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Thrombin-mediated Proteoglycan Synthesis Utilizes Both Protein-tyrosine Kinase and Serine/Threonine Kinase Receptor Transactivation in Vascular Smooth Muscle Cells*

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Micah L. Burch[‡§¶], **Robel Getachew**[‡], **Narin Osman**[‡¶], **Mark A. Febbraio**[§1], **and Peter J. Little**[‡¶2]

From the [‡]*Diabetes Complications Laboratory, Health Innovations Institute, Royal Melbourne Institute of Technology University, Bundoora, Victoria 3004, the* [§]*Cellular and Molecular Metabolism Laboratory, BakerIDI Heart and Diabetes Institute, Melbourne, 3004 Victoria, Australia, and the* [¶]*Department of Medicine, Central and Eastern Clinical School, Alfred Hospital, Monash University, Melbourne, 3004 Victoria, Australia*

Background: GPCR transactivation of PTKRs and TGF-βRs mediates proteoglycan synthesis in human VSMC.
Results: Transactivation of TGF-βRs is integrin-dependent, and inhibition of both transactivation pathways blocks proteoglycan synthesis.
Conclusion: GPCR utilize transactivation pathways and not classical signaling in proteoglycan synthesis.
Significance: GPCR transactivation of receptor kinase pathways may be broader and more significant than previously recognized.

G protein-coupled receptor signaling is mediated by three main mechanisms of action; these are the classical pathway, β-arrestin scaffold signaling, and the transactivation of protein-tyrosine kinase receptors such as those for EGF and PDGF. Recently, it has been demonstrated that G protein-coupled receptors can also mediate signals via transactivation of serine/threonine kinase receptors, most notably the transforming growth factor-β receptor family. Atherosclerosis is characterized by the development of lipid-laden plaques in blood vessel walls. Initiation of plaque development occurs via low density lipoprotein retention in the neointima of vessels due to binding with modified proteoglycans secreted by vascular smooth muscle cells. Here we show that transactivation of protein-tyrosine kinase receptors is mediated by matrix metalloproteinase triple membrane bypass signaling. In contrast, serine/threonine kinase receptor transactivation is mediated by a cytoskeletal rearrangement-Rho kinase-integrin system, and both protein-tyrosine kinase and serine/threonine kinase receptor transactivation concomitantly account for the total proteoglycan synthesis stimulated by thrombin in vascular smooth muscle. This work provides evidence of thrombin-mediated proteoglycan synthesis and paves the way for a potential therapeutic target for plaque development and atherosclerosis.

G protein-coupled receptors (GPCR)[3] represent the largest group of cell surface receptors in mammalian biology and are heavily implicated in physiology and pathology (1). They contribute to a diverse array of functions in various cell types such as migration and proliferation, contraction, and fibrosis and are prominent in many disease states such as cancer, fibrotic disorders, and cardiovascular disease (2). Cellular signaling by GPCR occurs via three main mechanisms. The first is the "classical" mechanism by which ligand activation leads to a conformational change in the receptor and the activation of intracellular G protein α and $\beta\gamma$ subunits and the propagation of signals through secondary messengers such as phospholipase C (3). The second is the utilization of β-arrestin molecules that form scaffold complexes and lead to intracellular signaling (4). Finally, they can also signal via various members of the protein-tyrosine kinase receptor (PTKR) family such as the very well documented EGF and PDGF receptors in a mechanism termed "transactivation." Transactivation results in the stimulation of Erk1/2 and PI3K pathways allowing the GPCR to elicit full mitogenic responses (5). Transactivation of PTKR was first described by Ullrich and colleagues (6) in 1996 and revealed the transactivation of the epidermal growth factor receptor (EGFR) by the GPCR agonists thrombin, angiotensin II, and endothelin-1 (6). Since then PTKR transactivation has gained much attention, and a more defined mechanism is beginning to emerge. However, recent evidence demonstrates that transactivation is not limited to PTKR but also includes serine/threonine kinase receptors (S/TKR), namely those of the transforming growth factor-(TGF)-β family. TGF-β signals via ligand engagement of a receptor complex containing the type I receptor Alk5, leading to phosphorylation of the immediate downstream Smad2/3 transcription factors (7). We and others have recently demonstrated that GPCR such as thrombin, angiotensin II, endothelin-1, and lysophosphatidic acid lead to the time-dependent generation of phosphorylated Smad2 (8–12).

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[1] A Senior Principal Research Fellow of the National Health and Medical Research Council of Australia.
[2] To whom correspondence should be addressed. Tel.: 61-3-8532-1203; Fax: 61-3-8535-1100; E-mail: peter.little@rmit.edu.au.
[3] The abbreviations used are: GPCR, G protein-coupled receptor(s); PTKR, protein-tyrosine kinase receptor(s); S/TKR, serine/threonine kinase receptor(s); EGFR, epidermal growth factor receptor; GAG, glycosaminoglycan; ROCK, Rho kinase; VSMC, vascular smooth muscle cell(s); ANOVA, analysis of variance; MMP, matrix metalloproteinase(s).

Atherosclerosis is the main underlying etiology in cardiovascular disease and is characterized by the formation of atheromatous plaques in blood vessel walls that can occlude the vessel or become unstable and rupture, resulting in heart attack or stroke and often death (13). The initiating event in plaque development is described in the "response to retention hypothesis" of atherogenesis in which retention of low density lipoproteins (LDL) in the neointimal layer of blood vessel walls is due to changes in the synthesis of extracellular matrix proteoglycans secreted by vascular smooth muscle cells (VSMC) migrating out of the medial layer (14, 15). VSMC exposed to various stimuli such as GPCR agonists, including thrombin, produce proteoglycans with elongated glycosaminoglycan (GAG) chains, which show higher binding affinity to LDL (16–21). We have also demonstrated that thrombin utilizes both PTKR and S/TKR transactivation in proteoglycan synthesis, but we had no indication of whether these are independent, redundant, or cooperative. Here we demonstrate that the two transactivation pathways act independently in thrombin-stimulated proteoglycan synthesis and that the two transactivation pathways, but not traditional pathways, account for almost all of the actions of thrombin on proteoglycan synthesis (see Fig. 8). Additionally, we show that the mechanism of thrombin-stimulated transactivation of TGF-β receptor signaling involves cytoskeletal rearrangement, Rho kinase (ROCK) signaling, and cell surface RGD binding integrins in human VSMC (see Fig. 8).

EXPERIMENTAL PROCEDURES

Materials—The following chemicals were purchased from Sigma: thrombin, EGF, benzamidine hydrochloride, 6-aminocaproic acid, DEAE-Sephacel, chondroitin sulfate, SB431542, GM6001, Y27632, AG1478, cycloheximide, and cytochalasin D. Dulbecco's modified Eagle's medium (DMEM) and glutamine were from Gibco; fetal bovine serum (FBS) and penicillin streptomycin fungizone were obtained from CSL (Parkville Australia); and carrier-free [^{35}S]SO_4 and [^{35}S]methionine/cysteine were obtained from MP Biomedicals. The synthetic RGD (cGRGDSP) and RGE (RGES) peptides were from Anaspec Inc. (Fremont, CA). Cetylpyridinium chloride was from Unilab Chemicals and Pharmaceuticals; Whatman 3MM chromatography paper was from Biolab (Mulgrave, Australia); Insta-Gel Plus scintillation fluid was from PerkinElmer Life Sciences; and Poly-Prep columns were from Bio-Rad. Human recombinant TGF-β and anti-phosphorylated Smad2 (phosphoSmad2), anti-total Smad2 (Smad2), anti-GAPDH (GAPDH), anti-phosphorylated Erk1/2 (phosphoErk1/2), anti-total Erk1/2 (Erk1/2), and anti-phosphorylated Ezrin, Radixin, and Moesin (phospho-ERM) rabbit monoclonal antibodies were from Cell Signaling Technology (Danvers, MA). ECL detection reagents and HRP-conjugated anti-rabbit IgG monoclonal antibody were from GE Healthcare (Birmingham, UK). Alexa Fluor 488-phalloidin and Hoechst stain were a generous gift from Dr. Simon Potocnik, RMIT University.

Culture of Human VSMC—Human VSMC were obtained by the explant method (22) from discarded sections of saphenous veins from coronary artery bypass grafting patients at the Alfred Hospital, with approval by the Alfred Ethics committee. VSMC were maintained in Dulbecco's modified Eagle's medium (DMEM) with 5 mM glucose, 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin-fungizone solution at 37 °C, 5% CO_2. For experiments, VSMC were seeded into 24- or 6-well plates, grown to confluence, and then rendered quiescent by serum deprivation for 48 h.

Western Blotting—Total cell lysates were collected and separated on 10% acrylamide gel SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk powder and then incubated with anti-phosphoSmad2, anti-Smad2, anti-GAPDH, anti-phosphoErk1/2, anti-Erk1/2, or anti-phosphoERM rabbit monoclonal antibody followed by HRP-anti-rabbit IgG and ECL detection. Blots were imaged using the Bio-Rad gel documentation system, and densitometry analysis was performed with Quantity One imaging software (Bio-Rad Laboratories) and normalized to basal for -fold change determination.

Quantitation of De Novo Protein Synthesis—Quiescent cells were changed to fresh medium containing 10 μg/ml cycloheximide, to which 10 μCi/ml was added, and the cells incubated for 4 h. After 4 h, thrombin was added, and the cells were incubated for a further 18 h. Cells were then washed with PBS and incubated with 10% w/v TCA for 30 min on ice. TCA was removed; cells were washed and then incubated with 0.1% SDS in 0.1 N NaOH for 30 min at 37 °C with agitation. Cells were then collected and added to scintillation fluid with 1% acetic acid (1 M) and counted.

Confocal Imaging of the Actin Cytoskeleton—Cells were grown on 1.5 grade glass coverslips and then rendered quiescent via serum deprivation. After treatments, cells were fixed in 2% paraformaldehyde in 1 N phosphate buffer and then permeabilized and blocked in 0.1% Triton X-100, 1% horse serum for 30 min. Cells were incubated with Alexa Flour 488-phalloidin (a generous gift from Dr. Simon Potocnik, RMIT University) and Hoechst stain for 30 min for the detection of the actin cytoskeleton and nucleus, respectively. Coverslips were then mounted on slides, and cells were imaged using a Nikon D-eclipse C1 confocal microscope.

Quantitation of Proteoglycan Synthesis—Quiescent cells were changed to fresh medium containing 50 μCi/ml [^{35}S]sulfate in the presence or absence of thrombin, TGF-β, or EGF for 24 h. Media from the cell cultures were harvested, and protease inhibitors (5 mM benzamidine in 0.1 M 6-aminocaproic acid) were added to prevent degradation. Incorporation of the radiolabel into proteoglycans was measured by cetylpyridinium chloride precipitation assay, as described previously (23).

SDS-PAGE Analysis of Proteoglycan Size—Proteoglycans labeled with [^{35}S]sulfate were prepared for SDS-PAGE by isolation through DEAE-Sephacel anionic exchange mini columns. Samples were added to pre-equilibrated columns and then washed extensively with low salt buffer (8 M urea, 0.25 M NaCl, 2 mM disodium EDTA, 0.5% Triton X-100). Proteoglycans were eluted with high salt buffer (8 M urea, 3 M NaCl, 2 mM disodium EDTA, 0.5% Triton X-100), and fractions containing the highest number of [^{35}S]sulfate cpm were pooled. Aliquots (25,000 cpm) were precipitated (1.3% potassium acetate, 95% ethanol), and chondroitin sulfate was added as a "cold carrier." Samples were resuspended in buffer (8 M urea, 2 mM disodium EDTA, pH 7.5), to which an equal volume of sample buffer was

added. Radiolabeled proteoglycans were separated on 4–13% acrylamide gels with a 3% stacking gel at 50 V overnight. Gels were then fixed and dried and exposed to a phosphorimaging screen (Fuji Photo Film Co.) for ~3 days and then scanned on a bio-imaging analyzer BAS-1000 MacBas (Fuji Photo Film Co.).

Statistical Analysis—Data were normalized and expressed as the mean ± S.E. Data were analyzed using a one-way ANOVA and deemed significant at $p < 0.05$.

RESULTS

We have previously demonstrated that the GPCR agonist thrombin transactivates the TGF-β receptor Alk5, leading to the generation of phosphoSmad2(Ser465/467), and that this plays a partial role in the synthesis of proteoglycans mediated by thrombin stimulation in human VSMC (8). However, we have no information on the intermediate mechanism that exists between the GPCR, PAR-1, and Alk5. Accordingly, we asked the question whether transactivation involves *de novo* protein synthesis. To explore the mechanism of transactivation, we utilized the inhibitor of translation, cycloheximide. Thrombin generated a temporal increase in levels of phospho-Smad2(Ser465/467), beginning at 1 h and reaching a maximum of 2.5-fold at 4 h ($p < 0.05$) (Fig. 1*A*, *lanes 1–4*). The generation of phosphoSmad2(Ser465/467) was maintained in the presence of cycloheximide (10 μg/ml) (Fig. 1*A*, *lanes 5–7*). As a control, we demonstrate that *de novo* protein synthesis is not required for the direct stimulation of phosphoSmad2(Ser465/467) by TGF-β at 1 h (Fig. 1*A*, *lanes 8* and *9*). To confirm that cycloheximide inhibits protein synthesis in these cells under the conditions of this experiment, a methionine/threonine radiolabel ([^{35}S]Met/Cys) was used. Cycloheximide (10 μg/ml) completely abolishes *de novo* protein synthesis in human VSMC both in the presence and in the absence of thrombin ($p < 0.01$) as assessed by [^{35}S]Met/Cys incorporation into total proteins in human VSMC. This experiment shows that the stimulation of phosphoSmad2 by thrombin does not require transcription/translation.

The transactivation of PTKR relies largely on the well characterized triple-membrane passing system. This involves the stimulation of cell surface matrix metalloproteinases (MMP), subsequently leading to the cleavage and generation of ligands that bind and activate the PTKR in an autocrine/paracrine fashion (24). It is known that in some cellular systems, membrane-bound TGF-β can be chemically cleaved and processed from its latent form, allowing it to act on its receptor in a similar manner (25). To determine the possibility that thrombin may be activating Alk5 via the activation of MMP, we utilized the broad spectrum MMP inhibitor GM6001. The 2-fold stimulation of phosphoSmad2(Ser465/467) by thrombin in VSMC ($p < 0.01$) occurred in either the presence or the absence of GM6001 (10 μM) (Fig. 2*A*, *lanes 1–8*). GM6001 did not inhibit TGF-β-stimulated phosphoSmad2(Ser465/367) at 1 h as a control (Fig. 2*A*, *last two separate lanes*). To confirm GM6001 as an MMP inhibitor, we directed it against the classical PTKR transactivation model using phosphoErk1/2(Thr202/Tyr204) as readout as phosphorylated Erk1/2 is typically described as the downstream product of EGFR transactivation. Thrombin stimulated a 2-fold increase in phosphoErk1/2(Thr202/Tyr204) at 5 min

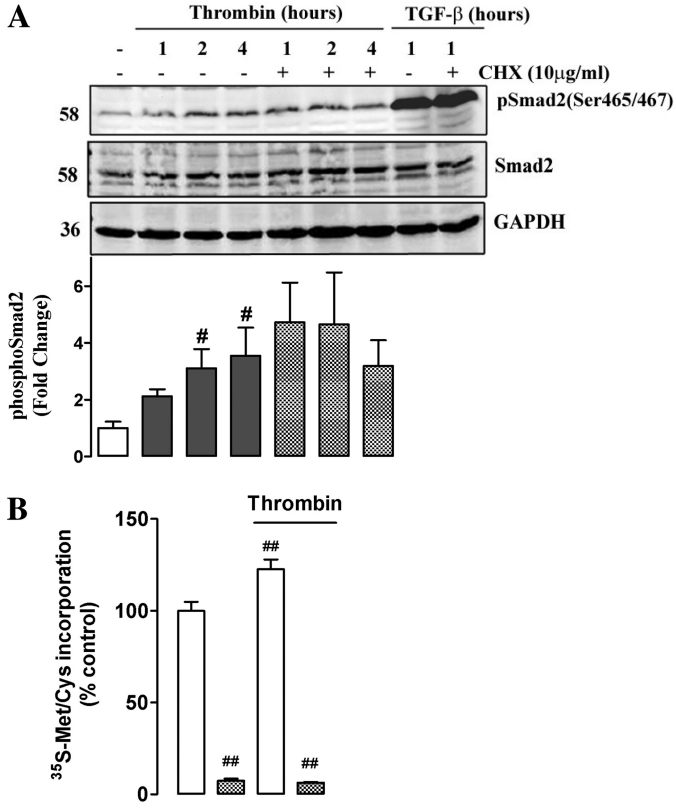

FIGURE 1. **Thrombin-mediated stimulation of phosphoSmad2(Ser465/467) does not require *de novo* protein synthesis in human VSMC.** *A*, VSMC were preincubated with cycloheximide (*CHX*) (10 μg/ml) or vehicle for 30 min and then treated with thrombin (10 units/ml) for 1, 2, and 4 h. VSMC stimulated with TGF-β (2 ng/ml) for 1 h in the presence or absence of cycloheximide (10 μg/ml) were used as controls. Cell lysates were collected, and proteins (50 μg) were resolved over 10% acrylamide gel SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was then incubated with anti-phosphoSmad2(Ser465/467) (*pSmad2(Ser465/467)*) monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody. Membranes were stripped and reincubated with anti-Smad2 monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody and anti-GAPDH HRP-conjugated monoclonal antibody (1:10,000) to determine equal loading. The gel is a representation of three separate experiments. Histograms represent band density as -fold over basal from at least three separate experiments. Data are expressed as mean ± S.E. #, $p < 0.05$ *versus* untreated control using a one-way ANOVA. Quantitation of TGF-β bands is not included in *panel A* due to intensity, but no significant decrease is detected. *B*, VSMC were incubated with cycloheximide (10 μg/ml) and [^{35}S]Met/Cys (10 μCi/ml) for 4 h and then with thrombin (10 units/ml) for an additional 16 h. Total proteins were purified and precipitated using TCA (10% w/v) followed by 0.1% SDS in 0.1 M NaOH, and radioactivity was measured. The histogram is representative of two separate experiments in triplicate. ##, $p < 0.01$ *versus* untreated control using a one-way ANOVA.

($p < 0.01$) in human VSMC (Fig. 2*B*, *lanes 1* and *2*). This stimulation was completely abolished in the presence of GM6001 (10 μM) ($p < 0.01$) with the level of phosphoErk1/2(Thr202/Tyr204) remaining at basal (Fig. 2*B*, *lane 3*). As a control, we also show that GM6001 (10 μM) has no effect on the ability of EGF to directly stimulate its cognate receptor as it fails to inhibit EGF stimulation of phosphoErk1/2(Thr202/Tyr204) at 5 min (Fig. 2*B*, *lanes 4* and *5*). Taken together, this indicates that thrombin does not transactivate Alk5 via MMP-driven catalytic cleavage and processing of TGF-β ligands in this context, or any other MMP-dependent mechanism.

Thrombin is a recognized inducer of cytoskeletal rearrangement. As such, we wished to determine the role of the cytoskel-

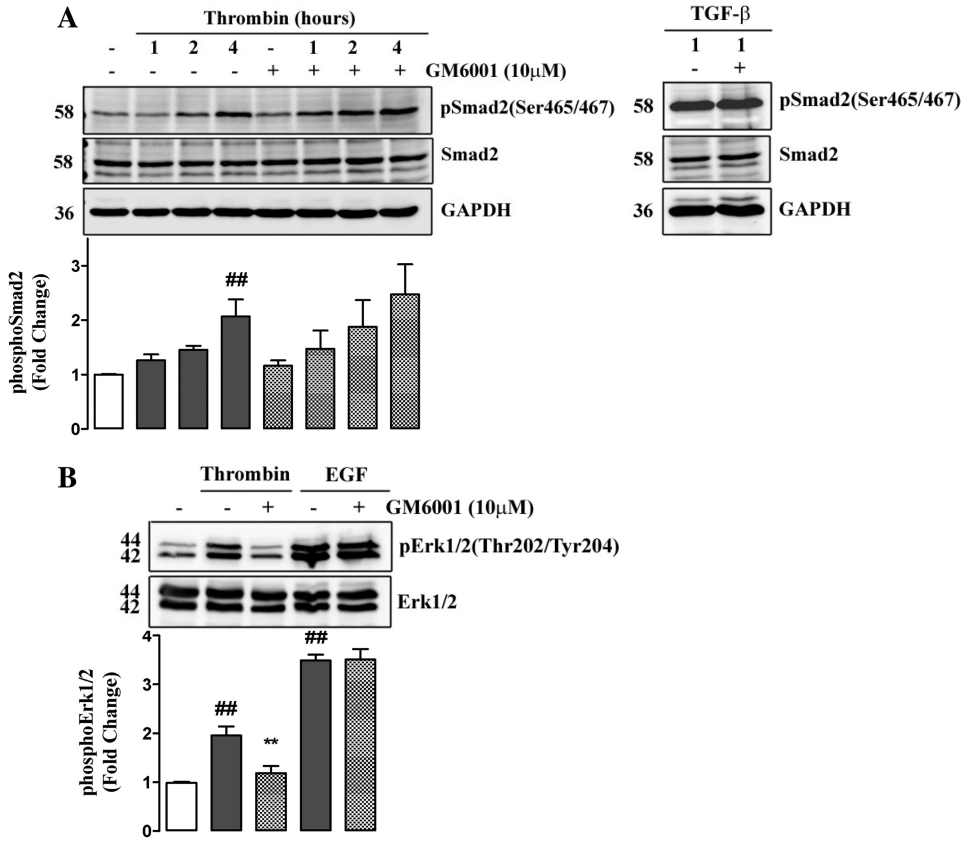

FIGURE 2. **Thrombin-mediated stimulation of phosphoSmad2(Ser465/467) is not dependent on MMP-induced shedding of TGF-β ligands in human VSMC.** *A*, VSMC were preincubated with GM6001 (10 μM) or vehicle for 30 min and then exposed to thrombin (10 units/ml) for 1, 2, and 4 h. VSMC stimulated with TGF-β (2 ng/ml) for 1 h in the presence or absence of GM6001 (10 μM) were used as controls. Cell lysates were collected, and proteins (50 μg) were resolved over 10% acrylamide gel SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was then incubated with anti-phosphoSmad2(Ser465/467) (*pSmad2(Ser465/467)*) monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody. Membranes were stripped and reincubated with anti-Smad2 monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody and anti-GAPDH HRP-conjugated monoclonal antibody (1:10,000) to determine equal loading. *B*, VSMC were preincubated with GM6001 (10 μM) or vehicle for 30 min and then exposed to thrombin (10 units/ml) or EGF (100 nM) for 5 min. Proteins were collected, separated, and transferred to membranes as above. The membrane was then incubated with anti-phosphoErk1/2(Thr202/Tyr204) (*pERK1/2(Thr202/Tyr204)*) monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody. Membranes were stripped and reincubated with anti-Erk1/2 monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody to determine equal loading. In both *A* and *B* are representatives of three separate experiments. Histograms represent band density as -fold over basal from at least three separate experiments. Data are expressed as mean ± S.E. ##, $p < 0.01$ *versus* untreated control. **, $p < 0.01$ *versus* thrombin treated using a one-way ANOVA. Quantitation of TGF-β bands is not included in *panel A* due to intensity, but no significant decrease is detected.

eton in the transactivation of Alk5 in VSMC. To test this, we employed cytochalasin D, which is a potent inhibitor of actin polymerization and also results in the destruction of the pre-existing cytoskeleton. We pretreated VSMC with cytochalasin D (10 μM) for 30 min and assessed the ability of thrombin to induce phosphorylation of Smad2. The stimulation of phosphoSmad2(Ser465/467) by thrombin was completely abolished at 1 and 2 h ($p < 0.05$ at 2 h) in the presence of cytochalasin D (Fig. 3*A*, *lanes 6* and *7*), suggesting that an intact cytoskeleton is required for the transactivation of Alk5 at least at these time points. We did not observe decrease in phosphoSmad2(Ser465/467) levels at 4 h in the presence of cytochalasin D (Fig. 3*A*, *lane 8*). As a control, cytochalasin D (10 μM) did not inhibit TGF-β stimulation of phosphoSmad2(Ser465/467) at 1 h in human VSMC (Fig. 3*A*, *last two separate lanes*). To confirm that cytochalasin D results in the destruction of the cytoskeleton in VSMC, we evaluated cytoskeletal integrity by tagging with an Alexa Fluor 488-phalloidin fusion protein that binds to all cytosolic actin, in both the presence and the absence of cytochalasin D (10 μM). Confluent, quiescent VSMC in culture display typical morphology with actin filaments seen as an ordered striated architecture within the cells, visualized using confocal microscopy (Fig. 3*B*, *panel i*). However, incubation with cytochalasin D at 1, 2, and 4 h results in complete destruction of the actin cytoskeleton as evidenced by the unordered, globular appearance of the actin, which is no longer filamentous in morphology (Fig. 3*B*, *panels ii–iv*). Thus, cytochalasin D (10 μM) is successful in destroying the cytoskeleton in human VSMC as early as 1 h and is maintained to at least 4 h.

The family of small Rho GTPases and their downstream target ROCK are implicated in cytoskeletal signaling induced by thrombin (26). Having provided evidence for cytoskeletal involvement in Alk5 transactivation, we sought to determine a possible role of ROCK signaling. We used the small molecule inhibitor of ROCK, Y27632. VSMC were incubated with Y27632 (10 μM) for 30 min prior to thrombin exposure. Thrombin stimulation resulted in the temporal increase in phosphoSmad2(Ser465/467) at 1, 2, and 4 h ($p < 0.01$) (Fig. 4*A*, *lanes 1–4*). This stimulation was abolished in the presence of

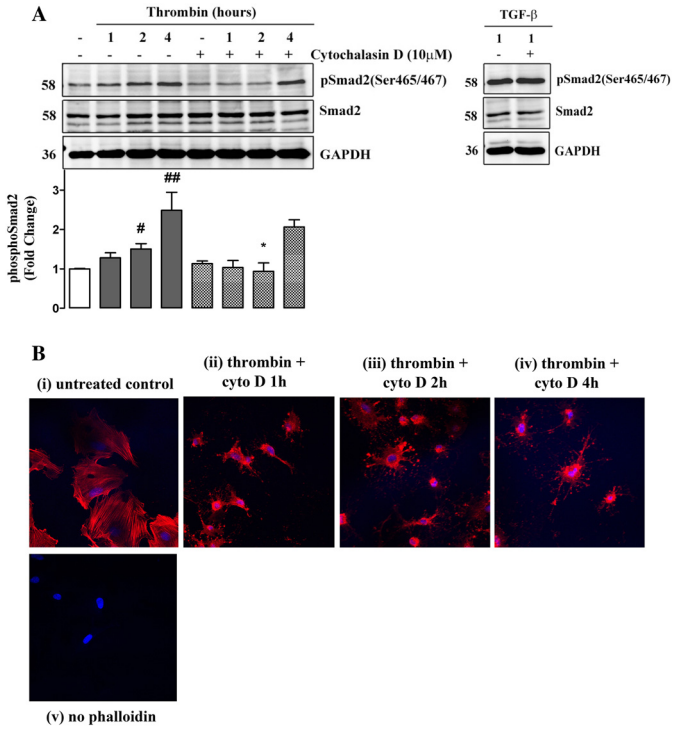

FIGURE 3. **Cytoskeletal integrity is required for thrombin-mediated stimulation of phosphoSmad2(Ser465/467) in human VSMC.** *A*, VSMC were preincubated with cytochalasin D (10 μM) or vehicle for 30 min and then exposed to thrombin (10 units/ml) for 1, 2, and 4 h. Stimulation by TGF-β (2 ng/ml) for 1 h in the presence or absence of cytochalasin D (10 μM) was used as control. Cell lysates were collected, and proteins (50 μg) were resolved over 10% acrylamide gel SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was then incubated with anti-phosphoSmad2(Ser465/467) (*pSmad2(Ser465/467)*) monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody. Membranes were stripped and reincubated with anti-Smad2 monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody and anti-GAPDH HRP-conjugated monoclonal antibody (1:10,000) to determine equal loading. The gel is a representation of three separate experiments. Histograms represent band density as -fold over basal from at least three separate experiments. Data are expressed as mean ± S.E. #, $p < 0.05$ *versus* untreated control, and ##, $p < 0.01$ *versus* untreated control. *, $p < 0.05$ *versus* thrombin-treated using a one-way ANOVA. Quantitation of TGF-β bands is not included in *panel A* due to intensity, but no significant decrease is detected. *B*, VSMC were grown on coverslips and treated with 10 μM cytochalasin D (*Cyto D*) for 1 (*panel ii*), 2 (*panel iii*), and 4 h (*panel iv*) or vehicle (*panel i*). Cells were fixed with 2% paraformaldehyde, permeabilized, and incubated with or without (*panel v*) Alexa Fluor 488-phalloidin and Hoechst nuclear counterstain. Cells were imaged using a Nikon D-eclipse C1 confocal microscope. Images are representative of two independent experiments.

Y27632 at all assayed time points ($p < 0.01$ at 4 h) (Fig. 4*A*, *lanes 5–7*). Y27632 had no effect on the ability of TGF-β to stimulate phosphoSmad2(Ser465/467) (Fig. 4*A*, *lanes 8* and *9*). To confirm that Y27632 successfully inhibits ROCK activity in these cells, we directed it against the immediate downstream product of ROCK, the group of three homologous proteins Ezrin, Radixin, and Moesin (ERM). Firstly, we established the time course of phosphoERM(Thr567/564/558) stimulation by thrombin. Thrombin stimulation results in rapid 10-fold generation of phosphoERM(Thr567/564/558) at 2, 5, and 10 min ($p < 0.01$), starting to fall at 30 min ($p < 0.05$) (Fig. 4*B*). We assessed the inhibitory effect of Y27632 at 2 min due to the early but strong stimulation of phosphoERM(Thr567/564/558). Thrombin stimulation of phosphoERM(Thr567/564/558) at 2 min ($p < 0.01$) is completely inhibited by Y27632 (10 μM) ($p < 0.01$), indicating that Y27632 is a competent inhibitor of ROCK and that ROCK signaling is involved in the thrombin-mediated transactivation of Alk5.

Latent TGF-β can be activated by various members of the integrin family of cell surface molecules (27–30). Inside out integrin signaling has been associated with cytoskeletal rearrangement and ROCK signaling cascades. Taking into account the relationship of the integrins and cytoskeleton/ROCK signaling and the RGD (Arg-Gly-Asp motif) binding properties, integrins make a likely candidate for a key player in the transactivation of Alk5. To assess this, we used a small synthetic RGD peptide that binds to RGD binding integrins, preventing them from binding to further RGD sites, disabling potential integrin extracellular cross-linking. We assessed the effect of integrin blockade using an RGD peptide on thrombin stimulation of phosphoSmad2(Ser465/467) at 1 and 4 h for early and late phase analysis. Treatment of VSMC with the RGD peptide had limited effect on thrombin-stimulated phosphoSmad2(Ser465/467) at 50 and 100 μM but displays a mild inhibitory effect at 1 h and almost full inhibition at 4 h at 200 μM ($p < 0.05$) (Fig. 5*A*, *lanes 1–9*). As a control, we utilized an analogous RGE (Arg-Gly-Glu) peptide, which is RGD binding-defective. The peptide failed to inhibit thrombin-generated phosphoSmad2(Ser465/467) (Fig. 5*A*, *lanes 10* and *11*). Additionally, we wished to determine whether PTKR transactivation was integrin-dependent to elucidate whether there is a common feature between PTKR and S/TKR transactivation or whether they are controlled by distinct mechanisms. Thrombin stimulated a 2-fold increase in phosphoErk1/2(Thr202/Tyr204) at 5 min that was maintained in the presence of RGD (200 μM) peptide preincubated for 30 min; this was also seen in the control RGE peptide (200 μM) at (Fig. 5*B*). Taken together, these data suggest that thrombin transactivation of Alk5 leading to the generation of phosphoSmad2(Ser465/467) is mediated by cytoskeletal rearrangement and the ROCK/integrin axis in human VSMC.

The modification of proteoglycans secreted by VSMC such that they exhibit increased GAG elongation is an initiating event in the development of atherosclerosis due to the binding and retention of LDL in the vessel wall (15). We have previously shown that thrombin-treated cells produce proteoglycans with increased GAG chain length when analyzed by SDS-PAGE and size exclusion chromatography of chemically liberated free GAG chains from core proteins or free GAG chains induced by the addition of exogenous xyloside (16). We have furthered this to show that thrombin-mediated GAG elongation is blocked by inhibition of both PTKR (EGFR) (16) and S/TKR (Alk5) (8) using the small molecule inhibitors AG1478 and SB431542, respectively. However, in either case, we have only observed an ~50% inhibition at the maximum concentration used with either inhibitor. This led us to investigate the possibility of the dual transactivation pathways working in tandem in thrombin-mediated proteoglycan synthesis. To assess the roles of the transactivation pathways, we used the inhibitors of EGFR and Alk5, AG1478 and SB431542, respectively. We commenced by confirming the validity of these inhibitors on their respective targets as well as the possibility of cross-

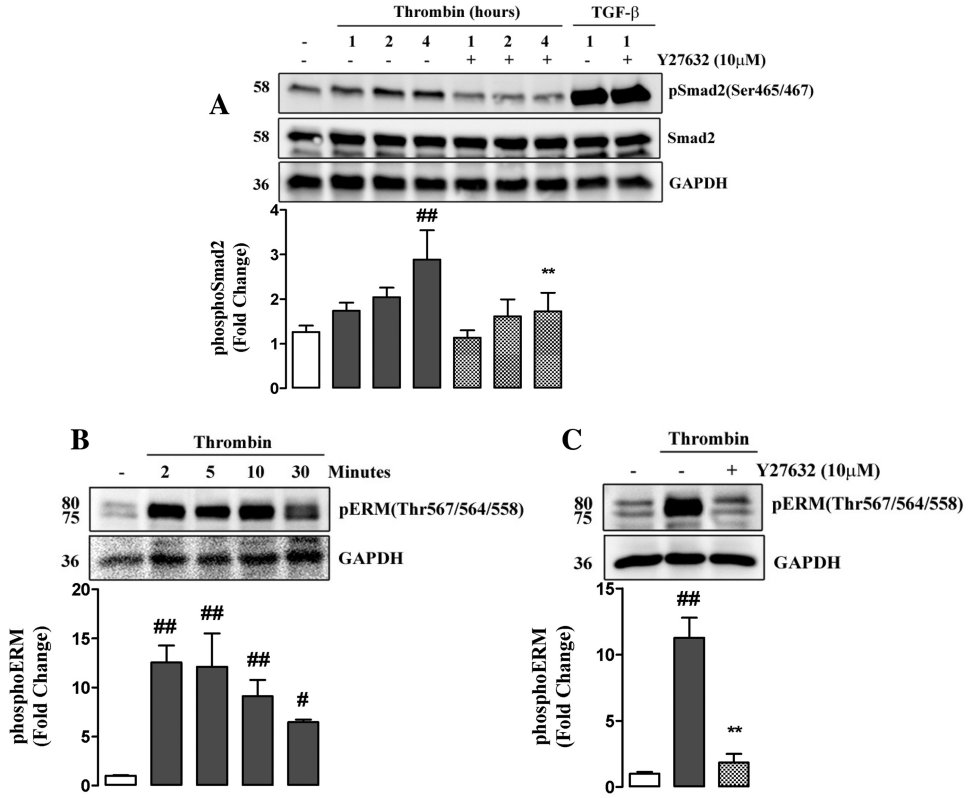

FIGURE 4. **Thrombin-mediated stimulation of phosphoSmad2(Ser465/467) requires ROCK signaling in human VSMC.** *A*, VSMC were preincubated with Y27632 (10 μM) or vehicle for 30 min and then exposed to thrombin (10 units/ml) for 1, 2, and 4 h. Stimulation by TGF-β (2 ng/ml) for 1 h in the presence or absence of Y27632 (10 μM) was used as control. Cell lysates were collected, and proteins (50 μg) were resolved over 10% acrylamide gel SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was then incubated with anti-phosphoSmad2(Ser465/467) (*pSmad2(Ser465/467)*) monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody. Membranes were stripped and reincubated with anti-Smad2 monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody and anti-GAPDH HRP-conjugated monoclonal antibody (1:10,000) to determine equal loading. *B*, VSMC were treated with thrombin (10 units/ml) for 2, 5, 10, and 30 min. *C*, VSMC were preincubated with Y27632 (10 μM) or vehicle and then treated with thrombin (10 units/ml) for 2 min. For gels *B* and *C*, cell lysates were collected, separated, and transferred to a nitrocellulose membrane as above. Membranes was then incubated with anti-phosphoERM(Thr567/564/558) (*pERM(Thr567/564/558)*) monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody and anti-GAPDH HRP-conjugated monoclonal antibody (1:10,000) to determine equal loading. *Panels A–C* are representative of at least three independent experiments. Histograms represent band density as -fold over basal from at least three separate experiments. Data are expressed as mean ± S.E. ##, $p < 0.01$ *versus* untreated control, and #, $p < 0.05$ *versus* untreated control. **, $p < 0.01$ *versus* thrombin-treated using a one-way ANOVA. Quantitation of TGF-β bands is not included in *panel A* due to intensity, but no significant decrease is detected

reactivity. Experiments showed that AG1478 (1 and 5 μM) inhibits thrombin-mediated phosphoErk1/2(hr202/Tyr204) stimulation ($p < 0.01$) (Fig. 6*A*, *lanes 1–4*) as a readout of PTKR transactivation, whereas SB431542 (3 μM) fails to cause inhibition (Fig. 6*A*, *lane 5*). As a positive control, AG1478 (5 μM) also successfully inhibited EGF-stimulated phosphoErk1/2(Thr202/Tyr204) at 5 min ($p < 0.01$) (Fig. 6*A*, *lanes 6* and *7*), indicating that it is an effective EGFR inhibitor. Conversely, thrombin stimulation of VSMC for 4 h results in a 2-fold increase in phosphoSmad2(Ser465/467) ($p < 0.01$), which is completely inhibited by SB431542 ($p < 0.01$) (1 and 3 μM, Fig. 6*B*, *lanes 1–4*) but is unaffected by AG1478 (5 μM, Fig. 6*B*, *lane 5*). As a control, we show that SB431542 (3 μM) inhibits TGF-β-stimulated phosphoSmad2(Ser465/467) ($p < 0.01$) (Fig. 6*B*, *lanes 6* and *7*), indicating that it is an effective Alk5 inhibitor. It is noteworthy that the response is far greater in the presence of TGF-β than that of thrombin; thus, SB431542 failed to completely inhibit TGF-β-stimulated phosphoSmad2(Ser465/467) at 3 μM. A concentration of 10 μM SB431542 would have been optimal for inhibition of TGF-β; however, we chose to use 3 μM to maintain consistency. Having shown that the inhibitors inhibit their respective targets but do not cross-react, we then used them concomitantly and measured thrombin-mediated proteoglycan synthesis.

Incorporation of radioactive sulfate ([^{35}S]sulfate) into secreted proteoglycans is used as a measure of total proteoglycan synthesis and represents the combination of core protein expression and incorporation of sulfate into GAG chains, giving an indication of GAG elongation (31). Treatment of VSMC with thrombin caused a 57% increase in [^{35}S]sulfate incorporation into secreted proteoglycans ($p < 0.01$), which was partially inhibited by 84% in the presence of AG1478 (5 μM) ($p < 0.01$) or 68% in the presence of SB431542 (3 μM) ($p < 0.01$) alone as described previously (8, 16). In the presence of both AG1478 (5 μM) and SB431542 (3 μM) simultaneously, the stimulation of [^{35}S]sulfate incorporation was reduced to the basal level ($p < 0.01$) (Fig. 7*A*). We used EGF and TGF-β stimulation as controls to show that both receptor types were active in these cells. Stimulation with EGF and TGF-β caused a 71 and 102% induction of [^{35}S]sulfate incorporation, respectively, in these cells ($p < 0.01$) (Fig. 7*A*) when compared with thrombin. The afore-

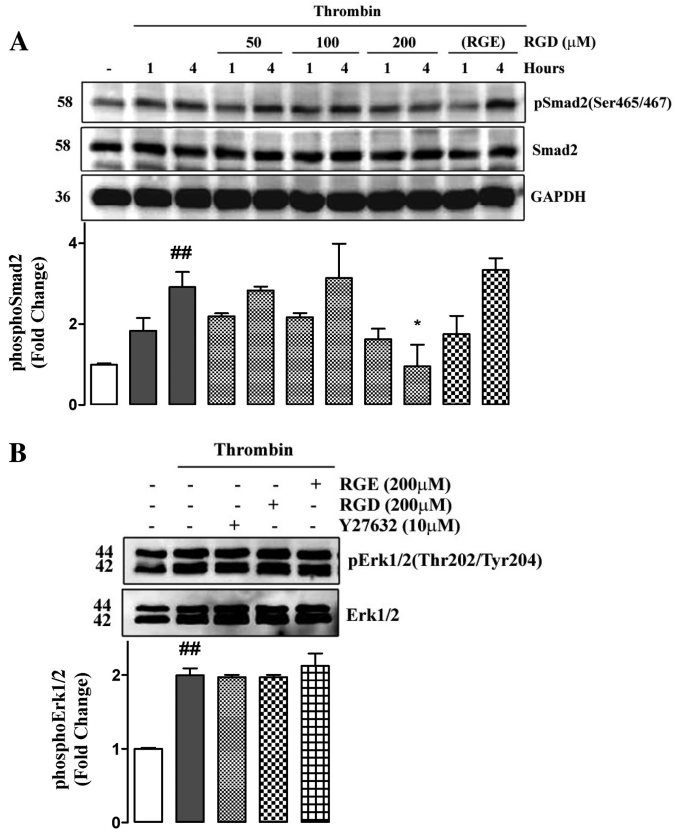

FIGURE 5. **Stimulation of phosphoSmad2(Ser465/467) but not phospho-Erk1/2(Thr202/Tyr204) by thrombin is mediated by cell surface integrins.** *A*, VSMC were preincubated with 50, 100, or 200 μM RGD peptide or vehicle for 30 min and then treated with thrombin (10 units/ml) for 1 or 4 h. VSMC preincubated with 200 μM RGE peptide for 30 min followed by thrombin (10 units/ml) stimulation were used as a negative control. Cell lysates were collected, and proteins (50 μg) were resolved over 10% acrylamide gel SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was then incubated with anti-phosphoSmad2(Ser465/467) (*pSmad2(Ser465/467)*) monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody. Membranes were stripped and reincubated with anti-Smad2 monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody and anti-GAPDH HRP-conjugated monoclonal antibody (1:10,000) to determine equal loading. *B*, VSMC were preincubated with 200 μM RGD, 200 μM RGE peptides, or vehicle for 30 min and then exposed to thrombin for 5 min. Proteins were harvested, separated, and transferred to membranes as above. The membrane was then incubated with anti-phosphoErk1/2(Thr202/Tyr204) (*pERK1/2(Thr202/Tyr204)*) monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody. Membranes were stripped and reincubated with anti-Erk1/2 (1:1000) monoclonal antibody followed by peroxidase-labeled anti-rabbit IgG secondary antibody to determine equal loading. *Panels A* and *B* are representative of at least three independent experiments. Histograms represent band density as -fold over basal from at least three separate experiments. Data are expressed as mean ± S.E. ##, $p < 0.01$ *versus* untreated control. *, $p < 0.05$ *versus* thrombin-treated using a one-way ANOVA.

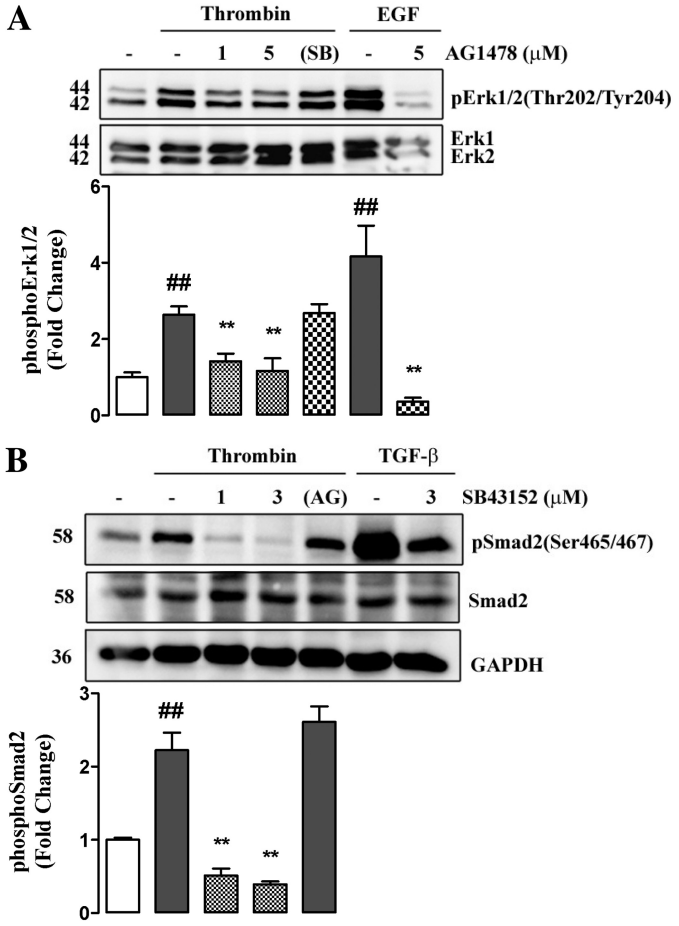

FIGURE 6. **AG1478 inhibits EGFR transactivation but not Alk5, and SB431542 inhibits Alk5 transactivation but not EGFR.** *A*, VSMC were preincubated with either 1 or 5 μM AG1478, 3 μM SB431542 (*SB*), or vehicle for 30 min followed by treatment with thrombin (10 units/ml) for 5 min. 5 min EGF (100 nM) stimulation in the presence or absence of 5 μM AG1478 was used as a positive control. Lysates were collected and separated (50 μg of protein) over 10% acrylamide gel SDS-PAGE followed by transfer onto a nitrocellulose membrane. The membrane was incubated with anti-phosphoErk1/2(Thr202/Tyr204) (*pERK1/2(Thr202/Tyr204)*) (1:1000) monoclonal antibody and then peroxidase-labeled anti-rabbit IgG secondary antibody. The membrane was stripped and reincubated with anti-Erk1/2 (1:1000) monoclonal antibody followed by peroxidase-labeled anti-rabbit IgG secondary antibody for equal loading determination. *B*, VSMC were preincubated with either 1 or 3 μM SB431542, 5 μM AG1478 (*AG*), or vehicle for 30 min followed by treatment with thrombin (10 units/ml) for 4 h. 1-h TGF-β (2 ng/ml) stimulation in the presence or absence of 3 μM SB431542 was used as a positive control. Lysates were collected, separated, and transferred to a nitrocellulose membrane as above. The membrane was then incubated with anti-phosphoSmad2(Ser465/467) (*pSmad2(Ser465/467)*) monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody. Membranes were stripped and reincubated with anti-Smad2 monoclonal antibody (1:1000) followed by peroxidase-labeled anti-rabbit IgG secondary antibody and anti-GAPDH HRP-conjugated monoclonal antibody (1:10,000) to determine equal loading. *Panels A* and *B* are representative of at least three independent experiments. Histograms represent band density as -fold over basal from at least three separate experiments. ##, $p < 0.01$ *versus* untreated control. **, $p < 0.01$ *versus* thrombin- or EGF-treated using a one-way ANOVA. Quantitation of TGF-β bands is not included in *panel B* due to intensity, but SB431542 caused a significant decrease in phosphoSmad2 when compared with TGF-β-treated ($p < 0.01$).

mentioned increase in [^{35}S]sulfate incorporation (Fig. 7*A*), as described before, results from an increase in expression of proteoglycan core proteins and GAG elongation. However, we chose only to examine the effect on GAG elongation as it relates to the increase in LDL binding and the development of atherosclerosis. To achieve this, proteoglycans were eluted using DEAE-Sepharose ion-exchange and resolved over SDS-PAGE. This demonstrates a change in size of the proteoglycans that can only be due to a shift in size of the GAG chains as core proteins have a conserved molecular weight. SDS-PAGE analysis of proteoglycan size reveals a similar pattern to that seen with the [^{35}S]sulfate incorporation (Fig. 7*A*), revealing that thrombin treatment of VSMC results in proteoglycans, specifically biglycan, with decreased electrophoretic mobility (Fig. 7*B*, *lanes 1* and *3*), corresponding to increased GAG elongation. This is also demonstrated with the co-incubation of the inhib-

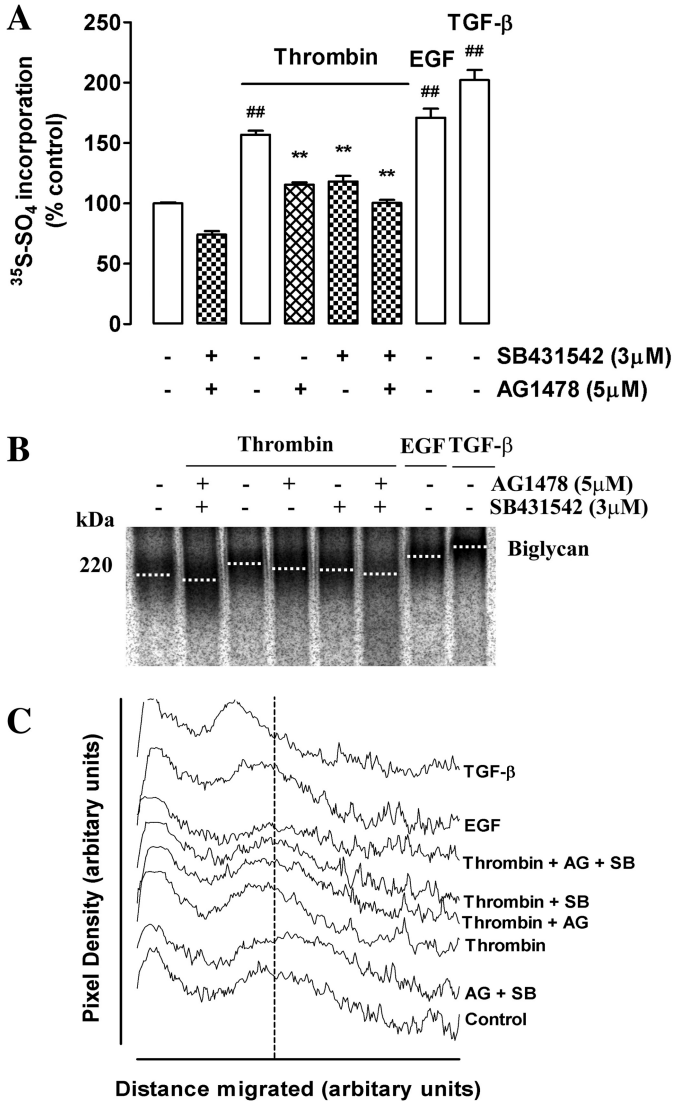

FIGURE 7. **Thrombin stimulation of GAG elongation on biglycan is mediated by cooperation of both PTKR and S/TKR pathways in human VSMC.** *A*, VSMC were treated with 3 μM SB431542, 5 μM AG1478, or in the presence of both thrombin (10 units/ml) and [^{35}S]SO$_4$ (50 μCi/ml) for 24 h. EGF (100 nM) and TGF-β (2 ng/ml) were used as positive controls. An equal proportion of media containing secreted proteoglycans was spotted onto Whatman paper and precipitated with cetylpyridinium chloride, as outlined under "Experimental Procedures" for assessment of radiolabel incorporation. *B*, secreted proteoglycans were purified from remaining media using DEAE ion-exchange chromatography and concentrated using ethanol/potassium acetate precipitation. Electrophoretic mobility relating to overall proteoglycan size was assessed by SDS-PAGE over a 4–13% acrylamide gradient gel. Biglycan is seen as the proteoglycan of interest and is representative of three identical experiments. Data are expressed as mean ± S.E. normalized to control from three separate experiments in triplicate. ## $p < 0.01$ *versus* untreated control. **, $p < 0.01$ *versus* thrombin-treated, using a one-way ANOVA. *C*, densitometric analysis of the gel in *B*. The *broken line* represents the mid-point in the peak of the control biglycan band, for comparison of the shift in the peak location from the treated samples.

itors AG1478 (5 μM) and SB431542 (3 μM), which results in biglycan with GAG chains similar to that of untreated control (Fig. 7*B*, *lane 6*). The controls TGF-β and EGF also result in GAG elongation (Fig. 7*B*, *lanes 7* and *8*). The SDS-PAGE gel was subject to densitometric analysis to quantify the shift in biglycan peak (Fig. 7*C*). The graph demonstrates that the mean peak for control is shifted with thrombin treatment, indicating an increase in proteoglycan size, but lines up with control in the presence of AG1478 and SB431542 (Fig. 7*C*). This demonstrates that thrombin utilizes both PTKR and S/TKR transactivation pathways in eliciting its full response with regard to GAG elongation in human VSMC.

DISCUSSION

Here we provide evidence that thrombin acting through its GPCR, PAR-1, mediates transactivation of both PTKR and S/TKR via distinct mechanisms and that both transactivation pathways contribute to proteoglycan synthesis (and GAG elongation) in human VSMC. The two transactivation pathways, tyrosine and serine/threonine kinase, account for all of the signaling of the action of thrombin on proteoglycan synthesis in these cells. We demonstrate that thrombin stimulation of phosphoSmad2(Ser465/467) requires an intact cytoskeleton and is abolished in the presence of the small molecule ROCK inhibitor Y27632. We also show that RGD binding integrins are involved in Alk5 transactivation as the presence of a synthetic RGD peptide blunts stimulation; however, the specific isoform remains to be determined.

In addition to classical signaling, GPCR can hijack and utilize PTKR to broaden the scope of their cellular responses. This phenomenon was first described in the mid-1990s with the discovery of EGFR transactivation (6) and since then has been expanded to include a variety of other receptors, including those for PDGF, insulin-like growth factor (IGF), NGF, and FGF and multiple mechanisms both ligand-dependent (24) and ligand-independent (32) that are generally cell- and context-specific. The transactivation of PTKR is relatively well characterized; however, work in the last decade has demonstrated transactivation of receptors from the TGF-β family, which signal through S/TK activity. Sheppard and colleagues (10, 11) have shown that in mouse lung epithelial cells, thrombin and lysophosphatidic acid stimulate phosphorylation of phosphoSmad2(Ser465/467) by 1 h and reaching a maximum at 4 h. This was demonstrated to be dependent on RhoA/ROCK and the αVβ6 integrin signaling. Furthermore, one study has provided evidence for serotonin transactivation of BMP receptors in mouse pulmonary artery smooth muscle cells (33). Here serotonin via its GPCR 5-hydroxytryptophan 1B/1D activates BMPR1A, leading to the rapid generation of phosphoSmad1, -5, and -8, which is also sensitive to ROCK inhibition, albeit a role for integrins was not examined (33). Our previous work has demonstrated that thrombin and endothelin-1 stimulate increases in phosphoSmad2(Ser465/467) in human VSMC similarly to that of mouse lung epithelial cells (8, 9); however, until now the mechanism by which this is mediated was unknown. This study provides evidence that thrombin stimulates phosphoSmad2 in human VSMC via a similar mechanism to that in mouse lung epithelial cells.

We attempted to evaluate the role of αVβ3 in this study but found that this integrin is unlikely to be involved as preincubation with an αVβ3-specific RGD peptide or blocking antibody failed to inhibit thrombin-stimulated phosphoSmad2(Ser465/467) (data not shown). We do not have information on the specific integrin that is responsible for Alk5 transactivation in these cells; however, the latency-associated peptide found in

the LLC can bind, in theory, to all RGD binding integrins including all αV integrins, αIIβ3, α5β1, and α8β1. LAP binding to integrins has officially been described for all αV integrins and α8β1 (although the latter fails to lead to activation) (34); thus, future studies should endeavor to uncover the specific integrin(s) required for Alk5 transactivation in human VSMC. We have shown that thrombin transactivates Alk5 via cytoskeletal rearrangement, ROCK, and integrin signaling, but we wished to determine the possibility that PTKR transactivation may also be mediated by this mechanism, giving both PTKR and S/TKR transactivation a common linking intermediate. δ-Opioid receptors have been shown to transactivate EGFR in HEK293 (35) cells and TrkA receptors in neuroblastoma cells (36), and in both situations, this was terminated with exogenous RGD peptides, indicating integrin involvement. However, thrombin treatment of human VSMC in the presence of Y27632 and RGD peptides failed to inhibit stimulation of phosphoErk1/2, suggesting that in these cells, PTKR transactivation is not ROCK/integrin-dependent. Thrombin stimulation of phosphoErk1/2(Thr202/Tyr204) was completely abolished by the MMP inhibitor GM6001 and the EGFR inhibitor AG1478, indicating that the PTKR transactivation in these cells follows the well characterized MMP-mediated triple-membrane passing system of activation. Conversely, thrombin stimulation of phosphoSmad2(Ser465/467) was unperturbed by GM6001, suggesting that Alk5 transactivation is not dependent on membrane shedding of TGF-β ligands. Together the data show that in human VSMC PTKR (EGF), transactivation by thrombin is mediated by the typical ligand-dependent mechanisms, whereas S/TKR (Alk5) transactivation is mediated by a cytoskeletal/ROCK/integrin axis. It is unknown exactly how integrin signaling manifests in Alk5 activation; however, a mechanism has been proposed that sees the pathway somewhat restricted to the extracellular surface and is as follows. The GPCR activates Rho/ROCK signaling via cytoskeletal rearrangement, leading to integrin activation via a shift in tensile force. Activated integrin binds to the LLC at the RGD site, leading to a conformational change in the LLC, exposing the TGF-β ligand. The ligand is then free to engage Alk5 and initiate downstream signaling (29).

GAG elongation of proteoglycans synthesized by VSMC such that they exhibit increased LDL binding is the initiating factor in atherosclerotic plaque development (15, 37, 38). Our laboratory has described the action of GPCR agonists in GAG elongation, and we have shown that thrombin mediates part of this response via Alk5 transactivation (8) and part of this response via PTKR transactivation but not via classical signaling (16). In this study, we reveal that simultaneous pharmacological inhibition of PTKR and Alk5 accounts for the total GAG elongation output by thrombin-treated human VSMC. We show that a physiological readout of proteoglycan synthesis is mediated by the synergistic actions of the independent transactivation pathways from the same GPCR. Knowing that these effects are additive yet share no common intermediate in GAG elongation means that the development of a therapeutic agent for the prevention of plaque development will have to consider these partial bypass mechanisms of the GPCR.

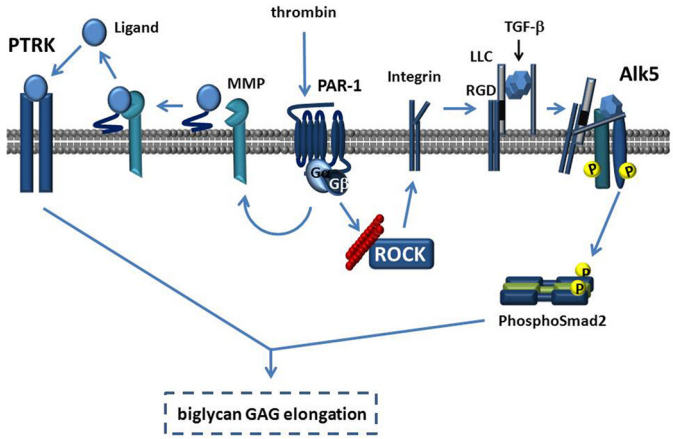

FIGURE 8. **Thrombin-mediated GAG elongation in human VSMC is mediated by concomitant transactivation of both PTKR and S/TKR pathways.** A schematic representation of the elongation of biglycan GAG chains mediated by thrombin in human VSMC is shown. Thrombin stimulation of human VSMC leads to the transactivation of PTKR via stimulation of cell surface by MMP and cleavage and release of PTKR ligands. Simultaneously, activation of the TGF-β receptor (Alk5) is achieved by cytoskeletal rearrangement, which activates ROCK signaling, leading to the activation of cell surface integrins, which bind to the latent TGF-β complex (*LLC*). This results in the mechanical denaturation of the complex, exposure of the TGF-β ligand to the receptor, and subsequent stimulation of phosphoSmad2. The transactivation of both of these signaling pathways results in the synthesis of biglycan molecules with elongated GAG chains from human VSMC and may play a role in the development of early atherosclerotic plaques.

In summary, this study demonstrates that thrombin activates the GPCR PAR-1, which transactivates PTKR and S/TKR via two distinct mechanisms in human VSMC (Fig. 8). We show here that PTKR (EGFR at least) is driven by the MMP-mediated ligand-dependent mechanism, whereas Alk5 transactivation is mediated by a dynamic cytoskeletal→ ROCK → integrin interplay (Fig. 8). Additionally, we provide evidence that thrombin utilizes both of these pathways in an additive manner in the GAG elongation of biglycan (Fig. 8), revealing that the transactivation pathways are the major signaling cascades employed in this regard. Notwithstanding that we have shown this in the context of biglycan synthesis, it surely has broader therapeutic utility in pathology such as acute lung injury (10, 11) and wound healing (39) in which these interactions are known to be highly active. This broadening in the current understanding of the GPCR transactivation phenomenon will provide a change in thinking in the way that medical therapies are developed, knowing that many more and indeed novel GPCR signaling pathways are activated than previously recognized, and this represents both a challenge and an opportunity for interrupting the pathophysiological actions of GPCR.

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Micah L. Burch, Robel Getachew, Narin Osman, Mark A. Febbraio and Peter J. Little

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