Shear stress regulates occludin content and phosphorylation

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DeMaio, Lucas, Yong S. Chang, Thomas W. Gardner, John M. Tarbell, and David A. Antonetti. Shear stress regulates occludin content and phosphorylation. Am J Physiol Heart Circ Physiol 281:H105–H113, 2001.—Previous studies determined that shear stress imposed on bovine aortic endothelial cell (BAEC) monolayers increased the hydraulic conductivity (Lp); however, the mechanism by which shear stress increases Lp remains unknown. This study tested the hypothesis that shear stress regulates paracellular transport by altering the expression and phosphorylation state of the tight junction protein occludin. The effect of shear stress on occludin content was examined by Western blot analysis. Ten dyn/cm² significantly reduced occludin content in a time-dependent manner such that after a 3-h exposure to shear, occludin content decreased to 44% of control. Twenty dyn/cm² decreased occludin content to 50% of control and increased Lp by 4.7-fold after 3 h. Occludin expression and Lp depend on tyrosine kinase activity because erbstatin A (10 μM) attenuated both the shear-induced decrease in occludin content and increase in Lp. Shear stress increased occludin phosphorylation after 5 min, 15 min, and 3 h exposures. The shear-induced increase in occludin phosphorylation was attenuated with dibutyryl cAMP (1 mM), a reagent previously shown to reverse the shear-induced increase in Lp. We conclude that shear stress rapidly (≤5 min) increases occludin phosphorylation and significantly decreases the expression of occludin over 1–4 h. Alterations in the occludin phosphorylation state and occludin total content are potential mechanisms by which shear stress increases Lp.

The vascular endothelium is the principal barrier to water and solute transport between blood and the underlying tissue. Starling (30) was the first to describe the pressure forces that drive filtration of these molecules into the tissue. Hydrostatic pressure was identified as the principle force driving water and solutes out of the vasculature, whereas oncotic pressure was defined as an opposing resorptive force. Subsequently, Kedem and Katchalsky (17) modified the Starling hypothesis to define the hydraulic conductivity (Lp) of the endothelium. Lp is the membrane transport property that describes the relationship between water flux (Jw) and the hydrostatic and oncotic pressure gradients. Traditionally, Lp has been regarded as a constant property of the membrane. However, previous studies demonstrated that Lp is sensitive to the mechanical and hormonal environment. The flow of blood through the vascular system imparts a frictional force on the endothelium known as shear stress. Sill et al. (29) have reported that shear stress increases Lp of bovine aortic endothelial cell (BAEC) monolayers grown on porous polycarbonate filters, whereas other investigators (20, 34) have provided evidence for shear-dependent changes in Lp by using in vivo models. Shear-dependent endothelial transport provides a physiological mechanism whereby vessels can regulate tissue supply of nutrients in response to changes in blood flow. However, significant hemodynamic deviations may lead to blood-tissue barrier deregulation, a hallmark of numerous pathologies, including atherosclerosis, hypertension, and diabetes (5, 14, 24, 26).

Arterial vascular transport may occur via at least two nonexclusive pathways: 1) a transcellular vesiculo-mediated pathway such as vesiculo-vacuolar organelles and 2) through the intercellular clefts between adjacent endothelial cells. Tight junctions form the seal between adjacent cells, creating a barrier to transendothelial flux through the intercellular cleft. Pressure-driven Jw may pass between endothelial cells through infrequent discontinuities in the tight junction strands or through a putative small-pore system along the outer leaflets of tight junctions (13). Tight junctions consist of an assembly of at least nine distinct proteins that likely contribute to the regulation of endothelial permeability. Thus, to understand shear-induced increases in paracellular transport, it is necessary to...
characterize molecular changes that occur in the tight junctions. We examined the effects of shear stress on the tight junction proteins occludin, a transmembrane protein, and zonula occludens-1 (ZO-1), a peripheral membrane-associated protein believed to play a central role in organizing tight junction proteins (10).

Several studies suggest that occludin plays a direct role in barrier transport modulation. Occludin, as well as the recently identified claudin family and junction adhesion molecule, are transmembrane proteins that localize precisely to tight junction contact sites (11). The vasculature in neural tissue contains high levels of occludin and forms a highly restrictive barrier, while in nonneural tissue, occludin expression is lower and discontinuous, forming a more leaky barrier (15). Overexpression of occludin increases transendothelial electrical resistance and the number of tight junction fibrils in Madin-Darby canine kidney monolayers (22). Occludin confers adhesiveness when expressed in fibroblasts (33) and microinjection of occludin decreases paracellular transport in Xenopus oocytes (8). Furthermore, antisense oligonucleotides to occludin in human arterial endothelial cells increase solute flux (18). Occludin contains two extracellular loops that are believed to form a junctional seal (2). Wong et al. (35) determined that loop-binding peptides caused a significant decrease in transendothelial electrical resistance and loss of occludin content in Xenopus epithelial cells.

Vascular endothelial cell growth factor (VEGF)/vascular permeability factor, a potent endothelial cell mitogen, induces vascular hyperpermeability as well as angiogenesis (28). Previously, we defined a model of the blood-retinal barrier by using bovine retinal endothelial cells (BRECs) and demonstrated that VEGF/vascular permeability factor increases occludin phosphorylation after 15 min (3) and decreases occludin content after 3–6 h (4). In the present study, we report that shear stress induced similar changes in BAEC occludin content and phosphorylation. The shear-induced increase in \( L_p \) and decrease in occludin content occurs through a tyrosine kinase activation pathway because erbstatin A, a tyrosine kinase inhibitor, attenuated both responses. Furthermore, we show that dibutyryl (DB) cAMP reversed the shear-induced increase in occludin phosphorylation over 30 min, coincident with the ability of this compound to reverse the shear-\( L_p \) response (29). Thus changes in occludin content and phosphorylation may mediate shear stress-induced changes in water transport.

**METHODS**

**Chemicals.** The following chemicals were obtained from Sigma (St. Louis, MO): bovine serum albumin (BSA) (30% solution, fraction V), trypsin, minimal essential medium (MEM) without 1-glutamine, L-glutamine, HEPES, sodium bicarbonate, fetal bovine serum (FBS), fibronectin, N°, DB-cAMP, erbstatin A, herbimycin A, and Hanks’ balanced salt solution (without sodium bicarbonate). A 100× antibiotic-antimycotic mixture (penicillin G sodium, streptomycin sul-fate, amphotericin B) was obtained from GIBCO-BRL (Rockville, MD). The following reagents were used for gel electrophoresis and transfer: polyacrylamide from Amresco (Dallas, TX), 3-cyclohexylamino-1-propane sulfonic acid buffer from Research Organics (Cleveland, OH), and nitrocellulose with 0.22-µm pore size from Micron Separations (Westborough, MA). Polyclonal rabbit anti-occludin and anti-ZO-1 antibodies were obtained from Zymed Laboratories (San Francisco, CA). Dr. Bruce Stevenson (Department of Cell Biology and Anatomy, University of Alberta; Edmonton, Alberta, Canada) provided the ZO-1 rat monoclonal antibody clone R40–176. Donkey anti-rabbit Cy3 and donkey anti-rat Cy2 antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). We received calf intestinal alkaline phosphatase from New England Biolabs (Beverly, MA). Polycarbonate filters (Transwell Chambers, 0.4-µm pore size, 24.5-mm diameter) were obtained from Costar (Cambridge, MA).

**Cell culture.** Bovine thoracic aortas, 20- to 30-cm in length, were obtained from a local meat-processing plant immediately after the animals died. The animals died from bullet-free stun gun wounds to the head. The aortas were placed in a sterile jar containing ice-cold Hanks’ balanced salt solution with 1% antibiotic-antimycotic solution (100×). BAECs were harvested from the thoracic aortas within 2 h (n = 5 total) and cultured in T-75 flasks containing MEM supplemented with 10% FBS, as described previously (27). Each aorta resulted in multiple vials of BAECs. Experiments were conducted with cells derived from different primary cultures to ensure reproducibility. Cells of passages 4–10 were seeded at a density of \( 2.5 \times 10^5 \) cells/cm² onto glass slides and cultured in T-75 flasks containing MEM supplemented with 10% FBS, as described previously (27). Each experiment was performed on monolayers 4–5 days postseeding on glass slides and 7–9 days postseeding on polycarbonate filters. The day of the experiment, MEM-10% FBS cell culture media was replaced with experimental MEM-1% BSA, as described previously (29).

**Measurement of \( L_p \).** A detailed description of the experimental apparatus used to measure endothelial \( L_p \) is given by Sills et al. (29). The entire apparatus is contained within a Plexiglas box that is kept at an ambient air temperature of 37°C by means of a temperature controller and hair dryer. A Transwell filter (0.4-µm pores) containing the endothelial monolayer is sealed between a two-piece polycarbonate assembly separating the luminal and abluminal compartments. The luminal compartment has a 5% CO₂–95% gas port, which maintains the pH of the media at 7.4. Tygon and borosilicate glass tubing connects the abluminal compartment to an abluminal reservoir, which can be raised or lowered with respect to the endothelial monolayer. In this fashion, hydrostatic pressure is generated, and this results in fluid flow across the endothelial monolayer. To calculate water transport, a bubble is inserted into the borosilicate tubing and tracked with a spectrophotometer as it moves with the fluid flow. A personal computer reads and composes the data from the bubble tracker to produce a record of time versus bubble displacement. Bubble displacement is converted to \( J_v \) according to the following equation

\[
J_v = (\Delta d/\Delta t) \cdot F
\]

where \( (\Delta d/\Delta t) \) represents bubble movement per unit time, and \( F \) is a tube calibration factor, i.e., fluid volume contained in a known length of tubing. Because the abluminal and...
luminal compartments contain the same media (MEM-1% BSA), there is a negligible osmotic gradient present. Therefore, hydraulic conductivity, \( L_p \), can be calculated as:

\[
L_p = J_V / A / \Delta P
\]

where \( \Delta P \) is the hydrostatic pressure differential across the monolayer (10 cm\( H_2O \)), and \( A \) is the area of the monolayer.

**Application of shear stress.** A cylindrical disk is within the luminal compartment, which imparts a defined rotational shear stress on the monolayer, given by

\[
\tau = \mu \omega R / h
\]

where \( R \) is the cylindrical disk radius (12.25 mm), \( \omega \) is the rotation frequency (2,827 or 5,654 rad/min for 10 or 20 dyn/cm\(^2\), respectively), and \( h \) is the gap distance between the rotating disk and the endothelial surface (487 \( \mu \)m). The absolute viscosity, \( \mu \), of MEM-1% BSA measured with a capillary viscosimeter (model H42, Canon-Fenske) is 8.434 \( \times 10^{-3} \) Poise at 37°C (29). The maximum shear stress (10 or 20 dyn/cm\(^2\)) occurs at the periphery of the disk, and decreases proportionately with distance to zero at the center of the disk. The average shear stress imposed by the rotating disk across the endothelial membrane is two-thirds of the maximum shear stress.

To examine the effect of shear stress on tight junction protein expression, cells were grown within a Transwell filter holder (24.5 mm diameter), which had been glued with the use of a silicone elastomer kit (Sylgard; Midland, MI) to a glass slide after removal of the polycarbonate filter. An aluminum plate lab jack supports the glass slide against the upper assembly, defining the luminal compartment. There is no abluminal compartment when shearing BAECs plated on glass slides. Monolayers treated with erstatin A or hemiycin A were incubated at 37°C for 1 h before shear exposure.

**Immunohistochemistry.** For immunohistochemical staining of occludin and ZO-1, BAECs on the glass slide were washed twice with phosphate-buffered saline (PBS), fixed in 1% paraformaldehyde for 10 min, permeabilized with PBS containing 0.2% Triton X-100 for 10 min, and blocked with PBS containing 10% BSA and 0.1% Triton X-100 for 1 h. Cells were incubated with anti-occludin polyclonal rabbit antibody (1:1,000) and anti-ZO-1 monoclonal rat antibody (1:1) for 1 h, then washed with PBS containing 0.1% Triton X-100 five times, and were incubated with donkey anti-rabbit Cy2 antibody (1:200) and donkey anti-rat Cy3 antibody (1:200). Slides were washed four times with PBS containing 0.1% Triton X-100 and coverslips were mounted with the use of Aqua Poly/Mount (Polysciences; Warrington, PA). A video microscope was used to capture digital images on a personal computer running Optimas software (Media Cybernetics; Silver Spring, MD). All images were handled identically.

**Immunoblotting.** Western blotting allowed quantification of relative changes in occludin content and identification of a posttranslational modification to occludin. After the monolayer was sheared on the glass slide, cells were immediately washed with ice-cold PBS and lysed in a SDS extraction buffer composed of 0.2% SDS, 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 2 mM EDTA, 10 mM HEPES, 10 mM NaF, 1 mM NaVO\(_4\), 1 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride, except in the experiments described in Fig. 2C, where cells were instead homogenized in a urea buffer composed of 6 M urea, 0.1% Triton X-100, 10 mM Tris pH 8.0, 1 mM dithiothreitol, 5 mM MgCl\(_2\), 5 mM EGTA, 150 mM NaCl, 10 mM NaF, 1 mM NaVO\(_4\), and 0.2 mM phenylmethylsulfonyl fluoride. Insoluble material was separated from lysate by centrifugation in a microfuge at 10,000 g for 10 min. Protein concentrations were determined by protein assay (Bio-Rad) and equal protein was loaded onto 7.5% or 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose (MSI; Westborough, MA), blocked with 5% milk in Tris-buffered saline, and immunoblotted with anti-occludin (1:3,000) or anti-ZO-1 (1:1,500) polyclonal antibody followed by anti-rabbit alkaline phosphatase-linked secondary antibody. Occludin and ZO-1 content were determined by using an enhanced chemiluminescence kit (Amer sham Pharmacia Biotech; Piscataway, NJ), a 595-fluorimeter, and Imagequant analysis software (Molecular Dynamics; Sunnyvale, CA).

**Statistical analysis.** Occludin content quantifications are normalized relative to controls and presented as means ± SE. Significance was determined by unpaired Student’s t-test between two groups of data or analysis of variance (ANOVA) with Student-Newman-Keuls multiple comparisons test between four or more groups of data (\( P < 0.05 \), \( P < 0.005 \)). \( L_p \) measurements were averaged at 5-min intervals, normalized with respect to the baseline value at 60 min, and presented as means ± SE. Significant differences between shear and shear + erstatin \( L_p \) means were analyzed by two-way ANOVA by using statistical analysis software with a Bonferroni correction (\( P < 0.05 \)). \( n \) was used in the statistical calculations and refers to the number of glass slides or Transwell filters.

**RESULTS**

**Localization of occludin and ZO-1 in BAEC.** Immunohistochemical staining was performed to examine the distribution of occludin and ZO-1 in BAEC monolayers. Fluorescent microscopy demonstrated that occludin immunoreactivity was observed within the cell cytoplasm but was concentrated predominantly along the cell border (Fig. 1A). ZO-1 immunoreactivity was discontinuous but appeared stronger at cell-cell contacts than occludin immunoreactivity (see Fig. 1B). Both ZO-1 and occludin immunoreactivity colocalized at cell-cell contacts. A negative control that excluded primary antibody incubation had little immunoreactivity (not shown).

**Effect of shear stress on occludin and ZO-1 protein content.** Formation of strong intercellular barriers is correlated with the expression of tight junctions. Western blotting was performed to determine whether the shear-induced increase in \( J_V \) is associated with altered occludin content in BAEC monolayers (Fig. 2A). Occludin migrates as two major bands at ~58 and ~60 kDa, which we have termed occludin-\( \alpha \) and occludin-\( \beta \), respectively (3). In BAECs that were lysed with an SDS-based extraction buffer, 3 h of 10 and 20 dyn/cm\(^2\) shear stress reduced total occludin content (\( \alpha + \beta \)) to 44% and 50% of control, respectively (Fig. 2B). The difference between control and sheared BAECs was significant (\( P < 0.005 \)); there was no significant difference between the two magnitudes of shear. To exclude the possibility that shear stress caused occludin to move to an insoluble pool, BAECs were lysed with the use of a urea-based extraction buffer after application of 10 dyn/cm\(^2\) for the indicated time exposures (Fig. 2C).
Shear stress decreased occludin content to 72%, 53%, 61%, and 26% of control after 1-, 2-, 3-, and 4-h exposures to 10 dyn/cm², respectively. The difference between control lysates and each time exposure was significant (P < 0.05). Because both extraction buffers yielded very similar results, shear stress most likely reduces occludin content rather than stimulating occludin movement to an insoluble pool. Interestingly, ZO-1 content was not affected by a 3-h exposure to 10 or 20 dyn/cm² shear stress (Fig. 2).

**Effect of tyrosine kinase inhibition on the shear-induced changes in L_p and occludin protein content.** A few studies (7, 31) indicate an increase in tyrosine phosphorylation in at least nine proteins between 30 and 220 kDa due to shear stress. None of the indicated phosphoproteins migrate in the same position as occludin. Figure 3 indicates an increase in protein tyrosine phosphorylation in at least nine proteins between 30 and 220 kDa due to shear stress. None of the indicated phosphoproteins migrate in the same position as occludin. Figure 3 illustrates the L_p response to 20 dyn/cm² with and without addition of erbstatin A (10 μM), a general tyrosine kinase inhibitor. Twenty dyn/cm² increased normalized mean L_p by 4.70 ± 0.20-fold after 3 h (baseline L_p was 2.48 ± 0.26 × 10⁻⁷ cm·s⁻¹·cmH₂O⁻¹).

Shear stress decreased occludin content to 72%, 53%, 61%, and 26% of control after 1-, 2-, 3-, and 4-h exposures to 10 dyn/cm², respectively. The difference between control lysates and each time exposure was significant (P < 0.05). Because both extraction buffers yielded very similar results, shear stress most likely reduces occludin content rather than stimulating occludin movement to an insoluble pool. Interestingly, ZO-1 content was not affected by a 3-h exposure to 10 or 20 dyn/cm² shear stress (Fig. 2D).
BAECs incubated with erbstatin A did not alter the shear- \( L_p \) response during the first 2 h of shear exposure. However, this agent significantly attenuated the shear- \( L_p \) during the third hour of shear exposure (\( P < 0.05 \)). At 240 min, normalized mean \( L_p \) increased by 3.66 \( \pm \) 0.66-fold (baseline \( L_p \) was 2.62 \( \pm \) 0.30 \( \times \) 10\(^{-7} \) cm s\(^{-1} \) cmH\(_2\)O\(^{-1} \)) in the presence of erbstatin A.

To implicate occludin content as a determinant of \( L_p \), we compared occludin content in control monolayers unexposed to shear and monolayers exposed to 20 dyn/cm\(^2\) for 3 h, with and without 10 \( \mu \)M of erbstatin A treatment by immunoblot analysis (Fig. 4B). Three-hour exposure to shear reduced occludin content to 51% of control, whereas in monolayers treated with erbstatin A, occludin content was decreased to 75% of control (Fig. 4C). Occludin content was significantly higher in sheared monolayers treated with erbstatin A than untreated sheared monolayers (\( P < 0.05 \)). Erbstatin A decreased occludin content in BAECs unexposed to shear compared with untreated static monolayers, but was not statistically significant. There was no statistically significant difference between static control monolayers treated with erbstatin A and sheared monolayers treated with erbstatin A. In Fig. 4D, the effect of 1 \( \mu \)M herbiycin A, another tyrosine kinase inhibitor was also examined between static and sheared monolayers. Four-hour exposure to 10 dyn/cm\(^2\) of shear stress reduced occludin content to 46% of control in untreated monolayers compared with 74% of control in herbiycin A-treated monolayers; the difference between these two groups was significant (\( P < 0.05 \)). There was no statistically significant difference between static control BAECs treated with herbiycin A and sheared BAECs treated with herbiycin A. Neither erbstatin A nor herbiycin A significantly altered the expression of occludin-\( \beta \) relative to occludin-\( \alpha \) (discussed below).

**Effect of 10 dyn/cm\(^2\) shear stress on endothelial occludin phosphorylation.** Shear stress altered the migration of BAEC occludin through SDS-polyacrylamide gels. We have previously shown that alkaline phosphatase treatment collapsed the slower migrating occludin bands in BREC monolayers and in retinal capillaries to a single band; therefore, the slower migrating 60 kDa band exists at a higher phosphorylation state than the 58 kDa band (3). Treatment of BAEC monolayers with alkaline phosphatase also collapsed the 60 kDa band to a single band at 58 kDa (blot not shown), indicating that occludin exists in multiple phosphorylation states in BAECs as well. Occludin phosphorylation was quantified as the ratio of occludin-\( \beta \) content to occludin-\( \alpha \) content. The ratio of occludin-\( \beta / \alpha \) increased significantly after 5-min, 15-min, and 3-h exposures to 10 dyn/cm\(^2\) shear stress (Fig. 5B; \( P < 0.005 \)), indicating that occludin phosphorylation is rapid and sustained for at least 3 h. Five- and fifteen-min exposures to shear did not alter total occludin content compared with static controls (data not shown). Neither erbstatin A nor herbiycin A attenuated the shear-induced increase in occludin phosphorylation (data not shown).

**Effect of DBcAMP on shear-induced changes in occludin phosphorylation and content.** Increased endothelial cell cAMP decreases capillary \( L_p \) (1) and protects against endothelial barrier dysfunction in response to proinflammatory mediators (21). Previously, we established that DBcAMP, a membrane-permeable cAMP analog, rapidly reversed the shear-\( L_p \) response. To correlate DBcAMP-induced decreases in \( L_p \) with occludin phosphorylation and total content, DBcAMP (1 mM) was added to the monolayer after a 3-h exposure to 10 dyn/cm\(^2\) and the experiment was continued for an additional 30 min. Afterwards, BAEC monolayers were lysed in a SDS-based extraction buffer and immunoblotted for occludin (Fig. 6A). \( \beta / \alpha \) was significantly lower in monolayers treated with DBcAMP during the last 30 min of a 3.5-h shear exposure period than in untreated monolayers exposed to 3.5 h of shear stress and was not statistically different from control (static) monolayers (Fig. 6C; \( P < 0.05 \)). DBcAMP did not reverse the shear-induced decrease in occludin content within 30 min.

**DISCUSSION**

The flow of blood through the vascular system imparts two physical forces on the vascular wall: transmural pressure, a normal force imposing a circumferential stress on the endothelium, and shear stress, a tangential friction force acting on the luminal membrane of the endothelium. It is now clear that both of these forces modulate transendothelial fluid flux. Tight junctions form very close contacts between adjacent cells, creating a rate-limiting barrier to paracellular transport. In this study, we present evidence that shear stress alters tight junction proteins coincident with changes in \( J_V \). Immunocytochemistry demon-
strates that occludin and ZO-1 localize to the cell border of in vitro BAECs, which is consistent with the formation of tight junctions. The intensity and continuity of tight junction immunoreactivity at cell-cell contacts is likely less than that observed in high-resistance cell monolayers, such as brain endothelial cells (15) or Madin-Darby canine kidney cells (12). Nonetheless, by using our BAEC in vitro model, we report that shear stress rapidly increases occludin phosphorylation state and, over 1–4 h, decreases occludin content. Changes in occludin phosphorylation state and occludin content are possible mechanisms by which shear stress increases transendothelial L

Occludin likely plays a functional role in the modulation of tight junction barrier integrity by shear stress. Our data shows that shear stress decreased occludin expression in BAECs in a time-dependent manner. This reduction in occludin content may decrease paracellular resistance, causing an increase in fluid flux. Both 10 and 20 dyn/cm² reduced occludin content by ~50% after 3 h. Therefore, occludin sensitivity to shear stress in our model system is independent of shear magnitude between 10 and 20 dyn/cm². Because the observed change in occludin content was similar by using either SDS or urea-based extraction buffers, it is unlikely that the observed decrease in occludin content is due to the movement of occludin to an insoluble pool.

In contrast to the effect of shear on occludin, 3-h exposure of BAEC monolayers to 10 dyn/cm² did not reduce total ZO-1 protein content. It is likely that ZO-1 and occludin are differentially regulated in response to shear stress and immunoblotted with anti-occludin. Slower-migrating 60-kDa band (occludin-β) exists at a higher phosphorylation state than the 58-kDa band (occludin-α), thus occludin phosphorylation was quantified as the ratio of occludin-β to occludin-α content. B: exposure to 10 dyn/cm² increased the ratio of occludin-β/α at all time points investigated (n = 5 glass slides; n = 1 thoracic aorta). Five - and 15-min exposures to shear stress did not significantly alter occludin content compared with static controls. *=P < 0.005 vs. static control.

Fig. 5. A: 10 dyn/cm² shear stress rapidly increased occludin phosphorylation. Monolayers were lysed in SDS extraction buffer after the indicated time exposures to shear stress and immunoblotted with anti-occludin. Slower-migrating 60-kDa band (occludin-β) exists at a higher phosphorylation state than the 58-kDa band (occludin-α), thus occludin phosphorylation was quantified as the ratio of occludin-β to occludin-α content. B: exposure to 10 dyn/cm² increased the ratio of occludin-β/α at all time points investigated (n = 5 glass slides; n = 1 thoracic aorta). Five- and 15-min exposures to shear stress did not significantly alter occludin content compared with static controls. *=P < 0.005 vs. static control.

Fig. 4. Tyrosine kinase inhibition attenuated both the shear-induced increase in L

and decrease in occludin content. A: L
response to 20 dyn/cm² with and without erbstatin A treatment (10 μM) is shown. At time 0, a hydrostatic pressure gradient of 10 cmH₂O was applied and L
was measured for 1 h to establish a baseline. Addition of 20 dyn/cm² at 60 min elicited a time-dependent increase in L
(n = 8 Transwell filters; n = 2 thoracic aortas). Compared with shear stress controls without the tyrosine kinase inhibitor, erbstatin A significantly inhibited the shear-L
response during the last hour of shear exposure (n = 4 Transwell filters; n = 1 thoracic aorta). *P < 0.05 vs. untreated sheared condition in A. B: immunoblot probed for occludin in BAECs unexposed to shear (lane C) and BAECs exposed to 20 dyn/cm² for 3 h (lane 3h) with and without erbstatin A (Erb) treatment. C: Erb significantly attenuated the shear-induced decrease in occludin content (n = 6 glass slides; n = 1 thoracic aorta). There was no statistically significant difference in occludin content between control monolayers (lane C) and control monolayers treated with erbstatin A (lane C + Erb). D: BAEC monolayers were exposed to 10 dyn/cm² for 4 h with and without 1 μM treatment of herbimycin A (Hrb), another tyrosine kinase inhibitor (blot not shown). Hrb also attenuated the shear-induced decrease in occludin content (n = 8 glass slides/n = 1 thoracic aorta). *P < 0.05 and **P < 0.005 vs. static control.
regulation of paracellular permeability by shear stress involves degradation of occludin but not of ZO-1.

At present, the molecular mechanism by which shear stress increases hydraulic conductivity remains to be determined. However, shear stress activation of at least two receptor tyrosine kinases for VEGF, fms-like tyrosine kinase (32) and fetal liver adhesion kinase-1/kinase-insert domain receptor (7), as well as protein-tyrosine kinases at focal adhesion sites such as focal adhesion kinase and c-Src (16) are early events of mechanotransduction. Therefore, shear stress may increase vascular permeability by stimulating tyrosine kinase activation pathways that originate from both the luminal and abluminal membranes of endothelial cells. Furthermore, shear stress increases the association of the adaptor protein Shc with both fetal liver adhesion kinase-1/kinase-insert domain receptor and mechanosensitive integrins at focal adhesion sites (7). We determined that erbstatin A, a tyrosine kinase inhibitor, partially attenuated both the increase in $L_p$ and decrease in occludin content induced by shear stress after 3 h. These results support our hypothesis that reduction of occludin content is a mechanism by which shear stress increases $L_p$.

However, the shear-$L_p$ response may only partially depend on tyrosine kinase activation. Previously we determined that the shear-$L_p$ response in BAECs is also mediated by a nitric oxide-dependent mechanism (6). It is possible that shear stress triggers a NO signaling cascade that is independent of tyrosine kinase activation. Furthermore, the shear-induced increase in occludin phosphorylation was not reversed by erbstatin A, suggesting that occludin phosphorylation may initiate an increase in $L_p$ without tyrosine kinase activation. Alternatively, the tyrosine kinase inhibitors used may not have effectively inhibited the tyrosine kinase activation pathways relevant to occludin phosphorylation. Finally, occludin phosphorylation may initiate degradation or other changes in tight junction assembly, which are themselves dependent on tyrosine kinase activity, and lead to further increases in paracellular $J_v$.

Previously, we showed that 10 dyn/cm$^2$ shear stress caused a 2.9-fold increase in $L_p$ after 3 h (29). Addition of DBcAMP to the monolayer after 3 h of shear stress reversed $L_p$ from 2.9 to 1.7 times its baseline over 30 min (42% reduction in $J_v$). In the present study, we repeated this time course and examined the effect of DBcAMP on occludin phosphorylation state and content. DBcAMP caused a significant reduction in occludin phosphorylation compared with untreated monolayers, but did not reverse the shear-induced decrease in total occludin content within 30 min. Intracellular cAMP regulates serine/threonine kinases and phosphatases and therefore may regulate occludin phosphorylation state (25). These results suggest that DBcAMP may decrease endothelial $L_p$ by reducing occludin phosphorylation. DBcAMP restored occludin phosphorylation levels to preshear values but only partially attenuated the shear-induced increase in $L_p$. This may be due to the inability of DBcAMP to reverse

Shear stress. Shear stress causes endothelial cells to undergo shape change, which may require degradation of transmembrane proteins, such as occludin, at cell-cell contacts. ZO-1, a peripheral membrane-associated protein, plays a central role in tight junction disassembly/reassembly (9, 10) and may not be degraded in the shear-induced reorganization of the cytoskeleton and cell-cell contacts. In a study examining the effect of shear stress on endothelial cell adherens junctions, Noria et al. (23) showed that an 8.5-h exposure to 15 dyn/cm$^2$ reduced transmembrane vascular endothelial-cadherin protein levels but not cytoplasmic α- and β-catenin levels. Therefore, it is plausible that the
the shear-induced decrease in occludin content in this short time course. Less occludin content may increase the frequency of discontinuities between tight junctions, preventing the complete return to preshear baseline $L_P$. Nonetheless, changes in occludin phosphorylation alone may allow for efficient $J_V$ modulation.

In conclusion, it is likely that blood flow more acutely regulates occludin phosphorylation than total occludin content. In our BAEC model system, shear stress increases occludin phosphorylation within 5 min and significantly increases $L_P$ after 30 min (29), before decreasing occludin content. Similarly, VEGF increases occludin phosphorylation in BREC monolayers by 15 min (3) and increases water transport through BREC after 30 min (19). Therefore, both mechanical and hormonal changes within the vasculature may increase occludin phosphorylation, resulting in increased paracellular transport before a decrease in occludin content. In diseased states, chronic alterations in vessel wall shear stress or elevated levels of VEGF likely affect both tight junction phosphorylation states and total content which, in turn, contribute to blood-tissue barrier breakdown and increased transendothelial transport.

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