Stretch of the Vascular Wall Induces Smooth Muscle Differentiation by Promoting Actin Polymerization*

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Stretch of the vascular wall by the intraluminal blood pressure stimulates protein synthesis and contributes to the maintenance of the smooth muscle contractile phenotype. The expression of most smooth muscle specific genes has been shown to be regulated by serum response factor and stimulated by increased actin polymerization. Hence we hypothesized that stretch-induced differentiation is promoted by actin polymerization. Intact mouse portal veins were cultured under longitudinal stress and compared with unstretched controls. In unstretched veins the rates of synthesis of several proteins associated with the contractile/cytoskeletal system (α-actin, calponin, SM22α, tropomyosin, and desmin) were dramatically lower than in stretched veins, whereas other proteins (β-actin and heat shock proteins) were synthesized at similar rates. The cytoskeletal proteins γ-actin and vimentin were weakly stretch-sensitive. Inhibition of Rho-associated kinase by Y-27632. The F/G-actin ratio after 24 h of culture was significantly greater in stretched than in unstretched veins, as shown by both ultracentrifugation and confocal imaging with phalloidin/DNase I labeling. The results show that stretch of the vascular wall stimulates increased actin polymerization, activating synthesis of smooth muscle-specific proteins. The effect is partially, but probably not completely, mediated via Rho-associated kinase and cofilin downstream of Rho.

Modulation of smooth muscle phenotype is of major importance for a number of disease states in the cardiovascular, respiratory, and visceral organs, and its molecular mechanisms are rapidly being elucidated (1, 2). Both intrinsic and extrinsic factors contribute to smooth muscle differentiation, marked by the expression of a limited number of proteins, primarily associated with the contractile/cytoskeletal apparatus (3). It is likely that mechanical stress in the walls of hollow organs is one of the key factors that regulate smooth muscle development as well as growth and phenotypic differentiation (4, 5). The signal mechanisms of stretch-dependent gene expression involve cell-cell contacts, the extracellular matrix, and integrins. This indicates that the intact tissue environment is critical for determining cell differentiation.

The effects of wall stress have been extensively studied in blood vessels, which respond to altered transmural pressure with growth and remodeling to normalize the mechanical stress in the tissue (4). In this process the smooth muscle cells are maintained in a contractile phenotype, which contrasts with the loss of differentiation that occurs at sites of vascular injury, as in atherosclerosis and restenosis after angioplasty (6). In organ culture of intact vessels, stretch has been shown to promote the expression of h-caldesmon and filamin in the rabbit aorta (7) and of SM22α, α-actin, and calponin in the rat or mouse portal vein (8, 9).

Although several transcription factors are likely to cooperate in promoting the expression of smooth muscle-specific genes (2), serum response factor (SRF) is so far the only one that has been shown to regulate nearly all of the smooth muscle specific genes. The promoter region of most smooth muscle specific genes contains one or more sites (CArG boxes) binding SRF (10). It is now clear that additional factors complement SRF binding in conferring smooth muscle specificity (2, 10). These include the recently identified proteins myocardin (11) and myocardin-related transcription factors (12). Molecular interactions between SRF, myocardin, and myocardin-related transcription factors seem to regulate binding to DNA, which suggests that cytoplasmic signals may influence transcriptional activity via nuclear translocation of any of these factors. Specifically, actin polymerization via RhoA activity regulates promoter activity of the prototypical differentiation markers SM22α and α-actin (13, 14). This is suggested to be due to nuclear translocation of the myocardin-related transcription factor known alternatively as MAL/MKL1 (megakaryocytic acute leukemia/megakaryoblastic leukemia 1), which is promoted by a decrease in the cytoplasmic concentration of monomeric G-actin caused by actin polymerization (15, 16).

Because extensive evidence suggests that factors influencing actin polymerization regulate smooth muscle differentiation, the present challenge is to determine whether such a mechanism operates in native cells in the intact tissue. The effects of stretch on smooth muscle differentiation suggest that this may be one condition where a physiological stimulus affects gene

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The abbreviations used in this paper are: SRF, serum response factor; ROCK, Rho-associated kinase; RBD, Rho-binding domain of rhotekin; HSP, heat shock protein; GTPγS, guanosine 5′-O-(3-thiotriphosphate); PIPES, 1,4-piperazineethanesulfonic acid; PBS, phosphate-buffered saline.
Stretches and Smooth Muscle Differentiation

Expression via actin polymerization. One preparation that has been studied with respect to stretch-induced protein synthesis both in vivo and in vitro is the rat portal vein, which rapidly develops hypertrophy with preserved contractility in response to increased pressure (17). Organ culture of the portal vein under maintained stretch (simulating pressure) reproduces many of the in vivo findings, including greater contractility and rates of protein synthesis than those of unstretched preparations (18). This response involves autocrine production of angiotensin II and of endothelin-1 and is blocked by inhibitors of extracellular signal-regulated kinase 1/2 and of actin polymerization (8, 18, 19). The present study was designed to investigate the pattern of stretch-sensitive protein synthesis and to correlate this with direct determinants of actin polymerization and with the activity of its regulating pathways. The results indicate that stretch promotes actin polymerization in the intact tissue, which activates synthesis of smooth muscle differentiation marker proteins.

EXPERIMENTAL PROCEDURES

Preparation of Portal Veins and Organ Culture—Female NMRI mice (30–35 g) and Sprague-Dawley rats (200–250 g) were killed by cervical dislocation. Inactivation of CO2, as approved by the regional Animal Ethics Committee. The portal vein was removed and dissected free from fat and connective tissue under sterile conditions. Rat portal veins were split longitudinally into two strips, whereas mouse vessels were used intact. The veins were then incubated at 37 °C in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) with 2% dialyzed fetal calf serum and 10 nM insulin as described previously (8). To stretch the vessel wall, one mouse portal vein was loaded with a 0.3-g weight, and one strip of rat portal vein was loaded with a 0.6-g weight. These loads cause extension approximately representing the optimal rate of protein synthesis than those of unstretched vessels. Most of the supernatant was diluted 1:2 with Laemmli buffer, whereas the pellet was resuspended in cold distilled H2O with 1 μM cytochalasin D and sonicated for 10 s. The pellet fraction was kept on ice for 45 min and then diluted 1:4 with Laemmli buffer. Both fractions were then boiled for 10 min and centrifuged at 14,000 × g for 10 min at 4 °C to remove remaining connective tissue. The same relative amounts of supernatant and pellet fractions (2:1) were loaded on 12.5% polyacrylamide gels and analyzed by Western blot using an anti-actin antibody (1:500, Sigma).

Fluorescence Microscopy—Mouse portal veins were cultured for 24 h and then incubated for 5 min at 4 °C in 150 mM NaCl, 10 mM Tris (pH 7.4), 2 mM MgCl2, 5 mM MgCl2, 5 mM GTP, 1 mM EGTA, 1 mM ATP, 5% glycerol, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, and 1:100-proteinase inhibitor mixture (Sigma). The F-actin and G-actin pools were separated by ultracentrifugation at 100,000 × g at 30 °C. The supernatant was diluted 1:2 with Laemmli buffer, whereas the pellet was resuspended in cold distilled H2O with 1 μM cytochalasin D and sonicated for 10 s. The pellet fraction was kept on ice for 45 min and then diluted 1:4 with Laemmli buffer. Both fractions were then boiled for 10 min and centrifuged at 14,000 × g for 10 min at 4 °C to remove remaining connective tissue. The same relative amounts of supernatant and pellet fractions (2:1) were loaded on 12.5% polyacrylamide gels and analyzed by Western blot using an anti-actin antibody (1:500, Sigma).

RESULTS

Effects of Stretch on Protein Synthesis in the Portal Vein—Fig. 1A shows silver-stained two-dimensional gels (pH 4–7) and corresponding autoradiographs of 35S)methionine incorporation in a stretched and an unstretched mouse portal vein. The culture time used here (72 h) is not sufficient to cause more than moderate effects on the relative contents of individual proteins, as seen from the silver-stained gels. However, α-actin, α- and β-tropomyosin, and desmin show dramatic stretch sensitivity on the autoradiographs, whereas γ-actin, like the intermediate filament protein vimentin, was weakly stretch-sensitive. The ubiquitous β-actin isoform was not stretch-sensitive. Three proteins that were clearly resolved and did not show any stretch sensitivity were identified as heat shock proteins (HSPA5, HSPA8, and HSPD1).

Summarized data on protein composition and synthesis rates are shown in Fig. 1B. Data for SM22α and calponin were determined from pH 6–11 or nonlinear 3–10 gels. The results are expressed as relative protein contents and radiolabel incorporation in unstretched versus stretched veins. Most of the proteins with stretch-sensitive synthesis rates, which are pre-
dominantly those associated with the contractile/cytoskeletal apparatus, appear in lower amounts by varying degree on the silver-stained gels from the unstretched veins. This does not include any contribution from greater overall protein contents in stretched veins because gels were loaded with equal amounts of total protein. The range of synthesis rates is, however, much wider than that of protein contents, with dramatic decrease of the rate for some proteins (<5% remaining) in unstretched vessels, whereas others were unaffected. Among the known differentiation marker proteins shown in Fig. 1, desmin (Des), vimentin (Vim), and HSP70 (HSPA8) appear in lower amounts by varying degree on the silver-stained gels. In contrast to desmin and vimentin, which dissociates F-actin filaments at their pointed (α) and γ ends, it is a muscle-specific protein (22). Partial depolymerization of F-actin is important for the differentiation of smooth muscle cells (23). Cofilin-2 belongs to a family of actin-severing proteins, which dissociates F-actin filaments at their pointed (−) end. In contrast to cofilin-1, it is a muscle-specific protein (22). The severing activity of cofilins is inhibited by phosphorylation by LIM kinase (21). We determined the time course of the effect of stretch on cofilin-2 phosphorylation by using a phospho-specific antibody (Fig. 5A). There was no change during the first hour of stretch, but then phosphorylation gradually increased during the following 24 h. A maximally 2-fold greater cofilin-2 phosphorylation in stretched versus unstretched veins was observed at 24 h. The effect of stretch on phospho-cofilin-2 (total cofilin-2 ratio at 72 h) was reduced compared with the earlier time point. However, at 72 h but not at earlier time points, stretch also affected the levels of total cofilin-2, which
was 113 ± 6% greater in stretched than in unstretched portal veins. Because cofilin-2 is a downstream effector of ROCK, it is likely to mediate the effect of Y-27632 on stretch-induced protein synthesis. In stretched veins, Y-27632 markedly reduced cofilin-2 phosphorylation after 24 h, whereas there was no significant reduction in unstretched veins (Fig. 5B).

Stretch-induced Actin Polymerization—Phosphorylation of cofilin inhibits its filament severing activity and thereby directly affects the structure of actin filaments. However, other signaling pathways could compensate for the stretch-induced inhibition of cofilin. Therefore, stretch-induced effects on actin dynamics were studied by measurement of F/G-actin ratios in the portal vein. The F- and G-actin pools were separated by ultracentrifugation and analyzed by Western blot. A 40% increase in the F/G-actin ratio was observed after 24 h of stretch (Fig. 6A). However, the effect was reduced in veins stretched for 48 or 72 h (data not shown). The F-actin stabilizing agent jasplakinolide increased the F/G-actin ratio in unstretched veins, whereas the actin depolymerizing agent latrunculin B had the expected opposite effect and decreased the F/G-actin ratio in stretched vessels.

Measurements of the F/G-actin ratio by ultracentrifugation represent average values that might be affected by tissue orientation, because the portal vein contains, in addition to longitudinal muscle, a thin circular muscle layer that will be differently affected by the longitudinal stretch. We therefore examined actin polymerization by an alternative and independent method, using confocal microscopy after labeling F- and G-actin with, respectively, phalloidin and DNase I conjugated to fluorescent probes. After a 24-h culture, the mouse portal veins were fixed, and slides were prepared for histochemistry. Fig. 6B shows representative images of a stretched (panel a), an unstretched (panel b), and a latrunculin B-treated stretched (panel c) portal vein in transverse sections, demonstrating that the majority of smooth muscle cells are longitudinally arranged, i.e. in the direction of the applied stretch. Analysis indicated a 55% higher F/G-actin ratio in stretched versus unstretched veins (Fig. 6B, panel d). Using both meth-
The effects of stretch on cofilin phosphorylation, as well as increase in total cofilin-2 in stretched cells, were prominent after 24 h of stretch but tended to decrease later. This may be due to tissue remodeling or other compensatory mechanisms, such as the increase in total cofilin-2 in stretched versus unstretched veins evident at 72 h but not at earlier time points. Nevertheless, a clear effect of stretch on cofilin phosphorylation, as well as synthesis of differentiation marker proteins, is apparent up to the latest time point studied here (72 h). The effect of stretch was slow in onset, because a clear increase in Rho activity was seen after 24 h but not after 15 min of stretch, and cofilin phosphorylation was unaltered at 1 h. The slow onset of Rho activity suggests that upstream mechanisms coupling to Rho may also be relatively slow in onset. The correlation of differentiation marker expression with alterations in actin dynamics, produced by stretch as well as pharmacological inhibition (Y-27632 and latrunculin B) and potentiation (jasplakinolide) of actin polymerization, strongly suggests regulation of smooth muscle differentiation in the vascular wall by the actin cytoskeleton.

The portal vein contains predominantly longitudinal muscle, and in this study was longitudinally distended to approximately the optimal length for force development. The distension can be considered to represent physiological conditions in terms of mechanical stress on the tissue, whereas the nondistended state is clearly nonphysiological. Possible hypertrophy stimulated by an extra load was not investigated here but would be expected to occur in a maintained contractile phenotype. Experimental portal hypertension in rats causes hypertrophy primarily of the longitudinal muscle layer, with increased total protein contents but a composition similar to that of control veins, the most notable difference being an increase of desmin contents (17). In our previous study an approximately proportional increase in SM22α contents was found in the rat portal vein following pressure-induced hypertrophy in vivo (8). In the present work, the desmin contents in unstretched mouse portal veins following culture were reduced to ~65% compared with stretched veins, whereas its rate of synthesis was <8% of that in stretched veins. A similar reduction in synthesis rate was seen for SM22α, whereas the total contents were better preserved (~85%). The tissue levels reflect the balance of synthesis and breakdown, but little is known about the effects of mechanical forces on breakdown rates of specific smooth muscle proteins.

All of the stretch-sensitive proteins examined here (α-actin, γ-actin, SM22α, calponin, α-β-tropomyosin, and desmin) have a CARG box containing promoters. Except for α-tropomyosin, they have been confirmed to be SRF-regulated (for references, see Ref. 10). Smooth muscle α-tropomyosin has been shown to be regulated coordinately with h-caldesmon and to undergo conversion to fibroblast isoforms in dedifferentiating smooth muscle cells (23). The γ-actin spot was only weakly stretch-sensitive, similar to vimentin, but may represent a mixture of smooth muscle and nonmuscle α-actin. Considering the diversity of smooth muscle, it is interesting that a limited number of contractile and cytoskeletal proteins seem to be the common defining elements of smooth muscle cells and that these are reversibly regulated by exposure to mechanical forces, the main physiological function of muscle. In the context of the putative role of MAL/MKL1 in transmitting signals from Rho and the actin cytoskeleton to the nucleus (15, 16, 24), it is significant that this ubiquitously expressed protein can activate SRF-dependent expression of endogenous smooth muscle genes in undifferentiated embryonic stem cells (16). The effects of stretch on smooth muscle development (5) may thus involve signals impinging on actin dynamics.

Stretch, in addition to its effect on Rho-dependent signaling, activates the mitogen-activated protein kinase pathway as demonstrated in a number of vascular preparations including the portal vein (18, 25–27). This would be expected to stimulate protein synthesis associated with cell proliferation by virtue of ternary complex factor-dependent c-fos activation by SRF (28). It is therefore notable that the proteins found to be strongly
Stretch and Smooth Muscle Differentiation

stretch-sensitive are those regulated by SRF binding independent of ternary complex factor. The ternary complex factor Elk-1 has recently been shown to compete with myocardin for binding to SRF and thereby to act as a repressor of differentiation (29). Because MAL/MK1 is a potent activator of smooth muscle genes and acts synergistically with myocardin (12, 16), it is possible that increased actin polymerization in response to stretch also increases the nuclear translocation of this factor enough to override an inhibitory effect of ternary complex factor. This might, on the other hand, not be the case in other instances of growth factor-dependent signaling, such as cell growth and proliferation in response to vascular injury.

Although SRF is a likely candidate for mediating smooth muscle-specific gene transcription via Rho activation and actin polymerization, it should be pointed out that several other transcription factors are activated in response to stretch (4). With respect to smooth muscle differentiation, one potential mechanism that has been shown to be present in intact vessels is nuclear translocation of the calcineurin-dependent transcription factor NFAT (nuclear factor of activated T cells) in response to pressurization of intact cerebral arteries (30). A calcineurin- and GATA 6-dependent pathway for α-actin and myosin heavy chain promoter activity in vascular smooth muscle cells has been demonstrated (31).

No previous study has to our knowledge investigated the stretch-induced coordinate effects on Rho activation, actin polymerization, and protein synthesis in intact vessels. Studies in cultured smooth muscle cells of Rho activation and actin polymerization, it should be pointed out that several other transcription factors are activated in response to stretch (4). This contrasts with the much greater effects on the synthesis of actin, which were reduced to 55, 33, and 25%, respectively.

This study shows that stretch of the blood vessel wall is critical for maintaining the smooth muscle contractile phenotype, expressing contractile and cytoskeletal proteins. The signaling mechanisms of this response include Rho activation and actin polymerization, partly but probably not exclusively mediated via ROCK activity and cofilin phosphorylation.

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