Cholesterol Dependence of Vascular ERK1/2 Activation and Growth in Response to Stretch
Role of Endothelin-1

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Objective—Stretch-induced growth of the vascular wall plays a role in hypertension and neointima formation. Its signal pathways involve integrins, cytoskeleton, membrane receptors, and ion channels, some of which are organized in cholesterol-rich, membrane domains such as lipid rafts or caveolae. This study tested the role of rafts/caveolae in stretch-induced vascular growth by manipulation of membrane cholesterol contents.

Methods and Results—Growth and protein synthesis were induced by mechanical stretch of rat portal veins in vitro. Sucrose gradient centrifugation showed stretch-induced tyrosine phosphorylation primarily in fractions containing caveolin-1. Disruption of membrane caveolae with use of methyl-β-cyclodextrin (mβcd) reduced weight gain, protein synthesis, and DNA synthesis to levels in unstretched, control veins. These effects were partially reversed by restoration of cellular cholesterol contents. Inhibited growth was associated with abolished activation of extracellular signal–regulated kinase (ERK) 1/2 in response to stretch and endothelin-1 (ET-1) but not to angiotensin II. Inhibition of ET-1 type A (ETₐA) receptors by RF139317 or endothelin-converting enzyme by phosphoramidone abolished stretch-induced ERK1/2 activation, which was, however, unaffected by removal of the endothelium.

Conclusions—Stretch-induced growth signaling in vascular smooth muscle depends on cholesterol-rich, membrane microdomains by a mechanism involving ETₐA receptors that respond to endogenous ET-1 production. (Arterioscler Thromb Vasc Biol. 2003;23:1528-1534.)

Key Words: hypertrophy □ portal vein □ caveolae □ ERK1/2 □ cyclodextrin

Blood vessels subjected to increased wall stress undergo structural and biochemical changes, which result in hypertrophy/hyperplasia of vascular smooth muscle. This leads to clinically important alterations in structure and function, as seen in hypertension and graft restenosis.1-3 A complex interaction of smooth muscle and the endothelium, which involves mechanical stimuli such as pressure and flow as well as growth factors and the extracellular matrix, controls vascular growth and differentiation.4,5

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The sequence of events that cause stretch-induced vascular growth is largely unknown, although several illuminating observations have been made. Extracellular matrix components and integrins, as well as the cytoskeleton, play important roles as mechanical sensors.6,7 Outside-in signaling by integrins depends on Src family kinases,8 and inhibition of Src decreases extracellular signal–regulated kinase (ERK) 1/2 activation and protein synthesis in blood vessels exposed to increased wall stress.9,10 In parallel with integrin signaling, growth factors produced in response to stretch, eg, angiotensin II (Ang II), endothelin-1 (ET-1), and platelet derived growth factor, act in a paracrine manner.11-14 Activation of the protein kinases Raf, MEK, and ERK1/2 is essential for the resulting growth response.9,10,12,15,16

The distribution of growth factor receptors and Src in the membrane is influenced by the presence of cholesterol-rich, membrane domains.17-20 Lipid rafts are aggregates of cholesterol and glycosphingolipids that lack a defined structure.19 Caveolae have a similar composition and appear as 50- to 100-nm, flask-shaped membrane invaginations.20 The protein caveolin binds cholesterol and stabilizes caveolae.21,22 Purified rafts/caveolae contain receptors, G proteins, and ERK1/2 in addition Src.20 It is therefore reasonable to hypothesize that these specialized membrane domains play a role in stretch signaling. Indeed, evidence for signaling of shear stress through rafts/caveolae has been obtained in endothelial cells.23-25 Such mechanotransduction in the endothelium might modulate contractility and also possibly growth and differentiation of the underlying vascular smooth muscle cells.3 A direct effect of stretch on smooth muscle growth and differentiation is, however, also present,4 and investigation of the role(s) of lipid rafts/caveolae in this response is expected to elucidate mechanisms of vascular hypertrophy.

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We have investigated the role of membrane cholesterol in stretch-dependent vascular growth of the rat portal vein in vitro. This vessel has spontaneous activity similar to the myogenic tone of precapillary resistance vessels, and because of its longitudinally oriented smooth muscle cells, the vessel wall might be conveniently stretched by weight loading. Culture under load causes greater contractility, protein synthesis, and cell cross-sectional area relative to unloaded, control veins. The stretch response promotes expression of differentiation-related proteins and depends on endogenous production of Ang II and cytoskeletal dynamics. Here, we tested whether stretch increases the phosphorytrosine (P-Tyr) contents in caveolin-containing membrane microdomains and examined the effects on growth of cholesterol depletion by cyclodextrin, which disrupts rafts and caveolae.

Methods

Animals, Dissection, and Cholesterol Depletion

Female Sprague-Dawley rats were killed by cervical dislocation, as approved by the Animal Ethics Committee, Lund University. Portal veins were dissected under sterile conditions and cut longitudinally into halves. For cholesterol depletion, strips were incubated with 5 mmol/L methyl-β-cyclodextrin (mβcd) or vehicle in Krebs’ solution (in mmol/L: NaCl 135.5, KCl 5.9, CaCl 2 2.5, MgCl 2 1.2, HEPES 11.6, and glucose 11.5, pH 7.4) for 1 to 3 hours at 37°C. The 1-hour treatment was used as the standard incubation time, because longer incubations impaired contractility. Cholesterol was reloaded into depleted strips by cholesterol-saturated mβcd (water-soluble cholesterol, Sigma) in Krebs’ solution for 3 hours at 37°C. Before culture, strips were gently blotted and weighed as described.

Organ Culture

After cholesterol depletion, strips were transferred to culture medium (Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium [1:1, vol/vol], containing 2% diazylated fetal calf serum, 10 mmol/L insulin, 50 μg/mL penicillin, and 50 μg/mL streptomycin) and either left undistended or loaded with a 0.6-g gold weight, which represents a preload approximately optimal for active force development. The passive length-tension relation is quite steep, so that a 10% shortening from the loaded length eliminates most of the passive force. The culture period was 24 hours, after which the strips were again weighed. No effects of mβcd on growth were observed with longer culture (3 days), presumably reflecting recovery from cholesterol depletion.

Mechanical Recording

Strips were mounted, immersed in 0.4 mL Krebs’ solution, and stretched to a passive tension of 2 mN. After equilibration (45 minutes), experimental protocols were started. Contraction was elicited by replacing NaCl with KCl to a final concentration of 500 mmol/L Na2CO3 with protease inhibitors (5 μg/mL Sigma), pH 11. The homogenate was adjusted to 45% sucrose with 1 mL of 90% sucrose in MBS (25 mmol/L MES and 0.15 mol/L NaCl, pH 6.5) and overlaid with 4 mL of 35% sucrose and 4 mL of 5% sucrose, both in MBS with 250 mmol/L Na2CO3. After centrifugation at 39 000 rpm for 22 hours (SW 41, Beckman Instruments), ten 1-mL fractions were collected. For detection of P-Tyr, protein was precipitated with 20% trichloroacetic acid, washed with acetone, and solubilized in sample buffer. When stretched and control veins were compared, the volumes loaded on the gel were adjusted to give equal protein contents in fraction 5. Protein synthesis rates are stretch dependent, but measurable differences in overall protein composition between loaded and unloaded veins do not appear during organ culture for several days, and hence, increases in P-Tyr during a 10-minute loading period represent increased phosphorylation of preformed proteins and not increased protein contents.

Electron Microscopy

Veins were fixed (1 to 2 hours) in 2% glutaraldehyde and 1% depolymerized paraformaldehyde in phosphate-buffered saline (pH 7.4, 300 mOsm). After being rinsed in phosphate-buffered saline, veins were postfixed in 1% OsO4 for 60 minutes, rinsed, dehydrated in graded ethanol (70%, 95%, and 100%), and embedded in mounting medium (Durcupan ACM, Fluka). Ultrathin sections were cut, mounted on copper mesh grids, and counterstained with uranyl acetate and lead citrate. Sections were examined in a transmission electron microscope, and photomicrographs were obtained with a CCD camera (Gatan 791 MultiScan). Caveolae, including completely and partially invaginated vesicles (50- to 100-nm diameter) and those with dilated necks found within 100 nm of the plasma membrane, were counted on coded photomicrographs. Membrane length was determined with commercially available software (ImageJ). The photomicrographs used for calculation of caveolar density were obtained randomly and in a similar way for all conditions, but because we concentrated on areas containing either caveolae or their remnants, we cannot exclude a bias toward regions rich in caveolae.

Statistics

Data are presented as mean±SE. Student’s t test was used to test statistical significance. For multiple comparisons, 1-way ANOVA was used. P<0.05 was considered significant.

Results

Stretch Rapidly Increases P-Tyr in Caveolin-1–Containing Membrane Domains

Mechanical stimuli rapidly increase P-Tyr of cellular proteins. To investigate whether stretch increases P-Tyr in caveolae of the intact rat portal vein, a detergent-free method for purification of caveolae by sucrose gradient fractionation was used. Ten minutes of stretch increased P-Tyr of several high-molecular-weight proteins (Figure 1A and 1B,a). P-Tyr increased almost exclusively in the fraction containing the caveolar marker caveolin-1 (Figure 1A). Comparisons (fold differences) of P-Tyr of several protein bands in fraction 5 of stretched versus unstretched veins are shown in Figure 1B,a. Caveolin-1 contents and protein composition in the fractions were the same (Figure 1B,b and Methods). This indicates that
the stretch signal involves P-Tyr of several proteins that are located in caveolae.

**Cholesterol Depletion Disrupts Caveolar Structure**

Cholesterol was extracted with mβcd (5 mmol/L, 1 to 3 hours),22,28 and membrane profiles were examined by electron microscopy. Caveolae were found as clusters at the cell ends and also along the longitudinal cell axes (Figure 1C,a). After incubation with mβcd for 1 hour, areas of flattened caveolae with dilated openings were present (Figure 1C,b), but in some regions, caveolae appeared normal. After longer treatment (2 to 3 hours), caveolae were more extensively flattened and dilated, and no caveolae of normal appearance were found (Figure 1C,c). The density of caveolae, irrespective of structure, was 3.8±0.4/μm membrane in controls versus 3.8±0.8, 4.3±0.2, and 1.5±0.3 (P<0.01) in preparations treated for 1, 2, and 3 hours, respectively (data from ≥8 photomicrographs per condition in 2 experiments).

**Effect of Cholesterol Depletion on Stretch-Induced Growth and Protein Synthesis**

Portal veins were cultured for 24 hours under loaded and unloaded conditions after cholesterol depletion for 1 hour. The weight of loaded, control vessels increased under these conditions,10,16 whereas unloaded veins lost weight (Figure 2A). Depletion of cellular cholesterol resulted in weight loss of loaded veins, similar to unloaded controls. Cholesterol-depleted, unloaded veins behaved as unloaded controls.

The disruption of caveolar structure with mβcd can be reversed by incubation with cholesterol-saturated mβcd (1 mmol/L cholesterol:5 mmol/L mβcd).28 Incubation with cholesterol-saturated mβcd without prior depletion did not affect weight gain in either loaded or unloaded veins (see online Figure I; available at http://atvb.ahajournals.org), demonstrating that mβcd itself has no effect on growth besides that caused by the extraction of cholesterol. Incubation with cholesterol-saturated mβcd for 3 hours after depletion (1 hour) partially restored the stretch-induced weight gain during the following culture (Figure 2A).

Protein and DNA syntheses were measured by [3H]leucine and [3H]thymidine incorporation, respectively, during culture (Figure 2B and 2C). Cholesterol depletion reduced protein and DNA syntheses in loaded veins toward the levels of unloaded controls. Synthesis recovered significantly, but not completely, when depleted veins were incubated with cholesterol-saturated mβcd before culture. Cholesterol-saturated mβcd alone did not influence leucine incorporation in loaded and unloaded veins, again excluding the possibility of nonspecific effects of mβcd on growth (see online Figure I; available at http://atvb.ahajournals.org). Neither protein synthesis nor DNA synthesis was affected by cholesterol depletion in unloaded veins. Extraction with mβcd had no effect on force in response to high-K+ depolarization in fresh strips (13±2 vs 11±2 mN/mm²; n=10, P=NS) or in veins cultured under load after extraction (8±3 vs 6±1 mN/mm²; n=10, P=NS). However, the amplitude of the regular spontaneous activity characteristic of the portal vein declined after cholesterol depletion (not shown).

**Effect of Cholesterol Depletion on Stretch-Induced ERK1/2 Activation**

Stretch-induced growth of the portal vein requires ERK1/2 phosphorylation,10 which is likely to be represented by the P-Tyr labeling in the 43-kDa region of the caveolar fraction in Figure 1A and 1B. Veins were therefore stretched for 5 minutes, and ERK1/2 phosphorylation was determined by
Western blotting. We first verified that mβcd did not have any nonspecific effect on ERK1/2 activation by using cholesterol-saturated mβcd (Figure 3A). Cholesterol depletion, on the other hand, inhibited ERK1/2 phosphorylation in stretched veins by 70% (Figure 3B). Significant recovery of stretch activation was observed after replenishing the cholesterol in depleted veins (Figure 3B).

**Effect of Cholesterol Depletion on ERK1/2 Activation by Ang II and ET-1**

Part of the stretch-induced ERK1/2 activation in the portal vein depends on endogenous release of Ang II. We therefore investigated the effect of cholesterol depletion on responses to Ang II. Addition of Ang II (10 μmol/L, 5 minutes) increased ERK1/2 phosphorylation in loaded and unloaded control as well as cholesterol-depleted veins. The effects of load and Ang II were additive (Figure 4). Although cholesterol depletion inhibited stretch-induced activation of ERK1/2, the additional response to Ang II in both loaded and unloaded veins was unaffected.

Addition of ET-1 increased ERK1/2 phosphorylation in both stretched and unstretched preparations, whereas the ET-1 type A (ET_A) receptor blocker RF139317 (1 μmol/L) reduced ERK1/2 phosphorylation of both stretched and unstretched preparations below their respective control values (Figure 5). This suggests that endogenous ET-1 production contributes to ERK1/2 activity, as also shown by the finding that the endothelin-converting enzyme inhibitor phosphoramidon (PA, 10 μmol/L) inhibited activation of ERK1/2 by stretch (loaded control, 100%; unloaded, 39±4%; loaded plus PA, 40±4%; unloaded plus PA, 34±3%; n=4 for all, *P<0.05 for effect of PA). Although ERK1/2 activation due to endogenous
production of Ang II and ET-1 seems to play a role in the stretch response, their dependence on membrane cholesterol differs, because mβcd completely inhibited activation of ERK1/2 by exogenous ET-1 as well as by stretch (Figure 5).

Role of the Endothelium

In view of the reported role of caveolae as mechanosensors in endothelial cells, the endothelium was removed, and the effect on stretch-induced ERK1/2 activation was examined. Scraping of the luminal vessel surface abolished endothelial nitric oxide synthase labeling, whereas stretch-induced ERK1/2 activation remained unaffected (Figure 6A).

Effect of Cholesterol Extraction on Subcellular Distribution of ERK1/2

With the use of sucrose gradient fractionation, ERK1/2 was found to copurify with the caveolar marker protein caveolin-1 (Figure 6B). Cholesterol depletion did not appear to alter the distribution of either caveolin-1 or ERK1/2 (Figure 6C).

Discussion

The observation that stretch increases P-Tyr preferentially in membrane microdomains containing caveolin-1 prompted us to examine the growth response after manipulation of caveolae. The effects of mβcd are specific for cholesterol extraction, because cholesterol-saturated mβcd had no effect on the growth response to stretch. The partial recovery of growth after reloading with cholesterol after depletion is likely due to incomplete restoration of cholesterol contents, because delivery of cholesterol is several-fold less effective than depletion by cyclodextrins.29

Even though cholesterol depletion influences caveolar structure and much evidence suggests that this affects signaling mechanisms located in lipid rafts/caveolae, the possibility remains that its functional consequences are linked, at least partly, to altered membrane fluidity. Removal of cholesterol from the membrane will increase its stiffness and thus perhaps the degree of mechanical deformation in response to an applied load. It is notable that limited extraction (1 hour) of cholesterol caused only regional disruption of caveolae yet inhibited growth completely. This might indicate that the effects on growth do not involve the caveolar structure per se but rather the liquid-ordered state normally present in either caveolae or rafts,17 which might influence mechanical forces acting on resident proteins.

Strain on the extracellular matrix and membrane integrins, which are attached to the cytoskeleton, influences gene expression, which accordingly is affected by manipulation of either matrix/integrin interactions,6 formation and receptor binding of endogenous growth factors,12,14,16 or cytoskeletal integrity.7,16 The present study implicates cholesterol-rich, membrane domains in the sequence. Mechanical stress is transmitted from contractile and cytoskeletal filaments to the plasma membrane, where adherens junctions constitute specific anchorage sites.30 Even though cholesterol depletion might influence signaling pathways involving the cytoskeleton, our results demonstrate that active force generated by a stimulus independent of caveolar signaling was unaffected by
cholesterol depletion, indicating that both the myofilaments and the force transmission from them function normally.

ERK1/2 activation by stretch in the rat portal vein is partly inhibited by candesartan, a blocker of the type 1 angiotensin (AT1) receptors, and by captopril, an inhibitor of angiotensin-converting enzyme, showing that endogenous Ang II plays an important role in ERK1/2 activation. Selective MEK inhibition by PD 98059 reduces ERK phosphorylation by 70% and completely inhibits stretch-sensitive growth in the portal vein. Thus, the reduced ERK1/2 phosphorylation after cholesterol depletion is sufficient to inhibit the growth response. Because AT1 receptors translocate to caveolae on ligand binding, we speculated that ERK1/2 activation was inhibited at this level. However, exogenous Ang II still activated ERK1/2 in the portal vein after cholesterol depletion, indicating that both the myofilaments and the force transmission from them function normally.

In cultured vascular smooth muscle cells, transactivation of epidermal growth factor receptors by Ang II was inhibited by cholesterol depletion, whereas c-Src activation and intracellular Ca²⁺ response to Ang II were unaffected. These results suggest that pathways downstream of the AT1 receptor might have a different dependence on cholesterol and/or caveolin-1.

In the portal vein, ERK1/2 copurified with the caveolar marker protein caveolin-1. Cholesterol depletion did not, however, affect the position of either caveolin-1 or ERK1/2 in the sucrose gradient, suggesting that the inhibition of stretch-mediated ERK1/2 activation after cholesterol depletion was not due to disrupted membrane targeting of ERK1/2. The unimpaired ERK1/2 response to Ang II also suggests that the function of the kinase was unaffected by cholesterol depletion.

The role of ET-1 in vascular growth seems to be partially linked to that of Ang II, because Ang II infusion causes increased ET-1 expression, and the ensuing hypertension is corrected by an ETA antagonist. Endogenous ET-1 release might thus account for some of the vascular effects ascribed to Ang II. In our experiments, both an ETA receptor antagonist (RF139317) and an endothelin-converting enzyme inhibitor reduced ERK1/2 phosphorylation in stretched veins. A small effect of RF139317 in unloaded veins was also observed, suggesting that basal ERK1/2 phosphorylation is associated with constitutive ET-1 signaling. Stretch activated ERK1/2 when portal veins were bathed with a high ET-1 concentration, implying that whereas ET-1 is critical, it is not the only relevant factor for activation of ERK1/2 by stretch. Using a similar approach, we have shown that a saturating concentration of Ang II does not eliminate stretch-dependent growth in the portal vein. Whereas ERK1/2 activation by exogenous ET-1 was highly sensitive to cholesterol depletion, this was not the case for Ang II. It is therefore unlikely that ET-1 acts downstream of Ang II, but rather in parallel, while possibly being released via a common mechanism. The cholesterol sensitivity of ERK 1/2 activation by ET-1 is consistent with a similar sensitivity of contractile responses and with a reported localization of ETA receptors in caveolae.

ERK1/2 activation by stretch was found to be insensitive to removal of the endothelium, and thus, the stretch-sensing mechanisms that affect gene expression in smooth muscle cells seem to primarily reside in these cells themselves. Substantial evidence indicates that endothelial cells are sensitive to shear stress in a manner dependent on intact caveolae. Thus, mechanical sensing in the vascular wall involves 2 separate modalities, wall tension and shear stress, which are transmitted through similar cholesterol-rich structures in entirely different cell types. This similarity argues that a role for caveolae in mechanotransduction might be a general phenomenon.

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References


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Supplement I. Cholesterol-saturated mβcd does not influence growth of the rat portal vein. Portal vein strips, treated with cholesterol-saturated mβcd (Chol.: 1mM cholesterol:5 mM mβcd) as indicated, were subjected to mechanical loading in culture and the change in wet weight (A) and leucine incorporation (B) determined. Results are means ± S.E. of two independent experiments.