Rapid Mechanotransduction in Situ at the Luminal Cell Surface of Vascular Endothelium and Its Caveolae*

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Victor Rizzo, Arthur Sung, Phil Oh, and Jan E. Schnitzer‡

From the Department of Pathology, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215

The vascular endothelium is uniquely positioned between the blood and tissue compartments to receive directly the fluid forces generated by the blood flowing through the vasculature. These forces invoke specific responses within endothelial cells and serve to modulate their intrinsic structure and function. The mechanisms by which hemodynamic forces are detected and converted by endothelia into a sequence of biological and even pathological responses are presently unknown. By purifying and subfractionating the luminal endothelial cell plasma membrane from tissue, we show, for the first time, that not only does mechanotransduction occur at the endothelial cell surface directly exposed to vascular flow in vivo but also increased flow in situ induces rapid tyrosine phosphorylation of luminal endothelial cell surface proteins located primarily in the plasmalemmal invaginations called caveolae. Increased flow induces the translocation of signaling molecules primarily to caveolae, ultimately activating the Ras-Raf-mitogen-activated protein kinase pathway. This signaling appears to require intact caveolae. Filipin-induced disassembly of caveolae inhibits both proximal signaling events at the cell surface and downstream activation of the mitogen-activated protein kinase pathway. With the molecular machinery required for mediating rapid flow-induced responses as seen in endothelium, caveolae may be flow-sensing organelles converting mechanical stimuli into chemical signals transmitted into the cell.

The vascular endothelium is continually challenged by hemodynamic forces such as pressure and shear stress, which play important roles in the acute and chronic regulation of vascular tone (1, 2) and vessel remodeling (3, 4) as well as in the development of various vascular diseases (1, 5, 6). Investigating the effects of such forces on endothelium in vivo has been rather difficult, at least in part, because of technical limitations. Thus, mostly in vitro experiments designed to mimic the forces imposed on the endothelium in vivo have been performed to demonstrate the importance of flow in determining cell morphology (7), activating second messenger signaling pathways (8–12) releasing vasoactivators such as nitric oxide (13, 14), and inducing specific gene expression (6, 15). Based on logistical and theoretical hemodynamic arguments, the luminal cell surface of the endothelium directly exposed to the circulatory system is expected to be a critical interface immediately involved in the transduction of fluid forces in vivo. The existence of a flow-sensing, mechanotransducing element on this endothelial plasma membrane seems likely but, as yet, remains unproven.

Caveolae are specialized invaginated microdomains that appear on the cell surface on many cell types and are especially abundant in certain vascular endothelia. They contain key protein mediators of vesicle formation, docking, and fusion (16) and have been shown to form discrete carrier vesicles capable of budding directly from the luminal plasma membrane (17, 18). Thus, caveolae may serve as dynamic carriers for intracellular and transvascular transport of blood macromolecules within endothelial cells. In addition, caveolae have been implicated in the organization of cell surface signaling molecules (17, 19–21). The localization of signaling molecules within a small microdomain is likely to provide the proximity necessary for rapid, efficient, and specific propagation of signals to downstream targets.

Endothelial cells have remarkable plasticity with the ability to modulate their constitutive phenotype in response to local tissue milieu (22) so that when grown in culture, they adopt a “dedifferentiated” phenotype. Although endothelial cells are investigated best under “native” conditions, which cannot be duplicated in culture, they constitute, even in most highly vascularized organs, only a small percentage of the tissue cells, which makes isolating their plasma membranes using classic techniques impractical. Here, we use a silica-coating methodology to purify and then subfractionate the endothelial plasma membrane directly from tissue to focus on the effects of fluid flow directly on the luminal endothelial cell surface in situ. We find that increased fluid flow through the rat lung vasculature in situ induced very rapid tyrosine phosphorylation of several proteins on the luminal endothelial cell surface, with the majority of these proteins residing within the caveolar microdomain. This induced signaling cascade appears to require intact assembled caveolae and local translocation of key signaling molecules, leading to activation of the Ras-Raf-MAP kinase

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‡ To whom correspondence should be addressed: Dept. of Pathology, Harvard Medical School, 330 Brookline Ave., Boston, MA 02215. Tel.: 617-667-3577; Fax: 617-667-3591; E-mail: jschnitz@bidmc.harvard.edu.

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1 The abbreviations used are: MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; PLC, phospholipase C; EGF, epidermal growth factor; PKA, protein kinase A; P, phosphorylation; MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; PLC, phospholipase C; EGF, epidermal growth factor; PKA, protein kinase A; P, phosphorylation; PKC, protein kinase C; P-V, plasma membranes stripped of caveolae; eNOS, endothelial nitric oxide synthase.
pathway. Thus, mechanotransduction does occur in tissue at the luminal endothelial cell surface and may require caveolae for efficient signal propagation. These studies are the first to describe mechanotransduction events as they occur on the luminal endothelial cell surface and its caveolae in situ and thus provide a new focus for defining the molecular events mediating mechanotransduction.

MATERIALS AND METHODS

In situ Vascular Perfusion—Male Sprague-Dawley rats (150–200 g) were anesthetized with a 5:1 mixture of 10 mg/ml ketamine and 10 mg/ml xylazine. The body weight of the animals was recorded prior to tracheostomy. The lungs were inflated with 3/4 tidal volume of mammalian Ringer's saline. The right atrium was cut, and a small cut was made in the right ventricle, through which a catheter was fed and secured into the pulmonary artery. The left atrium was cut to allow for outflow, and vascular fluid flow (mammalian Ringer's solution at 37 °C) was immediately established through the rat lungs at a predetermined rate and time of delivery using a variable speed pump (Bio-Rad) connected to a buffer reservoir. At the conclusion of the perfusion experiments, the vasculature was immediately cooled to 10 °C by perfusion with 20 mM MES-buffered saline (pH 6.0) (23). In some experiments, rat lungs were perfused with or without filipin (5 μg/ml for 0.5 hr) prior to flow stimulation. Pressure and flow rates were measured during perfusion of the lung. A flow rate of 4–5 ml/min produced pressures of 8–10 mmHg, whereas 10 and 12 ml/min gave 18–20 and 22–24 mmHg, respectively. For each independent experiment, rat lung samples were acquired on the same day, and up to four independent experiments were performed for each study, as indicated.

Purification of Endothelial Cell Luminal Plasma Membranes and Caveolae—Immediately following perfusion and cooling of the rat lung vasculature as described above, the luminal endothelial cell plasma membranes and then their caveolae were purified using an in situ silica-coating procedure described previously (23). Briefly, a positively charged colloidal silica suspension was perfused through the rat lung vasculature to selectively coat the luminal endothelial cell plasma membranes. Cross-linking of the silica particles by subsequent perfusion with polyacrylic acid created a stable adherent silica pellicle that marked this specific membrane of interest. This coating was firmly attached to the plasma membrane, greatly increased its density, and thus permitted purification by centrifugation of the silica-coated endothelial cell plasma membranes. Cross-linking of the noncaveolar markers (22–24). The remaining silica-coated membrane pellet stripped of caveolae was labeled P. Immuno-affinity Isolation of Caveolae—Membrane vesicles were separated from the plasma membranes, as described in our past work (16, 21). Briefly, PY-20 antibodies (10 μg; Transduction Laboratories, Lexington, KY) were pre-absorbed onto goat anti-mouse IgG-coated magnetic beads (Dynal, Oslo, Norway) by incubation for 4 h at 4 °C. The antibody-head conjugates were subsequently washed three times by sedimentation and resuspension to remove excess antibody and blocked with 0.1% bovine serum albumin for 1 h at 4 °C. Purified caveolae (10–15 μg total protein) derived from different flow conditions were added to the conjugates and incubated for 1 h at 4 °C. Beads with any attached membranes (B) were separated magnetically from unbound material (U) and then processed for SDS-PAGE and immunoblotting with anti-caveolin antibodies.

Western Analysis—As in our past work (16, 21), proteins (2–15 μg) from tissue fractions, such as H, P, V, and P-V, were analyzed by SDS-PAGE (5–15% gradient gels) followed by electrotransfer to nitrocellulose membranes before immunoblotting using primary and reported antibody detected by enhanced chemiluminescence. Primary antibodies used included PY-20, Ras, Raf, Lyn, and caveolin-1 (Transduction Laboratories). For examination of mitogen-activated protein kinase, 30 μg of H was separated, transferred, and probed using pan-ERK (Transduction Laboratories) or active mitogen-activated protein kinase (Promega) primary antibodies. Autoradiograms were scanned and digitized (Molecular Dynamics), and band intensities were quantified (16, 22, 23). The digital images were transferred to a Macintosh Power PC and printed using Adobe Photoshop software.

RESULTS AND DISCUSSION

Flow-induced Tyrosine Phosphorylation of Luminal Endothelial Cell Surface Proteins—We developed and characterized an in situ system designed to examine signal transduction events initiated in response to changes in fluid mechanical forces within vascular endothelium. The rat lung vasculature was perfused in situ via the pulmonary artery within the normal physiological range of pressure (0–24 mmHg) resulting in a range of flow rates from 0 to 12 ml/min. After rapid cooling, we used the silica-coating technique to purify the luminal endothelial cell plasma membranes (P) directly from the tissue (see “Materials and Methods”). This strategy allowed us to focus on the effects of increased flow on the vascular endothelium under physiologically relevant conditions found in the body. It avoids potential artifacts induced by isolating and growing endothelial cells in culture outside their normal tissue environment.

We found that several proteins residing on the luminal endothelial cell plasma membranes (P) became tyrosine phosphorylation in response to increasing flow (Fig. 1A). This phosphorylation was difficult to detect in the corresponding starting whole lung homogenates (H) of the same rat lung sample, consistent with the known enrichment of endothelial cell surface proteins in P (22–24). The protein-tyrosine phosphorylation detected in P increased with greater flow rates and pressures. Both the number of proteins phosphorylated and the degree to which they became phosphorylated increased significantly on the luminal endothelial cell surface (see arrows in Fig. 1A). Densitometry revealed that even small increases in flow rate and pressure from 4–5 ml/min to 6 ml/min (8–10 mmHg to 12–14 mmHg) caused an overall average increase of 1.9 ± 0.6-fold in total protein-tyrosine phosphorylation detected in P. Doubling flow rates and pressures to 9 ml/min (18–20 mmHg) caused an average increase of 8.8 ± 1.7-fold that did not increase further at 12 ml/min (22–24 mmHg). Under no flow conditions, the detected phosphorylation was minimal, which suggested that normal flow rate and pressure might maintain a basal level of endothelial cell surface stimulation in situ. The protein-tyrosine phosphorylation induced by flow occurred very rapidly and required only 1 min to achieve a maximum total response, as quantified and depicted in Fig. 1B. Analysis of the individual endothelial cell surface proteins as resolved by SDS-PAGE and immunoblotting revealed that the phosphorylation of a group of proteins with apparent molecular masses of 180, 120, 90, 58, and 48/50 kDa was acutely sensitive to high purity (21, 23), the few proteins containing immunoreactive antibodies detected by enhanced chemiluminescence detection. This strategy allowed us to focus on the effects of increased flow on the vascular endothelium under physiologically relevant conditions found in the body. It avoids potential artifacts induced by isolating and growing endothelial cells in culture outside their normal tissue environment.

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Mechanotransduction in Caveolae—Using a selective subfractionation procedure developed for isolating caveolae from P to high purity (21, 23), the few proteins containing immunode-
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**Fig. 1.** Flow-induced tyrosine phosphorylation of proteins on the luminal endothelial cell surface in situ. A, dependence on flow rate. Rat lungs were perfused in situ for 10 min at 4, 6, 9, or 12 ml/min (pressure range from 8 to 24 mmHg) prior to purification of the luminal endothelial cell plasma membranes (P) from the whole lung homogenates (H). For the 0 or “no flow” condition, the lungs were flushed at 4 ml/min for 1 min to remove the blood followed by 10 min of no flow stasis. Arrows denote proteins phosphorylated by flow. The immunoblot is representative of four independent experiments. For each experiment performed, one rat lung was perfused at each of the indicated flow rates on the same day. B, rapid increase in protein-tyrosine phosphorylation at the endothelial cell surface. The lung vasculature was perfused at a rate of 10 ml/min (pressure of 18–20 mmHg) for 0, 10, 30, 60, 180, or 600 s. The total protein-tyrosine phosphorylation detected in P at each time point was quantified densitometrically. Average values ± S.E. are plotted as a percentage of the maximum signal detected (*p < 0.05; n = 3). C, quantification of flow-induced tyrosine phosphorylation of individual endothelial cell surface proteins. The relative amount of tyrosine phosphorylation of protein bands distinctly resolved by SDS-PAGE and immunoblotting was quantified densitometrically and plotted as the fold-increase in signal over flow of 4–5 ml/min (8–10 mmHg). Values were calculated as mean ± S.E., which are provided in the text (n = 3).

Detectable phosphotyrosine under 4 ml/min flow conditions were localized primarily within caveolae (Fig. 2, left panel, lane V). These proteins, as well as others, seemed to become significantly more phosphorylated with increased flow. The majority of proteins that were rapidly phosphorylated in response to enhanced flow could be found in the caveolae (Fig. 2, right panel, lane V). Comparisons, by densitometry, of total protein-tyrosine phosphorylation between endothelial luminal membrane subfractions isolated from rat lungs subjected to high flow (10 ml/min) revealed that an average of 8.4 ± 1.7 more signal in the caveolae (V) than P as well as a 20-fold increase relative to the plasma membrane stripped of caveolae (P-V).

**Fig. 2.** Rapid flow-induced protein-tyrosine phosphorylation in caveolae. Silica-coated luminal endothelial cell plasma membranes were purified after perfusing the rat lungs at either 4 or 10 ml/min (8–10 and 18–20 mmHg, respectively) for more than 1 min and then subfractionated to purify their caveolae. Proteins (2 μg) from the indicated fractions were resolved by SDS-PAGE before electrotransfer to nitrocellulose filters for immunoblotting with phosphotyrosine antibody. The plasma membrane proteins (arrows) phosphorylated in response to enhanced fluid flow and pressure were primarily found in the caveolae (V). Due to the intense phosphotyrosine signal observed for p48/50 at exposure times required to detect the majority of tyrosine-phosphorylated proteins in V, an exposure within the linear range of film sensitivity is provided to allow for a more accurate assessment of flow-induced protein-tyrosine phosphorylation for these proteins. The immunoblot illustrates membrane fractions derived from the same experimental animal and is representative of four independent experiments. For each experiment, three rat lungs were perfused at each of the indicated flow rates on the same day.

The phosphotyrosine signal present in P was, on average, 2.8 ± 1.3-fold greater than that found in P-V in these same high flow samples, further substantiating the observation that the majority of flow-sensitive proteins reside in the caveolae. The few phosphorylated proteins remaining in P-V were also detected in V and thus may represent flow-sensitive proteins residing within the residual caveolae (about 5–10%) that remain associated with the luminal plasma membrane following subfractionation (16, 23). Alternatively, these proteins may be common to both caveolae and the luminal endothelial cell surface proper. In addition, the enrichment ratio (V/P-V) for individual proteins varied from an average of 3.3 ± 1.1 for the 120-kDa protein to 26.4 ± 4.8 for the 48/50-kDa proteins. The more slowly phosphorylated proteins at 25 and 15 kDa, when detected in P, could not be detected in V and remained in P-V (data not shown). As reported previously (16, 23), caveolin was enriched 15–25-fold in V relative to P. Consistent with our previous observations for many proteins found enriched in caveolae (23), the profile of phosphorylated proteins in caveolae remained quite similar whether we purified the caveolae in the presence or absence of detergent (data not shown), indicating a significant resistance of the phosphorylated proteins to Triton X-100 solubilization.

Immu-no-affinity isolations performed on the caveolae in V confirmed that the phosphorylated proteins existed in the same caveolar vesicles rich in caveolin (Fig. 5). Consistent with the protein phosphorylation detected at 8–10 mmHg with 4–5 ml/min of flow (Figs. 1A and 2), rapidly 25–40% of the caveolae were able to bind to the phosphotyrosine antibody beads. After increased pressure and flow (18–20 mmHg at 10 ml/min), the percentage of caveolae in the bound fraction of the immunosolubilates increased to >95%. Thus, the tyrosine phosphorylation of endothelial cell surface proteins induced by increased flow, and pressure seemed to occur in nearly all the caveolae found in V, consistent with the vast majority of the endothelial cell surface caveolae exhibiting mechanosensitivity. In addition,
caveolae imuno-isolated using caveolin antibody also contained the flow-induced tyrosine-phosphorylated proteins of the same apparent molecular weight as those observed in Fig. 2 (data not shown).

It is interesting that many of the endothelial cell surface proteins that are tyrosine phosphorylated by ligands (21) seem to be similar to those observed in these studies, which suggests a parallel between ligand-induced signaling and mechanotransduction at the endothelial luminal cell surface. We have reported that endothelial cell mitogens and chemokines such as VEGF, basic fibroblast growth factor, insulin, endothelin-1, and platelet-derived growth factor were effective in producing protein-tyrosine phosphorylation (21). Even though platelet-derived growth factor caused the greatest degree of ligand-induced phosphorylation, we found that only about half of endothelial cell caveolae in V contained tyrosine-phosphorylated proteins after platelet-derived growth factor stimulation (21). These data suggest that only a subset of caveolae is involved in ligand-specific signaling and contains the ligand’s receptor, which is capable of initiating signaling within these caveolae. However, flow-induced signaling seems to occur in nearly all of the caveolae (Fig. 3), which distinguishes mechanotransduction apart from growth-factor-stimulated signaling. Thus, flow-induced signaling within caveolae may be the result of the activation of a single, yet to be identified, mechanosensory molecule found in all caveolae or, more likely, the summation of several, yet unidentified, proteins that have mechanosensing and signaling capabilities.

**Caveolar Link to MAP Kinase Pathway and Gene Regulation**—Currently, the distal aspects of the signaling cascade elicited in response to mechanical stimuli, namely the effects of flow on molecular events at or near the nucleus, are better understood than more proximal signaling events at or near the plasma membrane. The role of shear stress response elements as well as flow-induced transcription factors and gene expression has been well described (6, 15, 25–27). Many of these nuclear events are regulated by the MAP kinase pathway, which can be activated in cultured endothelial cell monolayers by shear forces (12, 28). Little is known about flow-induced signaling events between the MAP kinase pathway and the cell surface, especially in situ, where it is unknown whether MAP kinase is stimulated by flow.

With this background in mind, we examined our preparations for flow-induced activation of the MAP kinase pathway and upstream effectors of this pathway. As shown in Fig. 4A, increased flow through the pulmonary artery did indeed activate MAP kinases in a time-dependent manner, as detected in the rat lung homogenate. This in situ activation was detected as early as 30 s, reached a maximum increase of 15-fold at 3 min, and quickly declined to 3-fold above baseline, which was sustained after 10 min. Past work has shown that the MAP kinase pathway could become activated when cytosolic Raf binds to activated GTP-charged Ras resulting in its translocation to the plasma membrane, clustering, and kinase activation (29, 30). Consistent with this activation process, we found that Raf was rapidly translocated to the plasma membrane (P) as a consequence of increased flow (Fig. 5A). More specifically, Raf was detected, along with caveolin, in the caveolae (V) subfraction (Fig. 5B). As quantified in Fig. 4B, a peak 10-fold increase of Raf was detected in P after 3 min of doubled flow and pressure (10 ml/min at 18–20 mmHg). Although the Raf signal rapidly decreased, it remained 3.2 times greater than lower pressure and flow (8–10 mmHg at 4–5 ml/min) after 10 min of increased flow (9–10 ml/min), whereas the caveolin signal remained unchanged (Fig. 5B). Thus, these results are consistent with flow-initiated signaling through caveolae via Raf translocation to the caveolae, which, in turn, activates MAP kinase.

**Disassembly of Caveolae Inhibits Mechanotransduction**—The organization or compartmentalization of molecules within caveolae might be necessary for efficient mechanotransduction. Cholesterol binding agents such as filipin cause reversible disassembly of caveolae, which effectively disperses the proteins normally organized in caveolae over the cell surface and inhibits its vesicular transport by caveolae but not clathrin-coated vesicles (31). Hence, we tested the effects of filipin on flow-induced phosphorylation at the luminal surface of the endothelium in situ and MAP kinase activation in the rat lung homogenates (H). Disassembly of caveolae greatly inhibited signaling at the cell surface as well as propagation of the signaling cascade into the cell. As shown in Fig. 6A, filipin reduced the protein-tyrosine phosphorylation detected on the endothelial cell surface by 73%. Moreover, downstream signal propagation was inhibited nearly 100% as indicated by the lack of flow-induced...
activation of MAP kinase after treatment (Fig. 6B). We previously showed that filipin does not have intrinsic kinase inhibitory activity (21). Lastly, we were unable to purify caveolae after perfusing filipin, which is consistent with our past work (31) showing nearly complete loss of caveolar invaginations on the endothelial cell surface. Thus, the disruption by filipin of caveolae as an organized subcompartment of the plasmalemma appeared to prevent the efficient and rapid conversion of the mechanical stimulus into a transduced signaling cascade necessary for regionalized protein-tyrosine phosphorylation and subsequent signal propagation, leading to MAP kinase activation.

Based on the rapid activation and concomitant Raf translocation at the endothelial cell caveolae, it is logical to assume that the MAP kinase activation that we detected in H was occurring within the rat lung vascular endothelium. We probed our P and V fractions for MAP kinase with the hope of ensuring its specificity in the endothelium. In our many attempts, we have been unable to detect MAP kinase in either membrane fraction. This result seems quite consistent with the known cytosolic localization of MAP kinase, which, upon activation, can translocate to the nucleus where it can exert its effects on gene transcription factors (32). However, other investigators have reported the presence of MAP kinase in caveolae (33). The difference may reflect the distinct experimental systems (particular cell type examined, culture versus in vivo) and the method of caveolae isolation. Our results suggest that intact caveolae are necessary for flow-induced signal propagation leading to MAP kinase activation, which provides further support for the hypothesis that caveolae play a role in the transduction of fluid mechanical forces.

**Other Mechanotransduction Machinery Associated with Caveolae**—Similar flow-induced increases are readily seen for other molecules including Ras, Src-like nonreceptor tyrosine kinases ( Lyn, Src, and Yes but not Lck), and the signaling adapter protein 14-3-3 but not angiotensin-converting enzyme, β-actin, PLC-γ, or caveolin (Fig. 5). Both Src-like kinases (34) and 14-3-3 play a major role in activating the Ras-Raf axis of the MAP kinase pathway (29). Furthermore, Src-like kinases are stimulated by various G protein-coupled cell surface events and are necessary for linking G protein activation to the Ras-Raf-MAP kinase pathway (35, 36). This linkage may be important because pertussis-sensitive flow responses exist in endothelial cells (37) and may be mediated via one or more G proteins that have been found in endothelial cell caveolae (16). Caveolae contain other molecules implicated in rapid endothelial cell responses to flow. For instance, the production of prostacyclin and nitric oxide in endothelial cells is mediated by flow activation of G proteins, ion channels, intracellular calcium, inositol triphosphate, and protein kinases (for reviews, see Refs. 1 and 14). Many of these response effectors may be found in caveolae (21). Flow-induced calcium fluxes (38, 39) also exist at the cell surface as well as stretch-activated Ca<sup>2+</sup> or K<sup>+</sup> channels (40, 41). G proteins may modulate shear-sensitive K<sup>+</sup> channels (41) or PLC to generate inositol 1,4,5-trisphosphate and diacylglycerol in mechanically stimulated endothelial cells (9), which may lead to elevated intracellular calcium and activation of protein kinase C. Consistent with these functional findings, Ca<sup>2+</sup> ATPase and inositol 1,4,5-trisphosphate-activated channels on the endothelial cell surface reside concentrated in caveolae (24, 42, 43). In addition, both protein kinase C and PLC are enriched in caveolae in situ, and PLCβ is translocated from the cytosol to caveolae with increased flow.

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2 V. Rizzo, A. Sung, P. Oh, and J. E. Schnitzer, unpublished data.
Although our in situ system does not differentiate between the effects of increased pressure and shearing forces, changing hemodynamic conditions in vivo are likely to reflect more than one type of mechanical stressor. Indeed, it is becoming apparent that both fluid shear and pressure can elicit many of the same endothelial cell responses using what may be similar mechanisms (1, 44, 45). Increasing the viscosity of the perfusion medium while maintaining a low constant pressure and flow rate through the rat lung vasculature may serve to differentiate between shear and pressure forces. Pilot studies in which the rat lung vasculature was perfused with a more viscous solution (5% dextran in mammalian Ringer’s) demonstrated induced protein-tyrosine phosphorylation at the luminal endothelial cell surface similar to those reported above for the increased flow and pressure conditions. Thus, it seems that increased shear is indeed stimulatory in situ. Whether increases in pressure alone can initiate these same responses remains to be tested.

Models for the Role of Caveolae in Mechanotransduction—
Based primarily on their location and appropriate molecular composition, a role for caveolae in mechanotransduction was proposed in 1995 (22, 46). Here, we show for the first time that mechanotransduction can occur at the luminal endothelial cell surface primarily through the caveolae. We localized, in caveolae, tyrosine phosphorylation and translocation events activating at least the downstream Ras-Raf-MAP kinase axis, which is known to turn on gene transcription. Disassembly of caveolae significantly reduces the level of endothelial cell surface protein phosphorylation as well as activation of MAP kinase, suggesting an important role for organized caveolae in the mechanosensing process. It appears from the available data that the caveolae have the necessary molecular machinery required for converting mechanical stimuli at the cell surface into the rapid chemical responses seen in the endothelium.

Our findings do not imply that other sites sensitive to flow do not exist. For instance, microdomains such as the focal adhesion sites located on the abluminal endothelial cell surface next to the extracellular matrix may play a role in the cellular response to flow. Interestingly, they may require significantly more time to become activated (10–60 min) (1, 47) and therefore are unlikely to mediate the most acute responses. Caveolae may specialize in early, rapid mechanotransduction events, especially occurring at the luminal cell surface.

Inherently, all endothelium are subjected to hemodynamic stressors. Yet, not all endothelium contain the same density of caveolae. Having caveolae may be especially relevant in tissues that undergo a high degree of stretch such as the lung and heart. Morphological studies show that the endothelium within these tissues contains a large population of caveolae (48). Caveolae may have a special role, perhaps by being more or less sensitive to particular types of forces that prevail in these tissues. In contrast, in tissue where repetitive oscillating stretch or large changes in flow are less often encountered, little need for a specialized flow sensor is required and thus may contain far fewer endothelial cell caveolae. Differential endothelial cell responses to flow in vivo as well as whether endothelium with few, if any, caveolae utilize alternate mechanisms to sense hemodynamic forces remain to be investigated.

Although the exact mechanisms whereby caveolae function in mechanotransduction requires further elucidation, several models can readily be constructed from the current data. In part, because caveolin may interact directly with lipid-anchored molecules such as Ras, G proteins (49), and eNOS (50), it may play a direct role in mechanotransduction. Caveolin polymerizes to create the invaginated form of caveolae (52, 53), which may act as a loaded tension-bearing coiled spring acutely responsive to changes in membrane tension. Hemodynamic forces are known to impose a strain on caveolae, which can distort them (54) and may, for instance, modulate caveolin conformation sufficiently to permit local activation and translocation events. Stress-induced alteration in caveolin conformation may permit the dissociation of caveolin-associated signaling molecules such as Ras, G proteins, Src-like kinases, and/or eNOS, thereby releasing them from the caveolin inhibitory clamp.

Recently, we have examined the effects of flow on eNOS activity in our system and find considerable eNOS activity within the caveolae, consistent with its caveolar localization (46, 55, 56). More importantly, incremental increases in vascular flow rate activate eNOS in caveolae in just 1 min (56). Flow-induced eNOS activation appeared to cause rapid dissociation from caveolin as well as rapid association with calmodulin. The association of eNOS and caveolin inhibits eNOS activity (51, 57, 58) Thus, it is possible that mechanical stress alters the conformation of caveolin, which releases eNOS from its inhibitory clamp, caveolin. This dissociation may, in itself, activate eNOS directly and/or may allow rapid activation through the binding of other positive protein regulators such as Ca²⁺/calmodulin, thereby providing a physiologically important mechanism for flow-regulated NO production. These studies provide additional evidence for the physiological relevance of caveolae in rapidly transducing mechanical stimuli such as flow stressors in endothelium in vivo.

Other structural components of caveolae may serve as mechanosensors. Recently, we have reported that dynamin forms an oligomeric structural collar around the neck of caveolae that functions in the fission and internalization of caveolae (18). Molecules located at the caveolar neck would reside adjacent to the luminal surface, which may place them in a position more suitable for reception of fluid shear forces. Molecular “sensing” at the neck of caveolae may propagate into the caveolae, resulting in direct or indirect activation of key caveolar signaling molecules such as Ras and eNOS or even ion channels. Whether an entity endowed with the potential to initiate the observed signaling events is located at or very near to the neck region of the caveolae is unknown.

The ability of caveolae to internalize may be important in signaling and mechanotransduction. Vesicular trafficking may move the signal originating in or near caveolae to new sites that may serve to enhance, reduce, or otherwise change the overall signaling event. Our data here provide little evidence of a loss of caveolae at the cell surface after flow stimulation up to 10 min, as suggested by the unchanging caveolin signal in P. It is possible, however, that internalization does occur with efficient recycling, thereby maintaining levels constant for caveolin/caveolae at the cell surface. It is also conceivable that endothelial cells may modulate their “sensitivity” to mechanical force by reducing the number of caveolae on their cell surface.

It remains to be seen whether caveolae act as flow-sensing organelles on the endothelial cell surface through their structure and overall organization (our preference at this time) or just contain a single flow-sensing protein that on its own is sufficient to initiate signaling events organized in the caveolae. One must consider the possible existence of multiple cell surface signaling molecules with “flow-sensing” capabilities that in the end may require appropriate organization and positioning on the cell surface in microdomains such as caveolae to reach an effective threshold level and propagate an effective and meaningful signaling cascade into the cell. Our findings here discover a new function for caveolae and thereby provide a new concept for defining the molecular events mediating mechanotransduction in endothelium.
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