Plasma Membrane Cholesterol Is a Key Molecule in Shear Stress-dependent Activation of Extracellular Signal-regulated Kinase*

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Shear stress, the dragging force generated by fluid flow, differentially activates extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK) in bovine aortic endothelial cells (BAEC) (Jo, H., Sipos, K., Go, Y. M., Law, R., Rong, J., and McDonald, J. M. (1997) J. Biol. Chem. 272, 1385–1401). Here, we examine whether cholesterol-enriched compartments in the plasma membrane are responsible for such differential regulation. Pretreatment of BAEC with a cholesterol-binding antibiotic, filipin, did not inhibit shear-dependent activation of JNK. In contrast, filipin and other membrane-permeable cholesterol-binding agents (digitonin and nystatin), but not the lipid-binding agent xylazine, inhibited shear-dependent activation of ERK. The effect of cholesterol-binding drugs did not appear to be due to membrane permeabilization, since treatment of BAEC with a detergent, Triton X-100 which also permeabilizes membranes, did not inhibit shear-dependent activation of ERK. Furthermore, shear-dependent activation of ERK, but not JNK, was inhibited by cyclodextrin, a membrane-impermeable cholesterol-binding agent, which removes cell-surface cholesterol. Moreover, the effects of cyclodextrin were prevented by adding cholesterol during the incubation. These results indicate that cholesterol or cholesterol-sensitive compartments in the plasma membrane play a selective and essential role in activation of ERK, but not JNK, by shear stress. Although exposure to shear stress (1 h) increased the number of caveolae by 3-fold, treatment with filipin had no effect in either control or shear-exposed cells suggesting that caveolae density is not a crucial determinant in shear-dependent ERK activation. In summary, the current study suggests that cholesterol-sensitive microdomains in the plasma membrane, such as caveolae-like domains, play a critical role in differential activation of ERK and JNK by shear stress.

Vascular endothelial cells recognize shear stress by unknown mechanosensing system(s) that respond both acutely and chronically to flow by producing autocrine and paracrine factors (1). Through these endothelial responses, shear stress controls vascular tone, vessel wall remodeling, binding of blood cells to endothelium, and hemostasis (1). Shear stress selectively and differentially regulates expression of many genes that are important in the pathophysiology of vessel wall function (2–10). Furthermore, a conserved cis-acting shear stress response element has been identified in many shear-sensitive genes including platelet-derived growth factor-B, intercellular adhesion molecule-1, tissue plasminogen activator, and transforming growth factor-β-1, suggesting its broad implication in shear-dependent regulation of gene expressions (6, 8). Shear stress also transiently activates nuclear factor-xB, immediate early response genes, and transcription factors that are likely to be involved in the regulation of shear-dependent gene expression (8, 11).

How does shear stress selectively and differentially regulate such a diverse range of nuclear responses? At least some of these responses appeared to be mediated through regulation of MAP3 kinases (12–16). Members of the MAP kinase family, ERK, JNK (also known as stress-activated protein kinase), and p38 kinase, have been proposed as important signaling components linking extracellular stimuli to cellular responses including cellular growth, death, differentiation, and metabolic regulation (17, 18). Recently, shear stress has been shown to differentially regulate activation of ERK and JNK, and at least some of shear-stimulated gene expressions are regulated by MAP kinases (15, 16).

Shear stress activates ERK in a rapid and transient manner (maximum by 5 min which returns to basal by 30 min shear exposure), whereas JNK activation occurs over a much slower and prolonged time course (requiring at least 30 min and returning to basal levels after 1 day shear exposure) than that of ERK (15). In addition, ERK activation is shear force-dependent (minimum and maximum at 1 and 10 dyn/cm2 shear stress, respectively) whereas that of JNK activity is either “turned off” under no shear condition or “turned on” maximally by even a relatively low shear force (0.5 dyn/cm2) (15). Further studies demonstrated that Gαs/tyrosine kinase(s)/Ras and Gβγ/tyrosine kinase(s)/Ras are upstream regulators of shear-dependent activation of ERK and JNK, respectively (15). How do endothelial cells control activation of specific signaling pathways lead-3

1 The abbreviations used are: MAP kinase, mitogen-activated protein kinase; BAEC, bovine aortic endothelial cells; ERK, extracellular signal regulated kinase; GST-c-Jun, c-Jun (amino acids 5–89) fused to glutathione S-transferase; JNK, NH2-terminal Jun kinase; PFS, fetal calf serum; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FAK, focal adhesion kinase; TEM, transmission electron microscopy.
ing to differential activation of ERK and JNK in response to various shear regimens? We hypothesized that there may be a spatial and/or temporal compartmentalization of signaling complexes in microdomains, which enable an orderly and efficient regulation of signaling cascades in response to shear stress. Cholesterol-enriched compartments such as caveolae and sphingolipid-cholesterol rafts (also called as "caveolae-like domains" or "pre-caveolae") are candidate micro-signaling domains (19–22).

Caveolae are non-coated, flask-shaped invaginations of the plasma membrane (50–100 nm in diameter) and are found in most cell types including endothelial cells, fibroblasts, smooth muscle cells and adipocytes (19, 20, 23). However, "flat" caveolae (formed by dynamic clustering of sphingolipids and cholesterol in the cell membrane and caveolae-specific proteins such as caveolin) do exist, although their physiologic significance has not been clearly defined (19–22). Caveolae are found in both the apical and basal plasma membranes and carry out at least two different functions as follows: 1) transport large and small molecules (transcytosis of macromolecules and potocytosis of ions and the vitamin folate), and 2) act as transmembrane signaling microdomains (20–24). Caveolae appear to be formed by self-packaging of "caveolin" along with highly concentrated cholesterol and glycolipids (25–27). In addition, caveolae contain signaling molecules such as Gαs, Src-like kinases, Ras, Raf, and even ERK itself (20, 28–33).

The present study was designed to examine whether cholesterol-sensitive membrane microdomains are involved in the signaling pathways responsible for the differential activation of ERK and JNK in response to shear stress. This study shows evidence that cholesterol or cholesterol-sensitive microdomains in the plasma membrane play a critical role in shear-dependent activation of ERK but not in the JNK pathway.

MATERIALS AND METHODS

Cell Culture and Drug Treatment—BAEC harvested from descending thoracic aortas were maintained (37 °C, 5% CO2) in a growth medium (DMEM (1 g/liter glucose, Life Technologies, Inc.) containing 20% fetal calf serum (FCS, Atlanta Biologicals) without antibiotics (34). Cells used in this study were between passages 5 and 10. For shear experiments, 1 million cells per glass slide (75 × 38 mm, Fisher) were seeded in growth medium. The next day, the medium was changed to a starvation medium (phenol red-free DMEM containing 0.5% FCS and 25 mM HEPES) and incubated for 18 h. Stock solutions (1 mg/ml each freshly prepared in 95% ethanol) of filipin, digitonin, nystatin, xylazine (Sigma) and up to 0.5% of ethanol as a vehicle control were added to the starvation medium 10 min before subjecting cells to shear stress. Methyl-β-cyclodextrin (prepared in distilled H2O, Sigma) was added to the starvation medium containing lipoprotein-free FCS (35) and incubated with cells for 60 min. In some studies, 1.3 mM cholesterol (Avanti) was added during the 1-h cyclodextrin treatment to block chelation of cholesterol from cell membrane (36). To test whether the effect of filipin on shear-dependent activation of ERK is reversible, cells were first preincubated with filipin (1 μg/ml) for 10 min, washed in starvation medium, and then incubated in the fresh medium without filipin for up to another hour before subjecting cells to shear stress.

Membrane Integrity Assay Using Calcein AM—BAEC were incubated with 0.5 μg/ml calcein AM (Molecular Probe) for 30 min at 37° in DMEM, 0.5% FCS. The calcein-loaded cells were washed three times with DMEM containing 0.5% FCS and 25 mM HEPES-buffered saline (MediaTech) followed by incubation with 0–0.1% Triton X-100 in DMEM containing 0.5% FCS for 10 min at 37 °C. Then, the medium was collected, and cells were harvested in phosphate-buffered saline (PBS). The amount of calcein that leaked out with cells was determined in a Perkin-Elmer fluorimeter (excitation at 488 nm and emission at 530 nm) as described (37). Goat anti-rabbit IgG-conjugated to alkaline phosphatase was used as a secondary antibody, and the membrane was developed with a chemiluminescent detection method (15).

Immune Complex-JNK Assay—Activity of JNK1 was measured by an immune complex kinase assay using an antibody specific for JNK1 (PharMingen, clone number G151-333) and e-Jun (amino acids 5–89) fused to glutathione S-transferase (GST-e-Jun) as the substrate as described (15). Briefly, Triton-soluble cell lysates (100 μg each) were incubated with JNK1 antibody (0.25 μg) for 1 h at 4 °C, followed by an additional 1 h incubation with a protein G-agarose. The immune complex was washed four times in the lysis buffer and twice in buffer A (20 mM HEPES, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 0.1 mM vanadate, and 2 mM dithiothreitol). The washed immunocomplexes were incubated in 20 μl of buffer A containing GST-e-Jun (5 μg each), 20 μM ATP, and 5 μCi of [γ-32P]ATP for 20 min at 30 °C. The reaction was terminated by boiling in 10× Laemmli sample buffer, resolved by 10% SDS-PAGE, and electropherograms were transferred to a polyvinylidene difluoride membrane. Autoradiograms were obtained from the dried blot, and radioactivity incorporated into GST-e-Jun was quantitated by cutting and counting each band in a scintillation counter.

Quantitative immunoprecipitation of JNK1 was examined by probing the same blot with a polyclonal JNK antibody (38) (kindly provided by Drs. C. C. Franklin and A. S. Kraft at the University of Colorado).

Cytotoxic Staining of Cholesterol—Filipin is fluorescent and has been widely used to localize cellular cholesterol (40). Briefly, BAEC grown on glass slides were washed with ice-cold PBS, fixed with 1% glutaraldehyde on ice for 15 min, rinsed with PBS, and incubated with filipin (50 μg/ml) for 30 min at room temperature. The cells were then rinsed with PBS and air-dried. Filipin was present during shear. Following shear, cells were fixed for 1 h at 4 °C on ice with 1.6% paraformaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, washed with 0.1 M sodium cacodylate and 3.5% sucrose buffer, pH 7.3, and then post-fixed for 1 h with 1% Palade’s OsO4. Cells were stained en bloc with Kellenberger’s uranyl acetate, dehydrated, embedded in epoxy resin, and sectioned. Ultra-thin sections were examined by TEM, and 26–67 different random fields (each field containing part of 1 or 2 cells) were photographed. As suggested by Schnitzer et al. (23), only distinctly flask-shaped, non-coated vesicles (50–100 nm in diameter) found on or within 100 nm of both apical and basal plasma membranes were scored as caveolae.

RESULTS

Filipin Does Not Inhibit Shear-dependent Activation of JNK1—To examine the role of cholesterol in the shear-dependent activation of JNK, BAEC were pretreated with increasing concentrations of filipin or vehicle prior to shear exposure. Treatment of BAEC with filipin did not have any significant effect on static or shear-induced protein kinase activation of JNK (Fig. 1, B and C). In some experiments, cells that were pretreated with filipin were subjected to shear stress in the shear medium containing no filipin. The presence or absence of Filipin (1 μg/ml) in the shear medium during a period of 1 h shear had no effect on shear-dependent activation of JNK (data not shown). As shown previously (15), exposure of BAEC...
Membrane Cholesterol in Shear-sensitive ERK Activation

Filipin does not inhibit shear-dependent activation of JNK1. BAEC were preincubated for 10 min with filipin (0–5 μg/ml) and further incubated under no flow condition (Static) or exposed to shear stress (5 dyn/cm²) for 1 h. Cell lysates were incubated with a JNK1 antibody (PharMingen) and protein G-agarose, and the immune complex was used to phosphorylate GST-c-Jun (15). Phosphorylated proteins were detected by 10% SDS-PAGE followed by electrotransfer to a polyvinylidene difluoride membrane and autoradiography (B). The same membrane was probed with a rabbit JNK antibody to demonstrate that similar amounts of JNK1 were used in each lane (A). The radioactivity of phosphorylated GST-c-Jun was quantified by cutting and counting each band (C). A representative blot showing HA-JNK1 (~48 kDa) (A) and an autoradiogram of GST-c-Jun phosphorylation (B) are shown. The line graph shown in C is the mean ± S.E. of results obtained from 3 to 6 independent experiments. When cells were preincubated with 5 μg/ml filipin and subsequently exposed to shear stress for 1 h, all cells detached from the glass plate so that JNK activity could not be measured. Filipin treatment did not have any statistically significant effect on the shear-dependent activation of JNK.

Filipin Inhibits Shear Stress-dependent Activation of ERK—Unlike the case for JNK, pretreatment of cells with filipin (0–1 μg/ml for 10 min) inhibited shear-dependent activation of ERK in a concentration-dependent manner (Fig. 2B and closed circles in C). As shown previously (15), exposure of BAEC to shear stress (10 dyn/cm²) for 5 min stimulated ERK activity by 5–6-fold over the no shear (static) control as measured by Western blot analysis with an antibody specific to phospho-ERK (pERK) (Fig. 2B). Filipin had minimal effects reaching up to 2-fold activation of ERK over vehicle control in the static control cells (p = 0.08 to 0.2, paired Student’s t tests, see also Figs. 3 and 4).

The Inhibitory Effect of Filipin on Shear Activation of ERK Is Reversible—Next, we tested whether the inhibitory effect of filipin was reversible. BAEC that were pretreated with filipin (1 μg/ml for 10 min) were washed and incubated in a filipin-free starvation medium for up to 1 h before exposure to shear stress. After 60 min incubation in filipin-free media, shear-stimulated activity of ERK was restored to ~70% of control (filipin-untreated group) (Fig. 3).

Other Cholesterol-binding Reagents Inhibit Shear-stimulated Activity of ERK—Two other membrane-permeable cholesterol-binding agents, digitonin and nystatin, and a lipid-binding agent, xylazine (as a control), were used to examine the specific functional role of cholesterol in the shear-dependent activation of ERK. Digitonin, a cardiac glycoside (42), inhibited shear-dependent activation of ERK, while raising basal ERK phosphorylation (Fig. 4A). Nystatin, a polynye macrolide antibiotic (43), partially inhibited (by 42%) shear-dependent activation of ERK while having no effect on the basal activity. In contrast, xylazine enhanced the shear-dependent activation of ERK by 45% without any significant effect on the basal activity (Fig. 4A). Next, the effects of these agents on JNK activity were also examined. Unlike ERK activity, digitonin, nystatin, and xylazine did not have any significant effect on shear-activated JNK activity (Fig. 4B). Nystatin and xylazine also had little effect on basal JNK activity, whereas digitonin increased it somewhat (Fig. 4B). At present, the underlying mechanisms for the differential effects of each cholesterol-binding compound on basal ERK activity are not known. Nevertheless, these results demonstrate that removal of cholesterol has specific inhibitory effects on ERK activation, but not JNK, in response to shear stress.

Permeabilization of Membrane Alone Does Not Lead to Inhibition of Shear-dependent ERK Activation—Filipin, digitonin, and nystatin can create transmembrane pores and alter membrane permeability. To determine whether the effect of these drugs was due to their nonspecific effect on the membrane permeability, we chose to use a detergent, Triton X-100, to permeabilize the cell membrane in a non-cholesterol-dependent manner. As demonstrated in Fig. 5B, treatment of BAEC with 0.001–0.01% Triton X-100 effectively increased the leakage of calcein without significantly depleting the total cellular protein level. Under similar conditions, total ERK levels did not change under static conditions (lanes 1–3, Fig. 5A) unless the Triton-treated cells were further subjected to shear stress (lane 7, Fig. 5A). The Triton treatment did not inhibit the shear-dependent activation of ERK, even when there was a significant loss of ERK1/2 (lane 7, Fig. 5A). Although it is relatively minor compared with the robust fold stimulation of ERK by shear stress, basal ERK activity was somewhat increased by Triton permeabilization of cells (Figs. 5A and 6B).

Next, we examined the effect of Triton permeabilization on JNK activity. Similar to the results of ERK, the detergent treatment did not have any effect on JNK activation induced by shear stress while raising basal JNK activity somewhat (Fig. 6C). Again, the effect of Triton on basal JNK activity was
relatively minor compared with the robust stimulation of JNK by shear stress (Fig. 6C). Taken together, these results suggested that nonspecific alteration of membrane permeability alone could not account for the inhibitory effect of the cholesterol-binding drugs on shear-dependent ERK activation.

Plasma Membrane Cholesterol Is a Key Component in Shear-dependent Activation of ERK—To examine whether cholesterol in the plasma membrane plays an essential role in the shear-dependent ERK signaling pathway, a membrane-impermeable cholesterol-binding drug cyclodextrin was used. Cyclodextrin (10 mM) treatment of BAEC prevented the shear-dependent activation of ERK (Fig. 6A). This inhibitory effect was prevented if cholesterol was added back to the incubation medium during the cyclodextrin treatment (Fig. 6A). Time course studies using 5–10 mM cyclodextrin showed that it was necessary to incubate cells with the drug for 1 h (data not shown) suggesting that the cholesterol targeted by cyclodextrin may not initially be in the exofacial plasma membrane where it could be readily accessible by the drug. This slow kinetics could be explained if cholesterol affected by cyclodextrin was present in the cytoplasmic plasma membrane which translocates slowly to the exofacial side, where it could be removed out of the membrane by binding to cyclodextrin as proposed (44). Treatment of BAEC with 5 mM cyclodextrin also substantially inhibited shear-dependent activation of ERK, although its inhibitory effect was lower than that induced by 10 mM (compare Fig. 6, A and B). However, if cyclodextrin was delivered to the interior of the cell after the cells were Triton-permeabilized, then 5 mM cyclodextrin completely prevented the shear-dependent activation of ERK.

Next, we examined whether the JNK pathway is inhibited by removal of membrane cholesterol using cyclodextrin. Identical to the result obtained with filipin (Fig. 1), cyclodextrin treatment in the absence or presence of Triton X-100 had no significant effect on shear-activated JNK activity (Fig. 6C). Taken together these results shown in Fig. 6 clearly demonstrated that removal of cholesterol by cyclodextrin inhibits shear-dependent activation of ERK but not JNK.

To study the effect of cyclodextrin on plasma membrane cholesterol, the fluorogenic characteristic of filipin (used at 50 μg/ml) was used to cytochemically stain cholesterol and examined by epi-fluorescent microscopy (40). In control cells (Fig. 7A), cholesterol on the cell surface appeared as fine lines, especially visible at borders between cells (marked with arrowheads). Treatment with cyclodextrin eliminated cholesterol staining in the plasma membrane as indicated by arrowheads placed at the border lines between the cells (Fig. 7B). If cholesterol was added during the cyclodextrin treatment, however, the plasma membrane cholesterol was clearly visible (arrowheads) again as in control cells (Fig. 7C). When cells were
permeabilized by Triton X-100 and subsequently incubated with cyclodextrin, then the cholesterol staining was no longer visible (Fig. 1D). This complete chelation of cellular cholesterol by cyclodextrin + Triton was not due to the loss of cells as BAEC were still intact (see the phase photomicroscope, Fig. 7E). Moreover, the treatment of BAEC with 0.01% Triton X-100 alone did not alter the cholesterol-staining pattern (data not shown). The results of the cytochemical cholesterol staining studies support that cyclodextrin is membrane-impermeable and its effect is mediated by chelating cholesterol in the plasma membrane. Combined with the results shown in Fig. 6, these findings clearly indicate that cholesterol in the plasma membrane is the target of cyclodextrin and a key element in the shear-dependent ERK signaling pathway.

**Effect of Filipin and Shear Stress on the Number of “Invaginated” Caveolae**—Based on the previous reports in bovine lung microvascular endothelial cells and fibroblasts (19, 23), we speculated that the inhibitory effect of filipin on shear-dependent activation of ERK would be due to loss of “invaginated” caveolae. Consistent with previous reports (23, 45), caveolae were found as single as well as clusters of non-coated vesicles (see arrowheads in Fig. 8A) on or near the apical and basal plasma membranes of BAEC. There were ~0.15 caveolae/µm in control cells as quantified by counting the number of “invaginated” caveolae seen by TEM using the morphological criteria suggested by Schnitzer and colleagues (23, 45). To our surprise, however, filipin treatment (up to 5 µg/ml for up to 1 h at 37 °C) did not reduce the number of invaginated caveolae in static controls and shear-exposed groups (Fig. 8D). On the other hand, exposure of BAEC to shear stress for 1 h increased invaginated caveolae by 3-fold (Fig. 8B).

**DISCUSSION**

Early atherosclerotic plaques tend to develop in curved and branched arterial regions, which are associated with disturbed and/or low shear stress conditions, whereas areas exposed to stable and high shear forces are relatively well protected (46, 47). Similarly, various shear regimens (laminar, turbulent, step, and gradual changes) induce different endothelial responses including gene expression, NO release, and activation of MAP kinases over different time courses (1, 3, 9, 15, 38, 48). How do endothelial cells recognize the differences in shear stress and respond accordingly? The results presented in this study suggest that endothelial cells contain cholesterol-sensitive structures and/or signaling modules that may render signaling specificity leading to selective activation of ERK without affecting that of JNK pathway in response to shear stress.

**Chelation of Cholesterol Prevents Shear-dependent Activation of ERK but Not JNK**—In this study, we presented several lines of evidence supporting the concept that the removal of cholesterol in the plasma membrane results in selective inhibition of the shear-sensitive ERK pathway. First, filipin treatment only blocked activation of ERK, but not JNK, in response to shear stress (Figs. 1 and 2). Second, other membrane-permeable cholesterol-binding drugs (digitonin and nystatin), but not a lipid-binding drug (xylazine), inhibited activation of ERK, but not JNK, by shear stress (Fig. 4). Third, the specific nature of these drugs was also supported by demonstrating the reversibility of filipin effect (Fig. 3). Fourth, the lack of Triton’s ability to inhibit the shear-sensitive ERK activation makes it unlikely that the inhibitory effects of filipin, digitonin, and nystatin could be solely attributed to their characteristic as detergents (Fig. 5). Finally, cyclodextrin, a cell-impermeant agent that sequesters plasma membrane cholesterol by inducing its efflux, selectively inhibited the shear-dependent activation of ERK but not JNK. Moreover, the inhibitory effect of cyclodextrin was reversed by adding exogenous cholesterol (Figs. 6 and 7).

**Plasma Membrane Cholesterol Is Essential to the Shear-dependent Activation of ERK**—It has been shown that ~85% of free cholesterol is present within the plasma membrane, and the majority (75–97%) of these cholesterol molecules are found in the cytofacial leaflet in mammalian cells (44, 49). Fielding and Fielding (44) proposed a classification of the cellular free cholesterol into three pools as follows: (a) the “fast pool” present in the exofacial plasma membrane, (b) an “intermediate pool” representing the cytofacial free cholesterol in transit toward the exofacial plasma membrane, and (c) a “slow pool” that is found intracellularly. During our study, we observed that the inhibitory effects of cyclodextrin (5 to 10 µM) were observed only after a relatively long incubation period (30–60 min). The exofacial cholesterol (3–25% of total cholesterol in the plasma membrane) is directly accessible by cyclodextrin and is expected to be removed quickly (fast pool) (44, 50). However, we have not been able to block the shear-sensitive ERK by less than 30 min cyclodextrin treatment (data not shown) suggesting that the exofacial cholesterol is not likely to be a key component in this shear-signaling pathway. It seems more
likely, however, that the slow time course of cyclodextrin action could be explained by the slow movement of the cytofacial cholesterol to the exofacial plasma membrane. Therefore, the cholesterol or cholesterol-enriched compartments present in the cytofacial plasma membrane could be responsible for the regulation of shear-sensitive ERK activation.

A specific role for cholesterol in the proper functioning of receptors and other membrane proteins, including G-proteins, has been reported. For example, receptors for 5-methyltetrahydrofolate, rhodopsin, oxytocin, cholecystokinin, transferrin, and acetylcholine have all been shown to require cholesterol for optimal functioning (36, 51–54). As early as 1975, Limbird and Lefkowitz (55) showed that the proper coupling of β-adrenergic receptors to adenylate cyclase was uncoupled by removing cholesterol. The role of cholesterol could also be to regulate membrane fluidity or rigidity (36). Since cholesterol directly binds to caveolin-1, cholesterol could directly influence the function of caveolin-1, which interacts and regulates many signaling molecules including G-proteins, Src, and Ras (20, 27, 56).

One question raised by the present study is whether cholesterol-sensitive structures such as caveolae or sphingolipid-cholesterol rafts play a role in the shear-dependent activation of ERK. In support of the role for these membrane domains, Anderson and co-workers (32) have shown that ERK activation is acti-
and confirmed that filipin treatment inhibited shear activation of ERK. Studies were used for Western blot analysis with a pERK1/2 antibody. Portions of monolayers that were saved before fixing them for EM were thin sectioned and examined by electron microscopy as described under “Materials and Methods.” Two to four independently prepared endothelial monolayers were used to photograph 26–62 different randomly chosen fields from each group. Only distinctly invaginated vesicles (50–100 nm diameter) found within 100 nm of both apical and basal plasma membranes were counted. Shown in A are examples of caveolae (arrowheads) obtained from control BAEC, and shown in B are average caveolae numbers per μm² ± S.E. (*, p < 0.05; **, p < 0.02; *** p < 0.001 as determined by Student’s t tests). Note the 3-fold increase in caveolae numbers after 60 min shear stress over static control. Some portions of monolayers that were saved before fixing them for EM studies were used for Western blot analysis with a pERK1/2 antibody and confirmed that filipin treatment inhibited shear activation of ERK as shown in Fig. 2.

Fig. 8. Filipin does not decrease the number of invaginated caveolae, but shear stress increases their numbers in BAEC. BAEC were preincubated with vehicle or filipin (5 μg/ml for 0- and 5-min shear groups and 1 μg/ml for 1-h group). Following shear, cells were thin sectioned and examined by electron microscopy as described under “Materials and Methods.” Two to four independently prepared endothelial monolayers were used to photograph 26–62 different randomly chosen fields from each group. Only distinctly invaginated vesicles (50–100 nm diameter) found within 100 nm of both apical and basal plasma membranes were counted. Shown in A are examples of caveolae (arrowheads) obtained from control BAEC, and shown in B are average caveolae numbers per μm² ± S.E. (*, p < 0.05; **, p < 0.02; *** p < 0.001 as determined by Student’s t tests). Note the 3-fold increase in caveolae numbers after 60 min shear stress over static control. Some portions of monolayers that were saved before fixing them for EM studies were used for Western blot analysis with a pERK1/2 antibody and confirmed that filipin treatment inhibited shear activation of ERK as shown in Fig. 2.

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REFERENCES
1. Davies, P. F. (1995) Physiol. Rev. 75, 519–560
2. Diamond, S. L., Eskin, S. G., and McIntire, L. V. (1989) Science 243, 1483–1485
3. Malek, A. M., Greene, A. L., and Izumo, S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5999–6003
4. Nishida, K., Harrison, D. G., Navas, J. P., Fisher, A. A., Dockery, S. P., Uematsu, M., Neron, R. M., Alexander, R. W., and Murphy, T. J. (1992) J. Clin. Invest. 90, 2092–2096
5. Malek, A. M., Gibbons, G. H., Dzau, V. J., and Izumo, S. (1993) J. Clin. Invest. 92, 2013–2021
6. Resnick, N., Collins, T., Atkinson, W., Bonthron, D. T., Dewey, C. F., Jr., and...
