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Translocation of caveolin regulates stretch-induced ERK activity in vascular smooth muscle cells

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Kawabe, Jun-ichi, Satoshi Okumura, Ming-Chih Lee, Junichi Sadoshima, and Yoshihiro Ishikawa. Translocation of caveolin regulates stretch-induced ERK activity in vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 286: H1845–H1852, 2004; 10.1152/ajpheart.00593.2003.—Mechanical stress contributes to vascular disease related to hypertension. Activation of ERK is key to mediating cellular proliferation and vascular remodeling in response to stretch stress. However, the mechanism by which stretch mediates ERK activation in the vascular tissue is still unclear. Caveolin, a major component of a flasklike invaginated caveolae, acts as an adaptor protein for an integrin-mediated signaling pathway. We found that cyclic stretch transiently induced translocation of caveolin from caveolae to noncaveolar membrane sites in vascular smooth muscle cells (VSMCs). This translocation of caveolin was determined by detergent solubility, sucrose gradient fractionation, and immunocytochemistry. Cyclic stretch induced ERK activation; the activity peaked at 5 min (the early phase), decreased gradually, but persisted up to 120 min (the late phase). Disruption of caveolae by methyl-β-cyclodextrin, decreasing the caveolar caveolin and accumulating the noncaveolar caveolin, enhanced ERK activation in both the early and late phases. When endogenous caveolins were downregulated, however, the late-phase ERK activation was subsided completely. Caveolin, which was translocated to noncaveolar sites in response to stretch, is associated with β_1-integrins as well as with Fyn and Shc, components required for ERK activation. Taken together, caveolin in caveolae may keep ERK inactive, but when caveolin is translocated to noncaveolar sites in response to stretch stress, caveolin mediates stretch-induced ERK activation through an association with β_1-integrins/Fyn/Shc. We suggest that stretch-induced translocation of caveolin to noncaveolar sites plays an important role in mediating stretch-induced ERK activation in VSMCs.

integrins; caveolae

CAVEOLINS are the principal structural components of the caveolae membrane domain. Caveolin acts as a scaffolding protein, thereby assembling a variety of signaling molecules via a modular protein domain, termed the caveolin-scaffolding domain. Caveolin inhibits the activity of tyrosine and serine/threonine kinases, including Src family tyrosine kinases, EGF receptor, ERK, protein kinase C, and protein kinase A (21, 27, 48). For example, overexpression of caveolin dramatically inhibits the ERK signaling cascade (6), whereas targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the ERK signal cascade (8). It has been speculated that caveolin inhibits these kinases by stabilizing the molecules in an inactive conformation within caveolae (28).

Growing lines of evidence suggest that mechanical force is one of the most important factors regulating proliferation and differentiation of vascular smooth muscle cells (VSMCs) (20, 46). It has been shown that ERK, which is an important mitogenic signal, is activated by stretch in vivo and vitro in VSMCs (10, 25, 47). Activation of integrins is one of the key events in the initiation of mechanotransduction (24, 31). Interaction between the extracellular matrix (ECM) and integrins induces several intracellular signal pathways, including focal adhesion kinase (FAK) (32) and ERK (9, 20, 46). Recently, two research groups independently reported that caveolin-1 associates with integrins, thereby mediating activation of Fyn and Shc (43–45). This sequence of events allows integrins to activate the Ras-ERK pathway and promotes cell cycle progression. Thus these results suggest that caveolin plays an essential role in mediating integrin-induced ERK activation. Interestingly, in these previous studies (42, 43), the interaction of caveolin and integrins was observed in a detergent-soluble fraction, implying that the interaction takes place at noncaveolar sites. However, caveolin is usually localized within detergent-insoluble caveolae in many tissues and cells, including VSMCs (2, 45). Thus it is not clear whether caveolin that is localized at noncaveolar sites but not within caveolae actually mediates an integrin/ERK signal pathway, nor is it clear how the displacement of caveolin to noncaveolar sites occurs. Furthermore, it is not clear how caveolin, which is a general kinase inhibitor, mediates activation of the ERK signal pathway through integrins.

In this study, we examined whether caveolin and integrins play an important role in mediating the stretch-mediated ERK signal pathway in VSMCs. Specifically, we examined whether cyclic stretch induces translocation of caveolin from the caveolae to noncaveolar sites. To investigate the role of caveolins, which was translocated to noncaveolar sites in stretch-induced ERK activation, we used VSMCs in which caveolin was downregulated or whose caveolae structure is disrupted by the cholesterol-chelating agent β-cyclodextrin. Our results suggest that transient translocation of caveolin outside caveolae is an important mechanism to mediate stretch-induced ERK activation in VSMCs.

MATERIALS AND METHODS

Cell culture and application of cyclic stretch. We handled animals according to rules proposed by the Association for Assessment and Accreditation of Laboratory Animal Care International. VSMCs were isolated from the thoracic aorta of rats as described (1, 18). After cells

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were grown to subconfluence, the medium was replaced with DMEM containing 0.05% bovine serum albumin for 48 h. Cells grown in collagen-coated silicone elastomer-bottomed plates were subjected to cyclic stretch in a FX-4000T Flexercell Strain Unit (Flexcell). VSMCs were exposed to an equiaxial stretch of 15% at a frequency of 1 Hz.

Antisense caveolin isoform adenovirus vectors. For construction of adenoviral vectors, full-length cDNA encoding rat caveolin-1 and -3 (kindly given by Dr. M. Lisanti) were inserted in an antisense orientation into the shuttle vector for construction of adenoviral vectors harboring antisense caveolin-1 and -3 using the AdenoX adenovirus construction kit (Clontech). Adenovirus-mediated transduction was performed as described (35). For a control study, adenovirus vectors harboring green fluorescent protein (GFP) were used. All experiments were performed at 48 h after transduction.

Detergent solubility. Extraction of the detergent (Triton X-100)-soluble proteins was performed as described (33) with a modification. Briefly, cells were resuspended in *buffer A* [containing (in mmol/l) 20 Tris·HCl (pH 7.5), 1 EDTA, and 1 PMSF with 10 mg/ml leupeptin] containing 1% Triton X-100 and incubated on ice for 30 min. After the protein concentration was adjusted, the lysate was centrifugated at 100,000 *g* for 20 min. The pellet was resuspended in 100 mmol/l NaOH (detergent-insoluble fraction). The proteins in the supernatant (detergent-soluble fraction) were precipitated by centrifugation in the presence of 7.2% (wt/vol) trichloroacetic acid and 0.015% (wt/vol) deoxycholic acid and resuspended in 100 mmol/l NaOH. We confirmed that >95% of proteins of the supernatant can be recovered by this method.

Sucrose gradient centrifugation. Caveolae fractions were separated by a previously optimized method using sodium carbonate (19, 27). Briefly, a 5–35% discontinuous sucrose gradient was formed and centrifuged at 39,000 rpm for 16 h at 4°C. From the top, 12 fractions were collected. *Fractions 4–6* were used as caveolae fractions, and *fractions 10–12* were used as noncaveolae fractions.

Immunoprecipitation and Western blot analysis. Immunoprecipitation was carried out as described (18) using the detergent-soluble fraction. Briefly, after stretch, the cells were lysed in *buffer A* containing 1% Triton X-100 and (in mmol/l) 10 pyrophosphate, 2 Na_3VO_4, and 10 NaF, and the detergent-soluble fraction was prepared as described above. This sample was incubated with anti-caveolin-1 antibody (2 μg/ml) at 4°C for overnight. Immunocomplexes collected on protein A/G agar beads (Sigma) were then washed three times with *buffer A* and separated by SDS-PAGE (4–20% gradient gel), followed by Western blot analysis. The following antibodies were used as 1:1,000 diluted solutions unless otherwise specified: anti-caveolin-1, -2, and -3; anti-Shc; anti-ERK (Transduction Labs); anti-phospho-ERK (Cell Signaling Technology); anti-β_1 integrin (Santa Cruz Biotechnology); and anti-Fyn (Upstate Biotechnology). Anti-Ig [F(ab′)$_2$] conjugated with peroxidase (Amersham) was used as second antibody to visualize the signals.

ERK activation assay. The activated ERK was measured as described (18). After normalization of protein concentration, activation of ERK was determined by Western blot analyses using anti-phospho-ERK and anti-ERK antibodies. The relative intensity of phospho-ERK to ERK was quantified by densitometry.

Immunofluorescence microscopy. Cells grown on collagen-coated glass slides were fixed with 3.7% formaldehyde in PBS (pH 7.2). Cells were then pretreated with 0.3% Triton X-100 in PBS for 10 min. Nonspecific binding was blocked with 1% bovine serum albumin in PBS. Primary antibodies at optimized concentration were incubated overnight at 4°C, followed by FITC-conjugated second antibodies. The staining was viewed and evaluated with a laser scanning microscope (Eclipse 500, Nikon) and a confocal microscope (Zeiss 500, Carl Zeiss).

Statistics. Results are presented as means ± SE. Statistical significance was determined by one-way ANOVA, and values of $P < 0.05$ were considered significant.

RESULTS

Stretch increases detergent solubility of caveolins. The detergent insolubility of caveolin is a well-established criterion for identifying the subcellular fractions containing the caveolae structure (2). First, to test whether the caveolae structure, and hence the localization of caveolins, is altered in response to cyclic stretch, we measured the detergent solubility of caveolins in VSMCs. After the cells were stretched for the indicated times, both detergent-soluble and -insoluble caveolin were detected by Western blot analysis. Before stretch, as consistent with previous studies (2, 33), >90% of caveolins existed in detergent-insoluble fractions (Fig. 1*A*). However, cyclic stretch increased the amount of detergent-soluble caveolin-1 and -3. This increase was apparent after 5 min of stretch, and the level of detergent-soluble caveolin reached a maximum after 30 min of stretch and then slowly decreased to baseline during the stretch for 2–4 h (Fig. 1*B*). In an inverse proportion to the increase in detergent-soluble caveolins, detergent-insoluble caveolins were decreased by 20% compared with that of nonstretch control. Expression level of caveolin was not changed by stretch for up to 12 h (data not shown). Thus these data suggest that caveolins translocate from caveolae to outside of caveolae in response to cyclic stretch.

Stretch induces translocation of caveolins from caveolae to noncaveolar sites. Caveolae have a low buoyant density and can be separated by sucrose gradient centrifugation (2). Thus to confirm whether the increased detergent solubility of caveolins by cyclic stretch reflects the translocation of caveolin from

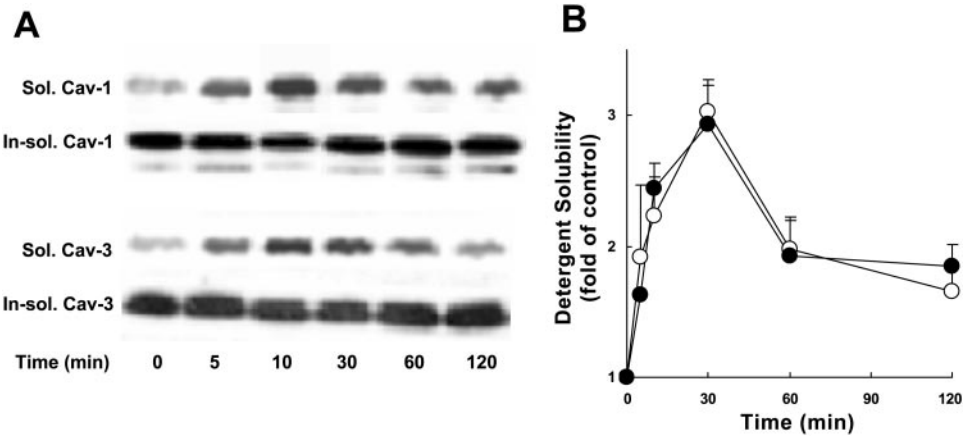

Fig. 1. Effect of cyclic stretch on detergent solubility of caveolins in vascular smooth muscle cells (VSMCs). Serum-starved cells were stimulated by cyclic stretch (15% elongation at 1 Hz) for indicated times. Caveolins in detergent-soluble (Sol) and -insoluble (In-sol) fractions were detected by Western blot analysis. *A*: representative blots of caveolin-1 (Cav-1) and -3 (Cav-3) in Sol and In-sol fractions. *B*: densitometric quantification of caveolin-1 (●) and caveolin-3 (○) bands in the Sol fraction. The value in the nonstretched sample was taken as 1. Data represent means ± SE of 4–6 experiments.

caveolae to noncaveolar sites, we separated caveolae and noncaveolar fractions by sucrose gradient centrifugation. After VSMCs were treated with cyclic stretch for 30 min, the cell homogenates were fractionated on a discontinuous sucrose gradient. As shown in Fig. 2, in nonstretched cells, most of caveolin (95.6 ± 1.4%, $n = 4$) was localized in *fractions 4–6*, termed as "caveolar fractions." The total amount of caveolin was not changed, but the ratio of caveolin in noncaveolar fractions to total caveolin was significantly increased in response to cyclic stretch (from 4.4 ± 1.4 to 29.1 ± 6.2%, $P < 0.05$, $n = 4$).

Stretch changes the localization of caveolin within plasma membrane in VSMCs. We next performed immunocytochemical studies to observe the subcellular localization of caveolin before and after stretch stimulation. It is well documented that caveolar caveolin can be easily detected as a punctuate pattern (33, 40). Caveolin-1 was observed as a punctuate distribution, indicating that caveolin-1 is localized at caveolae membrane domains (Fig. 3*A*). Interestingly, after 30 min of exposure to cyclic stretch, the number of punctuate caveolin spots was decreased, and the staining of caveolin was found throughout the cells in a diffuse pattern (Fig. 3*B*). We also observed the subcellular localization of caveolin using confocal microscopy. Most caveolin was located in the plasma membrane sites, although some was also detected within intracellular space (Fig. 3*C*), as consistent with the previous studies (2). However, the staining pattern of caveolins in intracellular spaces and the subcellular distribution of caveolins between the plasma membrane and intracellular spaces were not altered in response to cyclic stretch (Fig. 3*D*). These data suggest that cyclic stretch alters the staining pattern of caveolin within the plasma membrane but does not induce the translocation of caveolin from the plasma membrane to the intracellular spaces.

Intact network of actin fibers is necessary for stretch-induced translocation of caveolins. It has been suggested that the network of actin fibers is one of the most important candidates for the mechanosensor (13). Rho family small G proteins, Rho, Rac, and Cdc42, play a central role in the organization of the actin cytoskeleton (11). Recently, it has been reported that Rho and intact actin filaments are required for mechanotransduction (25). To explore the idea that the actin cytoskeleton involves stretch-induced translocation of caveolin, cells were pretreated with cytochalasin D, which selectively disrupts the network of actin filaments, and *Clostridium difficile* toxin B (toxin B), which inactivates Rho (3). Cytochalasin D and toxin B clearly inhibited the increases in detergent-soluble caveolin in response to stretch (Fig. 4). These data suggest that actin cytoskeletal organization is important for stretch-induced translocation of caveolins.

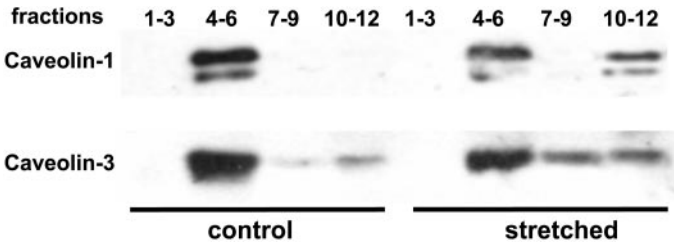

Fig. 2. Effect of cyclic stretch on subcellular localization of caveolins in VSMCs. Cells were stimulated by cyclic stretch for 30 min, and caveolae and noncaveolar fractions were separated by sucrose gradient centrifugation. Nonstretched cells were used as a control. Samples in caveolae (*fractions 4–6*) and noncaveolar fractions (*7–12*) were separated by SDS-PAGE, and caveolins in each fraction were detected by Western blot analysis. See MATERIALS AND METHODS for information on *fractions 1–3*.

Caveolae disruption enhances stretch-induced ERK activation. Next, we studied the role of caveolin in stretch-induced activation of the ERK signal pathway. Because stretch causes partial translocation of caveolins from caveolae to noncaveolar fractions (Fig. 1), we hypothesized that caveolar and noncaveolar caveolins may have different effects on stretch-induced ERK activation. The cholesterol chelating agent methyl-β-cyclodextrin disrupts the caveolae membrane domains and eventually decreases caveolar caveolin and increases noncaveolar caveolin (7, 29). Thus we tested the effects of methyl-β-cyclodextrin on stretch-induced ERK activation. When the cells were pretreated with 10 mmol/l methyl-β-cyclodextrin for 1 h, ~60% of detergent-insoluble caveolins were shifted to the detergent-soluble fraction without changes in the amount of total caveolins (data not shown). Cyclic stretch caused transient ERK activation: the activity peaked within 5 min of stretch (early-phase activity) and then gradually decreased to baseline over 120 min (late-phase activity) in control VSMCs. Methyl-β-cyclodextrin slightly increased basal ERK activity and had a striking effect on stretch-induced ERK activity, causing about twofold increases at the early phase, and its enhancement remained during the late phase (Fig. 5). These effects were subsided by adding exogenous cholesterol during the methyl-β-cyclodextrin treatment (Fig. 5), indicating that the effect of methyl-β-cyclodextrin was specific. These data suggest that the ERK activity is enhanced due to decreases in caveolar caveolin and/or due to increases in noncaveolar caveolin.

Downregulation of caveolin attenuates the late phase of ERK activation in response to stretch. To further examine the role of caveolin on stretch-mediated ERK activation, the expression of endogenous caveolins was attenuated by antisense genes. We confirmed that caveolin-1 and -3 were selectively downregulated by the transduction of adenoviral vectors harboring antisense caveolin-1 and -3 genes, respectively (Fig. 6*A*). When either endogenous caveolin-1 or -3 was downregulated, ERK activity at basal and after 5 min of stretch was increased compared with that of control cells, and downregulation of both caveolin-1 and -3 showed additive a much greater effect (Fig. 6, *B* and *C*). Importantly, however, stretch-induced increases in the ERK activity (fold increased vs. basal level) were not enhanced at 5 min of stretch and were significantly attenuated in the late phase (>10 min of stretch) when caveolin-1 and -3 were downregulated (Fig. 6, *B* and *C*). GFP-harboring adenovirus was used for control, but the cells transfected with GFP-harboring adenovirus did not affect stretch-induced ERK activation. Therefore, these data strongly suggest that caveolin mediates stretch-induced ERK activation at the late phase.

Noncaveolar caveolin associates with integrins and ERK signal molecules. Recently, it has been reported that caveolin acts as an adaptor protein to mediate the integrin/ERK signal pathway (43, 44). β_1-Integrins activate the tyrosine kinase Src family and Fyn through caveolin. Activated Fyn phosphorylates Shc, creating a binding site for the complex

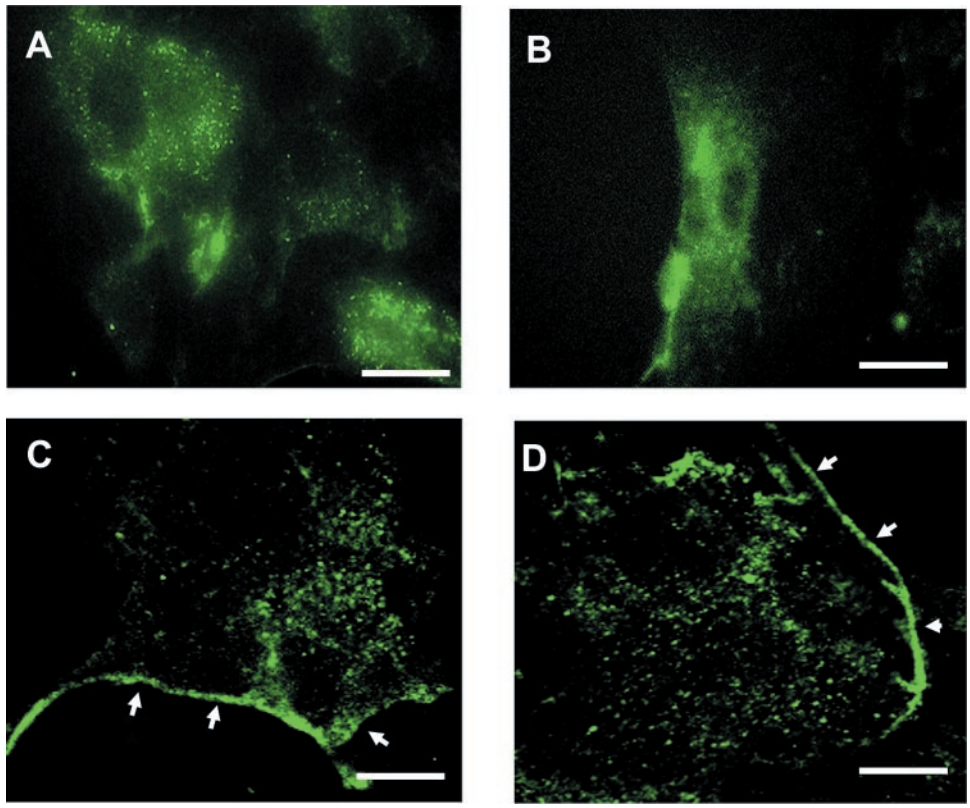

Fig. 3. Effect of cyclic stretch on immunostaining of caveolin-1 in VSMCs. Cells were stimulated by cyclic stretch for 30 min. The corresponding cells before (*A* and *C*) and after stretch (*B* and *D*) were fixed, and cellular caveolin-1 was stained using FITC-conjugated antibodies and observed by fluorescence microscopy (*A* and *B*) and confocal microscopy (*C* and *D*). Arrows indicate plasma membrane sites. Representative fluorescence images are shown. Bars = 10 μm.

of the adaptor protein Grb2, leading to activation of ERK (9). To this end, we examined whether the localization of β_1-integrins and Fyn, as well as caveolins, was altered in response to stretch. As shown in Fig. 7*A*, most of the β_1-integrins and Fyn were detected in the caveolae fraction together with caveolins. After cyclic stretch, β_1-integrins and Fyn were translocated from caveolae to noncaveolar fractions in parallel with caveolins. Furthermore, we performed immunoprecipitation of caveolin using the detergent-soluble fractions, i.e., noncaveolar fractions (Fig. 7*B*). After cyclic stretch for 30 min, significantly more caveolin-1 was immunoprecipitated in noncaveolar fractions compared with no stretch, consistent with the data shown above. Interestingly, β_1-integrins, Fyn, and Shc were coprecipitated with caveolin-1 in noncaveolar fractions obtained from stretched VSMCs. Immunoprecipitation without antibodies for caveolin-1 was used as a negative control, and nonspecific precipitation of these proteins was not observed (Fig. 7*B*).

DISCUSSION

We demonstrated that caveolin is translocated from caveolae to noncaveolar sites within plasma membrane in response to cyclic stretch using multiple methods, namely, detergent-mediated fractionation, sucrose gradient fractionation, and immunocytochemistry. In particular, in the immunostaining study, we found that the punctuate pattern of caveolin-1 was significantly reduced after cyclic stretch (Fig. 3). In addition, we also showed that translocated caveolin was associated with β_1-integrins, which exist at the plasma membrane (Fig. 6). Therefore, it is most likely that caveolins in caveolae are translocated into noncaveolar sites within the plasma membrane in response to cyclic stretch. It is known that caveolin is also dynamically translocated from caveolae to noncaveolar sites in response to several stimuli, such as cholesterol oxidation (36), heat shock, and hyperosmotic stress (17). However, translocation by those stimuli is quite different from that induced by stretch reported in this study. First, caveolins are moved to intracellular sites such as the Golgi apparatus and perinucleus portions in response to cholesterol oxidation and heat shock (17, 36). Second, the detergent insolubility of caveolins is not altered even after they move to intracellular portions after heat shock and hyperosmotic stress (17). Therefore, translocation of caveolin in response to stretch might be caused by different mechanisms distinct from those stimuli.

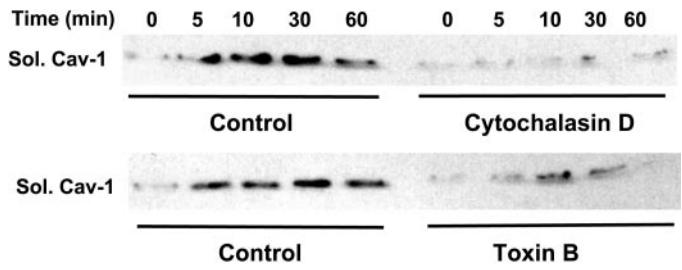

Fig. 4. Effect of disruption of the actin network on the stretch-induced detergent solubility of caveolin. VSMCs were incubated in the absence (control) or presence of 10 μmol/l cytochalasin D for 15 min or 10 ng/ml toxin B for 4 h. Cells were then stimulated with cyclic stretch for indicated times. Caveolin-1 in the Sol fraction was detected by Western blot analysis.

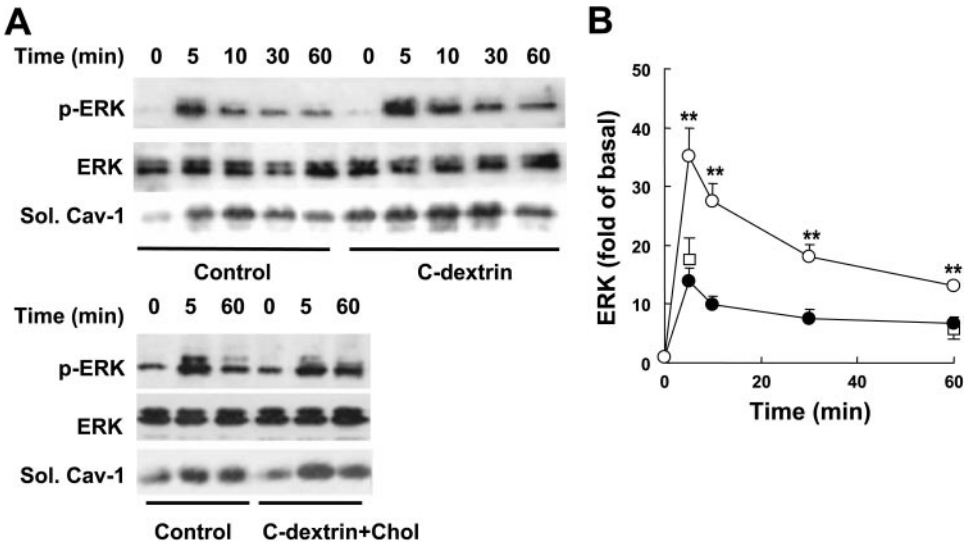

Fig. 5. Effect of disruption of caveolae structure on stretch-induced ERK activation. VSMCs were incubated in the absence (control) or presence of 10 mmol/l cyclodextrin (c-dextrin) or 16 μg/ml cholesterol (Chol) for 1 h. Cells were then stimulated with cyclic stretch for the indicated times. ERK activities and the detergent solubility of caveolin were determined. *A*: representative blots of phospho-ERK (p-ERK) and caveolin-1 in the Sol fractions are shown with ERK for comparison of loading. *B*: relative intensity of p-ERK to ERK was shown as ERK activity. ●, Control; ○, c-dextrin treated; □, c-dextrin + Chol treated. The basal value in the nonstretch sample was taken as 1 (means ± SE; $n = 4$–6). $^{**}P < 0.01$ vs. control at each time.

Recent studies reported that the actin-binding protein filamin, associated with caveolin, and that Rho, which mediates the organization of actin cytoskeleton, caused reorganization of the caveolae structure (37, 38). These findings imply that the actin network is involved in the organization of caveolae membrane domains. We found that stretch-induced translocation of caveolin was dependent on the intact actin fiber network (Fig. 4). However, at present, we do not know the molecular mechanisms by which stretch induces translocation of caveolin from caveolae to the noncaveolar sites. Cellular stresses, such as high osmolarity, induce tyrosine phosphorylation of caveolin in 3T3 cells (41). The detergent solubility of caveolin is affected by the phosphorylation status of caveolin in endothelial cells (39). However, in this study, there was no difference in the ratio of tyrosine-phosphorylated caveolin to caveolin in both caveolae and noncaveolar fractions (data not shown) after stretch. Thus phosphorylation of caveolin may not be the mechanism responsible for the translocation of caveolin in response to stretch.

Integrins are the major receptors connecting cells to ECM and mediating the mechanotransduction (9, 24, 31). Increasing lines of evidence suggest that the binding of integrins to their ligand, ECM, and activation of integrins require intracellular "inside-out" signaling (4). Cytoskeletal proteins and their modulator, Rho, are possible molecules that modulate the inside-out signaling for integrin activation (4, 34). Recent studies reported that actin cytoskeleton disruption with cytochalasin D abrogated stretch-induced ERK activation (14, 25). However, the molecular mechanism by which the actin network induces ERK activation in response to mechanical stress and the mechanism behind inside-out signaling remain to be elucidated. We showed that most caveolins and β_1-integrins were colocalized within caveolae in nonstretched VSMCs (Fig. 7*A*). Because caveolae are flask-shaped invaginations located at the cell surface (2), it is unlikely that integrins bind ECM when integrins are located within caveolae. Thus translocation of the integrin/caveolin complex from caveolae to noncaveolar sites must occur for the activation of integrins, and the translocation

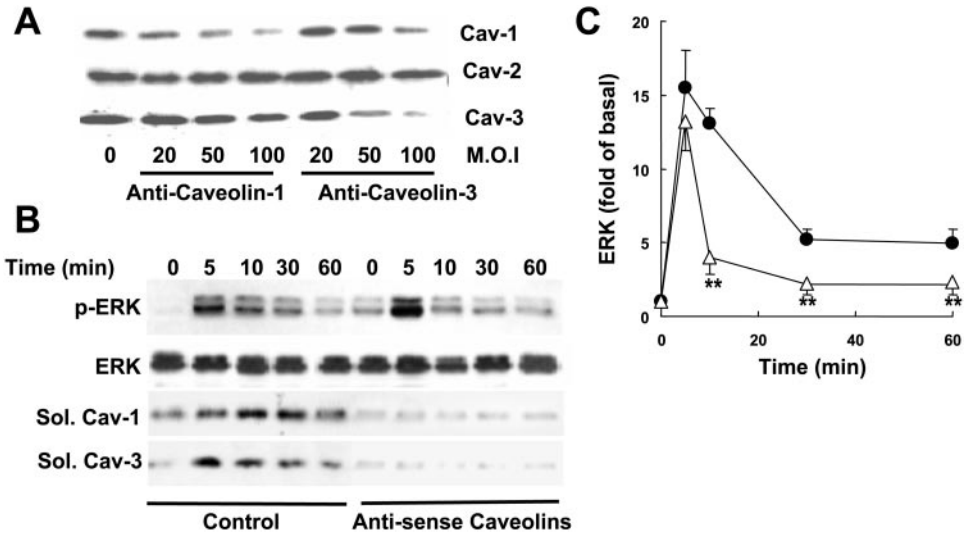

Fig. 6. Effect of downregulation of caveolin on stretch-induced ERK activation. For downregulation of caveolins, adenovirus vectors harboring antisense genes of caveolin-1 and -3 at a multiplicity of infection (MOI) of 0–100 were transfected and incubated for 48 h. The expression of endogenous caveolins was detected by Western blot analysis (*A*). Downregulation of caveolins was achieved by antisense caveolin-1 and -3 adenovirus vectors at a MOI of 100, and the cells were then stimulated with cyclic stretch for indicated times. Adenoviral vector harboring the green fluorescent protein gene was used as a control. *B*: representative blots of p-ERK and caveolin-1 and -3 in the Sol fraction are shown with ERK for comparison of loading. *C*: relative intensity of p-ERK to ERK was shown as ERK activity. ●, Control; △, antisense caveolins. The basal value in the nonstretch sample was taken as 1 (means ± SE; $n = 4$). $^{*}P < 0.01$ vs. control at each time.

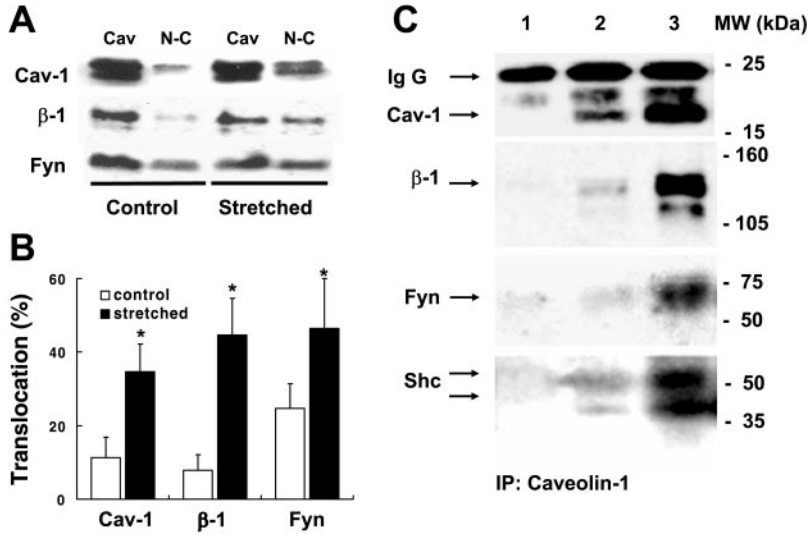

Fig. 7. Effect of cyclic stretch on subcellular localization of caveolin, integrins, and ERK signal molecules. VSMCs were stimulated with cyclic stretch for 30 min, and caveolae (Cav) and noncaveolar (N-C) fractions were separated by sucrose gradient centrifugation. *A*: caveolae and noncaveolar fractions were separated by 4–20% SDS-PAGE and applied to Western blot analysis using specific antibodies against caveolin-1, β_1-integrin (β-1), and Fyn. Representative immunoblots are shown. *B*: densitometric quantification of caveolin-1, β_1-integrin, and Fyn in both caveolae and noncaveolar fractions were performed, and the relative intensity of those in noncaveolar fraction (translocation) of nonstretched (control; open bars) and stretched (closed bars) samples are shown (means ± SE; n = 3–4). *$P < 0.05$ vs. control. *C*: before (*lane 2*) and after (*lanes 1* and *3*) cyclic stretch for 30 min, cavelin-1 was immunoprecipitated (IP) from the detergent-soluble fraction. The immunocomplex was subjected to Western blot analysis. MW, molecular mass markers. Immunoprecipitation without antibody is shown as a control (*lane 1*).

of the integrin/caveolin complex may contribute to the inside-out signaling to activate integrins.

Stretch evoked both rapid (within 5–10 min, the early-phase activity) and sustained (for several hours, the late-phase activity) activation of ERK in many cells including VSMCs (10, 15, 24, 30). Stretch-induced ERK activation may be composed by multiple mechanisms in which integrins and other signal pathways are involved (46). Hu et al. (12) reported that cyclic stretch induced the activation of the PDGF receptor in a ligand-independent manner and activated PDGF receptor-induced ERK activation within 4 min of stretch in VSMCs. It has been shown that integrin-induced ERK activation occurs more slowly (15–60 min after stimulation) in endothelial cells, corresponding to the stretch-induced late-phase ERK activity (15). Thus the role of caveolin in stretch-mediated ERK seems time dependent.

It has been shown that disruption of the caveolae structure by β-cyclodextrin enhances basal ERK activity and activity evoked by several stimuli, such as PDGF in different cells (7, 29), indicating that caveolin negatively regulates ERK signals within caveolae (6, 8). In this study, we also showed that β-cyclodextrin enhanced the stretch-induced ERK activation in VSMC (Fig. 5), consistent with previous studies, implying that caveolin within caveolae inhibits stretch-induced ERK activity. However, it has not been clear whether or not stretch-induced increases in the ERK activity are mediated by the increase of noncaveolar caveolin in addition to the decrease of caveolar caveolin. To clarify this question, we studied the cells in which endogenous caveolin was downregulated. Interestingly, downregulation of caveolin attenuated the stretch-induced ERK activation, especially in late-phase activity (Fig. 6*B*). The time course of the translocation of caveolin, which peaked at 30 min after stretch (Fig. 1), coincided well with the late-phase ERK activation. Therefore, these results strongly suggest that noncaveolar caveolin contributes to the late-phase ERK activity during the stretch.

To confirm this idea, we further showed that a part of β_1-integrins/caveolins/Fyn translocates from caveolae to noncaveolar sites in response to stretch (Fig. 7*A*), and then these molecules formed a complex and associated with Shc, which mediates ERK activation (9) (Fig. 7*B*). Therefore, caveolin may play an important role in mediating stretch-mediated ERK activation, especially in the late phase, rather than generally inhibiting ERK activation. In this study, however, we did not detect the association of ERK proteins with the integrin/caveolin complex in noncaveolar sites by means of immunoprecipitation using anti-caveolin antibody. It may be due to the weak association of ERK and caveolins, because of their indirect association, or the amount of caveolin-associated ERK was not great enough to detect by immunoblotting.

Iwasaki et al. (16) demonstrated that mechanical stretch stimulated ERK activation via transactivation of EGF (16). It is well recognized that caveolin acts as an important role in the growth factor receptor signal pathways (5, 22, 48). Furthermore, it has been reported that stretch induced the activation of Src (23), and the specific Src inhibitor PP2 blocked stretch-induced ERK activation (26). However, it is still unclear whether any particular Src family members are important for the stretch-ERK signal pathway. Therefore, it remains to be clarified that translocation of caveolin contributes to the stretch-mediated ERK activation through growth factor receptors and/or other members of the Src family in addition to the integrin/Fyn pathway.

Downregulation of caveolin increased basal ERK activity (Fig. 6), suggesting that caveolin acts as negative regulator for the ERK signaling. Accordingly, stretch-induced ERK activation at the early phase might be enhanced by downregulation of caveolin within caveolae. However, in contrast to the effect of methyl-β-cyclodextrin, stretch-induced ERK activity in the early phase was not enhanced by downregulation of caveolin. This may be due to the loss of caveolin-dependent ERK activation, because translocation of caveolin to outside caveolae occurred even at 5–10 min of stretch.

In conclusion, in the present study, we have shown that stretch stress induces translocation of caveolin from caveolae to noncaveolar membrane sites, and noncaveolar caveolin is critical in mediating the integrin/ERK signaling pathway. Thus our study may provide the important mechanical basis by which caveolin plays an essential role in mediating mechanotransduction in VSMCs.

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