Mechanosensitivity of mouse tracheal ciliary beat frequency: roles for Ca\(^{2+}\), purinergic signaling, tonicity, and viscosity

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Winters SL, Davis CW, Boucher RC. Mechanosensitivity of mouse tracheal ciliary beat frequency: roles for Ca\(^{2+}\), purinergic signaling, tonicity, and viscosity. Am J Physiol Lung Cell Mol Physiol 292: L614–L624, 2007. First published September 8, 2006; doi:10.1152/ajplung.00288.2005.—Mechanosensitivity is hypothesized to participate in the regulation of ciliary beat frequency (CBF) in airway epithelia. To investigate this hypothesis, CBF in excised mouse trachea was monitored (microscopy image analysis) while varying mucosal shear (perfusate velocity and/or viscosity; planar flow). CBF increased within minutes of step increase to steady shear stress as small as 10\(^{-3}\) Pa and decreased within minutes of shear reduction (≥10\(^{-4}\) Pa). CBF response was directional, being less with cephalad vs. caudal flow, and was reduced in trachea from mutant mice lacking P2Y2 receptors, as well as by administration of the Ca\(^{2+}\) chelator EGTA, the Ca\(^{2+}\) channel inhibitor La\(^{3+}\), the nucleotide phosphohydrolase apyrase, the metabolically stabilized adenosine receptor antagonist 5’-(3-thiotriphosphate), 0.1 mM inhibited CBF response to shear. Furthermore, neither ATP nor ATP\(_{\text{S}}\) substantially increased CBF in the relative absence of shear. With viscosity increase or shear withdrawal apyrase evoked CBF stimulation, inhibitable by the adenosine receptor antagonist 8-(p-sulfophenyl)theophylline. Thus CBF response to shear is finely tuned, directional, La\(^{3+}\) sensitive, likely dependent on extracellular Ca\(^{2+}\) and ATP, involving P2Y2 and adenosine receptor activations, influenced by shear history, tonicity, viscosity, and metabolism/exposure of ATP, and thus reflective of a complex interplay of physical and biochemical actions.

Shear; ATP; osmotic; stretch activated; mucociliary

The response of airway epithelia to mechanical force is presumed to be a fundamental factor in mucociliary defense, but it has not been established to what extent and by what means mechanosensitivity participates in the regulation of ciliary beat frequency (CBF). It has been suggested that mechanosensitive mechanisms might self-regulate mucociliary propulsion by adjusting CBF in response to increases or decreases in the load to be transported within a region, correcting for local deficiencies or excesses in mucus transport (37). On a more-global level, mechanosensitive mechanisms are likely involved in the stimulation of mucociliary clearance by chest physiotherapy or cough. Ultimately, both local and more global forces, including those generated by ciliary motion and fluid flow on the mucociliary escalator, are combined into defining shear stress (or shear) at the mucosal surface.

Prior studies have suggested that the airway is sensitive to shear within a limited range. For example, in ex vivo nasal mucosa, it was shown that CBF and ion transport varied with perfusion velocity across the apical epithelial surface in a nonlinear manner, between 30 and 100 μm/s velocities (21). Although a relationship of CBF to applied shear stress was not addressed in that study, we may, after making assumptions about flow geometry, speculate that this velocity range correlates to a shear stress range of 10\(^{-3}\) to 10\(^{-2}\) Pa. Average shear on the airway surface may also be estimated for various breathing patterns given airflow, time, and a relationship between airflow and shear. Thus we may relate changes in mucociliary clearance rates at tidal breathing to that with cough and relate no further change in rate with supramaximal cough (8, 4) to average shear stresses of ≤10\(^{-4}\) Pa, 4 × 10\(^{-3}\) Pa, and 1.5 × 10\(^{-2}\) Pa, respectively. Such a nonlinear response might result, in part, from the physical properties of the mucosal fluid. For example, mucus generally behaves as an elastic solid at shear stresses below 5 × 10\(^{-2}\) Pa shear (J. K. Sheehan, unpublished observations), but at higher shear, damping by internal mucus yielding might impede the translation of external shear into CBF response. Such a nonlinear response might also result from the constraints of messenger-receptor interactions (Michaelis-Menten kinetics).

Given their roles in the mechanosensitivity of endothelial and other tissues, ATP and Ca\(^{2+}\) are potential second messengers in the regulation of CBF by shear (49). In ciliated epithelia, mechanical stimulation is known to induce both extracellular Ca\(^{2+}\) influx (37) and noncytolytic intracellular ATP efflux (13), and both may increase CBF. In turn, intracellular Ca\(^{2+}\) mobilization may also be mediated by extracellular ATP ([ATP]\(_{\text{o}}\)) (9, 15, 28). Furthermore, shear on airway epithelial cultures has recently been shown to directly increase [ATP]o, presumably secondary to cellular ATP release, with [ATP]o approach to the EC\(_{50}\) for the P2Y2 receptor (P2Y2-R) at 2 × 10\(^{-3}\) Pa cyclic shear (time average) (41). Of course, CBF responses to shear may involve other agents, including nitric oxide (23), and the involvement of one mechanism does not exclude the potential involvement of other shear-induced signaling pathways. Yet, the roles of Ca\(^{2+}\) influx, adenine nucleotides, adenosine, and the P2Y2-R, in mechanosensitive CBF regulation, remain undefined.

A potential interaction of mucosal fluid properties and mechanical stress in CBF regulation is also logical but has not yet been explicitly established. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
been confirmed. For example, hypertonicity of the mucosal fluid likely influences epithelial turgor and cellular membrane stretch, and such properties are likely to affect mechanosensitive CBF regulation. Hypertonicity also induces cellular ATP release (1, 33), which is inducible as well by mechanical stress. In another example, viscosity of the mucosal fluid likely interacts with mechanical stress in CBF regulation. Viscosity not only influences the relationship between the fluid velocity (strain rate) and the shear stress at the epithelial surface, but it also influences the rate of ATP\textsubscript{2}\textsuperscript{-} hydrolysis (44), the potential convective/diffusive transport of mediators to receptors, and the viscous resistance to ciliary beat. Therefore, although it has recently been shown that some mechanosensitive responses in cultured endothelial cells (ATP release and Ca\textsuperscript{2+} influx) depend only on the magnitude of applied shear stress and are independent of strain rate (factors of flow rate and viscosity) (49), the response of airway CBF to mucosal fluid motion may depend on additional factors, besides shear stress alone, in this intricate system.

In these studies, we sought to quantify CBF response to shear, to consider the roles of some potential mediators and receptors in this response (Ca\textsuperscript{2+}, adenine nucleotides, adenosine, and the P2Y\textsubscript{2}-R), and to examine the capacity of hypertonicity and hyperviscosity to affect this regulation.

**MATERIALS AND METHODS**

Solutions, apparatus, and chemicals. The base perfusion solution used for all studies was culture medium solution from the University of North Carolina Advanced Cell Technologies Core, as this medium provided more prolonged tissue viability (several hours) and more consistent responses than physiological Ringer solution. This solution is described in Ref. 25, but briefly includes 50/50 mixture of DMEM with HEPES and LHC Basal Media (Biofluids, Rockville, MD), supplemented with insulin (5 μg/ml), hydrocortisone (0.72 μg/ml), EGF (0.5 ng/ml), 3,3,5-triiodo-L-thyronine (10\textsuperscript{-8} M), transferrin (10 μg/ml), epinephrine (0.6 μg/ml), phosphoethanolamine (0.5 μM), bovine pituitary extract (0.8%), BSA (0.5 mg/ml), CaCl\textsubscript{2} (80 μM), trace elements (1×, Biofluids), Stock 4 (1×, Biofluids), Stock 11 (1×, Biofluids), penicillin, streptomycin, and retinoic acid (5 \times 10\textsuperscript{-8} M). ATP was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Dextran T-500 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other reagents were purchased from Sigma Chemical (St. Louis, MO). Apyrase grade was selected for ATPase/ADPase ratio of ~1 (Sigma grade III). Solution viscosity was measured with falling ball viscometers from Gilmont Instruments (Barrington, IL).

Equipment. CBF was measured using a digital image analysis system, Sisson-Ammons Video Analysis of Cilia Beat Frequency (Ammons Engineering, Mt. Morris, MI), as described elsewhere (39). Differential interference microscopy microscopy was used. Specific components in this application included Axioskop 2 FS Research Microscope (Carl Zeiss Microscopy, Jena, Germany) with lens (Zeiss LD Achroplan \times 40/0.60 Korr Ph2, 44-08-65). Images were collected with Megaplus Camera Model ES 310 Turbo from Redlake (San Diego, CA) and interpreted with National Instruments IMAQ board PCI/PXI-1422 (Austin, TX). A computer was used for acquisition (Dell Dimension XPS T850r, Round Rock, TX). Fast-Fourier-Transform analysis of light intensity variation on collected images was used to estimate CBF, and values were recorded for each minute of study.

Tracheal tissue preparation. Mouse tracheal epithelium was chosen to study CBF response, given that freshly excised tissue is expected to be physiologically representative of in vivo response, that the predominant ciliary stroke orientation is established (potentially random in culture models), and that availability of mutant mouse strains allows specific investigation into the role of cell components in responses. Tissue harvesting and use of mouse tracheal tissue were approved by the Institutional Animal Care and Use Committee at the University of North Carolina. Redundant mice designated for euthanasia in other investigations were used. These mice were of mouse strain C57BL/6J in all studies, except for investigations of CBF response in P2Y\textsubscript{2}-R-deficient tracheal tissue and in age-matched control studies. Availability limitations demanded that these two sets of studies examine trachea from mouse strain 129S6/SvEvTac.

Carbon dioxide (CO\textsubscript{2}) euthanasia was used in accordance with the 2000 Report of the American Veterinary Medical Association Panel on Euthanasia (3). The euthanasia chamber was first charged with CO\textsubscript{2} from a pressurized tank for at least 30 s. The animals were then placed within the chamber, and additional CO\textsubscript{2} was sent through the chamber under pressure for 90 s. The mice were left within the chamber for at least an additional 3 min to ensure death; cessation of respiration and heartbeat were confirmed.

Mouse tracheal tissue was harvested immediately following euthanasia. Tracheal anatomic length in situ in the supine position was noted before excision. The excised trachea was divided axially along the anterior surface. Sutures were passed through the tissue to assist in flattening the posterior tracheal surface for microscopy imaging, as well as extending the elastic trachea back to anatomic length, ±1 mm or ~12%. Data collection generally commenced within 1 h of tissue harvest, following mounting of the tissue within the test fixture on the microscope, connecting perfusion piping, and equilibration of temperature.

Shear chamber. Shear chamber assembly provided rectangular duct flow across the mucosal tissue surface (Fig. 1). To exclude air from the chamber and piping, the chamber was assembled while submerged in excess perfusion fluid. A glass coverslip provided the top surface of the chamber, whereas mouse tracheal tissue, held flat by edge com-
pressure between two polycetal disks, comprised the bottom surface. The two surfaces were held 1-mm apart by a polycetal disk within which a 3-mm-wide slot was machined for a 6-mm length (Univ. of North Carolina Physiology Instrument Shop, see Fig. 1). Uniform perfusate flow along the length of this slot was facilitated by the presence of reservoir manifolds at the inlet and outlet slits (0.5 mm height), and uniform flow was visually confirmed by dye studies. The maximal Reynolds number corresponding to the highest flow rate used in these studies approached 10, supporting the assumption for flow characterization as laminar. The rate of shear change was damped by inherent tubing and system capacitance, but steady flow, following stepped change in pump setting, appeared to be achieved in less than 1 s, even in the increase from lowest to highest pump rate.

A combined push-and-pull syringe pump (PHD 2000; Harvard Apparatus, Holliston, MA) allowed precise control of volumetric flow rates as well as moderating chamber pressure and resolving a tracheal tissue distortion problem, as seen with flow change in preliminary studies with other pump types. Except for edge retention, the specimen itself was free on both the top and bottom surfaces, and measurements were focused on cilia near the posterior membrane (no tracheal cartilage rings) in the center of the slot width and between the center and outlet region of the slot length. Although not used in these studies, an identical slot was present below the specimen to allow separate perfusion of the serosal surface. As focus adjustment on the elastic tracheal tissue was rarely required, pressure fluctuations within the chamber were minimal throughout each study.

The chamber was sealed and mounted within a brass stage plate, and Peltier heat pumps (pump HT4-12-30-T1, controller MTCA; Melcor, Trenton, NJ) were coupled to the brass with zinc oxide-filled silicone oil. The temperature of the brass stage plate was monitored by thermistor (29F205; Newark Electronics, Gaffney, SC; Yellow Springs no. 4408, Yellow Springs, OH). Chamber temperature was maintained at 37 ± 1°C. In-line perfusion fluid temperature was also controlled (TC-344B; Warner Instrument, Harvard Apparatus).

The shear stress near the epithelial surface (τepith) was calculated by fluid dynamics law, given the known chamber dimensions (w, width; h, height), the viscosity of the perfusate (μ), and the volumetric velocity (Q), as τepith = μ × (6Q/wh²). Shear flows ranged from 10⁻⁵ Pa to 10⁻² Pa (Q = 0.0005 ml/min to 0.5 ml/min; average linear velocity 2.8 to 2,800 μm/s). The solution volume between the valve and the entrance to the rectangular duct was 0.25 cm³, and given the volumetric velocity, we estimated the length of time that the epithelial surface was exposed to an agent by simple arithmetic, albeit neglecting acceleration from forward diffusion of the agent through piping into the chamber and delay from limited diffusion-convection of the agent from central entry into the duct to the epithelial surface.

Protocols. Shear was applied to the epithelium by fluid perfusion through the mounting chamber for up to 150 min, using one of two general protocols. In the first, shear stress was increased sequentially every 30 min for 15 min (10⁻⁴, 10⁻³, or 10⁻² Pa; 1 dyn/cm² = 0.1 Pa) and then returned for 15 min to the low shear condition (10⁻² Pa) until all stress level trials were performed. In the second protocol, a higher low shear condition was used (10⁻⁴ Pa), which did not stimulate CBF but decreased the time required to exchange perfusing solutions, allowing more precise control of the duration of epithelial exposure to a particular agent. This second protocol used a single step change to a high shear condition (10⁻² Pa) for the longer duration of 30 min before returning to the low shear condition.

The effect of shear orientation with respect to ciliary stroke was examined by studying shear application in opposite directions. Except for this one investigation, all shear applications in these studies were applied in the caudal direction to control for this variable.

Data handling. Single-factor ANOVA was used in group comparisons. A statistical significance of P < 0.05 was used. Student’s two-sample t-test assuming equal variances was used for comparison to control data where appropriate.
similar manner with both 10 μM and 1 mM La^{3+}. The addition of 2 mM EGTA (Fig. 3B) also abolished the shear-induced CBF stimulation.

Interactions of shear and extracellular nucleotide signaling. We first considered whether perfusion with the candidate mediator ATP would enhance mechanosensitive CBF stimulation. A second protocol was used to better control ATP exposure time before shear. As shown in Fig. 4A, baseline control CBF was 9.1 ± 0.2 Hz at the beginning of this second protocol, rising to 19.7 ± 0.5 Hz with 10^{-2} Pa shear, in apparent similarity to the prior protocol. Administration of ATP (100 μM) was associated with only modest CBF stimulation during low shear flow (11.1 ± 0.3 Hz vs. control of 9.4 ± 0.4 Hz, P < 0.01, 10^{-4} Pa). However, with relatively short exogenous ATP exposure (20 min), CBF response to shear was markedly augmented (28.4 ± 2.1 Hz vs. control of 19.2 ± 0.5 Hz during the last 5 min of shear application, P < 0.01). Notably, this augmentation became even greater following reduction of shear back to 10^{-2} Pa (36.5 ± 1.7 Hz vs. control of 10.5 ± 2.5 Hz in the first 5 min after shear reduction), and this CBF stimulation persisted over the next 30 min.

We next considered whether the enhanced CBF response to shear might desensitize to [ATP]_o, as CBF desensitized to [ATP]_o over time with steady perfusion (30). Further increase in [ATP]_o (from 100 μM to 5 mM) did not stimulate CBF during low shear flow and actually was associated with less shear-induced increase in CBF (19.3 ± 0.9 Hz), producing a response that approached that of the control. As shown in Fig. 4B, further increase in the duration of epithelial ATP exposure (from 20 to 55 min) was also associated with decreased augmentation of shear-induced CBF stimulation. Not only was CBF stimulation less (23.6 ± 0.9 Hz in the last 5 min of shear application), but CBF increase upon shear reduction was also eliminated.

We then examined whether the modest CBF stimulation during low shear flow with ATP would be reproduced by the poorly hydrolysable ATP analog ATP_{5’}(3-thiotriphosphate). In endothelial cultures, [ATP]_o modulates shear-dependent increases in intracellular Ca^{2+} concentration ([Ca^{2+}]_i), which terminate upon cessation of flow, regardless of [ATP]_o. In substituting poorly hydrolysable ATP analogs for ATP, some have suggested that hydrolysis of ATP to adenosine by ectonucleotidases is responsible for the termination of [Ca^{2+}]_i responses at zero flow (28), but others have suggested that another portion of the signaling pathway besides P2 receptor (P2-R) activation may be responsible (9, 38). Yamamoto et al. (48) speculated that purinergic receptors might not only be affected by nucleotide binding, but also possess "shear transducer" properties. Thus, although ecto-

Fig. 2. Temporal plot of CBF and shear, cephalad or caudal application. CBF of mouse tracheal tissue (strain C57BL/6J) was monitored while shear was applied in either the caudal direction ("against" the predominant ciliary stroke direction) or in the cephalad direction ("with" the ciliary stroke), increasing at 15-30, 45–60, and 75–90 min as shown. CBF rise was greater when shear of 10^{-2} Pa was applied in the caudal direction vs. the cephalad direction. After each shear change, several minutes elapsed before a new, stable CBF response was attained. Symbols are means ± SE in 5-min intervals, n = 6–8 studies, *P < 0.05 for CBF results differing between the 2 shear directions.

Fig. 3. Effect of lanthanum chloride (La^{3+}) and EGTA on CBF mechanosen-sitivity. A: administration of La^{3+}, an inhibitor of cellular Ca^{2+} influx, inhibited the response of CBF to shear. The CBF plot from Fig. 2 (caudal direction) is replicated here for comparison as an experimental control. Differences (P < 0.05) are labeled between the 10 μM (*) or 1 mM (+) La^{3+} trials and the control. B: EGTA, a chelator of extracellular Ca^{2+}, also inhibited CBF response to shear. *P < 0.05. Symbols are means ± SE in 5-min intervals, n = 3–8 studies.
nucleotidases are expressed throughout the airway (35), hydrolysis of ATP may not be responsible for the minimal response of CBF to [ATP]o under low shear conditions. In both protocols (Fig. 5A, B), the initial administration of ATP/H9253S, under low shear conditions, induced minimal CBF stimulation, similar to the response with ATP. In the same trials, we examined whether ATP/H9253S might desensitize CBF response to shear to a greater extent than ATP. In a prior study of human nasal epithelial cultures, CBF stimulation induced by ATP/H9253S was largely transient, becoming desensitized to continuing ATPγS perfusion within minutes, and although CBF stimulation induced by ATP also declined over time, it desensitized to a lesser extent, likely secondary to hydrolysis of ATP, producing adenosine, activating nucleoside purinergic receptors (P1-R), and thereby indirectly stimulating and sustaining CBF (30). Using the first protocol (Fig. 5A), CBF response to shear was inhibited by including 100 μM ATPγS in the perfusate. With the use of similar perfusion in the second protocol (Fig. 5B), CBF response to shear was again reduced, although after the applied shear was reduced (from 10⁻² Pa to 10⁻³ Pa), CBF markedly increased.

**Interactions of P2-R and P1-R signaling.** We examined whether administering the nucleotide phosphohydrolase apyrase (reducing [ATP]o) might inhibit the CBF response to shear. In the studies where apyrase (5 U/ml) was included in the perfusion solution, CBF response to shear was dramatically reduced, as shown in Fig. 6A. The step increase of shear from

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**Fig. 4.** Effects of prolonged shear application of acutely increased [ATP]o, and of prolonged exposure to increased [ATP]o on CBF mechanosensitivity. A: several minutes elapsed between shear change and development of a stable CBF in response to the change, yet once developed, CBF response remained remarkably stable during prolonged 10⁻² Pa shear application (control). In the relative absence of shear (=10⁻⁴ Pa), CBF was relatively unaffected by exogenous ATP, but exogenous ATP markedly augmented CBF response to shear, if [ATP]o was moderate and if the duration of tissue exposure to ATP was brief, prior to shear challenge (100 μM, 20 min). B: this augmentation decreased, paradoxically, with higher [ATP]o, or longer ATP exposure (100 μM to 5 mM, 20–55 min). *P < 0.05 is labeled for 100 μM ATP (+) or 5 mM ATP (++) compared with the control. CBF for the control study is shifted along the time axis in the plot showing shear application delay (from 20–50 min to 55–85 min) to simplify comparison of results. Symbols are means ± SE in 5-min intervals, n = 3–8 studies.

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**Fig. 5.** Effect of adenosine 5’-(3-thiotriphosphate) (ATPγS) on CBF mechanosensitivity. A: the response of CBF to applied shear was inhibited by administration of the poorly hydrolysable ATPγS. B: after prolonged 10⁻² Pa shear application, CBF increased on reduction of shear back to 10⁻⁴ Pa. *P < 0.05. The plots for CBF control studies replicate control studies from Fig. 3 or Fig. 4 as appropriate. Symbols are means ± SE in 5-min intervals.
from $10^{-2}$ Pa to $10^{-3}$ Pa (4.7 ± 0.6 Hz control to 11.0 ± 0.1 Hz with apyrase present, 50–55 min).

We also examined whether addition of the adenosine receptor antagonist 8-($p$-sulfophenyl)theophylline (8-SPT) might inhibit CBF response to shear additively with apyrase, given that nucleotide metabolism may also stimulate ciliary activity indirectly through nucleoside purinergic receptors (P1-R) (30). Administration of 8-SPT alone (Fig. 6B) had little or no effect on CBF response to shear generally, and response following administration of both 8-SPT and apyrase (Fig. 6A, 5 U/ml and 100 μM, respectively) was not substantially different from that when apyrase alone was added, except following shear reduction from $10^{-2}$ Pa shear. CBF declined when both apyrase and 8-SPT were added vs. continued CBF stimulation following apyrase alone (20–25 min after step reduction from $10^{-2}$ Pa shear, 9.8 ± 0.4 vs. 14.5 ± 0.9 Hz, respectively, $P < 0.02$).

We then examined whether the administration of exogenous adenosine or the metabolically stabilized adenosine agonist 5′-(N-ethylcarboxamido)adenosine (NECA) would increase CBF and decrease CBF response to shear. In prior reports (neglecting shear effects), administration of adenosine may have decreased CBF in rabbit epithelial cultures (40), but in canine trachea in vivo, aerosolized adenosine both stimulated CBF and inhibited later CBF stimulation by P2-R agonists (47). Thus P1-R and P2-R signaling cross talk (29) may complicate these responses. Furthermore, exogenous adenosine, susceptible to metabolism, may not adequately represent the pharmacological activity of adenosine generated from nucleotides by ectonucleotidases locally near adenosine receptors (26). As shown in Fig. 7, adenosine, even at 5 mM concentration, had minimal effect on CBF shear response with these conditions (similar to 100 μM data, not shown). However, administration of NECA depressed CBF during shear ($10^{-2}$ Pa, 12.8 ± 0.2 vs. 19.2 ± 0.5 Hz for the control, $P < 10^{-6}$) and was associated with CBF stimulation after shear reduction.
Shear compared with that from wild-type mice (P2Y2-R) would display reduced CBF response to shear compared with that from wild-type mice [P2Y2(+/-)]. As seen in Fig. 8A, P2Y2(+/-) CBF was 8.9 ± 0.2 Hz in the first 5 min of study. CBF gradually declined over the next 40 min to 7.0 ± 0.4 Hz during subsequent 10^-5 Pa, 10^-4 Pa, and 10^-5 Pa shear stress applications, similar to prior studies. Tracheal tissue from P2Y2(-/-) mice also behaved similarly during the early period of the study, with CBF ranging from 9.9 ± 0.4 Hz in the first 5 min to 6.9 ± 0.6 Hz 40 min later.

Both P2Y2(-/-) and P2Y2(+/+) tracheas exhibited less sensitivity of CBF to shear than other results reported here (differences in CBF mechanosensitivity between mouse strains may have caused this discrepancy and may limit comparison of these results to other protocols). P2Y2(-/-) CBF responses were generally smaller than P2Y2(+/+) CBF responses, becoming more distinct during application of 10^-2 Pa shear and more than 10 min after step reduction from 10^-2 Pa shear (7.2 ± 0.1 vs. 10.7 ± 1.0 Hz, P < 0.01).

We also examined whether the CBF response to shear in P2Y2(-/-) trachea would be stimulated by apyrase (increasing adenosine) and be muted with both apyrase and 8-SPT. As seen in Fig. 8B, although all trials using trachea from this mutant mouse strain exhibited similar CBF in the first 5 min following step reduction from 10^-2 Pa shear, apyrase administration to P2Y2 (-/-) trachea was associated with marked increase of CBF for the next 15 min (17.2 ± 0.9 vs. 7.6 ± 0.5 Hz), an increase greater than CBF in (+/+) trachea (11.7 ± 0.7 Hz) and an increase that was reversible with further addition of 8-SPT (8.4 ± 0.4 Hz), suggesting apyrase-induced conversion of ATP to adenosine was responsible for the increase.

Shear and hypertonicity. We examined whether increasing the osmolality of the perfusate by addition of mannitol would inhibit CBF response to shear. Prior studies have examined CBF response to perturbations in osmolality (45), but no study has examined sensitivity of CBF to both mucosal osmolality and applied shear, presumed to cooperate if similar mechanisms are involved. Addition of mannitol to the perfusate inhibited stimulation of CBF by 10^-2 Pa shear (Fig. 9), and although not inhibiting response to 10^-2 Pa shear, mannitol addition led to prolonged CBF stimulation following the 10^-2 Pa shear challenge with respect to the control.

Shear and hyperviscosity. We examined whether increasing viscosity of the perfusate by adding dextran (Dextran T-500, D-glucopyranose homopolymer, mean molecular weight 500,000) would inhibit CBF response to shear. Dextran was chosen because dextran solutions maintain a linear relationship between fluid stress and strain (42), simplifying determination of the applied shear. CBF response to increased mucosal viscosity has been previously studied (16), and recently, the transient receptor potential vanilloid 4 channel has been suggested to have a role in this response (2), but the effect of increased viscosity on CBF response to shear has not been previously examined.

We examined the effect of viscosity increases from 7 × 10^-4 Pa-s to 2 × 10^-3 Pa-s (2% dextran) and to 1.4 × 10^-2 Pa-s (10% dextran). CBF responses were nominally similar during perfusion with both the 2% and 10% dextran solutions. Basal CBF trended toward a decrease with increased viscosity (40–45 min, control 6.8 ± 0.3 Hz; 2% dextran 5.7 ± 0.2, P > 0.1; 10% dextran 5.5 ± 0.4, P < 0.05), and no “autoregulatory” CBF increase was observed with these solutions (16). But the effect of increased viscosity on CBF response to shear was much more pronounced. As seen in Fig. 10, although CBF increased following application of 10^-2 Pa shear in the trials with increased viscosity (2% dextran 10.5 ± 0.8 Hz, 10%
Local fluid velocity, ranging between 30 and 100 m/s (equivalent to 1.1×10^{-3} to 3.7×10^{-3} Pa in vivo) in the propulsive force/area induced by cilia in the basal, unstimulated state.

**DISCUSSION**

Shear sensitivity, response time, and directionality. Our results suggest that the mechanosensitive regulation of CBF in airway epithelia is approximately 100 times more sensitive to shear than the mechanosensitive regulation of [Ca^{2+}]_i oscillations observed in excised lung capillary tissue (19) but not observed in cultured endothelial cells. Kuebler et al. (19) speculated that cells in the lung adapt to cyclic distortion by delaying responses to transient shear and that cells may lose this adaptation in cell culture over days, perhaps as membrane stiffness increases (5). The early CBF decline in many of our studies (shear ≤10^{-4} Pa) may also represent a trend away from the residual effects of chamber assembly and tissue manipulation (46). Importantly, the misconception that all CBF responses are instantaneous might mislead some observers to discount too quickly the effect of short, rapid perfusion if no immediate CBF stimulation is observed (22).

CBF increased more markedly when 10^{-2} Pa shear was opposed rather than aligned with ciliary propulsion, perhaps via facilitation of ciliary bending into the recovery stroke (12). The intense sensitivity suggested by these results predicts that, for an upright trachea, gravity force alone could stimulate CBF if fluid extended beyond the ciliary tips more than 1 μm. The minimal CBF stimulation by 10^{-2} Pa shear, when aligned with ciliary motion, suggests that this level of shear may be similar to the propulsive force/area induced by cilia in the basal, unstimulated state.

**Ca^{2+} dependence.** The differences between shear-induced CBF responses with medium containing EGTA or La3+ and the base medium (Fig. 3, A and B) support the hypothesis that Ca^{2+} influx, perhaps via a mechanosensitive channel, is required for a mechanosensitive CBF response (37). P2X-Rs have been suggested to participate (with the P2Y-R) in regulating the Ca^{2+} influx into rabbit airway ciliated cells (18) and in the ATP-induced increase in short-circuit current in rabbit airway epithelium (36). However, the similarity of ATP-induced [Ca^{2+}]_i responses in Ca^{2+}-free buffer and in the presence of 1.3 mM Ca^{2+}, in both wild-type and P2Y2-R(–/–) mice, makes a case against a major role for the P2X-R on the surface of murine tracheal epithelium (14).

**Interactions of shear and extracellular nucleotide signaling.** CBF response to [ATP], appears to be shear dependent, given the small CBF response during low shear perfusion with ATP (Fig. 4, A and B) or ATPγS (Fig. 5, A and B) and the unlikely removal of ATPγS during low shear perfusion by membrane-
bound ectonucleotidases. Although mechanosensitive responses of [Ca\textsuperscript{2+}]_i in endothelial cells are documented to depend on both [ATP]_o and shear (28), shear dependency of an ATP effect is a novel finding in the ciliary literature. Although [ATP]_o modulates CBF response to shear, it also modulates CBF response to [ATP]_o.

The maintenance of CBF responses over several minutes, without apparent fatigue or desensitization, is remarkable (Fig. 2 and Fig. 4, A and B), but this effect is not simple. Endogenous [ATP]_o, maintained by both basal secretion (20) and shear-stimulated release, is likely involved in these responses, which are inhibited by apyrase and augmented by exogenous ATP. Yet CBF responses to the joint stimuli of shear and exogenous [ATP]_o were not maintained over time (Fig. 4, A and B), likely secondary to purinergic receptor desensitization and resensitization (17, 23), which was apparently occurring even without evidence for receptor activation (CBF stimulation) while minimal shear was being applied. The more profound inhibition of CBF response to shear with ATPyS (Fig. 5, A and B) is consistent with more extensive P2-R desensitization by ATPyS (30). CBF increase after shear reduction with ATPyS may represent P2-R resensitization or cooperative CBF stimulation by adenosine (30) via metabolism of ATP released during the application of shear. Thus [ATP]_o is not a solitary mediator, but rather, the cooperation of variable [ATP]_o and variable P2-R activity, acting together, may regulate the “set point” of other cellular signaling pathways regulating and maintaining CBF response to shear (34).

Interactions of P2-R and P1-R signaling. CBF response to shear may be regulated by both P2-R and P1-R activations (29, 30, 27) as indicated by the following: 1) inhibition of CBF response to shear by apyrase (Fig. 6, A and B); 2) stimulation of CBF with apyrase on shear reduction (Fig. 6, A and B, and Fig. 8B) or viscosity increase (Fig. 10); 3) suppression of apyrase-induced CBF stimulation by the P1-R antagonist 8-SPT (Fig. 6A, Fig. 8B, and Fig. 10); and 4) inhibition of CBF response to shear by adenosine agonist NECA and stimulation of CBF with NECA on shear reduction (Fig. 7). Thus although P1-R may have no major role in murine CBF response to shear under some conditions (waterlike viscosity, isotonicity, and no recent shear), P1-R activation may have a more important role under other conditions.

CBF stimulation with NECA is consistent with a prior report (30), and the stimulation, both before and after shear challenge, suggests that CBF stimulation via the P1-R occurs independently of shear application. Inhibition of CBF response to shear with NECA may be consistent with inhibition of CBF stimulation by aerosolized P2-R agonists in canine trachea after pretreatment with adenosine (47). Other cell types may also show inhibited shear response by P1-R activation. In cultured airway smooth muscle cells, pretreatment with 100 \mu M NECA increased intracellular cAMP concentration ([cAMP]_i) and desensitized the P1-R (A2B-R) to further stimulation (31), and

Fig. 11. Mechanosensitive regulation of CBF. A system is proposed wherein shear induces cellular membrane stretch, ATP release, and Ca\textsuperscript{2+} influx, which may be inhibited by EGTA or La\textsuperscript{3+}. Apyrase enhances hydrolysis of extracellular ATP to adenosine (ADO), which is also subject to removal by metabolism, uptake, or diffusion-convection away from surface receptors. Adenosine and the metabolically stabilized NECA activate P1 receptors (P1-R), antagonized by 8-SPT. ATP and the metabolically stabilized ATPyS activate P2Y\textsubscript{2} (and other P2) receptors. CBF stimulation in response to shear involves Ca\textsuperscript{2+} influx, P2Y\textsubscript{2}-R activation, and possibly other stretch-induced responses. P2Y\textsubscript{2}-R activation in the absence of shear does not increase CBF but desensitizes the P2Y\textsubscript{2}-R to subsequent shear application. P1-R activation inhibits this desensitization but also inhibits CBF response to shear. P1-R activation stimulates CBF (not dependent on simultaneous application of shear), but prior recent P2Y\textsubscript{2}-R activation (and perhaps shear) enhances this stimulation. The drive to increase CBF is delayed and averaged over time, reducing excessive fluctuations in response to cyclic breathing and other transient distortions. Hypertonicity desensitizes the system to subsequent shear application (perhaps via ATP release). Hyperviscosity influences the shear stress applied at a given flow, but also affects ciliary propulsion and likely the convection, diffusion, and metabolism of extracellular mediators. Thus this complex system likely includes components dependent on shear (Ca\textsuperscript{2+} influx, ATP release) but also components that do not depend on shear stress alone (convection, diffusion, and viscous resistance to ciliary propulsion).
in cultured fibroblasts, increased [cAMP]i is associated with decreased mechano- and ATP-sensitivities (11).

**P2Y2-R dependence and other mechanisms.** Although trachea from P2Y2-R-deficient mice and their control group (both strain 129S6/SvEvTac) exhibited minor differences in CBF response to shear (attributed to lack or presence of the P2Y2-R), markedly different (but internally coherent) responses in trachea from a different mouse strain (C57BL/6J) suggest that other inherited differences (beyond P2Y2-Rs) may more strongly affect CBF response to shear.

Although the P2Y2-R is a primary component of CBF stimulation in response to shear (Fig. 8A), a separate P2-R, a P1-R, and shear itself may also participate in CBF regulation. The enhanced sensitivity of CBF to shear (at 10⁻³ Pa) after apyrase and 8-SPT administration to trachea already deficient in the P2Y2-R (Fig. 8B) suggests that 1) a nonpurinergic mechanosensitive mechanism is present and 2) an inhibitory P2-R is present. CBF stimulation following withdrawal of 10⁻² Pa shear in the presence of apyrase, but with CBF inhibition in the presence of both apyrase and 8-SPT, suggests the P1-R may also have a role in CBF stimulation, at least following the application of shear. This stimulation is likely secondary to P1-R activation, following metabolism of [ATP]o to adenosine (enhanced by apyrase). It is speculated that adenosine nucleotides, released at the cell surface, might accumulate near the cell surface when convective flow away from the surface is reduced. If CBF stimulation by [ATP]o depends on the presence of shear, [ATP]o alone might not be capable of stimulating CBF after the withdrawal of shear. But metabolism of [ATP]o to adenosine (whether enhanced by exogenous apyrase or endogenous ectonucleotidases) might provide a means for accumulated [ATP]o to indirectly maintain elevated CBF, even after flow ceases.

**Shear and hypertonicity.** Hypertonic mucosal fluid had minimal effect on basal CBF in the relative absence of shear but larger effects on CBF response to shear (Fig. 9). The minor effect of mannitol addition on baseline CBF observed here correlates to slight CBF decrease with hypertonic challenge of canine CBF (45). As ATP is likely released by cells in response to shear and hypertonicity (1, 33), the inhibition of CBF response to shear (10⁻³ Pa) following hypertonic perfusion may again indicate desensitization of a P2-R (P2Y2-R) before shear. The persistent CBF stimulation after shear reduction (from 10⁻² Pa) in the presence of hypertonicity may again represent coupling of P2-R and P1-R signaling via ATP metabolism.

**Shear and hyperviscosity.** Increasing mucosal fluid viscosity had a minimal effect on CBF in the relative absence of shear (Fig. 10) but decreased CBF response to shear, and surprisingly, increased CBF in the presence of apyrase. The CBF decline we observed correlates with reported similar CBF decreases with higher dextran concentrations, although shear flow may also have affected responses in that report (2). The reduced CBF response to shear with increased viscosity may be simply a physical effect, but desensitization of a P2-R (P2Y2-R) before shear challenge may also contribute, as increased viscous resistance to ciliary motion may also increase ATP release. CBF stimulation following addition of apyrase and dextran vs. dextran alone is likely secondary to apyrase action on released adenine nucleotides to increase extracellular adenosine concentration and P1-R activation, since this CBF stimulation was inhibitable by 8-SPT. Although it was not observed here, it is speculated that endogenous ectonucleotidases (similar to apyrase) might provide a means for CBF autoregulation in response to increased viscosity (16). This CBF stimulation is also consistent with the notion that P1-R activation, raising [cAMP]i, and increasing the rate of CFTR-dependent Cl⁻ secretion (41), may dilute the viscous resistance to ciliary beating, thereby allowing CBF to increase.

**Shear vs. viscosity/flow effects.** If CBF response to mucosal fluid movement involved only the shear stress near the mucosal surface, then any combination of fluid velocity and viscosity resulting in similar mucosal shear stress would produce the same CBF response; this was not the case (compare Figs. 2 and 10). Given the difference in CBF response to shear for the control (0%) and 2% dextran solutions, CBF responses to shear within this viscosity range arose from both shear stress and other effects. Shear-dependent effects include cell membrane tension and perhaps the opening of stretch-activated ion channels. Effects that are not dependent on shear stress alone include the convection of an extracellular mediator to a membrane-bound receptor, a viscosity-dependent rate of ATP hydrolysis (44), viscosity-dependent changes in membrane dynamics (43), and, perhaps most importantly, change in viscous resistance to ciliary beating. Given that the 2% dextran and 10% dextran solutions produced a stable CBF response over this significant viscosity range, viscosity and velocity effects overshadow the effect of shear stress on CBF over this range. Therefore, although mechanosensitive ATP release and Ca²⁺ influx in cultured endothelial cells depend only on the magnitude of applied shear stress (49), other effects participate in the response of CBF to shear.

**Summary.** Figure 11 presents a potential regulatory system for airway CBF in response to mechanical shear. This response is sensitive to shear magnitude (as small as 10⁻³ Pa), shear direction (caudal or cephalad), and time of shear application (taking minutes to develop). The response appears to require influx of Ca²⁺ through La³⁺-sensitive channels. Shear-induced ATP release and activation of the P2Y2-R likely contribute, since the response is inhibited by apyrase, augmented acutely by exogenous ATP, and diminished in trachea lacking the P2Y2-R. Although exogenous P2-R agonists are insufficient to modulate CBF in the relative absence of shear, they may inhibit CBF response to subsequent shear application. With viscosity increased or after stimulatory shear is withdrawn, apyrase administration may stimulate CBF through receptor activation by adenosine, a metabolic product of ATP released in response to shear. Thus local mechanosensitive regulation of ciliary motility reflects a complex interplay of physical and biochemical actions.

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**REFERENCES**
