Original Contributions

Mechanotransduction of Rat Aortic Vascular Smooth Muscle Cells Requires RhoA and Intact Actin Filaments

Kotaro Numaguchi, Satoru Eguchi, Tadashi Yamakawa, Evangeline D. Motley, Tadashi Inagami

Abstract—The growth-promoting effect of mechanical stress on vascular smooth muscle cells (VSMCs) has been implicated in the progress of vascular disease in hypertension. Extracellular signal–regulated kinases (ERKs) have been implicated in cellular responses, such as vascular remodeling, induced by mechanical stretch. However, it remains to be determined how mechanical stretch activates ERKs. The cytoskeleton seems the most likely candidate for force transmission into the interior of the cell. Therefore, we examined (1) whether the cytoskeleton involves mechanical stretch–induced signaling, (2) whether Rho is activated by stretch, and (3) whether Rho mediates the stretch-induced signaling in rat cultured VSMCs. Mechanical stretch activated ERKs, with a peak response observed at 20 minutes, followed by a significant increase in DNA synthesis. Treatment with the ERK kinase-1 inhibitor, PD98059, inhibited the stretch-induced increase in DNA synthesis. Cytochalasin D, which selectively disrupts the network of actin filaments, markedly inhibited stretch-induced ERK activation. In the control state, RhoA was observed predominantly in the cytosolic fraction, but it was translocated in part to the particulate fraction in response to mechanical stretch. Botulinum C3 exoenzyme, which inactivates Rho p21 (known to participate in the reorganization of the actin cytoskeleton), attenuated stretch-induced ERK activation. Inhibition of Rho kinase (p160ROCK) also suppressed stretch-induced ERK activation dose dependently. Our results suggest that mechanotransduction in VSMCs is dependent on intact actin filaments, that Rho is activated by stretch, and that Rho/p160ROCK mediates stretch-induced ERK activation and vascular hyperplasia. (Circ Res. 1999;85:5-11.)

Key Words: actin filament ﬂ cell growth ﬂ mitogen-activated protein kinase ﬂ RhoA ﬂ vascular smooth muscle cell

Excessive hemodynamic forces play an important role in vascular remodeling in hypertension and the genesis of atherosclerosis in vivo.1,2 In hypertension, mechanical strain on the vessel wall is increased by as much as 15%.3 Previous studies have shown that mechanical stress such as hydrostatic pressure4 and stretch5,6 induces DNA synthesis and proto-oncogene expression in vascular smooth muscle cells (VSMCs). These growth-promoting events are induced partly through an autocrine secretion of platelet-derived growth factor-AA (PDGF-AA) in neonatal VSMCs.7 Recently, it has been shown that extracellular signal–regulated kinase (ERK)-1/ERK-2, one of the important mitogenic signals, is activated by stretch in vivo and in vitro in VSMCs.8–10 However, the underlying mechanisms by which mechanical events are converted into biochemical signals in VSMCs remain unknown.

Physical deformation of endothelial and other cell types is associated with cytoskeletal reorganization and adaptive changes in the area of cell–matrix contact.11,12 All eukaryotic cells express actin, which often constitutes as much as 50% of the total cellular protein. Although actin filaments are dispersed throughout the cell, they are most highly concentrated just beneath the plasma membrane. Rearrangement of the actin cytoskeleton mediates, at least in part, adaptive changes of cell shape and the degree of cell attachment to the substratum in response to environmental signals. Cytoskeletal proteins are also known to modulate ion channel activity in neural tissue and fibroblasts.13,14 It has been reported that changes in the cytoskeleton are closely associated with some specific gene expression.15,16 Point mutations in adducin heterodimer, which could affect the assembly of the actin-based cytoskeleton, are involved in a form of rat primary hypertension.17 Therefore, the cytoskeleton seems the most likely candidate for force transmission into the interior of the cell.

Previous studies have shown that Rho-family small G proteins (Rho, Rac, and Cdc42) play a central role in the organization of the actin cytoskeleton18 and that Rho is required for Gq-induced hypertrophic gene expression in cardiomyocytes.19–21 Recently, Rho-associated protein kinase (p160ROCK), a downstream target of Rho, has been shown to mediate stress fiber formation in Swiss 3T3 fibroblasts.22 However, little is known about the involvement of Rho-family small G proteins in mechanotransduction in VSMCs.
In the present study, we investigated whether the cytoskeleton is involved in stretch-induced biochemical signals of VSMCs. We also examined the role of Rho and p160ROCK in mechanotransduction in VSMCs.

**Materials and Methods**

**Materials**

DMEM, FCS, penicillin, and streptomycin were obtained from Life Technologies, Inc. Cytochalasin D, colchicine, and angiotensin II were purchased from Sigma. PDGF-AA was purchased from Upstate Biotechnology Inc. Botulinum C3 exoenzyme was purchased from Calbiochem. PD98059 was purchased from New England Biolabs. [γ-33P]ATP was purchased from NEN. Y-27632 was a gift from Yoshitomi Pharmaceutical Industries Ltd.

**Cell Culture and Stretch Protocol**

VSMCs were prepared from the thoracic aorta of 12-week-old Sprague-Dawley rats (Charles River Laboratories) by the explant method and cultured in DMEM containing 10% FCS, penicillin, and streptomycin. Subcultured VSMCs from passages 3 to 15, used in the experiments, showed >99% positive immunostaining of smooth muscle α-actin antibody (Sigma) and were negative for mycoplasma infection by the polymerase chain reaction kit (Stratagene).

The cells were seeded at 5 × 10⁴ cells per culture well on type I collagen–coated Flex I and Flex II plates (Flexercell International Corp) and grown under nonstretch conditions. Cells were maintained in complete medium for 4 days, achieving ~80% confluence. The medium was then changed to serum-free DMEM for 3 days to allow the cells to become quiescent before application of stretch. The Flex I plates containing a flexible silicone elastomer substratum were then mounted in the Flexcell Strain Unit (Flexercell International Corp), which consists of a computer-controlled vacuum unit that applies cyclic vacuum (15 to 20 kPa, 1 Hz) to the rubber-bottomed dishes in a humidified incubator with 5% CO₂ at 37°C (10% elongation did not increase thymidine uptake). Control Flex II plates, containing the same collagen-coated silicone elastomer substratum plus a rigid polystyrene bottom, were grown in parallel but not mounted in the strain unit.

**ERK Activity**

After stimulation, the reaction was terminated by the replacement of medium with the ice-cold lysis buffer containing (in mmol/L) Tris-HCl (pH 7.4) 10, NaCl 20, EGTA 2, DTT 2, Na₃VO₄ 1, and phenylmethylsulfonyl fluoride (PMSF) 1, as well as leupeptin and aprotinin (10 μg/mL each). After brief sonication (10 seconds), the samples were centrifuged at 14 000g for 5 minutes, and the supernatant was assayed for ERK activity with an assay kit (Amersham Corp) that measures the incorporation of [γ-33P]ATP into a synthetic peptide (KRELVEPLTAEPPNQALLR) as a specific ERK substrate. The reaction was carried out with the cell lysate (~1 μg of protein) in 75 mmol/L HEPES buffer (pH 7.4), containing (in mmol/L) MgCl₂ 1.2, substrate peptide 2, and ATP 1.2, and 1 μCi of [γ-33P]ATP for 30 minutes at 30°C. The resultant solution was applied to a phosphocellulose membrane and washed in 1% acetic acid and then H₂O. The radioactivity trapped on the membrane was measured by liquid scintillation counting.
Subcellular Fractionation

Cell-free lysates were prepared by adding 100 μL lysis buffer containing (in mmol/L) Tris (pH 8.0) 20, sucrose 250, and PMSF 1, as well as aprotinin and leupeptin (10 μg/mL each). After 3 cycles of freeze and thaw, samples were centrifuged at 100,000 g at 4°C for 60 minutes. The supernatant was saved as a “soluble” fraction. Pellets were washed twice by the same lysis buffer and resuspended in 100 μL of lysis buffer supplemented with 1% Triton X-100 and 0.1% SDS. Cell debris was separated by centrifugation (14,000 rpm at 4°C for 20 minutes), and the supernatant was saved as a “particulate” fraction. The protein content of each fraction was determined by the Lowry method.

Immunoblotting

In separate experiments, the reaction was terminated by the replacement of medium with SDS-PAGE buffer, pH 6.8, containing 62.5 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 50 mmol/L DTT, and 0.1% bromphenol blue after stimulation. After brief sonication (5 seconds), samples were boiled for 5 minutes at 95°C and centrifuged (14,000 rpm at 4°C for 20 minutes), and the supernatant was saved as a “particulate” fraction. The protein content of each fraction was determined by the Lowry method.

[3H]Thymidine Incorporation

After 72 hours in serum-free medium, cells were stimulated by stretch. After stretching for 24 hours, [3H]thymidine (1 μCi/mL, Amersham LifeScience) was added for the next 24 hours. Cells were then washed twice with PBS, and 5% trichloroacetic acid was added at 4°C for 30 minutes to precipitate the protein. Trichloroacetic acid–precipitable radioactivity was counted by a scintillation counter.

Statistics

Data are given as mean±SE. Statistical analyses were performed using ANOVA. A post hoc test was performed by the method of Bonferroni. Significance was accepted at the P<0.05 level.

Results

Mechanical Stretch Activated ERKs and Increased DNA Synthesis

In response to mechanical stretch, there was an apparent phosphorylation of both ERK1 and ERK2, which are p44 and p42 MAPKs, respectively, with peak activity at 20 minutes followed by return to baseline (Figure 1A). The time course of ERK activity induced by stretch was similar to that of phosphorylation (Figure 1B, open bar). The cells in the middle of the dish show little strain, but they constitute <15% of the total cells. We performed control experiments...
using dishes in which cells were excluded from the central, low-stretch area by placing a 9-mm stainless-steel cloning cup in the center of the well. Results in ERK activation using these dishes (Figure 1B, hatched bar) showed results closely analogous with those of stimulation of cells grown on the entire well. Therefore, we can assume that the small-percentage presence of cells showing little strain has little effect. It has been shown that mechanical stretch induces growth of neonatal VSMCs via autocrine production of PDGF. To investigate the possible role of prestored growth factor, which might be released from the cells during stretching, we applied conditioned medium harvested from stretched cells to other wells containing quiescent VSMCs. However, the conditioned medium had no effect on ERK activation, which suggests that the contribution of a paracrine release of growth factors is minimal in the present system (Figure 1C).

Mechanical stretch also significantly increased \(^{[3]H}\)thymidine incorporation. PD98059 (25 \(\mu\)mol/L), a highly selective ERK kinase-1 inhibitor that binds to the inactive form of ERK kinase-1 and prevents its activation, abolished the increase in thymidine incorporation by stretch (Figure 2). These observations suggest a correlation between mitogenesis and the activity of ERK.

**Stretch-Induced but Not Agonist-Induced ERK Activation Is Dependent on Intact Actin Filaments**

Cytoskeletons mainly comprise actin filaments, microtubules, and intermediate filaments. To test whether the cytoskeleton is important in MAPK activation by mechanical stretch, phosphorylation of ERKs and their kinase activities at 20 minutes were determined in the absence and presence of cytochalasin D or colchicine. Cytochalasin D is known to depolymerize actin microfilament bundles, and colchicine is known to bind tubulin and inhibit its assembly to form microtubules. Disruption of actin filaments with cytochalasin D (0.4 \(\mu\)mol/L) markedly inhibited ERK activation by mechanical stretch in rat VSMCs, whereas inhibition of microtubule polymerization with colchicine had no inhibitory effect (Figure 3A and 3B). To exclude the nonspecific action of cytochalasin D, we examined its effect on ERK activation by 2 different agonists. Angiotensin II is reportedly released from cardiac myocytes during stretching. PDGF-AA is reported to be important for strain-induced growth of neonatal rat VSMCs. Both agonists induced apparent phosphorylation of ERK-1/ERK-2. However, the dose of cytochalasin D used in the present study had little effect on angiotensin II–induced and PDGF-AA–induced phosphorylation of ERK (Figure 4). Therefore, the effect of cytochalasin D is specific for mechanical stretch–induced signaling.

**Mechanical Stretch Induces Partial Translocation of RhoA From the Soluble to the Particulate Fraction in VSMCs**

Because Rho p21, one of the Rho-family small GTP-binding proteins, has been reported to be critical in actin reorganization and formation of focal adhesion, we investigated whether Rho is involved in mechanical stretch–induced signaling in VSMCs. It has been shown that Rho and Rac partially translocate from the soluble to the particulate fraction on their activation. To study whether mechanical stretch activates RhoA in VSMCs, the subcellular localization of RhoA before and after mechanical stretch was examined. Before stimulation, the majority of RhoA was detected in the soluble fraction, consistent with previous reports. Parallel immunoblot analysis indicates that the amount of RhoA detectable in total lysate did not change significantly before and after mechanical stretch. These results suggest that part of RhoA translocates from the soluble to the particulate fraction in response to mechanical stretch.
Botulinum C3 Exoenzyme and Inhibition of Rho-Associated Protein Kinase Suppressed Stretch-Induced ERK Activation

Exoenzyme C3 has been a useful and well-established tool in studying the function of Rho, because it causes ADP ribosylation at Asn41 of Rho and, hence, specifically inactivates Rho. To assess the involvement of Rho p21 in the mechanical stretch–induced ERK activation, VSMCs were treated with 4 μg/mL of C3 for 48 hours. Pretreatment with C3 significantly attenuated mechanical stretch–induced ERK activation (Figure 6). p160ROCK is known as one of the main direct target molecules of Rho. Recently, Uehata et al. have developed a pyridine derivative, Y-27632, which suppresses Rho-induced, p160ROCK-mediated formation of stress fibers and have shown that p160ROCK is involved in various models of hypertension. Pretreatment with Y-27632 dose-dependently inhibited mechanical stretch–induced ERK activation in our system, suggesting that p160ROCK mediates stretch-induced signaling in rat VSMCs (Figure 7).

Discussion

The present study demonstrates for the first time that mechanical stretch–induced ERK activation in VSMCs requires intact actin filaments. Our results also suggest that mechanical stretch activates Rho, and Rho/p160ROCK mediates mechanical stretch–induced ERK activation.

When a member of the small GTP-binding proteins is activated, the GTP-bound form is supposedly increased. However, the guanine nucleotide binding assay and determination of the guanine nucleotide exchange activity for Rho

Figure 6. Effect of exoenzyme C3 on stretch-induced ERK activation. After pretreatment with 4 μg/mL C3 for 48 hours (+) or without the pretreatment (−), VSMCs were stretched by 20% for 20 minutes. ERK activities were measured as described in Materials and Methods. Data are expressed as fold ERK activity, in which ERK activity in unstimulated cells was defined as 1.0. Values shown represent mean±SE from 3 separate experiments, each performed in triplicate. **P<0.01 vs stretch only.

Figure 7. Effect of p160ROCK inhibitor (Y-27632) on stretch-induced ERK activation. After pretreatment with indicated doses of Y-27632 for 30 minutes or without pretreatment (−), VSMCs were stretched by 20% for 20 minutes. ERK activities were measured as described in Materials and Methods. Data are expressed as fold ERK activity, in which ERK activity in unstimulated cells was defined as 1.0. Values shown represent mean±SE from 3 separate experiments, each performed in triplicate. **P<0.01 vs stretch only.

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have been technically difficult, because the currently available antibodies are not suitable for immunoprecipitating the native form of Rho in the presence of Mg
. On the other hand, Mg
 is required for preserving its guanine nucleotide binding of Rho. However, results from the present study support the conclusion that mechanical stretch activates Rho in VSMCs. First, mechanical stretch partially translocated Rho from the cytosolic to the membrane fraction determined by immunoblotting. A variety of mitogens, such as angiotensin II, endothelin, and lysophosphatidic acid, have been reported to induce actin filament organization and cause translocation of Rho from the cytosolic to the membrane fraction. The level of mechanical stretch–induced RhoA translocation was comparable with that of the known stimulators mentioned above. Second, activation of ERK was suppressed by C3 treatment and inhibition of Rho kinase. Taken together, these results are consistent with RhoA activation by mechanical stretch in VSMCs.

At present, we do not know how Rho is activated by mechanical stretch. Several growth factors, such as lysophosphatidic acid, endothelin, PDGF, and angiotensin II, have been reported to activate Rho. A previous study has also suggested that mechanical stretch induces growth of neonatal VSMCs via autocrine production of PDGF. Therefore, it is possible that the release of growth factors from stretched cells. However, this is less likely in our system, because the conditioned medium had no effect on ERK activation (Figure 1C) and [3H]thymidine uptake (data not shown). Another possible factor that can activate Rho is an integrin receptor. It has been shown that antibodies to α, β, and β integrins block thymidine uptake induced by mechanical stretch and that Rho involves integrin-mediated activation of ERK. Whether antibodies to integrins inhibit stretch-induced activation of Rho and ERK remains to be elucidated.

The downstream signaling pathway that can connect Rho to ERK constitutes another important question. Recently, several proteins, such as p160ROCK, showing selective binding to GTP-Rho have been isolated and proposed as potential Rho targets. p160ROCK, which is a 160-kDa protein serine/threonine kinase, has also been reported to work as a Rho effector in the formation of focal adhesions and stress fibers. It has been reported that Y-27632, a ROCK inhibitor, blocks stretch–induced ERK activation and cell growth. Another possibility is that Rho has many targets except p160ROCK. Also, we cannot exclude the presence of Rho/p160ROCK–independent pathways such as superoxide. Hishikawa et al have shown that pulsatile stretch stimulates superoxide production and antioxidants inhibit stretch-induced DNA synthesis. Rac, another GTP-binding protein of the Rho family, may affect the activity of NADPH. Therefore, we need to perform further studies on the Rho/p160ROCK–independent pathways in our system.

Details of the mechanotransduction pathway of mechanical stretch–induced cell growth remain unclear. The present study provides the first evidence that Rho, p160ROCK, and intact actin filaments may play an important role in mechanical stretch–induced ERK activation and cell growth.

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**References**


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