

Shear stress regulates aquaporin-5 and airway epithelial barrier function

Venkataramana K. Sidhaye*[†], Kelly S. Schweitzer*, Michael J. Caterina*[§], Larissa Shimoda*, and Landon S. King**

*Department of Medicine, Division of Pulmonary and Critical Care Medicine, and Departments of [†]Biological Chemistry and [§]Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

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As the interface with the outside world, the airway epithelial barrier is critical to lung defense. Because of respiratory efforts, the airways are exposed to shear stress; however, little is known regarding the effects of shear on epithelial function. We report that low-level shear stress enhances epithelial barrier function, an effect that requires serial activation of the transient receptor potential vanilloid (TRPV) 4 and L-type voltage-gated calcium channel (VGCC) and an increase in intracellular calcium. These changes lead to a selective decrease in aquaporin-5 (AQP5) abundance because of protein internalization and degradation. To determine whether AQP5 plays a role in mediating the shear effects on paracellular permeability, we overexpressed hAQP5 in 16HBE cells, an airway epithelial cell line without endogenous AQP5. We found that AQP5 expression was needed for shear-induced barrier enhancement. These findings have direct relevance to the regulation of epithelial barrier function, membrane permeability, and water homeostasis in the respiratory epithelia.

permeability | epithelium | transient receptor potential vanilloid | voltage-gated calcium channel | cytoskeleton

Mechanical forces elicit biologically relevant signals, best demonstrated in the cardiovascular and musculoskeletal systems, where stretch and shear alter differentiation and proliferation (1–5). The airway wall exists in a mechanically dynamic environment, and each breath causes circumferential and longitudinal expansion and contraction (6). Airway epithelial cells are exposed to luminal shear stress (frictional force per surface area) generated by airflow, which at rest breathing is 0.5–3 dynes/cm². Shear forces may be a log higher with exercise (7, 8) and may increase to nearly 1,700 dynes/cm² with cough and bronchospasm (8). In asthma, smooth muscle constriction produces airway folding, reducing luminal caliber producing heterogeneous shear at different parts along the airway (9).

Endothelial shear stress alters cytoskeletal organization, cell shape, and gene expression (10–20). Endothelial shear increases intracellular calcium concentration [Ca²⁺]_i, triggering nitric oxide production (20), changes in actin stress fiber formation, and altered paracellular permeability (21) in part due to activation of transient receptor potential vanilloid (TRPV) 4, a nonselective cation channel (22). Mechanical stimulation alters airway epithelial EGF signaling (23), surface liquid height (8), and ciliary beat frequency (24). TRPV4 is also present in airway epithelial cells and is activated by temperature, hypotonicity (25–28), and shear stress (22, 29–31). TRPV4 activation has been implicated in alveolar septal barrier function (32), but its role in airway epithelia remains undefined.

We find that low levels of shear stress enhance airway epithelial barrier function, an effect mediated through increases in [Ca²⁺]_i by sequential activation of TRPV4 and voltage-gated calcium channel (VGCC). Although VGCC has been implicated in lung vascular tone, its role in airway epithelia is unclear. Calcium influx triggers selective degradation of aquaporin-5 (AQP5), an apical channel with high selectivity for water (33). In salivary gland cells, AQP5 also may participate in the regulation of paracellular permeability (34). Similarly, we find that

altered AQP5 expression modulates paracellular permeability in response to shear stress in airway epithelial cells. These studies provide insight into a homeostatic mechanism likely central to airway epithelial function.

Results

Shear Stress Alters Epithelial Paracellular Permeability. To examine the effects of shear on epithelial barrier function, we differentiated primary human bronchial epithelial cells (NHBE) at an air-liquid interface, exposed them to shear for 1–2 h (see *Materials and Methods*), and then transferred the membrane to an intact transwell chamber for measurement of FITC-dextran permeability. Shear stress decreased paracellular permeability (Fig. 1A).

To examine the effects of shear on epithelial permeability in intact trachea, mouse trachea was resected and attached to a ventilator (see *Materials and Methods*) to create phasic airflow. After 1 h of ventilation, Evans blue dye-4% albumin, a commonly used tracer that, because of its size, is restricted to paracellular movement, was instilled in the trachea for 20 min. Then the trachea was flushed with PBS to eliminate residual dye, which was confirmed by visual inspection. Flows of 10 and 50 μl/s (1 and 5 dynes/cm²) decreased Evans blue permeation in tissue homogenates compared with static conditions (Fig. 1B), indicating that low levels of shear enhance barrier function in both human airway epithelial cells and mouse trachea.

Shear-Induced Changes in Permeability Are Mediated by TRPV4 and L-Type Calcium Channels. Endothelial shear increases [Ca²⁺]_i (35, 36), with subsequent actin reorganization and stress fiber formation (21). Similarly, fura-2-loaded NHBE cells exposed to flow (0.5 ml/min; 1.5 dynes/cm²) exhibited increased [Ca²⁺]_i (Fig. 2B). NHBE cells express both TRPV4 and TRPV1 (Fig. 2A). Addition of ruthenium red (RR), a nonselective TRPV inhibitor, blocked the increase in [Ca²⁺]_i. Neither capsaizepine, a TRPV1 inhibitor (data not shown), nor the T-type channel inhibitor, mibefredil (Fig. 2B), block the shear-induced increase in [Ca²⁺]_i. In contrast, the VGCC inhibitor, nifedipine, blocked the shear-induced increases in [Ca²⁺]_i. Increasing flow increased [Ca²⁺]_i (Fig. 2C). This response was blocked by perfusion with calcium-free buffer, RR, or the VGCC inhibitors (Fig. 2C).

To examine the relation between calcium and barrier function, we exposed NHBE cells to shear in the presence or absence of calcium-free medium, nifedipine, and RR: All three agents blocked the shear-induced changes in paracellular permeability (Fig. 3A). Similarly, treatment of trachea with nifedipine blocked the shear-induced enhancement of barrier function (reduction in

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The authors declare no conflict of interest.

[†]To whom correspondence should be addressed. E-mail: vsidhay1@jhmi.edu.

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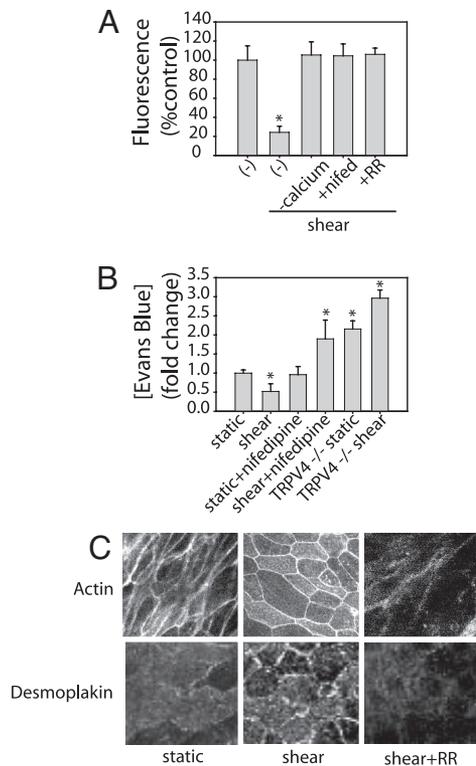


Fig. 3. TRPV4 or VGCC inhibition block shear-induced barrier enhancement. (A) NHBE cells were exposed to shear in the presence or absence of calcium-free Krebs, 20 μ M RR, or 10 μ M nifedipine. FITC-dextran permeability was measured by fluorescence. All three blocked the shear-induced reduction in paracellular permeability. (B) Trachea incubated in 10 μ M nifedipine or trachea isolated from TRPV4^{-/-} mice showed no reduction in paracellular permeability in response to shear stress. (C) In NHBE cells, shear stress alters actin expression and causes redistribution of desmoplakin. Both are prevented by the addition of 20 μ M RR to the perfusate ($n = 3$). *, $P < 0.05$ with ANOVA one-way Bonferroni correction.

Exposure in the opposite direction (from distal to proximal trachea) produced similar changes (prestenosis reduction in AQP5, poststenosis no change), indicating a primary effect of flow, rather than tracheal orientation (data not shown).

AQP5 and Paracellular Permeability. Because TRPV4 and VGCC mediated increases in $[Ca^{2+}]_i$ lead to both decreased AQP5 and decreased paracellular permeability, we examined the relation of AQP5 to paracellular permeability. 16HBE cells, which endogenously express TRPV4 but not AQP5 (data not shown), were grown on inserts to confluence and infected with either Adeno-GFP or Adeno-AQP5 (85–90% infection efficiency). As with NHBE, shear decreased AQP5 abundance in adeno-hAQP5 infected 16HBE cells (Fig. 7A). 16HBE cells expressing hAQP5 had increased baseline paracellular permeability, compared with either GFP-expressing cells or uninfected 16-HBE cells (Fig. 7B), and less cortical actin (Fig. 7C). In GFP-expressing cells, shear did not alter paracellular permeability (Fig. 7B). In AQP5-expressing cells, shear reduced paracellular permeability. In AQP5-expressing cells, shear stress induced a peripheral redistribution of both actin and desmoplakin (Fig. 7C).

Discussion

The airway epithelial barrier serves several functions. In addition to protecting against infectious and noninfectious respirable particles, the epithelium segregates signals initiated in the apical and basal compartments (38, 39). Disruption of the epithelial

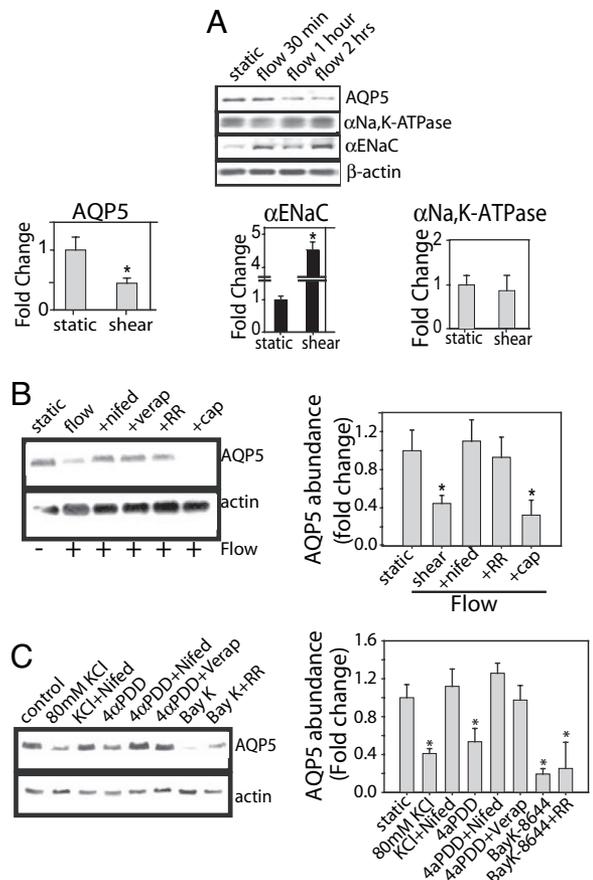


Fig. 4. Shear stress alters epithelial transporter expression. (A) In NHBE cells, shear stress decreased AQP5 abundance and increased α ENaC expression with no change in α Na, K-ATPase expression. (B) NHBE cells treated with inhibitor were exposed to shear stress for 2 h. TRPV4 inhibition with 20 μ M RR and VGCC inhibition with 10 μ M verapamil or 10 μ M nifedipine blocked shear-induced decreases in AQP5; the TRPV1 inhibitor, capsaizepine (50 μ M), had no effect. (C) In NHBE cells, under static conditions, TRPV4 activation with 4aPDD decreases AQP5, which was blocked by VGCC inhibition with nifedipine. VGCC activation with BayK-8644 decreased AQP5; the TRPV4 inhibitor, RR, did not prevent the decrease ($n = 3$). *, $P < 0.05$ with ANOVA one-way Bonferroni correction.

barrier as occurs during infection, inflammation, or asthma is believed to contribute to disease expression (39–42). Our findings indicate that shear forces can regulate barrier function and provide insight into an important homeostatic mechanism.

Using both cell culture and intact trachea, we show that shear stress enhances epithelial barrier function. Although transepithelial electrical resistance is often used as a surrogate for permeability, it measures instantaneous open probability of pores within tight junction strands, rather than the passage of solute and may diverge from solute permeability, highlighted when chicken occludin was expressed in MDCK cells, causing increased electrical resistance and solute permeability (43, 44). In our study, calcium influx followed sequential activation of TRPV4 and VGCC, and solute permeability was blocked by either channel. TRPV4 functions as a molecular integrator of biophysical stimuli relevant to the airway luminal environment, such as temperature, hypotonicity, and shear stress (28). In mammary epithelial cells (45) and in the lung vasculature (46), TRPV4 activation modulates paracellular permeability. It appears to play a similar role in the airways.

AQP5 abundance was altered by shear, and shear-induced modulation of paracellular permeability did not occur in the

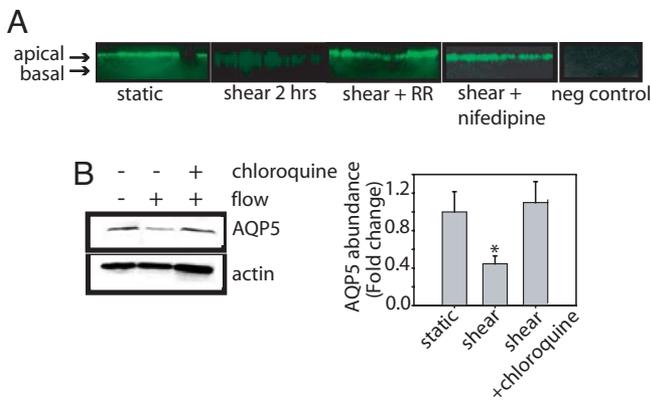


Fig. 5. Shear-induced AQP5 internalization and degradation. (A) Immunofluorescence reveals that in NHBE cells, 2 h of shear stress leads to AQP5 internalization, which was blocked by TRPV4 inhibitor, 20 μ M RR, or VGCC inhibitor, nifedipine (10 μ M), with preimmune serum control. (B) NHBE cells were exposed to shear stress in the presence of absence of the lysosome inhibitor, chloroquine (100 μ M), and harvested after 2 h. Chloroquine blocked the shear-induced reduction in AQP5 abundance ($n = 3$). *, $P < 0.05$ with ANOVA one-way Bonferroni correction.

absence of AQP5. Kawedia *et al.* (34) demonstrated decreased paracellular permeability in salivary gland cells from AQP5-null mice, suggesting a role for AQP5 in cell–cell interactions. Our findings are consistent with those observations. Shear provoked changes in cell-shape change, as well as actin and desmoplakin

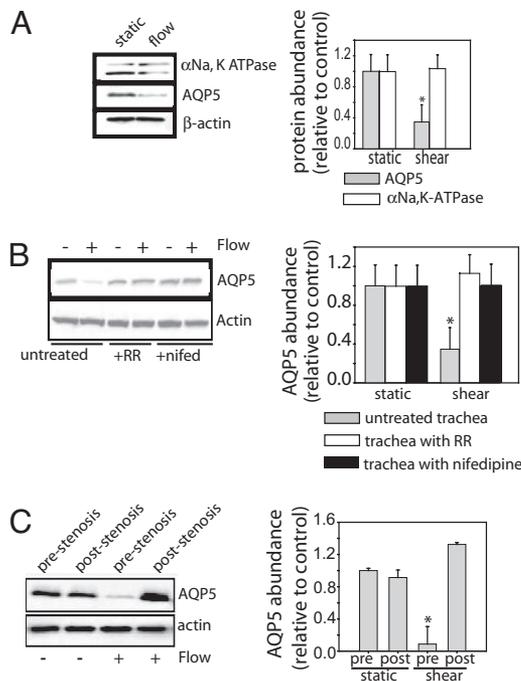


Fig. 6. Phasic shear stress created by airflow decreased AQP5 abundance in mouse trachea. (A) *Ex vivo* mouse trachea was exposed to either static or shear conditions for 1 h. Shear stress decreased AQP5 abundance without a change in α 1Na, K-ATPase. (B) Nifedipine (10 μ M) or RR (20 μ M) prevented the shear-induced decrease in AQP5 abundance. (C) Flow disruption by a midtracheal stenosis altered AQP5 expression. Laminar flow created greater shear stress proximal to the obstruction, whereas turbulent flow led to lower shear stress distally. Higher shear proximal to the obstruction caused less AQP5 expression compared with the distal, lower shear region ($n = 3$). *, $P < 0.05$ with ANOVA one-way Bonferroni correction.

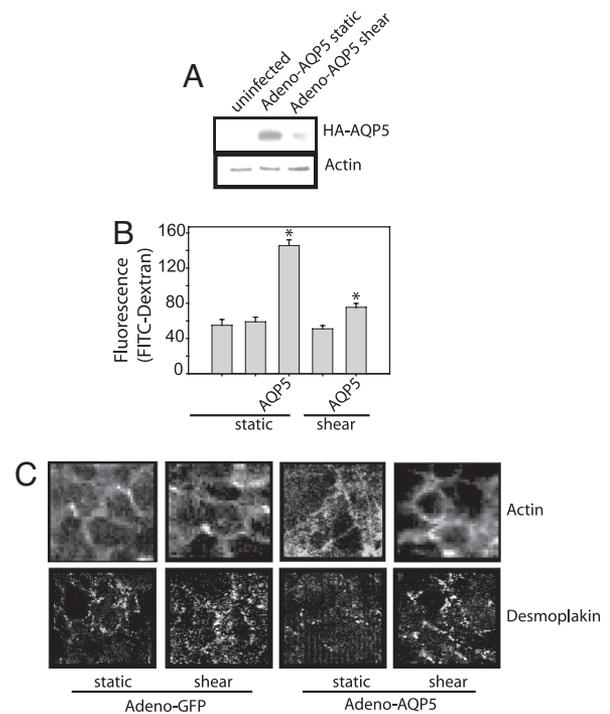


Fig. 7. The presence of AQP5 alters shear-induced changes in paracellular permeability. (A) 16HBE cells expressing Adeno-hAQP5 were exposed to static or shear conditions. AQP5 expression decreased after 2 h of shear stress. (B) 16HBE cells infected with Adeno-GFP virus or Adeno-hAQP5 were exposed to static or shear. Presence of Adeno-hAQP5 caused increased paracellular permeability, compared with either infection with a GFP control or an uninfected control. After the application of shear stress, Adeno-hAQP5-expressing cells had significantly decreased paracellular permeability, compared with static conditions ($n = 3$). *, $P < 0.05$ with ANOVA one-way Bonferroni correction. (C) 16HBE cells were infected with Adeno-GFP virus or Adeno-hAQP5 under static and shear condition. Actin fibers were assessed by using Texas-red phalloidin. Only hAQP5-expressing cells had altered actin expression and desmoplakin redistribution in response to shear stress.

redistribution. Desmoplakin, a component of desmosomes, binds to intermediate filaments within the cells and thereby contributes to the barrier function the monolayer. Shear effects on actin and desmoplakin were not seen in the absence of AQP5. Other AQPs also are linked to cytoskeletal changes. AQP2 binds actin (47) and AQP0 binds to intermediate filament proteins to alter cells shape and morphology (48).

We have provided evidence that shear modulates barrier function in airway epithelia by serial activation of TRPV4 and L-type VGCC, and with our findings support the recent proposal that AQP5 may contribute to regulation of paracellular permeability (34). These observations provide insight into a potentially important homeostatic mechanism in the respiratory tract. In addition, our studies suggest cross-talk between transcellular and paracellular pathways that may be relevant in multiple tissues.

Materials and Methods

See [supporting information \(SI\) Materials and Methods](#) for details.

Cell Culture. NHBE (Lonza) were grown on collagen-coated inserts (Falcon) at 37°C with 5% CO₂ in specified media and maintained at an air–liquid interface for 6–9 weeks before study; transepithelial resistance was >400 ohms when used. Inhibitors were used as pretreatment and were included in the perfusate. All experiments were repeated a minimum of three times. In specified studies, immortalized human bronchial epithelial cells (16HBE; a gift of Gary Cutting, The Johns Hopkins University School of Medicine) were cultured on inserts and infected with either GFP- or AQP5-expressing adenovirus.

Shear Stress. We calculated the rate of perfusate flow in an open-plate apparatus required to create shear stress comparable shear *in vivo*, assuming the perfusate is a Newtonian, noncompressible fluid with no-slip boundary conditions. Fluid flow rates of 0.5–1 ml/min provided a shear stress consistent with the magnitude for airway epithelial cells *in vivo*. Cells on inserts were placed on coverslips and perfused in a chamber (RH-2; Warner Instrument) at 0.5–1 ml/min with a Krebs solution (49). The perfusate was gassed with 16% O₂/5% CO₂ at 37°C. Chamber temperature was maintained with an inline heat exchanger, and heated platforms were controlled by a dual-channel servo controller (SF-28 and TC-344B; Warner Instrument). The cells were exposed to laminar shear stress (τ) calculated by $\tau = 6 \mu Q/wh^2$, where Q = the flow, at 0.5–1 ml/min. The chamber dimensions (w and h) are 12.5 mm and 1 mm; assuming that the Krebs solution has a similar viscosity (μ) to water at (temperature 37°C) $\mu = 0.00653$ dynes/cm², then shear stress is $\tau = 1.5$ –3 dynes/cm².

Sample Processing, Immunoblotting, and PCR. See *SI Materials and Methods* for details.

Animal Studies. Briefly, 8- to 10-week-old C57BL/6 mice (20–30 g; Charles River Laboratory) (approved by the Johns Hopkins Animal Care and Use Committee) were killed by 135/1.5 mg/kg i.p. ketamine/acepromazine. The trachea was resected and placed in a physiological Krebs bath under a heat lamp. Both ends of the excised trachea were cannulated with 18-gauge angiocaths, and one end was attached to a ventilator [tidal volume 200 μ l, rate 120/min MiniVent (Harvard Apparatus); Micro Flow meter 5–500 μ l/s (Cole-Parmer); 20 psi compressed air tank]. Air was humidified and warmed to 37°C before entry into the tracheal lumen. Laminar flow can be assumed given that the entry length of the angiocath before the trachea is greater than five times the length of the trachea (50). Flow rates were 10, 50, and 100 μ l/s, which corresponded to shear stresses of $\approx 1, 5$, and 10 dynes/cm² assuming laminar flow ($\tau = 4\mu Q/\pi r^3$, where μ is the viscosity, Q is the flow, and r is the radius of the tube). In some experiments, a midtracheal obstruction was created with hemoclips (M&I Medical Sales) to create stenosis. The trachea was ventilated

for 1 h, and results were compared with nonventilated trachea similarly mounted.

TRPV4^{-/-} mice (51) were generously provided by M. Suzuki and A. Mizuno (Jichi Medical School) and by Daiichi Pharmaceutical Company.

Calcium Imaging. Cells were incubated with 7.5 μ M Fura-2 AM (Molecular Probes) for 60 min at 37°C under an atmosphere of 5% CO₂/95% air and mounted in a heated chamber on the stage of a Nikon TSE 100 Ellipse inverted microscope. Fura-2 fluorescence ratios ($R = F_{340}/F_{380}$) allows estimation of [Ca²⁺]_i (see *SI Materials and Methods*).

Permeability Assay *in Vitro*. After shear, the excised insert was placed on the base of an intact transwell chamber, allowing for separation of the apical and basal compartments, with the cells on the apical surface of the top insert. The edges were sealed with gel (Dow Corning), and a rubber washer and 500 μ l of FITC-coupled dextran beads (4 or 70 kDa, 10 mg/ml; Calbiochem) were added to the upper well for 20 min. Then, 3 ml of medium was placed in the bottom well. The FITC concentration in 1 ml of the lower well was determined by using a fluorometer (excitation 490 nm, detection 530 nm) and compared with static and cell-free two insert controls. *Ex vivo*, after exposure to shear or static conditions for 1 h, we instilled 50 μ l of Evans blue dye-4% albumin into the lumen of the tracheal segments for 20 min. The lumen was gently flushed with PBS, and the tracheal segments were homogenized in formamide and incubated in a 60°C water bath for 18 h. The Evans blue dye concentration was measured by using a spectrophotometer and quantified by using predetermined standards.

Statistics. All statistical analyses described in figure legends were performed by using STATA 9 (Stata Corporation).

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