamycin suppressed both thrombin-induced VSMC DNA synthesis and migration. Adenovirus-mediated expression of dominant-negative Akt also inhibited thrombin-induced VSMC DNA synthesis and migration. Furthermore, thrombin induced the expression of Fra-1 in a sustained PI3K-Akt-dependent and mTOR-independent manner in VSMC. Suppression of Fra-1 by its small interfering RNA attenuated both thrombin-induced VSMC DNA synthesis and migration. Thrombin also induced the expression of FGF-2 in a PI3K-Akt-Fra-1-dependent and mTOR-independent manner, and neutralizing anti-FGF-2 antibodies inhibited thrombin-stimulated VSMC DNA synthesis and motility. In addition, thrombin stimulated the tyrosine phosphorylation of EGFR receptor (EGFR), and inhibition of its kinase activity significantly blocked Akt and S6K1 phosphorylation, Fra-1 and FGF-2 expression, DNA synthesis, and motility induced by thrombin in VSMC. Together these observations suggest that thrombin induces both VSMC DNA synthesis and motility via EGFR-dependent stimulation of PI3K/Akt signaling targeting in parallel the Fra-1-mediated FGF-2 expression and mTOR-S6K1 activation.

Thrombin, a serine protease, in addition to its essential role in coagulation, elicits both mitogenic and motogenic effects in several cell types, including vascular smooth muscle cells (VSMC) (6, 20, 36). Because thrombin is produced at the sites of vascular injury and influences both migration and proliferation of VSMC (20, 36), it is implicated in the pathogenesis of vessel wall diseases (14). In addition, the mechanism(s) by which thrombin influences its nonthrombotic actions, particularly the mitogenic effects in VSMC, is fairly well studied (4, 30, 33, 26, 31, 29, 16). Like many other G protein-coupled receptor (GPCR) agonists, thrombin, in addition to activation of its cognate protease-activated receptors, stimulates a number of signaling events in mediating its nonthrombotic effects. Specifically, it exhibits a requirement for activation of receptor tyrosine kinases (RTK) such as FGF receptor-1 (FGFR-1) and IGF-I receptor in mediating its mitogenic effects in VSMC (30, 33). In addition, a role for activation of nonreceptor tyrosine kinases (NRTK) such as Src and Jak in thrombin-induced VSMC proliferation has been demonstrated (26, 33). A requirement for activation of RTK and/or NRTK in the stimulation of serine/threonine kinases, such as ERK1/2, JNK1/2, and p38MAPK groups of mitogen-activated protein kinases, and their role in mediating the mitogenic effects of thrombin have also been investigated (16, 26, 29, 31, 33). Similar mechanisms have been reported to be involved in the mitogenic effects of thrombin in other cell types (8, 27, 28). However, the mechanisms by which thrombin elicits its mitogenic effects in VSMC are less clear.

Phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR)-S6K1 signaling plays an essential role in cell survival (40, 45, 46). Akt, an effector of PI3K via phosphorylation of a number of molecules, mediates antiapoptotic, mitogenic, motogenic, and thrombotic effects in a variety of target cells in response to the respective external cues (1, 5, 10, 13, 15, 19, 40, 45, 46). Despite the involvement of Akt in mediating antiapoptotic, mitogenic, motogenic, and thrombotic signaling events, its role in the nonthrombotic effects of thrombin was either less understood or disputed. For example, it was reported that thrombin causes a transient and weak activation of Akt in VSMC, and this mode of Akt stimulation was attributed to modulation of the regulation of expression of genes such as smooth muscle-specific myosin heavy chain gene enduring redifferentiation of VSMC (35). In contrast, in other cell types such as Chinese hamster fibroblasts, thrombin activated Akt in a sustained and robust manner, and this mode of Akt stimulation was shown to be associated with the proliferative capacity of the cell (17). In fact, Akt has been shown to be involved in the regulation of Fra-1, a protooncogene that plays an important role in the mediation of cell motility and proliferation (2, 39). The inability of thrombin to stimulate Akt in a sustained manner in VSMC was even thought to be a cause for its lack of effect as a mitogen to this cell type (35). However, work from various laboratories, including ours (30), has shown that thrombin elicits both mitogenic and motogenic effects in VSMC (4, 17, 20, 26, 33). To understand the signaling events of its nonthrombotic effects in VSMC, we have studied the role of PI3K-Akt-mTOR-S6K1 signaling. We found that thrombin activates Akt and one of its effectors, S6K1, in a sustained and PI3K-dependent manner in VSMC. Inhibition of Akt and S6K1 activation by pharmacological and genetic approaches suppressed both the mitogenic and motogenic effects of thrombin in VSMC. Thrombin in-
duced the expression of Fra-1 in a sustained PI3K-Akt-dependent and mTOR-independent manner in VSMC. Inhibition of expression of Fra-1 by its small interfering RNA (siRNA) attenuated both thrombin-induced VSMC DNA synthesis and motility. Thrombin also induced the expression of FGF-2 in a PI3K-Akt-dependent and mTOR-independent manner in VSMC. Neutralizing anti-FGF-2 antibodies suppressed both thrombin-induced VSMC DNA synthesis and motility. In addition, thrombin stimulated tyrosine phosphorylation of EGF receptor (EGFR), and inhibition of its kinase activity reduced Akt and S6K1 phosphorylation, Fra-1 and FGF-2 expression, DNA synthesis, and motility induced by thrombin in VSMC. Together these observations suggest that thrombin elicits both mitogenic and motogenic effects in VSMC, and these responses require EGFR-dependent stimulation of PI3K-Akt signaling targeting in parallel the Fra-1-mediated FGF-2 expression and mTOR-S6K1 activation.

MATERIALS AND METHODS

Reagents. Aprotinin, dithiothreitol, HEPEs, hydroxyurea, PMSF, sodium orthovanadate, sodium deoxycholate, leupeptin, and thrombin were purchased from Sigma (St. Louis, MO). Lipofectamine 2000 was bought from Invitrogen (Carlsbad, CA). Neutralizing anti-FGF-2 antibodies were purchased from PerkinElmer Life Sciences (Boston, MA). Fra-1 siRNA obtained from Dharmacon (Lafayette, CO). Adenoviral vector harboring green fluorescent protein (ad-GFP) was constructed as described previously (25). Adenoviral vector harboring dominant-negative (dn)Akt (ad-dnAkt) was constructed by Fujio et al. (12) and was generously provided to us by Dr. Kenneth Walsh (Boston University School of Medicine, Boston, MA) for use in our experiments. Anti-Akt (catalog no. 9272), anti-phospho-Akt (catalog no. 9271), and anti- phospho-S6K1 (catalog no. 9204) antibodies were procured from Cell Signaling Technology (Beverly, MA). Anti-EGFR (catalog no. SC-03) and anti-FGF-2 (catalog no. SC-079) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibodies (catalog no. 05-321, clone 4G10) were obtained from Upstate (Charlottesville, VA). Neutralizing anti-FGF-2 (AB-33-NA) antibodies were bought from R & D Systems (Minneapolis, MN). Lipofectamine 2000 was bought from Invitrogen (Carlsbad, CA). 3H-thymidine (specific activity 78 or 20 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). Fra-1 siRNA sense (5'-AAG UUC CAC CUU GUG GCA AGC dtTdT-3') and antisense (5'-GCU UGG CAC AAG GUG GAA CUU dtTdT-3') oligonucleotides were made by Dharmacon (Lafayette, CO). Adenoviral vector harboring dominant-negative (dn)Akt (ad-dnAkt) was constructed by Fujio et al. (12) and was generously provided to us by Dr. Kenneth Walsh (Boston University School of Medicine, Boston, MA) for use in our experiments. Adenoviral vector harboring green fluorescent protein (ad-GFP) was constructed as described previously (25).

Cell culture. VSMC were isolated from the thoracic aortas of 120- to 150-g male Sprague-Dawley rats by enzymatic dissociation as described previously (9). Cells were grown in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained at 37°C in a humidified 95% air-5% CO2 atmosphere. Cells were quiesced by incubating in DMEM containing 0.1% calf serum for 72 h and used to perform the experiments unless otherwise stated.

Cell motility. VSMC motility was measured by cell wound assay (9). Quiescent confluent monolayers of VSMC were wounded with a sterile pipette tip to generate a cell-free gap of ~1-mm width, and the wound location in the culture dish was marked. Cells were washed, and fresh serum-free DMEM was added and photographed to record the wound width at 0 h. To prevent replicative DNA synthesis, hydroxyurea was added to the medium to a final concentration of 5 mM just before the addition of agonist. Twenty-four hours after the appropriate treatments, photographs were taken again at the marked wound location. Cell migration was measured with the NIH Image 1.62 program, and cell motility was expressed as distance migrated in micrometers.

DNA synthesis. VSMC with and without appropriate treatments were labeled with 1 µCi/ml [3H]thymidine for the last 12 h of the 24-h incubation period. After being labeled, cells were washed with cold PBS, trypsinized, and collected by centrifugation. The cell pellet was suspended in cold 10% (wt/vol) TCA and vortexed vigorously to lyse cells. After standing on ice for 20 min, the cell lysate mixture was passed through a glass fiber filter (GF/C, Whatman). The filter was washed once with cold 5% TCA and once with cold 70% (vol/vol) ethanol. The filter was dried and placed in a liquid scintillation vial containing the scintillant fluid, and radioactivity was measured in a Beckman Coulter liquid scintillation counter (LS 6500).

Immunoprecipitation. After appropriate treatments, VSMC were rinsed with cold PBS and lysed in 500 µl of lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, 100 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM sodium orthovanadate) on ice for 20 min. The cell extracts were scraped into 1.5-ml Eppendorf tubes and cleared by centrifugation at 12,000 rpm for 20 min at 4°C. Cell extracts containing 500 µg of protein from control and each treatment were immunoprecipitated with 3 µg of anti-EGFR antibodies overnight at 4°C. The immunocomplexes were collected by incubation with 40 µl of 50% (wt/vol) protein A/G Sepharose beads, followed by serial washings with lysis buffer and PBS. The immunocomplexed protein A/G Sepharose beads were suspended in 50 µl of the Laemmli sample buffer, heated for 10 min in boiling water, resolved by 0.1% SDS-10% PAGE, and subjected to Western blot analysis using anti-phosphotyrosine antibodies.

Western blot analysis. After appropriate treatments, VSMC were rinsed with cold PBS and lysed in 250 µl of lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, 100 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM sodium orthovanadate) on ice for 20 min. The cell extracts were scraped into 1.5-ml Eppendorf tubes and cleared by centrifugation at 12,000 rpm for 20 min at 4°C. Cell extracts containing an equal amount of protein were resolved by electrophoresis on 0.1% SDS and 10% polyacrylamide gels. The proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond; Amersham Biosciences, Piscataway, NJ). After blocking in 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (wt/vol) 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 with a chemiluminescence reagent kit (Amer sham Biosciences).

Statistics. All experiments were performed three times with reproducible results. Data are presented as means ± SD, and the treatment effects were analyzed by one-way ANOVA, followed by Bonferroni’s multiple comparison test. Comparisons between two groups were performed using Student’s t-test. P values <0.05 were considered to be statistically significant. In the case of Western blot analysis, one representative set of data is presented.

RESULTS

To understand the mechanisms by which thrombin influences its nonthrombotic effects in VSMC, we have studied the role of Akt. Quiescent VSMC were treated with and without thrombin (0.5 µM) for the indicated times, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for phosphorylation of Akt and one of its effectors, S6K1, using their phosphospecific antibodies. Thrombin stimulated phosphorylation of both Akt (Ser473) and S6K1 (Thr421/Ser424) in a time-dependent manner, with a maximum of fivefold increase at 1 h of treatment and a return to near-basal levels by 8 h (Fig. 1, A and B). Akt and S6K1 are downstream effector molecules of PI3K (11, 22, 42). Therefore, to find out whether thrombin-
stimulated Akt and S6K1 phosphorylation depend on activation of PI3K, we tested the effect of its specific inhibitor, LY-294002 (41). As shown in Fig. 1, C and D, LY-294002 (25 μM) completely inhibited thrombin-induced phosphorylation of Akt and S6K1. This finding suggests that thrombin stimulates the phosphorylation of both Akt and S6K1 in a PI3K-dependent manner in VSMC. mTOR is a downstream effector of Akt and an upstream regulator of S6K1 (11, 22, 42). Therefore, to understand the order in which Akt and S6K1 phosphorylation occur, we next tested the effect of rapamycin, a potent inhibitor of mTOR (3). Rapamycin (50 ng/ml) also completely inhibited S6K1 phosphorylation induced by thrombin (Fig. 1, C and D). Rapamycin, however, alone increased Akt phosphorylation compared with control, and this response may reflect a compensatory loop in the activation of Akt, perhaps to override mTOR inhibition. Together these results indicate that thrombin activates PI3K-Akt-mTOR-S6K1 signaling in this order in VSMC.

Having found that thrombin activates Akt and S6K1 in a sustained manner, we next wanted to test their role in thrombin-induced DNA synthesis. Quiescent cells were treated with and without thrombin (0.5 U/ml) in the presence and absence of LY-294002 (25 μM) or rapamycin (50 ng/ml) for 24 h, and DNA synthesis was determined by labeling cells with 1 μCi/ml [3H]thymidine for the last 12 h of the 24-h incubation period, followed by measuring the TCA-precipitable radioactivity. Thrombin induced VSMC DNA synthesis by about fourfold compared with control, and LY-294002 and rapamycin, the PI3K and mTOR inhibitors, respectively, suppressed this effect (Fig. 2A). These results suggest that the mitogenic effect of thrombin requires activation of PI3K-Akt-mTOR-S6K1 signaling in VSMC. We next studied the role of PI3K-Akt-mTOR-S6K1 signaling in thrombin-induced VSMC migration. A cell-free gap was produced in the monolayer of quiescent VSMC, and then the cells were treated with and without thrombin (0.5 U/ml) in the presence and absence of LY-294002 (25 μM) or rapamycin (50 ng/ml) for 24 h and cell migration was measured as described in MATERIALS AND METHODS. As shown in Fig. 2B, thrombin induced VSMC migration by about twofold compared with control, and this effect was completely suppressed by both LY-294002 and rapamycin. This result indicates that thrombin-induced VSMC migration also depends on activation of PI3K-Akt-mTOR-S6K1 signaling.

A recent study showed that Fra-1 is an effector gene for PI3K-Akt signaling (39). To understand the potential mechanism(s) by which Akt and S6K1 modulate thrombin-induced VSMC DNA synthesis and migration, we first studied the time course effect of thrombin on Fra-1 expression. Quiescent VSMC were treated with and without thrombin (0.5 U/ml) for the indicated times, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for Fra-1 expression.
antibodies. Thrombin induced the expression of Fra-1 in a time-dependent manner, with a maximum increase of >20-fold at 8 h of treatment (Fig. 3, A and B). To determine whether thrombin-induced expression of Fra-1 is mediated by activation of PI3K-Akt-mTOR-S6K1 signaling, we next tested the effect of LY-294002 and rapamycin. Interestingly, only LY-294002 but not rapamycin blocked thrombin-induced Fra-1 expression by ~65% (Fig. 3, C and D). To find out the involvement of Fra-1 in thrombin-induced VSMC DNA synthesis and migration, we used a siRNA approach. VSMC that were transfected with mock or Fra-1 siRNA (200 nM) were quiesced and treated with and without thrombin (0.5 U/ml) for 8 h, and cell extracts were prepared and analyzed for Fra-1 levels as described above. In response to thrombin, Fra-1 expression was increased in mock-transfected VSMC, and these levels were reduced by 70% in cells that received Fra-1 siRNA (Fig. 4, A and B). Fra-1 siRNA had no effect on the levels of Fra-1-unrelated protein such as Akt. We then studied the effect of Fra-1 siRNA on thrombin-induced VSMC DNA synthesis and migration. VSMC that were transfected with mock or Fra-1 siRNA (200 nM) and quiesced. DNA synthesis and motility were measured by [3H]thymidine incorporation and wounding assay, respectively, in response to 0.5 U/ml thrombin. As shown in Fig. 4, C and D, Fra-1 siRNA substantially blocked both thrombin-induced VSMC DNA synthesis and migration.

To confirm the role of PI3K-Akt-mTOR-S6K1 signaling in thrombin-induced VSMC DNA synthesis and migration, we also used a dominant-negative mutant approach. VSMC were infected with adenovirus expressing either GFP or dnAkt at a multiplicity of infection (MOI) of 80, quiesced, and treated with and without thrombin (0.5 U/ml) for 1 or 8 h, and cell extracts were prepared. Equal amounts of protein from control and 1-h treatments were analyzed for phosphorylated levels of S6K1, whereas the samples of control and 8-h treatments were tested for Fra-1 expression by Western blotting using their specific antibodies. Adenovirus-mediated expression of dnAkt but not GFP completely blocked thrombin-induced S6K1 phosphorylation (Fig. 5, A and B). This result and the finding that rapamycin does not suppress thrombin-induced phosphorylation of Akt suggest that Akt is upstream from mTOR-S6K1 in VSMC. Adenovirus-mediated expression of dnAkt, but not GFP, also blocked thrombin-induced Fra-1 expression by ~70% (Fig. 5, A and B). This result, in concert with the inhibitory effect of LY-294002 and the lack of effect of rapamycin on thrombin-induced Fra-1 levels, reveals that thrombin induces Fra-1 expression in a PI3K-Akt-dependent and mTOR-independent manner in VSMC. To test the effect of dnAkt on thrombin-induced VSMC migration, cells at ~80% confluence were infected with ad-GFP or ad-dnAkt (MOI 80), quiesced, and treated with and without thrombin (0.5 U/ml) for 24 h, and DNA synthesis was measured by [3H]thymidine incorporation. Consistent with the effect of LY-294002 and rapamycin, adenovirus-mediated expression of dnAkt also blocked thrombin-induced VSMC DNA synthesis (Fig. 5C). To test the effect of dnAkt on thrombin-induced VSMC migration, cells at ~80% confluence were infected with ad-GFP or ad-dnAkt (MOI 80), quiesced, and a cell-free gap was produced in the quiescent

![Fig. 2. Inhibition of phosphatidylinositol 3-kinase (PI3K) or mammalian target of rapamycin (mTOR) suppresses thrombin-induced VSMC DNA synthesis and motility. A: quiescent VSMC were treated with and without thrombin (0.5 U/ml) in the presence and absence of LY-294002 (25 μM) or rapamycin (50 ng/ml) for 24 h, and DNA synthesis was measured by [3H]thymidine incorporation. B: a cell-free gap was produced in the quiescent VSMC monolayer, treated with and without thrombin (0.5 U/ml) in the presence and absence of LY-294002 (25 μM) or rapamycin (50 ng/ml) for 24 h, and cell migration was measured. *P < 0.01 vs. control; **P < 0.01 vs. thrombin treatment alone.](http://ajpcell.physiology.org/)
VSMC monolayer. The quiescent cells were then treated with and without thrombin (0.5 U/ml) for 24 h, and cell migration was measured as described above. VSMC infected with adenovirus expressing dnAkt but not GFP failed to respond to thrombin-induced migration (Fig. 5D). Inhibition of activation of PI3K and Akt or suppression of expression of Fra-1 caused a little more decrease in both VSMC DNA synthesis and motility compared with control, suggesting the importance of PI3K-Akt-Fra-1 signaling in the regulation of even the basal growth and migration capacity of these cells.

Previously we reported (32) that PI3K plays a role in prostaglandin F2α-induced FGF-2 expression in VSMC. To learn whether thrombin also induces FGF-2 expression and, if so, the role of PI3K-Akt-mTOR-S6K1 signaling, we first studied the time course effect. Quiescent VSMC were treated with and without thrombin (0.5 U/ml) for the indicated times, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for Fra-1 levels using its specific antibodies. Bar graphs in B and D show quantitative analysis of 3 independent experiments on the time course and the effect of LY-294002 and rapamycin on thrombin-induced expression of Fra-1, respectively. *P < 0.05 vs. control; **P < 0.01 vs. control; ***P < 0.05 vs. thrombin treatment alone.

Fig. 3. Thrombin induces Fra-1 expression in PI3K-dependent and mTOR-independent manner in VSMC. Quiescent VSMC were treated with and without thrombin (0.5 U/ml) for the indicated times (A) or with and without thrombin (0.5 U/ml) in the presence and absence of LY-294002 (25 μM) or rapamycin (50 ng/ml) for 8 h (C), and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for Fra-1 levels using its specific antibodies. Bar graphs in B and D show quantitative analysis of 3 independent experiments on the time course and the effect of LY-294002 and rapamycin on thrombin-induced expression of Fra-1, respectively. *P < 0.05 vs. control; **P < 0.01 vs. control; ***P < 0.05 vs. thrombin treatment alone.
completely blocked thrombin-induced FGF-2 expression (Fig. 6B). This result indicates that thrombin-induced FGF-2 expression requires activation of PI3K but not mTOR. To find out whether thrombin-induced FGF-2 expression involves Akt, we also determined the effect of dnAkt. Quiescent VSMC that were infected with ad-GFP or ad-dnAkt at a MOI of 80 and quiesced were treated with and without thrombin (0.5 U/ml) for 8 h, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for Fra-1 levels using its specific antibodies. Because Fra-1 is a component of AP-1 transcriptional factor and its expression in response to thrombin required activation of PI3K-Akt, we next wanted to determine the role of Fra-1 in thrombin-induced FGF-2 expression. VSMC that were transfected with mock or Fra-1 siRNA were quiesced, and DNA synthesis (C) and motility (D) in response to thrombin (0.5 U/ml) were measured as described in Fig. 2. *P < 0.01 vs. control; **P < 0.05 vs. thrombin treatment alone.

Fig. 4. Suppression of Fra-1 by small interfering RNA (siRNA) prevents thrombin-induced VSMC DNA synthesis and motility. A: VSMC that were transfected with mock or Fra-1 siRNA were quiesced and treated with and without thrombin (0.5 U/ml) for 8 h, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for Fra-1 levels using its specific antibodies. B: quantitative analysis of 3 independent experiments on the effect of Fra-1 siRNA on thrombin-induced Fra-1 expression. C and D: VSMC that were transfected with mock or Fra-1 siRNA were quiesced, and DNA synthesis (C) and motility (D) in response to thrombin (0.5 U/ml) were measured as described in Fig. 2. *P < 0.01 vs. control; **P < 0.05 vs. thrombin treatment alone.
Fra-1 siRNA reduced thrombin-induced FGF-2 levels by 60% (Fig. 6D). To understand the functional role of FGF-2, we then determined the effect of neutralizing anti-FGF-2 antibodies on thrombin-induced VSMC DNA synthesis and motility. Quiescent VSMC that were preincubated with neutralizing anti-FGF-2 antibodies (30 μg/ml) significantly failed either to incorporate [3H]thymidine into DNA or to migrate in response to thrombin (Fig. 7).

Fig. 5. Adenovirus-mediated expression of dominant-negative (dn)Akt but not GFP suppresses thrombin-induced S6K1 phosphorylation, Fra-1 expression, DNA synthesis, and motility. A: VSMC were infected with adenovirus expressing either GFP or dnAkt, quiesced, and treated with and without thrombin (0.5 U/ml) for 1 h or 8 h, and cell extracts were prepared. S6K1 phosphorylation and Fra-1 levels were measured by Western blot analysis using their specific antibodies. B: quantitative analysis of 3 independent experiments on the effect of dnAkt on thrombin-induced S6K1 phosphorylation and Fra-1 expression. C: VSMC that were infected with ad-GFP or ad-dnAkt and quiesced were treated with and without thrombin (0.5 U/ml) for 24 h, and DNA synthesis was measured by [3H]thymidine incorporation. D: VSMC that were infected with ad-GFP or ad-dnAkt were quiesced, and a cell-free gap was generated and treated with and without thrombin (0.5 U/ml) for 24 h. At the end of 24-h thrombin treatment, VSMC migration was measured as described in Fig. 2. *P < 0.01 vs. control; **P 0.01 vs. thrombin treatment alone; ***P < 0.05 vs. thrombin treatment alone.
Earlier studies from our laboratory (30) as well as others (7, 20) demonstrated that GPCR agonists require activation of RTK in mediating their cellular effects. To understand the upstream mechanisms by which thrombin activates PI3K-Akt-mTOR-S6K1 signaling, we studied the role of EGFR. Quiescent VSMC were treated with and without thrombin (0.5 U/ml) for the indicated times, and cell extracts were prepared. Five hundred micrograms of protein from control and each treatment were immunoprecipitated with three micrograms of anti-EGFR antibodies, and the resulting immunocomplexes were analyzed by Western blotting using anti-PY20 antibodies. A band with a molecular mass of 170 kDa was found to be tyrosine phosphorylated by about threefold in response to treatment with 0.5 U/ml thrombin for 10 min compared with control (Fig. 8A). The thrombin-induced increases in the tyrosine phosphorylation of this protein were gradually decreased thereafter, returning to basal levels by 2 h. Furthermore, thrombin-induced increases in the tyrosine phosphorylation of this protein were completely suppressed by 1 μM of AG-1478, a specific inhibitor of EGFR tyrosine kinase activity, suggesting the identity of this protein to be EGFR (Fig. 8A).

We next tested the effect of AG-1478 on thrombin-induced Akt and S6K1 phosphorylation, Fra-1 and FGF-2 expression, DNA synthesis, and motility. AG-1478 inhibited Akt and S6K1 phosphorylation, Fra-1 and FGF-2 expression, and DNA synthesis and motility in VSMC induced by thrombin (Fig. 8, B–F).

**DISCUSSION**

The important findings of the present study are as follows. 1) Thrombin, a serine protease and a GPCR agonist, stimulated the phosphorylation of Akt and S6K1 in a time- and PI3K-dependent manner in VSMC. 2) Thrombin induced VSMC DNA synthesis and motility, and both of these responses are mediated by PI3K-Akt-mTOR-S6K1 signaling. 3) Thrombin induced the expression of Fra-1 in a time- and PI3K-Akt-dependent and mTOR-independent manner in VSMC. 4) Similarly, thrombin induced the expression of FGF-2 in a time- and PI3K-Akt-Fra-1-dependent and mTOR-independent manner in VSMC. 5) Inhibition of Fra-1 levels by its siRNA suppressed both thrombin-induced VSMC DNA synthesis and motility. 6) Neutralizing anti-FGF-2 antibodies also blocked both thrombin-induced VSMC DNA synthesis and motility. 7) Furthermore, inhibition of EGFR activation attenuated thrombin-stimulated PI3K-Akt-mTOR-S6K1 signaling, Fra-1 and FGF-2 expression, DNA synthesis, and motility. It was reported that thrombin activates Akt in a weak and transient manner in VSMC, and this mode of Akt stimulation was thought to be one of the causes of its lack of effect as a mitogen to VSMC (35). In other cell types such as Chinese hamster embryonic...
fibroblasts, thrombin activated Akt in a sustained and biphase manner, and the second and prolonged phase of Akt stimulation accounted for thrombin-induced G1 phase progression (17). On the basis of these two reports, a sustained activation of Akt appears to be correlated with the mitogenic capacity of thrombin at least in Chinese hamster embryonic fibroblasts. However, previous findings from this laboratory (4, 16, 29–31) as well as other laboratories (26, 33, 36) and the present results clearly show that thrombin induces DNA synthesis in VSMC. The other discrepancy between the present study and that of Reusch et al. (35) is in regard to the capacity of thrombin in the activation of Akt in VSMC. Although the present observations clearly reveal a robust and sustained activation of Akt, the results of Reusch et al. (35) showed only a weak and transient activation of this serine/threonine kinase by thrombin in VSMC. In addition, our results show that activation of PI3K-Akt-mTOR-S6K1 signaling is needed for thrombin-induced VSMC DNA synthesis. Thrombin and lysophosphatidic acid, GPCR agonists, have been shown to activate RTK such as EGFR and FGFR-1 in mediating cell proliferation and migration (18, 20, 33). However, the role of these RTK in GPCR agonist-induced signaling events is less clear. In this regard, the present results show that thrombin, a GPCR agonist, stimulates PI3K-Akt-mTOR-S6K1 signaling via the involvement of EGFR transactivation leading to DNA synthesis in VSMC. Fra-1, a protooncogene, in concert with the Jun family of proteins forms the transcriptional factor activator protein-1 (AP-1) (37). AP-1 plays an indispensable role in the regulation of cell proliferation (37). In this regard it is interesting to note that thrombin induces the expression of Fra-1 in a sustained and EGFR-PI3K-Akt-dependent manner in VSMC. Furthermore, suppression of Fra-1 by its siRNA blocked thrombin-induced DNA synthesis. These findings indicate that Fra-1 is one of the effector transcriptional factors of EGFR-PI3K-Akt signaling and is involved in mediating thrombin-induced VSMC proliferation. Evidence in support of this conclusion comes from the finding that thrombin induces FGF-2 expression via a mechanism involving EGFR-PI3K-Akt-Fra-1 signaling and neutralizing anti-FGF-2 antibodies reduced thrombin-induced DNA synthesis. If this is the mechanism, why then
does the inhibition of mTOR, while preventing VSMC DNA synthesis, have no effect on thrombin-induced Fra-1 or FGF-2 expression? A role for mTOR in the cap-dependent regulation of gene expression has been well established (23). Because thrombin-induced expression of both Fra-1 and FGF-2 were not sensitive to mTOR suppression, it is quite possible that the regulation of expression of these molecules in response to thrombin occurs in a cap-independent manner. However, enhanced cap-independent translation of Fra-1 and FGF-2 in the absence of mTOR activation may not be sufficient to trigger the production of other factors, including the downstream mediators of Fra-1-FGF-2 signaling that are needed for DNA synthesis, because global cap-dependent protein synthesis is suppressed under these conditions. In view of these possibilities, we predict that Akt targets in parallel the Fra-1-mediated FGF-2 expression and mTOR-S6K1 activation, and both of these events are needed for thrombin-induced VSMC DNA synthesis.

The present results also reveal that stimulation of EGFR-P13K-Akt signaling targeting in parallel the Fra-1-mediated FGF-2 expression and mTOR-S6K1 activation is needed for thrombin-induced VSMC migration. Several studies have reported that activation of P13K-Akt-mTOR-S6K1 signaling is required for mediating cell motility in response to various stimuli (21, 38). However, the mechanisms by which this signaling contributes to cell motility are largely unclear. In this regard, it was recently reported that group 1B secretory phospholipase A2 mediates NIH3T3 fibroblast migration via P13K-Akt-dependent induction of expression of matrix metalloproteinase-2 (5). Matrix metalloproteinases such as matrix metalloproteinase-2 and -9, by catalyzing the degradation of the extracellular matrix proteins, play an important role in cell migration (5, 44). Furthermore, it was shown that P13K mediates the expression of matrix metalloproteinases such as matrix metalloproteinase-12 via the involvement of Fra-1 in human aortic smooth muscle cells derived from atherosclerotic lesions (43). A role for P13K-Akt in the regulation of AP-1 activity has also been reported (24). In addition, the present study shows that P13K-Akt-Fra-1 plays a role in thrombin-induced expression of FGF-2, a growth factor that has been shown to be a very potent chemoattractant for VSMC (34). On the basis of these observations, it is quite possible that EGFR-P13K-Akt signaling via the involvement of Fra-1 may lead to induction of expression of a variety of molecules such as FGF-2 and matrix metalloproteinases and thereby mediate thrombin-induced VSMC migration. Because inhibition of mTOR-S6K1 axis also prevents thrombin-induced VSMC migration, it is likely that, similar to their involvement in DNA synthesis, both Fra-1-mediated FGF-2 expression and mTOR-S6K1 activation subsequent to stimulation of EGFR-P13K-Akt signaling may be necessary for mediation of VSMC motility in response to thrombin.

In summary, our results demonstrate for the first time that thrombin-induced VSMC DNA synthesis and migration require activation of EGFR-P13K-Akt signaling targeting in parallel Fra-1-mediated FGF-2 expression and mTOR-S6K1 stimulation.

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