Stretch-induced contractile differentiation of vascular smooth muscle: sensitivity to actin polymerization inhibitors

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Stretch-induced contractile differentiation of vascular smooth muscle: sensitivity to actin polymerization inhibitors. Am J Physiol Cell Physiol 284: C1387–C1396, 2003. First published January 29, 2003; 10.1152/ajpcell.00508.2002.— Signaling mechanisms for stretch-dependent growth and differentiation of vascular smooth muscle were investigated in mechanically loaded rat portal veins in organ culture. Stretch-dependent protein synthesis was found to depend on endogenous release of angiotensin II. Autoradiography after [35S]methionine incorporation revealed stretch-dependent synthesis of several proteins, of which SM22 and actin were particularly prominent. Inhibition of RhoA activity by cell-permeant C3 toxin increased tissue mechanical compliance and reduced stretch-dependent extracellular signal-regulated kinase (ERK)1/2 activation, growth, and synthesis of actin and SM22, suggesting a role of the actin cytoskeleton. In contrast, inhibition of Rho-associated kinase by Y-27632 did not reduce ERK1/2 phosphorylation or actin and SM22 synthesis and did not affect tissue mechanical compliance but still inhibited overall growth. The actin polymerization inhibitors latrunculin B and cytochalasin D both inhibited growth and caused increased tissue compliance. Whereas latrunculin B concentration-dependently reduced actin and SM22 synthesis, cytochalasin D did so at low (10^{-6} M) but not at high (10^{-8} M) concentration. The results show that stretch stabilizes the contractile smooth muscle phenotype. Stretch-dependent differentiation marker expression requires an intact cytoskeleton for stretch sensing, control of protein expression via the level of unpolymerized G-actin, or both.

SM22; cytoskeleton; rat portal vein; RhoA; hypertension

THE MECHANICAL STRESS IMPOSED on the vascular wall by the intraluminal blood pressure is critical for regulating its growth and phenotypic differentiation, as shown by a massive body of experimental and clinical evidence. The contractile phenotype of the smooth muscle cells in the vessel media, characterized by contractile ability and little tendency to proliferation, is marked by the expression of several smooth muscle-specific proteins, including among others SM22, calponin, h-caldesmon, α-actin, and smooth muscle myosin heavy chain (27). These are decreased in cell culture and in cells reacting to injury and inflammatory mediators in the early atherosclerotic process (28). Understanding of the mechanisms maintaining cellular differentiation and of the influence on them by mechanical stress may thus clarify processes involved not only in hypertension but also in the development of atherosclerotic lesions, as well as suggest new approaches for preventing vascular disease.

Veins are exposed to lower intraluminal pressure than arteries but show considerable pressure-induced growth, as demonstrated by the hypertrophy, and often rapidly progressing atherosclerosis, of venous grafts exposed to arterial pressure (30). One vessel that has been extensively investigated is the rat portal vein, in which elevated intraluminal pressure in vivo over a few days causes hypertrophy and increased contractility (16, 21). In vitro, portal venous strips kept in organ culture under an applied load developed increased contractility, protein synthesis, and cell cross-sectional area compared with unloaded control strips (40). This model thus allows investigation of the effect of stretch on the pattern of protein expression and its relationship to contractile differentiation. The present study aimed to reveal signaling mechanisms involved in stretch-sensitive expression of differentiation marker proteins in the rat portal vein.

Earlier studies of stretch-sensitive protein expression in vascular tissue showed that intraluminal pressure in organ culture of rabbit aorta is essential for the maintenance of caldesmon and filamin contents (5), suggesting a specific role of mechanical factors in contractile differentiation. Recent evidence in cultured cells suggests that the expression of SM22 and several other proteins under the control of serum response factor (SRF) is negatively regulated by an increase in unpolymerized G-actin (9, 18, 34). It is thus possible that an influence of stretch on the expression of differentiation marker genes involves altered actin dynamics. Alterations in length-force relationships (remodeling) in response to altered mechanical stress under organ culture (2, 40) suggest that the cytoskeleton and contractile apparatus are restructured, and hence actin filament dynamics will be affected.

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Investigations of signal mechanisms involved in stretch-sensitive protein synthesis in the heart and vascular wall have concentrated on the roles of endogenous release of growth promoters such as angiotensin (ANG) II and on the roles of integrins and the cytoskeleton (1, 3, 26). In vascular tissue, both pressure (6, 39) and exogenous ANG II (23) activate extracellular signal-regulated kinase (ERK)1/2 (44 and 42 kDa, respectively). This in turn regulates the early-response gene c-fos and protein synthesis (36). Matrougui et al. (24) obtained evidence that disruption of the actin filament network by cytochalasin B attenuates both pressure- and ANG II-induced activation of ERK1/2 in rat mesenteric arteries, suggesting an influence of the cytoskeleton on the MAP kinase pathway. The role of such an interaction for cellular differentiation in response to mechanical strain may be elucidated by studies of the expression of smooth muscle-specific marker genes.

We have investigated the effect of mechanical stress on contractility, growth, and synthesis of actin and SM22 in organ culture of rat portal vein. To specifically address the interaction between the cytoskeleton and the ERK1/2-dependent pathway, inhibitors of the different pathways and of actin polymerization were used. The results suggest that stretch stabilizes the smooth muscle contractile phenotype by a mechanism involving the actin cytoskeleton.

MATERIALS AND METHODS

Preparation. Female Sprague-Dawley rats (200–250 g) were killed by cervical dislocation, as approved by the Animal Ethics Committee, Lund University. The portal veins were dissected under sterile conditions, cut longitudinally into two halves, gently blotted, and weighed as described previously (40).

In vivo experiments. Portal venous hypertension was induced as described previously (16). Experiments were approved by the Animal Ethics Committee, Lund University. The animals were anesthetized with ketamine (Ketalar; Parke-Davis) and xylazine (Rompun; Bayer), and two branches of the portal vein entering the liver were partially ligated with 4.0 surgical silk. Control animals were sham operated with a loose loop of silk placed around the vessel. After 7 days, animals were killed and the portal vein was dissected and extracted for protein separation.

Organ culture. Vascular strips were cultured hanging, unloaded or attached to a 0.6-g gold weight, in glass tubes filled with 2 ml of culture medium at 37°C in a humid atmosphere of 5% CO2 in air. The medium was DMEM–Ham’s F-12 (1:1) with 50 U/ml penicillin and 50 µg/ml streptomycin. This formulation contained methionine at a concentration of 17.4 mg/l. For autoradiography (see Protein turnover studies), low-methionine (2 mg/l) medium was used. Fetal calf serum (FCS) was dialyzed with a cutoff molecular mass of 6 kDa and added (2%) to the medium. Insulin (Sigma) was used at a concentration of 10 nM. After 3 days, except where noted, strips were removed, weighed as above, and mounted for force recording. Strips were frozen and stored at −80°C for protein analysis. Total protein concentration was determined with a Bio-Rad protein assay. Dry-to-wet weight ratios were determined after freeze-drying of the tissue.

Drugs used during culture to interfere with the local renin-angiotensin system were ANG II (Sigma) in 1% albumin buffer, the type 1 ANG (AT1) receptor blocker candesartan (Astra Zeneca), or the ANG I-converting enzyme inhibitor captopril (Sigma). To avoid degradation of ANG II or the blockers, the culture medium was changed every 24 h. The same procedures were used with the Rho kinase inhibitor Y-27632 (Yoshitomi Pharmaceutical). The C3-like ADP-ribosyltransferase from Clostridium limosum, which specifically inactivates RhoA (38), was delivered intracellularly by incubating preparations with the C21N-C3 fusion toxin (C2-C3; 100 ng/ml) together with the binding component C2II from C. botulinum, which increases the uptake efficiency several hundredfold (4). Actin polymerization was inhibited by the use of latrunculin B (0.5–50 nM; Calbiochem) and cytochalasin D (10 nM–1 µM; Calbiochem). To inhibit ERK1/2 phosphorylation, the MAP kinase kinase (MEK) inhibitor PD-98059 (10 µM; Calbiochem) was used.

Mechanical recording. Strips were attached to a force transducer (AE 801; SensoNor AS), stretched to a passive tension of 2 mN, and equilibrated for 45 min before experimental protocols were begun. The solution (0.4 ml, 37°C) had the following composition (mM): 135.5 NaCl, 5.9 KCl, 2.5 CaCl2, 1.2 MgCl2, 11.6 HEPES, and 11.5 glucose. In high-K+ solution, NaCl was isosmotically replaced by KCl. The cross-sectional area of strips was determined from the length and wet weight.

Immunoblotting. ERK1/2 phosphorylation was determined in tissues preincubated for 1 h in culture medium, with drugs added as indicated, and then loaded for 5 min. Control experiments indicated that stretch-induced ERK activity peaked at this time. ERK phosphorylation was determined by Western blot with an antibody against phosphorylated ERK1/2 (PhosphoPlus p44/p42 MAP kinase antibody; New England Biolab). The signals were visualized with enhanced chemiluminescence (Lumi-Glo; New England Biolab).

Protein turnover studies. Except for time course experiments (see Results), strips were kept in organ culture for 48 h and then exposed for an additional 24 h to [3,4,5-3H]leucine (Amersham) at an activity of 1 µCi/ml. Incorporation was determined as described previously (40). For autoradiography, [4,5-3H]leucine was exchanged for [35S]methionine (10 µCi/ml) in a low-methionine culture medium. For protein separation, frozen tissues were pulverized in liquid N2 and extracted. Protein patterns were evaluated on 12% SDS-polyacrylamide gels (Bio-Rad Mini-Protein system). Silver-stained gels were scanned with an imaging densitometer (GS710; Bio-Rad), and gels were then dried and exposed to film (Kodak X-Omat AR) at −80°C. The autoradiograms were scanned and analyzed with Quantity One gel analysis software (Bio-Rad).

Two-dimensional electrophoresis. Two-dimensional (2D) electrophoresis was performed according to standard procedures. Briefly, first-dimension isoelectric focusing was done with 11-cm strips with immobilized pH gradients (pH 6–11 or 3–10) in a Protein IEF Cell (Bio-Rad). After isoelectric focusing, the strips were equilibrated in a buffer containing SDS. Eight to sixteen percent SDS-PAGE gradient gels were used for separation in the second dimension. The gels were either silver stained, dried, and exposed to a film for autoradiography or fluorescence stained with Sypro Ruby (Bio-Rad) and then transferred onto nitrocellulose membranes for autoradiography and incubation with a polyclonal antibody for identification of SM22. The 2D gels and their autoradiograms were scanned and analyzed with gel analysis software (Bio-Rad).
Statistics. Values are presented as means ± SE. Student’s t-test was used for evaluation of statistical significance. For multiple comparisons one-way ANOVA was used. P < 0.05 was considered statistically significant.

RESULTS

Effects of stretch during organ culture on protein expression of rat portal vein. Rat portal veins cultured for 3 days under mechanical load in the presence of growth stimulants showed increased rate of protein synthesis compared with unloaded preparations. The pattern of protein synthesis was visualized with autoradiography after [35S]methionine incorporation and separation on SDS-polyacrylamide gels. Overall protein composition was not much affected by this period of culture, as revealed by silver staining (Fig. 1A, left). However, a generally higher incorporation of radioactivity was found in stretched than in unstretched preparations (Fig. 1A, right). One band, in the 22-kDa region, was most strikingly affected by stretch. This band comigrated with SM22 as determined by Western blotting (Fig. 1A, center). Although less prominent, several other bands showed greater synthesis rate in loaded preparations, and among these actin (42 kDa) was clearly evident.

To ensure identification of the 22-kDa protein, samples from stretched and unstretched preparations cultured in the presence of [35S]methionine were resolved on 2D polyacrylamide gels and then subjected to autoradiography. The enhanced radioactivity in the 22-kDa region was almost entirely accounted for by two basic spots, which represent two isoforms of SM22 as seen by Western blots of the same gels (Fig. 1B, right and center). Although there was no obvious difference in the amount of SM22 protein on gels from loaded and unloaded preparations (Fig. 1B, left), the amount of radiolabel incorporation was markedly greater in the stretched preparations (5.0 ± 0.6 fold; n = 3), indicating increased rate of SM22 synthesis.

SM22 in pressure-induced hypertrophy in vivo. Whereas SM22 expression is decreased or absent in smooth muscle cells in synthetic phenotype, such as in cell culture (32) and in atherosclerotic lesions (28, 31), its presence in tissue after culture under stretch is a sign of maintained differentiation. Conversely, the absence of stretch, representing a deviation from the normal pattern of cellular inputs, might induce a decrease in differentiation marker expression, indicating that cells tend to assume a less contractile, or even

Fig. 1. Stretch-sensitive synthesis of actin and SM22 (A and B) and SM22 contents after hypertrophy in vivo (C). Portal veins were cultured for 3 days under loaded (Lo) or unloaded (Unl) conditions. [35S]methionine was present during the last 24 h. Proteins were separated by 1-dimensional (A) or 2-dimensional (2D; B and C) SDS-PAGE. A: silver-stained gel (left) and autoradiogram (right) from 1 separation and Western blot for SM22 (center) from a parallel separation. B: regions from 2D gels corresponding to ~20–40 kDa and pH 7.8–9.1. Arrows indicate the position of SM22 isoforms. All gels are from the same loaded and unloaded cultured veins. Western blots for SM22 (center) were aligned with fluorescence stained spots from the same gels (see MATERIALS AND METHODS), but for clarity of illustration silver-stained (left) parallel gels and corresponding autoradiograms (right) are shown here. C: regions from silver-stained 2D gels of a control (left) and a hypertrophied (right) portal vein, obtained after experimental portal hypertension in vivo for 7 days. Equal amounts of protein were loaded on both gels. Arrow indicates 22 kDa.
synthetic, phenotype. After 3 days of organ culture there is not a marked effect of stretch on tissue concentrations of SM22, although synthesis rates are stretch sensitive (Fig. 1, A and B). To examine whether hypertrophic growth under in vivo conditions affects SM22 concentration, portal veins were analyzed after experimental portal hypertension (16). In 7 days, this treatment approximately doubles the amount of smooth muscle in the vessel wall, with a corresponding increase in force generation (21). Tissue concentrations of myosin and actin are essentially unaltered after hypertrophy, and thus the total amounts of these contractile proteins increase during hypertrophy in proportion to tissue mass (22). Figure 1C shows 2D gels of a normal and a hypertrophied portal vein. The gels are loaded with the same amount of protein, and it is seen that SM22 concentrations are essentially the same in both samples. By Western blot, no difference in SM22 concentration between normal and hypertrophied portal veins was found in three experiments. Thus the pressure-induced hypertrophy caused an increased synthesis of SM22 in proportion to the increase in smooth muscle mass and contractile proteins, confirming that hypertrophic growth occurs with cells maintained in a contractile phenotype.

Time course of SM22 synthesis. The rate of SM22 synthesis was evaluated with autoradiography of the 22-kDa band on 1D gels after culture for varying time periods. In this series of experiments, the radiolabel was present for the last 6 h of all culture periods. As shown in Fig. 2A, there was little difference in synthesis rates between stretched and unstretched veins for the first 6 h but then the rates fell in unstretched veins, to become 50% of that in stretched veins at 3 days. The rates of protein synthesis showed biphasic time dependence, increasing to a maximum at 3 days and then decreasing again at 5 and 7 days. The time course of SM22 protein concentration was biphasic as well but delayed relative to the rate of synthesis (Fig. 2B). Although there is only a small difference in SM22 concentrations at 3 days (cf. Fig. 1), continued higher synthesis rates in stretched veins have built up a significant difference at 5 days. At 7 days the difference is again diminished, reflecting the smaller stretch dependence of synthesis rates at 5 days and onward. In the remainder of this study, 3-day cultures were used to optimize conditions for detection of stretch-dependent protein synthesis, because values of protein concentration are affected by breakdown as well as synthesis.

Quantitation of stretch-dependent protein synthesis. For analysis of overall growth responses, portal vein preparations were weighed before and after culture and protein synthesis was evaluated with [3H]leucine incorporation (Fig. 3, A and B). Alterations in wet weight during culture closely parallel rates of protein synthesis, although relative changes in weight are also affected by altered water content of the tissue in response to growth factors. After 3 days of culture in the presence of growth factors the dry-to-wet weight ratio has decreased, whereas in serum-free culture or under the influence of growth inhibitors no swelling occurs (40). The dry-to-wet weight and protein-to-dry weight ratios are independent of loading conditions during culture, allowing evaluation of relative effects of loading based on wet weight alone. As seen in Fig. 3A, the wet weight of loaded veins increased by 28% whereas that of unloaded veins decreased by 6%. This is lower than the values (44% and 20%, respectively) obtained with 10% FCS as growth stimulant (40), suggesting that the conditions used here (2% FCS + 10 nM insulin) cause less than maximal growth. The dry-to-wet weight ratio in both loaded and unloaded cultured veins in this study was 0.16 ± 0.01 (n = 10), corresponding to 76% of the value in freshly dissected tissue (0.21). Thus the dry weight of loaded veins after culture was 97% of that before culture, whereas that of unloaded veins was 71%. Under the present conditions loaded veins are therefore maintained in an approximate steady state between protein synthesis and breakdown, whereas in unloaded veins breakdown is faster than synthesis.

Roles of ANG II- and Rho-dependent pathways. Part of the growth response to stretch was due to endogenous release of ANG II, as the AT1 receptor inhibitor candesartan (10 μM) inhibited growth and protein
synthesis of stretched preparations in the absence of added ANG II (Fig. 3, A and B). Further support for a role of endogenous ANG II production is the finding that the angiotensin-converting enzyme inhibitor captopril (1 μM) reduced stretch-dependent growth to the same extent (8 ± 2% growth; n = 5) as candesartan. The role of Rho-dependent signaling was investigated by using the chimeric toxin C2-C3, which inactivates RhoA (4, 38), and Y-27632, which is a selective inhibitor of Rho-associated kinase (8, 15). These interventions reduced weight gain of the portal vein to the level of unstretched control veins, although Y-27632 produced less inhibition than did C2-C3. No effect of inhibiting AT1 receptors, RhoA, or Rho-associated coiled-coil forming kinase (ROCK) was observed in the absence of an applied load (Fig. 3A). Effects on the incorporation of radioactive leucine in loaded veins closely paralleled the effects on weight gain (Fig. 3B).

To investigate whether all of the effects of stretch on growth can be attributed to stretch-dependent ANG II production, a saturating concentration of ANG II (10 μM) was added to either loaded or unloaded portal veins. This produced a growth response of 34 ± 2% (n = 5) in loaded and 5 ± 1% (n = 6) in unloaded veins, and thus excess ANG II cannot compensate for the lack of stretch during culture. This suggests that the effect of stretch on growth involves factors in addition to endogenous ANG II production.

Role of cytoskeleton. To inhibit actin filament assembly we used latrunculin B, which binds to G-actin and prevents its incorporation into F-actin. This causes disruption of the actin filament structure and increase in G-actin contents (35). Cytochalasin D, on the other hand, inhibits actin filament assembly by capping the barbed end of F-actin, thus preventing incorporation of G-actin, but also causes dimerization and nucleation of short F-actin filament segments (12). Inhibition of actin polymerization would be expected to cause net depolymerization of actin filaments. In the cultured portal vein preparations, both latrunculin B and cytochalasin D concentration-dependently decreased stretch-induced growth (Fig. 3, C and D). However, the effect of cytochalasin D was smaller than that of latrunculin B and growth was not totally inhibited at the highest concentration used (10^-6 M).

Contractility and tissue remodeling. During culture under stretch without growth stimulants (no FCS) active stress (developed force per cross-sectional area) is maintained at the level of freshly dissected tissue, whereas growth stimulation causes decreased active stress (40). This may be due partly to swelling without a compensating increase in contractile proteins, but it might also involve a change toward a less contractile cellular phenotype. Under all conditions, unloaded cultured veins produce substantially less force than their loaded counterparts. When the growth response of

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**Fig. 3.** Inhibitors of angiotensin (ANG) II signaling and of actin dynamics block stretch-dependent weight increase and protein synthesis. Effects of C2-C3 exoenzyme (C3; 100 ng/ml), Y-27632 (Y; 10 μM), and candesartan (Cand; 10 μM) on weight gain (A) and [3H]leucine incorporation (B) of loaded (Lo) or unloaded (Unl) portal vein strips cultured for 3 days in 2% fetal calf serum (FCS) + 10 nM insulin are shown. In B–D inhibitors were applied to loaded veins only. Concentration-dependent effects of latrunculin B and cytochalasin D on relative change in wet weight are shown in C and D, respectively (n = 5–6). *P < 0.05 vs. loaded control.
loaded preparations was inhibited by culture in the presence of candesartan, active stress in response to high-K⁺ stimulation was slightly greater than in growth-stimulated loaded veins (Fig. 4A), showing that contractility is well maintained during this treatment. In contrast, culture in the presence of either C2-C3 toxin or Y-27632 reduced active stress to the level seen after culture without load (Fig. 4A). Interference with the RhoA signaling system thus directly inhibits force production in addition to growth.

Two potential mechanisms behind the decreased force generation during inhibition of RhoA and ROCK are a lack of Ca²⁺ sensitization due to inhibited ROCK activity on myosin phosphatase (37) and impaired force transmission due to disruption of the cytoskeleton. To evaluate the mechanical function of the cytoskeleton, we determined the effect of culture on the length of the portal vein preparations under the standard load (0.6 g). This load is slightly above that optimal for force development (40). Shifts in length under this load during culture (Fig. 4B) correlate well with shifts in the optimum for force development as determined by complete length-force relationships (40). Thus unloaded culture causes remodeling toward shorter length, whereas culture under load causes ~20% elongation. The presence of Y-27632 during culture under load did not affect this remodeling, whereas C2-C3 toxin caused a further elongation (Fig. 4B). This suggests that disruption of cytoskeletal structure, causing increased mechanical compliance, is a factor behind the effects of RhoA inhibition on force development, whereas inhibition of the downstream effector ROCK does not affect tissue compliance but is likely to inhibit force production by Ca²⁺ desensitization.

To investigate whether direct inhibition of actin filament assembly affects tissue mechanical compliance, responses to latrunculin B and cytochalasin D were evaluated. Both agents caused concentration-related elongation over a similar range (Fig. 4, C and D).

Stretch-dependent ERK1/2 activation. Stretch of vascular strips for 5 min in culture medium containing 2% FCS + insulin doubled ERK1/2 phosphorylation compared with unloaded strips (Fig. 5). Pretreatment with Y-27632 (10 μM) did not affect stretch-induced ERK1/2 activation. In contrast, C2-C3 toxin and latrunculin B, as well as candesartan, markedly attenuated ERK1/2 activity to the level of unloaded preparations or below.

Actin filament dynamics determine stretch-dependent expression of actin and SM22. To quantitate the effects of the interventions affecting ANG II, RhoA/Rho-kinase signaling, and actin polymerization on the synthesis of actin and SM22, samples from veins cultured with inhibitors were analyzed by gel electrophoresis and autoradiography. Silver staining of the gels indicated that the band pattern differed negligibly between treatments when gels were loaded with equal amounts of total protein (data not shown; cf. Fig. 1). The amounts of radioactivity in the respective bands

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**Fig. 4. Remodeling by inhibitors of RhoA and of cytoskeletal assembly.** Labels are as in Fig. 3. Active stress elicited by high K⁺ after culture for 3 days under load with additions is shown in A. Length at constant load as a function of culture conditions is shown in B to indicate degree of remodeling. Concentration-dependent effects of latrunculin B and cytochalasin D on relative length change (Δ) are shown in C and D, respectively (n = 6). *P < 0.05 vs. loaded control.
were normalized to those of the loaded portal vein. C2-C3 toxin significantly reduced the synthesis of both actin and SM22, whereas Y-27632 had no effect and candesartan had an intermediate effect on the incorporation of radioactivity into either actin or SM22 (Fig. 6B). Generally, effects of stretch and inhibitors were greater in the SM22 than in the actin band. Also shown are data for the effect of MEK inhibition by PD-98059. This treatment, which reduces ERK phosphorylation and inhibits growth and protein synthesis (40), had a small inhibiting effect on actin and SM22 synthesis, similar to that of candesartan.

The fact that RhoA inhibition reduced actin and SM22 synthesis to the levels seen during culture of unloaded veins but inhibition of Rho-kinase had no such effect suggests that actin filament assembly is an important determinant of cell differentiation. Accordingly, the effects of latrunculin B and cytochalasin D on actin and SM22 synthesis were determined. The effects of these two inhibitors showed interesting differences (Fig. 7). Whereas latrunculin B caused a concentration-dependent decrease in actin and SM22 synthesis, the effect of cytochalasin D was greatest for the lowest concentration used ($10^{-8} \text{ M}$) and was abolished at the highest concentration ($10^{-6} \text{ M}$). Despite their similar effects on growth and tissue remodeling, these agents thus showed different effects on differentiation marker expression.

**DISCUSSION**

As shown in this study, maintained stretch promotes the differentiated contractile state of vascular smooth muscle in organ culture, as evidenced by contractility and by synthesis of the differentiation marker proteins SM22 and actin. Pressure-induced hypertrophic growth of the portal vein preserves the contractile phenotype, whereas absence of stretch causes decreased contractility and rates of SM22 and actin synthesis relative to overall protein turnover.

Extensive evidence indicates that actin dynamics regulate the expression of SRF-dependent proteins, crucial for cell differentiation (9, 18, 34). Whereas studies in cultured smooth muscle cells have shown that cyclic strain promotes actin polymerization (33) and expression of differentiation markers (29), there are no data from intact tissue linking strain-dependent actin polymerization and differentiation. However, Cipolla et al. (7) reported pressure-induced actin polymerization in cerebral arteries and suggested that decreased G-actin and increased F-actin levels with increased wall tension is a mechanism behind myogenic tone. Similarly, Mehta and Gunst (25) demonstrated increased actin polymerization in agonist-stimulated contractions of airway smooth muscle. Thus actin filament formation may be a component in the activation of contraction, which might also play a role in the phenomena studied here, because the portal vein has a prominent myogenic tone that is clearly sensitive to stretch (17). Recent evidence also indicates that thick (myosin) filament assembly may be promoted by con-

![Graph](image)
bladder epithelial cells it has been shown that although a low concentration (0.25 μM) of cytochalasin D causes actin depolymerization, higher concentrations (≥2 μM) actually cause net polymerization (10). These concentrations correspond well to those used here. Mehta and Gunst (25) observed that cytochalasin D (10 μM), in contrast to latrunculin, did not inhibit the decrease in G-actin on agonist stimulation of airway muscle. In agonist-stimulated cultured cells, latrunculin B but not cytochalasin D decreases SRF-stimulated SM22 promoter activity (18, 34). With the reservation that direct demonstration under the present conditions is needed, the results obtained here could tentatively be explained by an effect of the actin polymerization inhibitors on actin and SM22 synthesis in the portal vein mediated by altered G-actin concentration. It cannot be excluded that additional effects appear because of altered tissue mechanical properties, which may influence stretch-sensing mechanisms unrelated to any direct effect of G-actin on gene expression. However, it is notable that at high, as opposed to low, concentration of cytochalasin D the rates of synthesis of actin and SM22 are not decreased below control level, despite the disruption of the cytoskeleton expected under these conditions.

Inhibition of RhoA activity with C2-C3 toxin inhibited actin and SM22 synthesis and in addition prevented stretch-induced growth and caused increased tissue compliance. These effects are similar to those produced by latrunculin B and likely reflect the role of RhoA in regulating actin cytoskeletal assembly (13). Activation of RhoA by stretch has been demonstrated in cultured cells (26) but, to our knowledge, not in intact vascular tissue. In the present experiments C2-C3 toxin had no effect on weight or protein synthesis in unloaded tissue, whereas the growth responses to load during culture were inhibited. This would suggest that RhoA activity is involved in stretch-dependent protein synthesis. As in the case of actin polymerization inhibitors, several mechanisms of action are possible.

C2-C3, like latrunculin B, inhibited stretch-dependent ERK1/2 activation. Specific inhibition of ERK phosphorylation with PD-98059 inhibits growth (40) and actin and SM22 synthesis (this study), and thus reduced ERK1/2 activation might explain at least part of the decreased protein synthesis caused by inhibitors of RhoA and actin polymerization. Gineitis and Treisman (11) proposed two nonexclusive mechanisms for the control of SRF-dependent transcription, one involving RhoA in regulating actin cytoskeletal assembly (13). Activation of RhoA by stretch has been demonstrated in cultured cells (26) but, to our knowledge, not in intact vascular tissue. In the present experiments C2-C3 toxin had no effect on weight or protein synthesis in unloaded tissue, whereas the growth responses to load during culture were inhibited. This would suggest that RhoA activity is involved in stretch-dependent protein synthesis. As in the case of actin polymerization inhibitors, several mechanisms of action are possible.

Actin filament disruption by latrunculin B caused a concentration-dependent decrease in actin and SM22 expression in the portal vein and also increased tissue compliance, as shown by elongation during culture under load. In light of the known effect of latrunculin B to inhibit actin polymerization the elongation may be considered to reflect breakdown of load-bearing structures associated with the cytoskeleton, but it should be emphasized that the long-term treatment applied here might have effects other than inhibition of actin filament formation alone. For example, the decrease in protein synthesis might include decreased formation of extracellular matrix proteins, which contribute to tissue elasticity. Cytochalasin D causes actin dimerization and nucleation of short filaments, and in frog tractile activation and that this mechanism is most pronounced in smooth muscles with low myogenic tone, suggesting that maintained tone contributes to the preservation of filament organization (14). The present results show that stretch and altered actin dynamics both influence cell differentiation via mechanisms that have much in common. Whereas these results and evidence from cultured cells suggest that stretch preserves differentiation by promoting actin polymerization, more evidence on filament dynamics in intact tissue is needed to test this hypothesis and to clarify the mechanisms involved.

**Fig. 7. Effects of inhibitors of cytoskeletal assembly on actin and SM22 synthesis.** Concentration-dependent effects of latrunculin B (A) and cytochalasin D (B) were determined from autoradiograms and normalized to the loaded control (n = 3). Protein contents evaluated by silver-stained gels were similar for all treatments except for the highest concentration of latrunculin B (0.5 × 10⁻⁷ M), where a loss of protein was found. *P < 0.05 vs. loaded control.
stretch and altered actin filament organization. Analysis of the global pattern of protein expression may further elucidate the relative roles of ERK1/2 phosphorylation and of the actin filament structure in the regulation of stretch-induced growth and differentiation marker expression in intact vascular tissue.

Our results show that the ROCK inhibitor Y-27632 decreased overall protein synthesis but not ERK1/2 phosphorylation or actin and SM22 synthesis. Lack of effect of Y-27632 on ERK1/2 activation was also found in pressurized mesenteric arteries by Matrougui et al. (24), whereas activation was inhibited in aortic cells exposed to cyclic strain as described by Numaguchi et al. (26). Mack et al. (18) found decreased α-actin promoter activity and SM22 synthesis in cultured vascular cells exposed to Y-27632. It is possible that ROCK activity in cell cultures is high enough to significantly influence actin dynamics and associated protein expression, whereas this may not be the case in intact tissue composed of relatively quiescent cells in contractile phenotype.

In summary, stretch-induced growth in the rat portal vein is mediated in part by endogenous release of ANG II, acting on AT1 receptors in the plasma membrane. Stretch causes growth in a maintained contractile phenotype, and this requires RhoA activity and an intact cytoskeleton. Under normal conditions in situ and in portal vein hypertrophy, stretch-induced SM22 expression appears to be saturated. On the other hand, the absence of stretch represents a deviation toward less growth stimulation, and this results in diminished synthesis of SM22 relative to overall protein synthesis, correlating with decreased contractility. Although further studies are needed to establish causality, the evidence obtained here at the protein level in intact tissue is consistent with an effect of cytoskeletal dynamics on the synthesis of smooth muscle differentiation marker proteins, involving a role in stretch sensitivity actin dynamics and associated protein expression, whereas this may not be the case in intact tissue composed of relatively quiescent cells in contractile phenotype.

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