Role of integrins in angiotensin II-induced proliferation of vascular smooth muscle cells

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Bunni MA, Kramarenko II, Walker I, Raymond JR, Garnovskaya MN. Role of integrins in angiotensin II-induced proliferation of vascular smooth muscle cells. Am J Physiol Cell Physiol 300: C647–C656, 2011. First published December 9, 2010; doi:10.1152/ajpcell.00179.2010.—Angiotensin II (AII) binds to G protein-coupled receptor AT1 and stimulates extracellular signal-regulated kinase (ERK), leading to vascular smooth muscle cells (VSMC) proliferation. Proliferation of mammalian cells is tightly regulated by adhesion to the extracellular matrix, which occurs via integrins. To study cross-talk between G protein-coupled receptor and integrin-induced signaling, we hypothesized that integrins are involved in AII-induced proliferation of VSMC. Using Oligo GEArray and quantitative RT-PCR, we established that messages for α1-, α5-, αv-, and β1-integrins are predominant in VSMC. VSMC were cultured on plastic dishes or on plates coated with either extracellular matrix or poly-D-lysine (which promotes electrostatic cell attachment independent of integrins). AII significantly induced proliferation in VSMC grown on collagen I or fibronectin, and this effect was blocked by the ERK inhibitor PD-98059, suggesting that AII-induced proliferation requires ERK activity. VSMC grown on collagen I or on fibronectin demonstrated approximately three- and approximately sixfold increases in ERK phosphorylation after stimulation with 100 nM AII, respectively, whereas VSMC grown on poly-D-lysine demonstrated no significant ERK activation, supporting the importance of integrin-mediated adhesion. AII-induced ERK activation was reduced by >65% by synthetic peptides containing an RGD (arginine-glycine-aspartic acid) sequence that inhibit αβ-integrin, and by ~60% by the KTS (lysine-threonine-serine)-containing peptides specific for integrin-αβ. Furthermore, neutralizing antibody against β1-integrin and silencing of α1, α5, and β1 expression by transfecting VSMC with short interfering RNAs resulted in decreased AII-induced ERK activation. This work demonstrates roles for specific integrins (most likely αβ and αβ) in AII-induced proliferation of VSMC.

G protein-coupled receptors; signal transduction; extracellular signal-regulated protein kinase 1 and 2

ANGIOTENSIN II (AII), a potent mitogen for vascular smooth muscle cells (VSMC), mediates its effects via a specific plasma membrane AT1 receptor that belongs to the seven membrane-spanning G protein-coupled receptor (GPCR) family. AT1 receptor initiates multiple signaling pathways (reviewed in Ref. 36), including extracellular signal-regulated kinase (ERK) that leads to VSMC proliferation. Proliferation of mammalian cells is tightly regulated by adhesion to the extracellular matrix (ECM). Integrins are heterodimeric receptors for cell-surface adhesion molecules and ECM proteins that are composed of two subunits, α and β. Each αβ combination has specific signaling properties (reviewed in Ref. 12). To date, 18 α- and 8 β-subunits have been identified, which form at least 24 different αβ-integrins (10). Integrin-mediated cell attachment to the ECM regulates progression through G1 phase of the cell cycle (reviewed in Ref. 1).

Integrins are extremely important in the physiology of VSMC, playing roles in adhesion, migration, proliferation, contraction, differentiation, and apoptosis (reviewed in Ref. 23). β1-Integrins are predominant in VSMC in vivo and in culture and are considered to play a major role in adhesion. The major α-integrin subunits present in VSMC in vivo are α1, α3, and α5. However, expression of different integrins varies dramatically in VSMC with different phenotypes. Integrins have been shown to participate in several signaling pathways in VSMC. For example, integrins-αβ and -αβ, affect Ca2+ influx in arteriolar VSMC, suggesting a connection with Ca2+-dependent signaling pathways (40). The importance of cell adhesion in apoptosis of VSMC has been supported by studies in an animal model of vascular injury. It has been shown that the blockade of αvβ3-integrin reduced intimal thickening, which correlates with abundant apoptosis in injured vessels (6). Integrin-αβ has been implicated in VSMC proliferation induced by platelet-derived growth factor and insulin-like growth factor I (41, 21). Extensive cross-talk between pathways activated by integrins and by receptor tyrosine kinases, including epidermal growth factor receptor (EGFR), has been described previously (22, 32). The exact mechanisms of integrin and receptor tyrosine kinases cooperative effects are not clear, although the physical association between platelet-derived growth factor receptor and αvβ-integrin has been demonstrated in NIH/3T3 and Rat 1 fibroblast cells (31). Integrins also have been shown to form physical complexes with EGFR at the cell membrane and to trigger ligand-independent phosphorylation of Tyr845, Tyr1068, Tyr1086, and Tyr1173 residues in the EGFR molecule (25), and this integrin-dependent EGFR activation appeared necessary for full EGFR-dependent transcriptional responses (5).

Much less is known, however, about interactions between GPCRs and integrins. Some GPCRs (muscarnic receptors) stimulate tyrosine phosphorylation of focal adhesion kinase (FAK) via an integrin-dependent mechanism (34). Integrin-mediated cell anchorage also impacts on GPCR signaling to the ERK/MAPK pathway (7, 33). The precise molecular mechanisms underlying integrin-mediated GPCR signaling to ERK remain to be defined, but a likely possibility involves integrin-mediated recruitment of cytoskeletal components to form scaffolds that allow efficient assembly of the components of the signaling pathway. A connection between integrins and the angiotensin AT1 receptor has not been well studied. There are publications suggesting that prolonged (up to 48 h) treatments

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http://www.ajpcell.org
with AII modulate integrin expression in several cell models. Thus AII enhanced α1-, β1, β3, and β5 mRNA and protein levels, in rat cardiac fibroblasts (15). The positive correlation between expression of AT1 receptor and integrin-β1 has been demonstrated in cardiac myocytes, suggesting that expression of integrin-β1 may be modulated by AII via AT1 receptor (11). At the same time, treatment with AII did not change α5- and β1-integrin expression in human smooth muscle cells obtained from iliac arteries (13). Also, in AII-infused rats, AT1 receptors enhanced α8-1, β1-1, and β3-integrin subunit expression, without affecting expression of αα-integrin, and actually reduced α1-subunit expression in rat aorta (4). Another important in vivo study performed in mice lacking α1-integrin subunit demonstrated that signaling through α1β1-integrin and focal adhesions plays a role in AII-induced arterial wall hypertrophy (17). There is little evidence in the literature about possible roles of integrins in GPCR-induced proliferation of VSMC. One publication describes a synergistic interaction of integrins and AT1 receptor in the activation of the ERK pathway in VSMC (35). The authors showed that AII-induced ERK phosphorylation occurred even in suspended VSMC, but was enhanced by integrin activation in response to cell adhesion. This report suggested involvement of integrins in AII-induced proliferation of VSMC, but did not describe the specific integrin types involved, or the mechanism of their action (35).

The present study aimed to address this gap in our knowledge and to test the hypothesis that integrins are involved in AII-induced proliferation of cultured VSMC.

**EXPERIMENTAL PROCEDURES**

**Materials.** All and various chemicals were from Sigma (St. Louis, MO). Phospho-ERK antibodies were obtained from Cell Signaling Technology (Beverly, MA). All cell culture media and supplements were from Invitrogen (Carlsbad, CA). Cell culture 6-well, 12-well, or 96-well plates coated with collagen I, fibronectin, or poly-L-lysine were from BD Biosciences (San Jose, CA). Cyclic RGD (arginine-glycine-aspartic acid) and control RGD peptides were from Calbiochem (La Jolla, CA). KTS (lysin-threonine-serine)-containing and control peptides were from Peptides International (Louisville, KY). Monoclonal antibodies against α1-, 1-α-, and αα-integrin subunits and mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were from Millipore (Billerica, MA). Antibodies against α4-, α5-, 1-α-, 1-β-, and β3-integrin subunits; blocking antibodies against αα-, α5-, α3-, β1-, β3-, and β5-integrins; integrin-α1, -α5, and -β1 short interfering RNAs (siRNAs); and control (scrambled) siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). The RNA Stat-60 reagent was from Tel-Test (Friendswood, TX). The RT² Profiler PCR Array System for rat ECM and adhesion molecules, the True Labeling-AMP linear RNA amplification kit, and the Oligo GEArray kit for rat ECM and adhesion molecules were from SuperArray Bioscience (Frederick, MD). VisionBlue fluorescence cell viability assay kit was from BioVision (Mountain View, CA).

**Cell culture.** Rat aortic VSMCs were obtained as described previously (38) by terminal harvesting of aortas from male Sprague-Dawley rats (150–275 g; Charles River Laboratories, Wilmington, MA) using anesthesia under a protocol approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina. A 2-cm segment of artery cleaned of fat and adventitia was incubated in 1 mg/ml collagenase for 3 h at room temperature. The artery was then cut into small sections and fixed to a culture flask for explantation in minimal essential medium containing 10% FCS, 1% nonessential amino acids, 100 mU/ml of penicillin, and 100-μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air-5% CO₂. Medium was changed every 3–4 days, and cells were passaged every 6–8 days by harvesting with trypsin-EDTA. Our laboratory has previously characterized these cultures by immunostaining as positive for intracellular cytoskeletal fibrils of actin and smooth muscle cell-specific myosin, and negative for factor VIII antigens (37). VSMC isolated by this procedure were homogeneous and were used in all studies between passages 3 and 7.

**Integrin subunits expressed in VSMC.** To monitor the expression profile of integrins in VSMC, we employed the Oligo GEArray kit for rat ECM and adhesion molecules. We extracted total RNA from VSMC that were grown on plastic plates, using the RNA Stat-60 reagent, and converted RNA into biotin-labeled cRNA target probes for microarray hybridization using the True Labeling-AMP linear RNA amplification kit. The cRNA targets (2 μg of cRNA) were next hybridized with oligonucleotide probes, representing different genes, printed on a nylon membrane. The resulting products on arrayed membranes were detected by a chemiluminescent detection kit, and analyzed by GEArray Analyzer data analysis software. We also employed an RT² Profiler PCR Array System for rat ECM and adhesion molecules that validates the expression of 84 relevant genes for cell-cell and cell-matrix interactions, including eight α- and four β-integrin subunits to confirm microarray data, and to compare the expression of integrins in VSMC grown on different matrices. To evaluate the expression of α1-subunit, a customized RT² PCR Array was prepared (SuperArray Bioscience). Total RNA from VSMC grown on plastic, or on collagen-, fibronectin-, or poly-L-lysine-covered plates was isolated as described above, was converted to cDNA using the RT² Profiler PCR Array first-strand kit, and quantitative real-time-PCR was performed according to the manufacturer’s protocol. To demonstrate the presence of various integrin subunits on the protein level, we performed Western blotting of VSMC lysates with commercially available antibodies against integrins.

**ERK assays.** ERK phosphorylation was assessed using a phosphorylation state-specific ERK antibody, which specifically recognizes threonine382 and tyrosine385 phosphorylated ERK, as previously described (9, 26). Briefly, cells were cultured in 12-well plates, serum starved for 36 h, and stimulated with vehicle or AII. After treatment, cells were scraped into Laemmli buffer and subjected to SDS-PAGE using 4–20% precast gels (Invitrogen, Carlsbad, CA), and semidry transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked with BLOTTO buffer and incubated with the phospho-ERK antibody (1:1,000 dilution), followed by incubation with goat anti-rabbit alkaline phosphatase-conjugated IgG. Immunoreactive bands were visualized by a chemiluminescent method (CDP Star, New England Biolaboratories, Ipswich, MA) using preflashed Kodak X-AR film and quantified using ImageJ software (Image Processing and Analysis in Java, http://rsb.info.nih.gov/ij/index.html). The membranes were stripped using Re-Blot Plus antibody stripping solution (Millipore, Billerica, MA) and reprobed with the control ERK antibody, which recognizes equally well phosphorylated and nonphosphorylated ERK, to quantify total ERK. Results are presented as intensities of phospho-ERK bands relative to total ERK bands and expressed as fold of basal phosphorylation (nontreated cells).

**Proliferation assay.** Proliferation of VSMC was assessed by the VisionBlue fluorescence cell viability assay kit, which utilizes the redox dye, resazurin, which becomes fluorescent upon reduction by metabolically active cells. VSMC cells were seeded (10⁴/well) in 96-well black microplates with different cell attachment substrates (poly-L-lysine, fibronectin, and collagen I), and incubated in serum-free MEM for 36 h. Next, cells were preincubated for 1 h with 10 μM PD-98059 (MEK inhibitor) or vehicle before addition of 100 nM AII or 10 ng/ml EGF (positive control) or vehicle (negative control) for 24 h. After incubation with the assay solution for 2 h at 37°C, the fluorescence signal was monitored at the emission wavelength of 590 nm with an excitation wavelength of 540 nm using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA).
Transfections of VSMC integrin silencing. Transfections of VSMC were achieved by nucleofection with an Amza Biosystems instrument (Giessen, Germany) to transfer DNA or RNA directly into the nucleus of the cell. Cells (1 × 10⁶) were resuspended in 100 μl of Nucleofector solution for primary smooth muscle cells and nucleofected with either 100 nM of siRNA for integrin-α₁β₁, or integrin-α₅β₁ siRNA, or with the same amount of control (scrambled) siRNA using manufacturer’s protocol U-25. Forty-eight-hour postnucleofection cells were stimulated with AII or vehicle, lysed, and analyzed for integrin expression by Western blotting with an anti-integrin-α₁,-α₅, and -β₁ rabbit polyclonal antibodies, and for ERK activation. Blots were reprobed with a mouse monoclonal GAPDH antibody to control for protein loading and for silencing efficiency and specificity.

Data analysis. ERK assays were performed in duplicate and repeated at least three times. Data are presented as means + SE. Statistical evaluations of the data were performed using ANOVA or Student’s t-test as appropriate. Differences were considered significant at P < 0.05. Statistical probability (P) in figures is expressed as P < 0.05, P < 0.01, and P < 0.001 vs. vehicle-treated samples.

RESULTS

Expression profile of integrin subunits in VSMC. Because, to date, at least 18 α- and 8 β-integrin subunits have been identified (10), we determined to which integrins are present in VSMC to plan more specific experiments. First we employed the Oligo GEArray rat ECM and adhesion molecules microarray, which represents 111 genes encoding proteins important for the attachment of cells to their surroundings, including various types of cell adhesion molecules (such as the integrins, IgG superfamily members, cadherins and catenins, and selectins), as well as ECM proteins, proteases (such as the matrix metalloproteinases and the serine and cysteine proteinases), and their inhibitors. This array allowed us to determine simultaneously the expression profile of 17 α- and 8 β-integrin subunits to determine which isoforms are present in VSMC. Integrin subunits-α₁, -α₅, and -β₁ appeared to be the most abundant in VSMC. The messages for α₄, α₅, and β₃-integrin subunits were also present. The message for α₃-integrin, although detectable, was masked by a strong message for fibronectin-1 (spot no. 36), which is highly expressed in VSMC (Fig. 1A). To verify the microarray data, we employed an RT² Profiler PCR Array System for rat ECM and adhesion molecules that validates the expression of 84 relevant genes for cell-cell and cell-matrix interactions, including eight α- (α₂, α₃, α₄, α₅, α₁, α₂, α₃, and α₅) and four β-integrin (β₁, β₂, β₃, and β₁) subunits. We used a customized array to assess the expression of α₁-integrin. The expression of the GAPDH gene was used as control. Results revealed that the expression of α₁, α₃, α₄, α₅, α₇, β₁, and β₁ genes identified by the Oligo GEArray was confirmable by RT-PCR (Table 1). Furthermore, to compare the expression of integrins in VSMC grown on different matrices, we isolated total RNA from VSMC grown on collagen-, fibronectin-, or poly-L-lysine-covered plates, converted it to cDNA using the RT² Profiler PCR Array first-strand kit, and performed a quantitative real-time-PCR, according to the manufacturer’s protocol. We did not detect any significant changes in integrin messages in VSMC grown on different matrices (collagen, fibronectin, or poly-L-lysine) compared with the cells grown on plastic dishes (not shown). To confirm the mRNA data, we performed Western blotting on VSMC lysates using commercially available antibodies against α₁, α₃, α₄, α₅, α₇, β₁, and β₃-integrins. Results presented in Fig. 1B demonstrate the presence of all tested integrins in VSMC.

Table 1. Vascular smooth muscle cells express mRNA for α- and β-integrin subunits

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Threshold Cycle</th>
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<td>Itgα1</td>
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<td>24.6</td>
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<tr>
<td>Itgβ4</td>
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Total RNA was isolated from vascular smooth muscle cells grown onto plastic plates, as described in experimental procedures, and a quantitative real-time-PCR was performed according to the manufacturer’s protocol. Data were analyzed using the RT² Profiler PCR Array Analysis Web Portal (Super Array Bioscience). Messages with a threshold cycle value <20 are considered highly expressed, whereas values >20 are considered moderately expressed. Threshold cycle values >30 were used as a cutoff point and were reported as not detected.
VSMC proliferation and All-induced ERK activation are dependent on integrin-mediated anchorage. Because VSMC in vivo normally are surrounded by a basement membrane composed primarily of fibronectin and collagen that are known to play roles in controlling the growth and phenotype of VSMC (23), we tested the effect of ECM proteins on cell proliferation. VSMC were seeded (2 × 10^5/well) on control plastic six-well plates or on six-well plates coated with collagen I, fibronectin, or poly-D-lysine, and cultured in complete growth media (MEM medium containing 10% fetal bovine serum). Poly-D-lysine coating creates a uniform net positive charge on the plastic surface, thus promoting electrostatic cell attachment in an integrin-independent manner. We noted that VSMC were able to attach to all templates. After 48 h, VSMC were trypsinized, and the number of cells in each well was counted with a hemocytometer. Results are presented as number of cells per well (average of three independently counted wells). As shown in Fig. 2A, both ECM proteins (collagen I and fibronectin) supported the proliferation of VSMC, and the rate of proliferation was approximately two times higher than in cells cultured on plastic. Importantly, VSMC nonspecifically attached to poly-D-lysine did not proliferate. Actually, the number of viable cells in the samples grown onto poly-D-lysine-coated plates decreased after 48-h incubation in growth medium, suggesting that adhesion mediated by integrins, rather than through electrostatic binding, was necessary for VSMC proliferation.

Next, we evaluated the role of integrin-mediated cell anchorage in All-induced ERK activation. VSMC were cultured as described above, serum starved for 36 h, and stimulated with vehicle or with 100 nM All for 5 min. Cells were lysed and analyzed for ERK activation by Western blotting with a phospho-ERK antibody. To take into consideration the differences in density of VSMC grown onto different templates, the same membranes were stripped and reprobed with control ERK antibody, which equally well recognizes phosphorylated and nonphosphorylated ERK, to quantify total ERK. Results are presented as intensities of phospho-ERK bands relative to total ERK and expressed as fold of basal (cells without All treatment) phosphorylation (Fig. 2B). VSMC grown on plastic demonstrated approximately fivefold increase in ERK phosphorylation after stimulation with All. VSMC adherent via their integrins to fibronectin displayed even more pronounced All-induced ERK activation (~6-fold increase), but VSMC grown on collagen I demonstrated only approximately threefold ERK activation by All. VSMC nonspecifically adherent to poly-D-lysine, showed nonsignificant increases in ERK phosphorylation in response to All, suggesting the importance of integrin-mediated adhesion in the process of ERK activation (Fig. 2B). All-induced VSMC proliferation is dependent on ERK activation and integrin-mediated anchorage. The next series of experiments was aimed to establish the ability of All to stimulate VSMC proliferation and to study the role of ERK in this process. VSMC proliferation was assessed by the VisionBlue fluorescence cell viability assay kit, as described in Experimental procedures. VSMC grown on ECM (collagen I or fibronectin) demonstrated increased proliferation in response to All treatment (Fig. 3A). At the same time, All-induced increase in proliferation of VSMC grown on poly-D-lysine was not statistically significant. Treatment with EGF, which served as a positive control, stimulated proliferation of VSMC grown on all matrices. As previously shown, pretreatment of VSMC with 10 μM of PD-98059 completely blocked the activation of ERK kinase (MEK), thus preventing ERK phosphorylation (26). In our experiments, this treatment completely blocked All-induced VSMC proliferation, suggesting that ERK is essential for this process (Fig. 3A). Because it has been reported that All may enhance the expression of integrins in several models (4, 11, 15), we next tested the possibility that All modulates integrin expression in our cell model. We treated VSMC cells with 1 μM All for 6 or 24 h and assessed the expression of integrin subunits using an RT2 Profiler PCR Array. The concentration of All used in these experiments was chosen on the basis of concentration-response curve for All-
induced ERK activation, published previously by our group (26), and has been reported as optimal to study AII-induced protein expression in cultured VSMC (13, 24). No significant changes in any integrin messages, including α1-, α5-, or β1-integrin subunits, were detected in VSMC treated with AII compared with vehicle-treated cells (not shown).

To further study the involvement of integrins in AII-induced proliferation of VSMC, we employed synthetic integrin-binding peptides. Recent studies described KTS sequence as a biologically active motif relevant for the integrin-α1β1 (18). VSMC grown onto collagen I were pretreated for 2 h either with 200 μM KTS peptide, or with negative control peptide, and stimulated with AII for 24 h in the presence of peptides. As shown in Fig. 3B, treatment with KTS peptide blocked the effect of AII, suggesting an involvement of integrin-α1β1 in AII-induced proliferation in VSMC. Next, we employed RGD peptides. RGD was originally identified as the sequence in fibronectin that is a recognition site for α5β1-integrin, but it also serves as a recognition motif for other integrins, including α5β1, α5β3, α5β5, α5β6, and α2β1 (30). VSMC grown onto fibronectin were pretreated for 2 h with 200 μM of either control or RGD peptides, followed by stimulation with 100 nM AII for 24 h in the presence of RGD peptides. As demonstrated in Fig. 3C, RGD1 and RGD2 peptides completely prevented AII-induced proliferation, suggesting an importance of integrins with RGD recognition specificity in VSMC proliferation.

Effect of KTS and RGD peptides on AII-induced ERK phosphorylation. To test the involvement of integrins in AII-induced ERK phosphorylation in the next series of experiments, we employed KTS-containing peptides to test the possible involvement of integrin-α1β1 in AII-induced ERK activation in VSMC. Quiescent VSMC were pretreated for 2 h either with 200 μM KTS peptide, highly specific for integrin-α1β1, or with negative control peptide before stimulation with vehicle or AII for 5 min. As shown in Fig. 4A, KTS-containing peptide reduced AII-dependent ERK activation by ∼60%, suggesting that integrin-α1β1 is important in this process. To assess possible role of integrins with RGD recognition specificity in AII-induced ERK phosphorylation, in the next series of experiments, we employed RGD peptides. VSMC, grown in 12-well plates to 80% confluence and serum starved for 36 h, were pretreated for 2 h with either 200 μM control or RGD peptides before stimulation with 100 nM AII for 5 min. Control RGD peptides were without effect, whereas RGD1 and RGD2 peptides reduced the activation of ERK by AII by ∼65 and ∼69% correspondingly, suggesting that integrins with RGD recognition specificity (probably integrin-α1β1) are involved in AII-induced ERK phosphorylation (Fig. 4B).
Role of specific integrins in AII-induced ERK activation in VSMC. To further examine specific integrins that are involved in AII-induced ERK phosphorylation, we employed commercially available neutralizing antibodies against rat integrin subunits. Quiescent VSMC cultured on plastic 12-well plates were pretreated for 2 h with anti-integrin antibodies or normal mouse or goat IgG before stimulation with 100 nM AII for 5 min. Neutralizing antibody against α9 integrin when used in a concentration of 0.1 mg/ml significantly (50%) attenuated AII-induced phosphorylation of ERK in VSMC (Fig. 4C). At the same time, neutralizing antibody against α4, ανβ₃-integrin subunits was without effect, suggesting specificity in the integrin participation (not shown).

Transfection of VSMC with integrin siRNAs decreases AII-induced ERK activation. To further support the involvement of integrins in AII-induced ERK activation, we employed RNA-mediated interference to knock down the expression of α5β₁-integrins. VSMC were nucleofected with 100 nM of siRNA for integrin-α5, αvβ₃, or β3-integrin siRNAs demonstrated ~30% less AII-induced ERK activation than cells.

Fig. 4. AII-induced ERK phosphorylation is integrin dependent. Cells were cultured on plates coated with collagen I (A) or fibronectin (B), grown to 80% confluence, and serum starved for 36 h. Cells were pretreated for 2 h with 200 μM inactive peptides, cyclic RGD, or KTS peptides before stimulation with 100 nM AII for 5 min. ERK activity was assessed by Western blotting with phospho-specific ERK antibody. Values are expressed as fold of basal (vehicle-treated cells). C: quiescent VSMC were pretreated for 2 h with 100 μg/ml of anti-integrin antibodies or normal mouse or goat IgG before stimulation with vehicle or with 100 nM of AII for 5 min. Cells were lysed and analyzed by Western blotting for ERK activation. Results are presented as intensities of phospho-ERK bands relative to total ERK and expressed as fold of basal (cells without AII treatment) phosphorylation (means ± SE) of at least three independent experiments performed in duplicate. *P < 0.05, ***P < 0.001 vs. vehicle-treated samples.
transfected with control siRNA. Cells transfected with a combination of both $\alpha_1$- and $\beta_1$-integrin siRNAs demonstrated a further decrease ($\sim 50\%$) in AII-induced ERK phosphorylation (Fig. 5A). The effect was even more pronounced in VSMC grown on fibronectin, for which transfection with $\alpha_5$ or with $\beta_1$-integrin siRNAs caused $\sim 50\%$ decreases in AII-induced ERK phosphorylation, and silencing of both integrin subunits simultaneously resulted in $\sim 70\%$ inhibition of AII-induced ERK phosphorylation (Fig. 5B). Effective silencing of integrin expression in VSMC transfected with siRNAs was supported by immunoblotting (Fig. 5, bottom). These results support the hypothesis that integrins-$\alpha_5\beta_1$ and -$\alpha_1\beta_1$ mediate AII-induced signaling in cultured VSMC.

DISCUSSION

In our laboratory’s previous work, we investigated the relationship between the ERK cascade and the phosphorylation state of the gene product encoded by retinoblastoma in VSMC and demonstrated that the AT1 receptor-induced rapid phosphorylation of retinoblastoma-Ser$^{795}$ was functionally significant but insufficient to cause the transition of cells through the cell cycle (9). Therefore, we suggested that AT1 receptor collaborates with the other mechanisms to stimulate proliferation of VSMC and hypothesized a role of integrins in this process.

The present work provides strong evidence for the involvement of integrins in AII-induced signaling in VSMC. What is new about this work is that we have 1) characterized the repertoire of integrins in VSMC cells using Oligo GEArray detection, RT-PCR, and Western blotting; 2) implicated that VSMC proliferation and AII-induced ERK activation are dependent on integrin-mediated anchorage; and 3) provided evidence that integrins-$\alpha_5\beta_1$ and -$\alpha_1\beta_1$ are involved in AII-induced ERK activation based on results of experiments utilizing RGD and KTS peptides, neutralizing anti-integrin antibodies, and siRNA.

VSMC in vivo normally are surrounded by a basement membrane composed primarily of fibronectin and collagen that are known to play roles in controlling the growth and phenotype of VSMC, and the major integrin subunits present in VSMC in vivo are $\alpha_1$, $\alpha_3$, $\alpha_5$, and $\beta_1$ (23). Because expression of different integrins varies dramatically in VSMC with different phenotypes, we first studied which integrins are present in our cell model and established that messages for $\alpha_1$, $\alpha_5$, $\alpha_3$, and $\beta_1$-integrins are predominant (Fig. 1 and Table 1). The repertoire of integrins expressed in our cell model was not

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Fig. 5. Transfection of VSMC with integrin short interfering RNAs (siRNAs) decreases AII-induced ERK activation. A, top and middle: VSMC were nucleofected with 100 nM of siRNA for integrin $\alpha_1$ (-$\alpha_1$) or $\beta_1$ (-$\beta_1$) alone, or with combinations of both siRNA (-$\alpha_1\beta_1$), or with the same amount of scrambled siRNA (control). Transfections were performed as described in EXPERIMENTAL PROCEDURES. Bars represent intensities of phospho-ERK (p-ERK) bands relative to total ERK expressed as fold of basal (cells treated with vehicle). Experiments were performed at least 3 times in duplicate. Values are means $\pm$ SE. ANOVA-AII treated ($\alpha_1$ or $\beta_1$) compared with All treated (-$\alpha_1\beta_1$) was not significant. B, top and middle: VSMC were nucleofected with 100 nM of siRNA for integrin $\alpha_5$ (-$\alpha_5$) or $\beta_1$ (-$\beta_1$) alone, with combinations of both siRNA (-$\alpha_5\beta_1$), or with the same amount of scrambled siRNA (control). Forty-eight hours postnucleofection, cells were stimulated with vehicle or 100 nM AII for 5 min, lysed, and analyzed for ERK phosphorylation. **$P < 0.01$ vs. vehicle-treated samples. ANOVA-AII treated ($\alpha_5$ or $\beta_1$) compared with All treated (-$\alpha_5\beta_1$) was not significant. Bottom: Western blot analyses of lysates of VSMC cells transfected with scrambled (Scr) siRNA or siRNAs for $\alpha_1$, $\alpha_5$, and $\beta_1$-integrins (40 $\mu$g of total protein) were performed with commercially available antibodies against $\alpha_1$, $\alpha_5$, and $\beta_1$-integrin subunits, to demonstrate downregulation of these proteins. Blots were stripped and reprobed with an antibody against GAPDH to control the specificity of silencing and protein loading.
INTEGRINS AND PROLIFERATION OF SMOOTH MUSCLE CELLS

dependent on the matrix (plastic, collagen I, or fibronectin) on which VSMC were grown (data not shown). Importantly, according to our microarray data, VSMC express mRNA for several ECM proteins, including fibronectin (spot no. 36), and collagen I (spot no. 20), suggesting that these cells may produce ECM when grown on plastic dishes. However, VSMC grown onto collagen I and/or fibronectin proliferated approximately two times faster than VSMC cells cultured on plastic, whereas cells electrostatically attached to poly-γ-lysine did not proliferate (Fig. 2A), suggesting that integrin-mediated adhesion is important for VSMC proliferation. Similarly, VSMC nonspecifically adherent to poly-γ-lysine and showed only minor (−30%) increases in ERK phosphorylation in response to AII, whereas VSMC grown on fibronectin demonstrated prominent (−6-fold) AII-induced ERK activation (Fig. 2B). Surprisingly, at the same time, VSMC grown on collagen I demonstrated only approximately threefold increase in ERK phosphorylation in response to AII, whereas VSMC grown on fibronectin demonstrated prominent (−6-fold) AII-induced ERK activation (Fig. 2B).

The incomplete inhibition of AII-induced ERK activation by RGD and/or KTS peptides, neutralizing antibodies against α5- and/or β1-integrin subunits and by RNA-mediated interference, suggests that, most probably, several integrins are involved in AII-induced signaling. Another explanation could be the ability of various α- and β-integrins to substitute for each other in situations when expression of particular subunits is silenced. In that sense, an overexpression of α5-, αv-, and β1-integrins has been described in α1-null mice, suggesting that upregulation of these integrins may partly compensate for the absence of α1-integrin (17). Thus, although our results demonstrate specific roles for integrins-α5β1 and -αvβ1, we cannot exclude the possibility that other integrins could be involved in AII-induced signaling, and synergy between them should be considered.

This study is important in that there are not many reports regarding interactions between GPCR and integrin signaling. Thus β1- and β3-integrins have been shown to colocalize with the µ-opioid receptor and to regulate the signaling of this receptor in sensory neurons (3). The only GPCR that has been shown to interact directly with integrins, the P2Y2 nucleotide receptor, which contains an RGD motif in the first extracellular loop, has been shown to interact directly with αvβ3- and αvβ5-integrins (8). These interactions between the P2Y2 nucleotide receptor and αv-integrins are necessary for the P2Y2 nucleotide receptor to activate Gq and to initiate Gq-mediated signaling events leading to chemotaxis (2) and also are critical for astrocyte migration (39). Recent studies from our laboratory described the interaction of the bradykinin B2 receptor with α5β1-integrin and provided evidence that this interaction leads to transactivation of EGFR and ERK phosphorylation in cultured kidney mouse inner medullary collecting duct-3 cells (16). Only a few studies have suggested possible roles of integrins in AII-induced signaling. Thus, in cultured VSMC, activation of AT1 receptors resulted in phosphorylation of FAK (28). Tamura et al. (35) reported that simultaneous stimulation of integrin and AT1 receptors in VSMC caused synergistic interaction in the activation of ERK pathway, possibly via phosphorylation of FAK, and suggested that integrins may play a critical role in AII-mediated mitogenic response in VSMC. However, in adrenal glomerulosa cells, AII inhibited cell proliferation, acting at the level of integrin binding and disrupting the stress organization of actin filaments (29). A role of α1-integrin in AII-induced arterial wall hypertrophy was assessed in vivo using mice lacking α1-integrin subunit (17). The authors demonstrated that suppression of α1β1-integrin resulted in reduced mechanical strength of the vascular wall and in decrease of VSMC hypertrophy in response to AII and emphasized the importance of integrin-α1-dependent p38 MAPK and FAK phosphorylation in AII-induced vascular hypertrophy. At the same time, AII significantly increased ERK phosphorylation in aortas from both α1-integrin-null and wild-type mice treated with AII for 4 wk, suggesting that AII-induced activation of ERK is independent of α1-integrin. The differences between this study and our data may be related...
to the different model systems, as well as different time course of experiments. The time course for All-induced ERK phosphorylation in VSMC was peaking at 5 min and persisting for at least 1 h of treatment with 100 nM of All (9). No significant All-induced phosphorylation of p38 MAPK, and only relatively small increase (~50%) in p44/p42 MAPK phosphorylation, which occurred at 2 min and decreased to the basal level by 10 min of incubation with All, was detected under the same experimental conditions (M. N. Garnovskaya, unpublished observations).

In conclusion, these studies demonstrate roles for specific integrins (αvβ3 and αvβ1) in ERK-dependent, All-induced proliferation of VSMC. Because All is thought to mediate excessive vascular proliferation and restenosis after angioplasty (14), and because considerable evidence suggests that integrins are involved in both acute and chronic vascular control (19), these studies ultimately might lead to development of new strategies for treatment of vascular diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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