

# Molecular responses of rat tracheal epithelial cells to transmembrane pressure

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<sup>3</sup>Division of Pulmonary and Critical Care Medicine and <sup>4</sup>Cardiovascular Division, Brigham and Women's Hospital, Boston, Massachusetts 02115; and <sup>2</sup>Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

**Barbara Ressler, Richard T. Lee, Scott H. Randell, Jeffrey M. Drazen, and Roger D. Kamm.** Molecular responses of rat tracheal epithelial cells to transmembrane pressure. *Am J Physiol Lung Cell Mol Physiol* 278: L1264–L1272, 2000.—Smooth muscle constriction in asthma causes the airway to buckle into a rosette pattern, folding the epithelium into deep crevasses. The epithelial cells in these folds are pushed up against each other and thereby experience compressive stresses. To study the epithelial cell response to compressive stress, we subjected primary cultures of rat tracheal epithelial cells to constant elevated pressures on their apical surface (i.e., a transmembrane pressure) and examined changes in the expression of genes that are important for extracellular matrix production and maintenance of smooth muscle activation. Northern blot analysis of RNA extracted from cells subjected to transmembrane pressure showed induction of early growth response-1 (Egr-1), endothelin-1, and transforming growth factor- $\beta$ 1 in a pressure-dependent and time-dependent manner. Increases in Egr-1 protein were detected by immunohistochemistry. Our results demonstrate that airway epithelial cells respond rapidly to compressive stresses. Potential transduction mechanisms of transmembrane pressure were also investigated.

asthma; gene expression; mechanical stress; early growth response-1; endothelin-1; transforming growth factor- $\beta$ 1

IT IS NOW WELL ESTABLISHED that mechanical forces play a critical role in tissue remodeling and cellular homeostasis. Vascular endothelial cells (32), cardiac myocytes (37), osteoblasts (41), and glomerular mesangial cells (53) all function in mechanically dynamic environments; perturbations in mechanical stress are known to have physiological and pathological implications. Cells in the lung also experience variable mechanical stress and respond through numerous lung signal transduction pathways. For example, stretching alveolar epithelial cells (50) and mechanically perturbing tracheal epithelial cells (10) result in  $\text{Ca}^{2+}$  mobilization. Cyclic stretch of airway epithelium inhibits prostanoïd synthesis (39) and wound repair (38) in vitro.

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Cyclic stretch and fluid shear stress on pleural mesothelial cells has been shown to affect growth factor release (45).

Another important perturbation occurs in the airway when smooth muscle constricts and the airway wall consequently buckles into a rosette pattern, causing the epithelium to fold into deep crevasses (51). Epithelial cells along the folds of these rosettes are pushed up against each other (Fig. 1) and thus are subjected to a compressive stress (i.e., pressure or a stress perpendicular to their surface). Pressures exerted on the epithelium in an unconstricted airway during normal breathing are seldom  $> 1$   $\text{cmH}_2\text{O}$  (98 Pa) above atmospheric pressure (46) and are much lower than the compressive stresses exerted by constricting smooth muscle ( $>30$   $\text{cmH}_2\text{O}$ ; 15). We hypothesized that the compressive stress on airway epithelial cells during bronchoconstriction could stimulate the cells to modify their environment in response to this stress much as hemodynamic stresses induce biological responses in endothelial cells (32).

To test this hypothesis, we subjected primary cultures of rat tracheal epithelial cells to a physiological range of static pressures and measured changes in gene expression. We examined genes that are known to be transduced by mechanical forces in vascular endothelial cells and that also play a role in tissue fibrosis. Early growth response-1 (Egr-1), an immediate-early gene, activates transcription of platelet-derived growth factor (PDGF; 25), which stimulates fibroblast proliferation and collagen production (31). Endothelin-1 (ET-1) is a potent airway contractile agonist and also promotes collagen synthesis by fibroblasts (4). Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) is an important regulator of lung fibrosis (6) and has been shown to stimulate expression of fibronectin and collagen genes in fibroblasts (31). In this study, we demonstrated magnitude- and time-dependent differential expression of these genes in response to static transmembrane pressure. Our results suggest that the response of airway epithelial cells to compressive stresses in constricted airways may lead to airway fibrosis independent of inflammation in asthma.

## METHODS

**Cell culture.** DMEM-Ham's F-12 medium (DMEM-F-12) and penicillin-streptomycin were obtained from GIBCO BRL

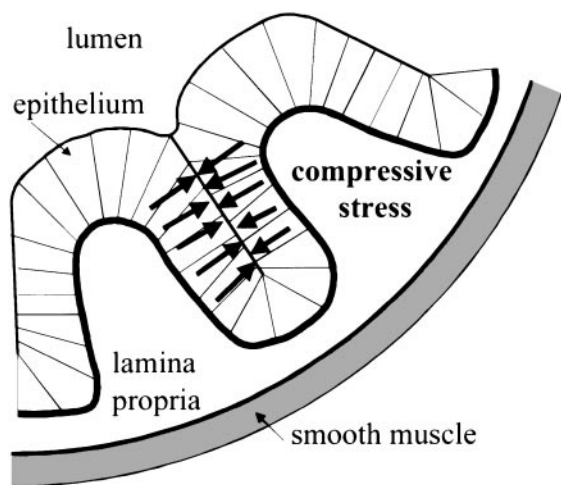


Fig. 1. Schematic of a highly constricted airway. Smooth muscle constriction causes the airway to buckle into folds. Epithelium within the folds of the lamina propria is subjected to a normal stress (i.e., a stress perpendicular to its surface).

(Life Technologies, Grand Island, NY). Transferrin and epidermal growth factor (EGF) were from Collaborative Research (Bedford, MA). Bovine pituitaries were purchased from Pel Freeze (Rogers, AR). All other culture medium additives were obtained from Sigma (St. Louis, MO). Cells were grown on Transwell-COL tissue culture inserts (0.4- $\mu$ m pore size, 24-mm diameter; Corning Costar, Cambridge, MA). The base of the culture insert consisted of a porous membrane made of polytetrafluoroethylene coated with type I and III collagen.

Rat tracheal epithelial (RTE) cells were cultured following the procedure of Kaartinen et al. (22) with slight modification. Cells were grown in complete medium (CM), which consisted of DMEM-F-12 supplemented with penicillin-streptomycin (100 U/ml and 100  $\mu$ g/ml, respectively), gentamicin (50  $\mu$ g/ml), HEPES (30 mM), insulin (10  $\mu$ g/ml), hydrocortisone (0.1  $\mu$ g/ml), cholera toxin (0.1  $\mu$ g/ml), transferrin (5  $\mu$ g/ml), phosphoethanolamine (10  $\mu$ M), ethanolamine (80  $\mu$ M), EGF (25 ng/ml), retinoic acid ( $5 \times 10^{-8}$  M), BSA (0.5 mg/ml), and bovine pituitary extract (1%).

Male Fischer 344 rats, 300–500 g body wt, were killed by CO<sub>2</sub> asphyxiation. Tracheae were sterilely excised, filled with a 1% protease solution (Sigma) in DMEM-F-12, and kept overnight at 4°C. Cells were flushed out of the tracheae with DMEM-F-12 containing 5% heat-inactivated FBS and collected by centrifugation (500 g, 10 min, 4°C). Cells were resuspended in a high-protein medium (CM with 3 mg/ml BSA), counted, and seeded on the top surface of the culture inserts at a density of  $\sim 5 \times 10^4$  cells/cm<sup>2</sup>. The lower chamber of the culture wells contained high-protein medium supplemented with 10% FBS. Cultures were incubated in a humid environment at 35°C with 3% CO<sub>2</sub> in air.

After 24 h, the medium was changed to CM without serum. The medium was changed every other day until *day 5*, after which it was changed daily. The culture conditions promoted growth of epithelial cells with a cobblestone morphology; there was no apparent growth of cells with a fibroblast morphology. After the cells were confluent (*day 7*), the medium on the apical surface was removed, and CM was added to the bottom compartment only to produce an air-liquid interface at the cell surface. Another 7 days elapsed during which the cells differentiated into a pseudostratified, mucus-secreting culture with cells of columnar and basal morphology. Experiments and interventions were conducted on *day 14*.

**Experimental procedure.** Approximately 12 h before the start of the experiment, the medium was changed to minimal medium (DMEM-F-12 plus antibiotics only), and the culture wells were prepared for pressure application in the incubator (Fig. 2). Stoppers fitted with connectors were tightly placed in the culture wells. Control culture wells were handled in the same manner and inserted in the apparatus but were not pressurized. To apply pressure, a large tank filled with incubator air was pressurized to the desired level with a sphygmomanometer bulb. At the start of the experiment, the valve from the tank to the cells was opened, and pressure over the cells ( $P_1$ ) was raised. This apparatus applied pressure only to the apical surface of the cells; the basal surface of the culture membrane and the culture medium were exposed to atmospheric pressure ( $P_2$ ) only. Thus the cells were subjected to a transmembrane pressure ( $P_1 > P_2$ ).

The pressures ( $P_1$ ) we selected to study were 2, 5, 10, and 20 cmH<sub>2</sub>O, which were in the range of pressures known to be generated by airway smooth muscle (15). Samples were collected after 0, 1, and 6 h. Cells harvested at the zero time point were not inserted in the apparatus and were minimally handled to examine the effects of manipulation on cell stimulation. At the appropriate time, cells were lysed for RNA extraction and medium samples were collected. Cell viability was evaluated by the amount of lactate dehydrogenase (LDH; Sigma) in the medium. Experimental LDH levels were normalized to LDH levels of completely lysed cells.

To explore the effects of elevated hydrostatic pressure on the cells (i.e.,  $P_1 = P_2$ , with both greater than atmospheric pressure), the culture plates were placed in a Plexiglas box that was sealed and then pressurized. Cells in the box experienced elevated pressure on both the apical and culture membrane surfaces. There was no strain of the culture membrane, and the pressures were equal on all surfaces of

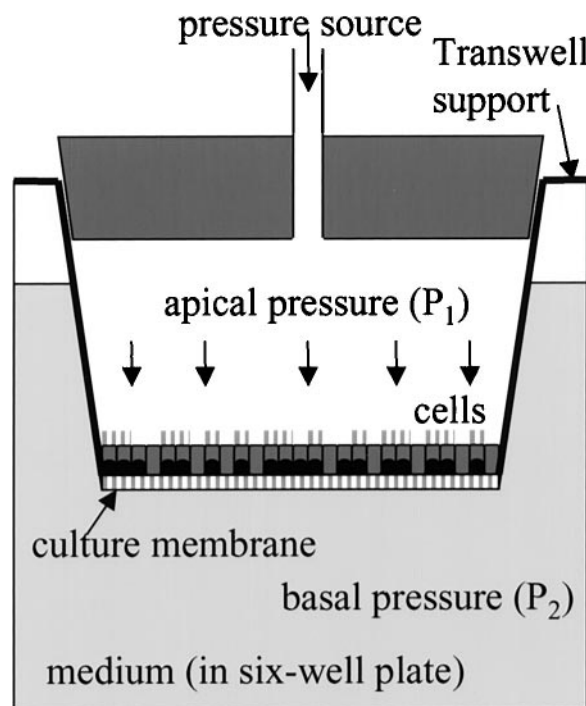


Fig. 2. Schematic of a culture well subjected to transmembrane pressure. Air in the sealed chamber above the cells was pressurized ( $P_1$ ) to apply a normal stress to the surface of cells and basal surface of membrane, and culture medium remained exposed to atmospheric pressure ( $P_2$ ). Thus cells experience a transmembrane pressure ( $P_1 > P_2$ ).

the culture; thus the cells in this experiment were subjected to hydrostatic pressure only.

To determine the amount of membrane strain that occurred as a result of transmembrane pressure, we obtained a digital image (Pulnix, Sunnyvale, CA) of the pressurized culture well and measured the maximum deflection at the membrane center ( $w_{max}$ ) with image-analysis software (NIH Image version 1.60, Bethesda, MD). Average membrane strain ( $\bar{\epsilon}$ ) was calculated according to the analysis of Williams et al. (49):  $\bar{\epsilon} = \frac{2}{3}(w_{max}/a)^2$ , where  $a$  is the culture membrane radius ( $a = 12$  mm for our Transwells).

To explore the effects of transmembrane pressure without the associated strain of the culture membrane, we placed a stiff plastic screen in the bottom of the culture dish and held the culture membrane flush against the screen during apical pressure application. Cells were thereby exposed to the medium at atmospheric pressure while strain of the culture membrane was minimized.

**RNA isolation and hybridization.** Total cellular RNA was isolated with commercially available kits (RNeasy, QIAGEN, Valencia, CA). After the RNA was concentrated and its purity and concentration were determined, 15  $\mu$ g of total RNA were fractionated on 1.2% agarose-6% formaldehyde gels. RNA was transferred overnight by capillary action onto nylon membranes (Stratagene, La Jolla, CA) and immobilized with ultraviolet radiation. Northern blot hybridization was performed with a random-primer  $^{32}$ P-labeled 3-kb *Eco*R I cDNA fragment of mouse Egr-1 (a kind gift from Dr. V. Sutakme, Beth Israel/Deaconess Medical Center, Boston, MA), a 600-bp *Eco*R I cDNA fragment of rat ET-1, a 1-kb *Xba* I-*Hind* III cDNA fragment of rat TGF- $\beta$ 1 (ET-1 and TGF- $\beta$ 1 were kind gifts from Dr. M.-A. Lee, Brigham and Women's Hospital, Boston, MA), and a 1.1-kb cDNA fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Clontech (Palo Alto, CA). Membranes were hybridized with ExpressHyb solution (Clontech) for 1 h at 68°C and then probed simultaneously for either Egr-1 and GAPDH or ET-1 and GAPDH, washed, and exposed to X-ray film for 1–3 days at –80°C with intensifying screens. Membranes probed with ET-1 and GAPDH were boiled in 0.5% SDS for 10 min and then reprobed for TGF- $\beta$ 1 following the same procedure. Autoradiograms were quantitatively analyzed by scanning densitometry and image analysis (Optimas, version 5.2, Bothell, WA). Egr-1, ET-1, and TGF- $\beta$ 1 signals were normalized to the corresponding GAPDH signal to correct for variations in loading and transfer. GAPDH was chosen because it has been shown not to be strongly regulated by other mechanical stimuli such as shear stress (27) or stretch (3).

**Immunohistochemistry.** Culture wells subjected to 10 or 20 cmH<sub>2</sub>O pressure for 1 or 6 h were fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.3% Triton (Sigma) for 5 min, and blocked with 10% goat serum. Cells were then incubated with rabbit anti-human EGR-1 antibody (1:100 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4°C. After being washed, cells were incubated with biotinylated goat anti-rabbit antibody (1:200 dilution; DAKO, Carpinteria, CA) for 1 h at room temperature. Then the cells were rinsed, incubated with streptavidin fluorescein (1:750 dilution; NEN, Boston, MA), washed, mounted, and examined by fluorescence microscopy. Staining specificity was determined by incubating the cells with nonspecific mouse IgG1 (1:100 dilution; Sigma) or with the biotinylated secondary antibody only.

**Statistics.** Data are expressed as means  $\pm$  SE unless indicated otherwise. Data were compared with an unpaired Student's *t*-test or a one-way ANOVA combined with Scheffé's test. *P* values < 0.05 were considered significant.

## RESULTS

**Northern analysis: transmembrane pressure experiments.** Figure 3 shows representative Northern blots of RNA collected from RTE cells subjected to 20 cmH<sub>2</sub>O pressure for 0, 1, and 6 h. Egr-1 hybridization signals were easily apparent after 1 h of stimulation and returned to baseline control levels after 6 h of steady pressure stimulation. ET-1 hybridization signals were elevated after both 1 and 6 h of stimulation. TGF- $\beta$ 1 hybridization signals were elevated after only 6 h of stimulation.

A summary of densitometric data from the Northern analysis is given in Fig. 4. After 1 h of steady pressure stimulation, Egr-1 mRNA levels were elevated above control levels: 18.5  $\pm$  4.7-fold ( $n = 9$  experiments;  $P < 0.0001$ ) with 20 cmH<sub>2</sub>O pressure, 6.8  $\pm$  1.2-fold ( $n = 8$  experiments;  $P < 0.0001$ ) with 10 cmH<sub>2</sub>O pressure, and 3.4  $\pm$  0.6-fold ( $n = 6$  experiments;  $P < 0.001$ ) with 5 cmH<sub>2</sub>O pressure. In all experiments, Egr-1 expression returned to baseline levels after 6 h of stimulation. Pressures of 2 cmH<sub>2</sub>O did not significantly stimulate Egr-1 expression. ET-1 mRNA levels were elevated above control only in the 20 cmH<sub>2</sub>O experiments in which ET-1 levels were 4.1  $\pm$  0.7-fold ( $n = 5$  experiments;  $P < 0.01$ ) over control level after 1 h and 4.7  $\pm$  1.0-fold ( $n = 5$  experiments;  $P < 0.01$ ) over control level after 6 h. TGF- $\beta$ 1 mRNA expression was increased over control level by a factor of 4.6  $\pm$  0.7 ( $n = 7$  experiments;  $P < 0.0001$ ) and 2.2  $\pm$  0.2 ( $n = 5$  experiments;  $P < 0.0001$ ) after 6 h of 20 and 10 cmH<sub>2</sub>O pressure stimulation, respectively; 5 and 2 cmH<sub>2</sub>O pressure failed to significantly induce TGF- $\beta$ 1 expression. Expression of

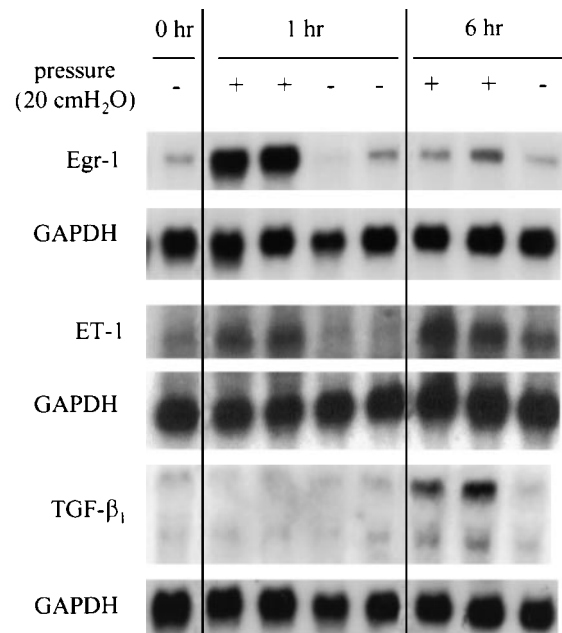


Fig. 3. Expression of early growth response-1 (Egr-1), endothelin-1 (ET-1), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) after 1–6 h of 20 cmH<sub>2</sub>O pressure stimulation. Egr-1 mRNA was upregulated after 1 h of pressure stimulation. ET-1 mRNA was elevated with 1 and 6 h of pressure. TGF- $\beta$ 1 mRNA was clearly present after 6 h of pressure application. +, With; –, without.

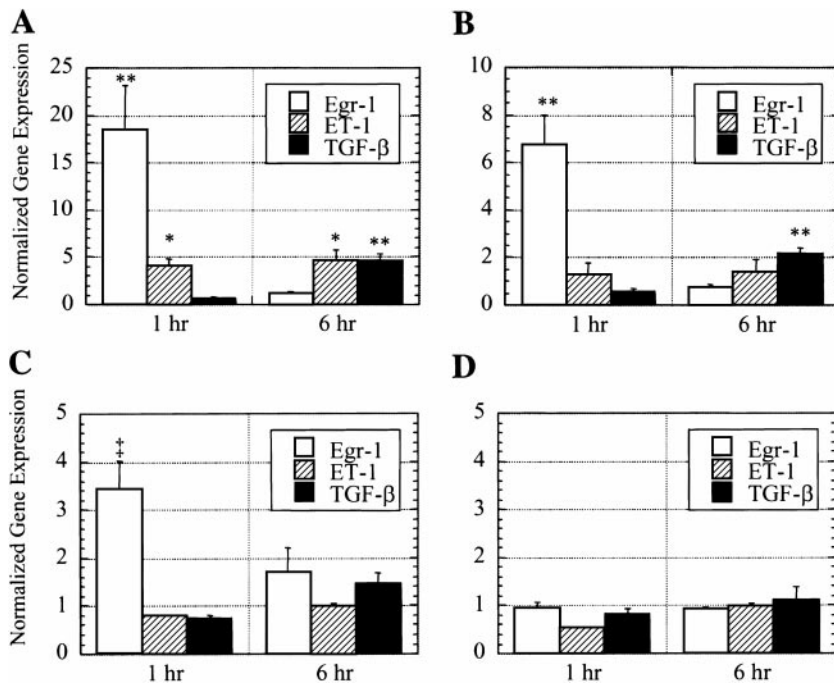


Fig. 4. Densitometric analysis of Egr-1, ET-1, and TGF- $\beta$ 1 at 20 (A), 10 (B), 5 (C), and 2 (D) cmH<sub>2</sub>O. Values were normalized to control levels. Note different scales of y-axes. Significantly greater than control level: \*\* $P < 0.0001$ ; † $P < 0.001$ ; \* $P < 0.01$ .

GADPH mRNA was highly consistent with ethidium bromide staining of the 18S bands and did not vary with the magnitude of pressure applied (data not shown).

**Northern analysis: hydrostatic pressure and no-strain experiments.** To study the effects of hydrostatic pressure on gene expression, we subjected RTE cells to 10 cmH<sub>2</sub>O transmembrane pressure or hydrostatic pressure for 0, 1, or 6 h. Hydrostatic pressure did not induce Egr-1 mRNA expression but transmembrane pressure did at the 1-h time point (Fig. 5A). Transmembrane pressure, but not hydrostatic pressure, also increased TGF- $\beta$ 1 mRNA expression approximately twofold after 6 h (Fig. 5B).

The average strain of the culture membrane ( $\bar{\epsilon}$ ) was calculated to be 2.3% at 20 cmH<sub>2</sub>O transmembrane pressure, 1.5% at 10 cmH<sub>2</sub>O, 1.0% at 5 cmH<sub>2</sub>O, and 0.5% at 2 cmH<sub>2</sub>O. Because the membrane was fixed at the edges, the strain pattern was anisotropic and nonuniform (49). To reduce membrane strain, a rigid mesh support with 800- $\mu$ m-diameter pores was inserted; membrane strain with the support in place was calculated to be less than one-tenth the average strain of the unsupported membrane (8).

Cells were subjected to 10 cmH<sub>2</sub>O transmembrane pressure with and without the mesh supports. Thus the cells were subjected to 1.5% average membrane strain without support or  $<0.15\%$  strain in the mesh pores with support. Representative Northern blots for Egr-1 mRNA in cells exposed to 10 cmH<sub>2</sub>O transmembrane pressure for 1 h with and without membrane strain are shown in Fig. 6. Cells subjected to 10 cmH<sub>2</sub>O transmembrane pressure without membrane strain for 1 h exhibited a  $4.8 \pm 0.5$ -fold ( $n = 5$  experiments) increase in Egr-1 mRNA. Cells under the same pressure but with membrane strain had a  $7.0 \pm 1.1$ -fold ( $n = 9$  experiments) increase in Egr-1 mRNA after 1 h. The differ-

ence in levels of Egr-1 mRNA between the strained and supported samples was not significant ( $P = 0.18$ ). TGF- $\beta$ 1 mRNA was also elevated in both strained (1.7-fold) and unstrained (2.1-fold) cells after 6 h of pressure stimulation, but too few experiments were performed to determine significance (data not shown).

**Immunohistochemistry.** Increased levels of Egr-1 protein were detected in cells subjected to 6 h of 20 (Fig. 7A) and 10 cmH<sub>2</sub>O (data not shown) pressure stimulation. There was no detectable immunofluorescence above background in cells subjected to 1 h of pressure stimulation (data not shown) or in control cells (Fig. 7B). Specificity of the staining was confirmed by the absence of staining with exposure to nonspecific mouse IgG1 (Fig. 7C) and to the biotinylated secondary antibody (data not shown).

**Cell death.** At 6 h and 20 cmH<sub>2</sub>O pressure, LDH levels indicated  $16 \pm 5\%$  (SD) cell death ( $n = 47$  experiments). At all other time points and pressures,  $<2\%$  of the cells died.

## DISCUSSION

**Implications for airway remodeling and asthma.** Cells in mechanically dynamic environments such as endothelial cells (32), osteoblasts (41), and fibroblasts (14) often cause stress adaptation in the surrounding tissue if the mechanical environment deviates from normal. We present evidence that mechanical forces equivalent to those resulting from bronchoconstriction can transduce signals in airway epithelial cells. In response to compressive stresses, which we believe mimic those exerted on epithelial cells in the folds of a highly constricted airway, rat tracheal epithelial cells differentially upregulated mRNA encoding expression of Egr-1, ET-1, and TGF- $\beta$ 1 in a magnitude- and time-dependent manner. In the case of Egr-1, we also

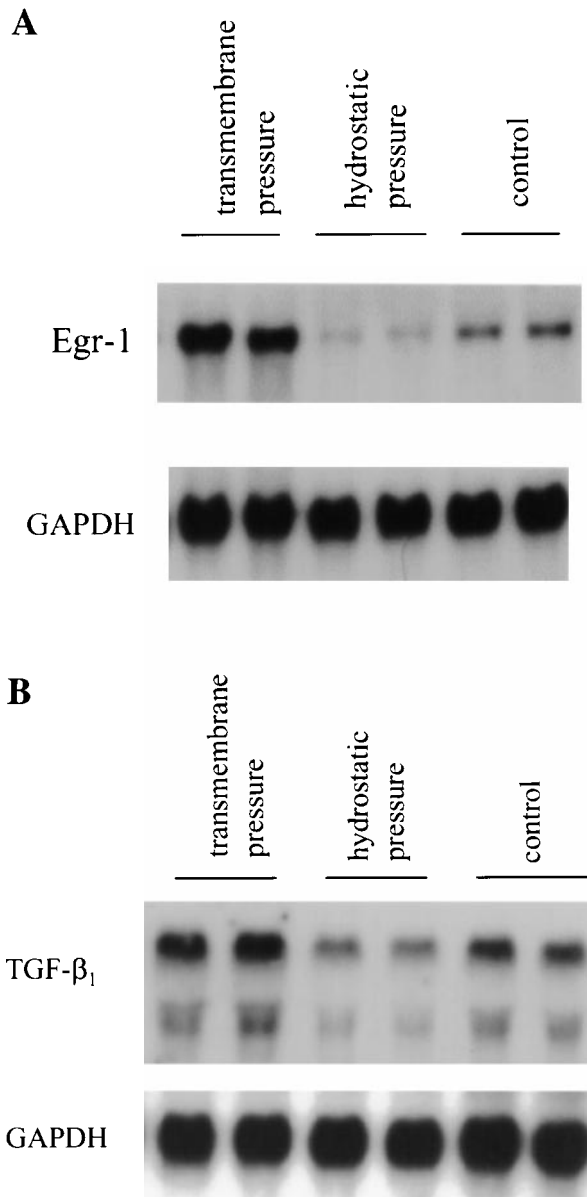


Fig. 5. *A*: Egr-1 mRNA expression after 1 h of 10 cmH<sub>2</sub>O transmembrane pressure or hydrostatic pressure application. *B*: TGF- $\beta_1$  mRNA expression after 6 h of 10 cmH<sub>2</sub>O transmembrane pressure or hydrostatic pressure application.

demonstrated by immunohistochemistry that elevated mRNA levels are accompanied by elevated Egr-1 protein levels.

The compressive stresses applied were estimated to be in the physiological range of those generated by smooth muscle. For example, canine airway smooth muscle has been shown to generate intraluminal pressures > 30 cmH<sub>2</sub>O in small bronchi with ACh stimulation (15). The magnitude of compressive stress acting on the epithelium of a constricted airway (such as that depicted in Ref. 51) can be estimated by modeling the airway as a thick-walled cylinder under external pressure. A simple force balance shows that the mean circumferential wall stress ( $\bar{\sigma}$ ) is related to the effective external pressure due to smooth muscle constriction

( $P_{sm}$ ) through the following expression:  $\bar{\sigma} \cong (R/t + 1)P_{sm}$ , where  $R$  is the inside radius and  $t$  is the wall thickness, both of the constricted airway (43). Using the estimated value of  $R/t \approx 0.5$  (51) and substituting  $P_{sm} = 30$  cmH<sub>2</sub>O gives  $\bar{\sigma} \approx 45$  cmH<sub>2</sub>O. A thick-walled cylinder is a crude model of a buckled airway and does not provide any details of the stress pattern on the epithelium. Extension of previous finite-element models of the airway (48) leads to a more accurate estimation of the type and magnitude of stresses experienced by the epithelium during smooth muscle constriction that are consistent with this rough estimate (33).

After rapid smooth muscle constriction, the airway tissue may initially be regarded as incompressible and the epithelial cells within the folds would experience no transcellular pressure difference. However, if constriction continues, fluid within the high-pressure regions of the airway wall may shift to regions of lower fluid pressure within the wall or in the luminal space. As this occurs, stresses within the constricted airway would be increasingly borne by the matrix, and the epithelial cells within the folds would eventually experience a transcellular fluid pressure gradient such as that imposed in our in vitro system. Employing poroelastic computational models of the airway wall would be helpful in determining the time-varying fluid stresses within a constricted airway.

In the absence of smooth muscle constriction, the airway is unbuckled and the epithelium only experiences those stresses associated with airway wall strain and variations in air pressure. Wall strain is difficult to estimate, but it has been reported that the circumference of the supporting basement membrane changes little during changes in airway caliber (19). Variations in air pressure in the airways amount to only  $\sim 1$  cmH<sub>2</sub>O with quiet breathing (47). Thus compressive stresses acting on the epithelium in an unconstricted airway during normal breathing are likely to be well below those in a constricted airway and also below those that resulted in signal transduction in our experiments. During positive-pressure ventilation, however,

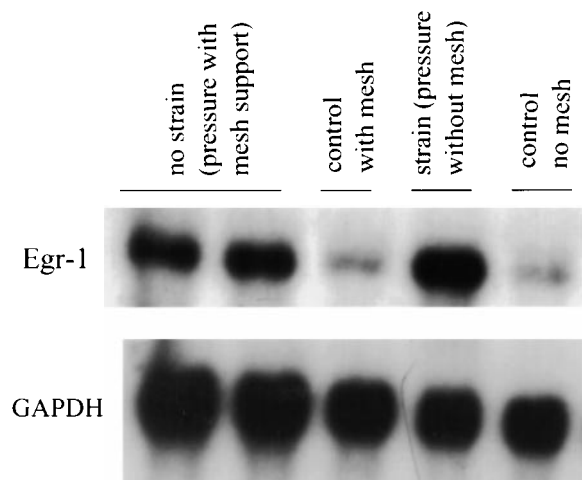


Fig. 6. Effect of membrane strain on Egr-1 mRNA expression. Experiment was 10 cmH<sub>2</sub>O pressure applied for 1 h with and without a mesh support to remove membrane strain.

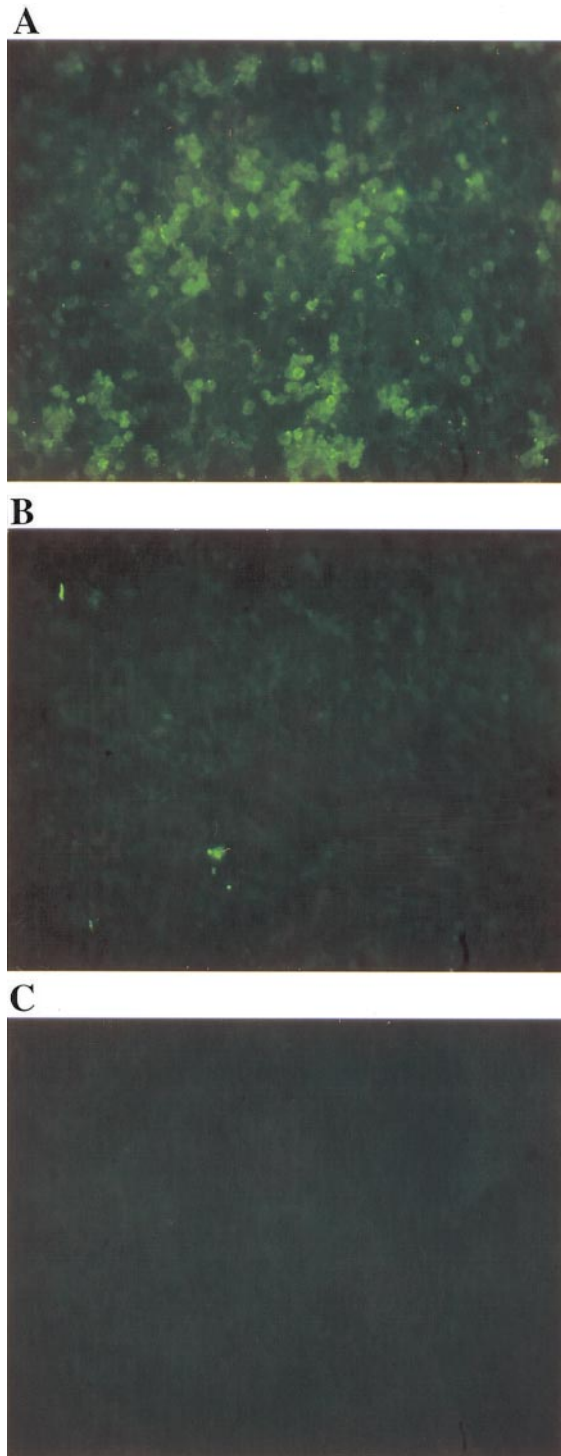


Fig. 7. Immunofluorescence of Egr-1. Cells exposed to 20 cmH<sub>2</sub>O for 6 h (A) showed increased Egr-1 protein immunostaining. B: control cells with no pressure. C: cells stained with irrelevant mouse IgG antibody.

airway pressures reach levels in excess of 35 cmH<sub>2</sub>O. This has led others to suggest that mechanical ventilation may lead to trauma and remodeling of the lung parenchyma (5, 13), although the cause may be high alveolar distension (13), high pulmonary capillary pressures (5), or high oscillatory airway pressures.

We chose not to study pressures higher than 20 cmH<sub>2</sub>O to avoid rupturing the culture membrane and because of the decrease in cell viability observed at 20 cmH<sub>2</sub>O. The cause of this cell death is unknown. Vital staining of the culture wells after 6 h of 20 cmH<sub>2</sub>O transmembrane pressure showed predominantly live cells, with small clusters of dead cells scattered throughout the culture well (data not shown). The late onset of cell death in the high-pressure experiments suggests an apoptotic rather than a necrotic pathway, but this was not determined in these experiments. There is intriguing evidence of a link between cell viability and cytoskeletal integrity. Disruption of cytoskeletal components has been shown to lead to apoptosis either by liberation of enzymes or by loss of mechanical tension at points of cell contact (see Ref. 21 for review). If the stress of transmembrane pressure application disrupted the cytoskeleton of the RTE cells, then the resulting cell death may have been a natural consequence of this disruption. Because mRNA upregulation was observed at pressures where cell death was undetectable and at the early time point in the high-pressure experiments, gene transcription is not believed to depend on cell death.

Bronchoconstriction in asthma may occur rapidly after exposure to antigen or may occur several hours after antigen exposure and last several hours to several days (46). Thus epithelial cells in a constricted airway may be exposed to mechanical stresses for periods much longer than 6 h, the maximum exposure time in our experiments. We limited our study to a time course consistent with the duration of smooth muscle constriction during an acute asthma attack. Our results show that epithelial cells respond rapidly to mechanical stresses and suggest that repeated bouts of bronchoconstriction, even for relatively short times, may indeed result in accumulation of extracellular matrix in the airway wall.

RTE cells were used as a model system to study the cellular response to forces in constricting bronchi. The epithelial cell phenotypes vary along the length of the bronchial tree, so it is not known whether rat bronchial epithelial cells would respond to compressive stresses in the same manner as tracheal epithelial cells. Further experiments would be required to determine regional differences in the response of epithelial cells to mechanical stress.

This study focused on genes thought to influence the asthmatic airway. Egr-1 is an immediate-early gene and zinc finger transcription factor. It has been shown to play a role in cell proliferation (17), has been detected at sites of vascular injury (24), and is upregulated by increased cardiac ventricular loads (pressures) in cat heart muscle (36). Egr-1 also activates transcription of PDGF (24, 25), which stimulates fibroblast proliferation and collagen production (31). Other genes such as TGF- $\beta$ 1 and Egr-1 itself also have nucleotide recognition sites for Egr-1. Egr-1 is known to be sensitive to mechanical stimulation (29); our experiments showed that Egr-1 was particularly sensitive to the magnitude of normal stress and was upregulated with as little as 5

cmH<sub>2</sub>O of pressure. The lack of upregulation at 2 cmH<sub>2</sub>O indicates that the manipulations required to apply the steady pressure did not initiate mechanotransduction. The time course for transcription was similar to that in endothelial cells subjected to shear stress, with early (30 min to 1 h) enhanced transcription and a decrease to baseline by 6 h (40).

ET-1 is a potent airway contractile agonist that also promotes collagen synthesis by fibroblasts (4). Its transcription appears to be highly dependent on the type and magnitude of stress. Physiological levels of fluid shear stress on vascular endothelial cells cause rapid downregulation of ET-1 mRNA in a dose-dependent manner (27). Stretch, on the other hand, has been shown to either upregulate ET-1 mRNA expression (44) or have no effect (27) in endothelial cell cultures. Our experiments showed a different pattern of ET-1 mRNA expression, with rapid (1 h) and sustained (6 h) elevation when 20 cmH<sub>2</sub>O was used as the stimulus. Interpretation of this finding is complicated, however, by the cell death observed in experiments conducted at 20 cmH<sub>2</sub>O.

Stretching mesangial cell cultures caused induction of TGF- $\beta$ 1 mRNA, but long periods of stretch (36–48 h) were required (34, 53). In the present experiments, TGF- $\beta$ 1 mRNA levels were clearly increased after only 6 h of pressure stimulation. TGF- $\beta$ 1 mRNA was elevated more than four times the control level with high pressure (20 cmH<sub>2</sub>O) and was doubled with 10 cmH<sub>2</sub>O pressure. Thus our results are consistent with those of Berg et al. (5), who reported that TGF- $\beta$ 1 mRNA expression levels in rabbit lung parenchyma were increased by a factor of four after only 4 h of high airway pressure ventilation. Our results also raise the possibility that airway epithelial cells were the source of the increased TGF- $\beta$ 1 mRNA levels noted by Berg et al. Because of the relatively short duration of our experiments, we did not investigate whether transmembrane pressure increased production of ET-1 and TGF- $\beta$ 1 proteins because there would likely be a delay of several hours between expression of message and production of protein. Further experiments would be necessary to determine whether transmembrane pressure also leads to production, secretion, and activation of these proteins.

*Mechanisms of gene induction.* Although it is unclear exactly how transmembrane pressure stimulates gene expression in epithelial cell cultures, we are able to eliminate certain potential causes. In our experiments, we observed average culture membrane strains as high as 2.3%. Although cyclic strains as low as 1% are adequate to produce changes in gene expression (52), no studies have shown any effect of such small static strains. Our own experiments demonstrated elevated levels of Egr-1 mRNA even when membrane strain was reduced 10-fold. Therefore, membrane strain is not responsible for our findings.

Experiments were also performed to eliminate the effects of hydrostatic pressure as a mechanism for gene induction in our cultures. Although hydrostatic pressures in the range of 10–170 cmH<sub>2</sub>O have been shown

to enhance cell proliferation (23), DNA synthesis (16), or release of cellular protein (1), there are no examples of hydrostatic pressure affecting gene expression levels. Our experiments did not show any changes in mRNA expression with 10 cmH<sub>2</sub>O hydrostatic pressure. It is unclear how hydrostatic pressure would stimulate the cells because this would not lead to cell deformation. It is possible that changes in dissolved gas partial pressures induced the changes seen in the experiments by Acevedo et al. (1) with elevated hydrostatic pressure. However, in other studies in which pressure was elevated with the use of an inert gas and medium pH, PO<sub>2</sub>, and PCO<sub>2</sub> were carefully monitored, similar responses were seen (16, 23). In our experiments, pressurization would cause a small (<1%) increase in the gas partial pressures above the cells, but pressure in the culture medium was not elevated. If changes in the gas partial pressure had induced gene expression, then we would have expected to also observe upregulation in the hydrostatic pressure experiments. Because we did not, we conclude that the slight changes in gas partial pressures were not responsible for the changes in gene expression that occurred with transmembrane pressure application.

We can only speculate on how the cell is deformed to transduce the signal, assuming that deformation, leading to protein conformational changes, is ultimately responsible. Raising the pressure above the cells might cause liquid to be expelled from the cells, raising intracellular osmotic pressure and inducing cell volume regulation. Using osmotic water permeabilities of 40–50  $\mu$ m/s for distal airway epithelium (12) in the analysis of Fischbarg et al. (11) and assuming no concentration gradients to retard water flux (for maximum cell volume change) give a change in cell layer height of  $5\text{--}7 \times 10^{-4}$   $\mu$ m/s with 20 cmH<sub>2</sub>O pressure application. These changes could cause a transient reduction in cell layer thickness before volume regulation and might therefore cause sufficient deformation to transduce a biological signal.

Transmembrane pressure could also force liquid through the intercellular gap, producing a shear stress on the cells. In an experiment similar to ours, Dull et al. (9) measured solute and albumin fluxes across endothelial cell monolayers by pressurizing the medium on the apical surface of the cells. Dull et al. also grew their cells on Transwell inserts, and the pressures they used ranged from 5 to 10 cmH<sub>2</sub>O. Volume fluxes of medium without albumin ranged from  $3 \times 10^{-5}$  to  $1.4 \times 10^{-4}$  ml/s, which would be enough to force 1 ml of liquid across the cells in 2–9 h. The amount of surface liquid initially covering our cell cultures would be inadequate to produce such high volume fluxes, and the surface of our cells did not appear dry after 6 h of pressure application.

If fluid flux did occur, we may estimate the magnitude of shear stress ( $\tau_w$ ) by a force balance equating the pressure gradient ( $\Delta P$ ) acting over the cell layer and the shear stress in the intercellular space:  $\Delta P \cdot h \approx 2\tau_w L$ , where  $L$  is the thickness of the cell layer and  $h$  is the average intercellular gap size. The maximum  $\Delta P$

(20 cmH<sub>2</sub>O) applied across a 20- $\mu$ m-thick cell layer with an  $h$  of 200 nm (estimated from the transmission electron micrographs) may produce a shear stress of  $\sim$ 0.1 cmH<sub>2</sub>O (or 100 dyn/cm<sup>2</sup>) on the cells. Shear stresses as low as 10–25 dyn/cm<sup>2</sup> have been shown to induce gene expression in endothelial cell cultures (28, 32), so this mechanism of stimulation cannot be ruled out.

Pressure applied to the apical surface might also cause the cells to be deformed into the pores of the culture membrane. One study using hollow fiber membranes for separation of stem cells from bone marrow showed that long extensions of the cell membrane and cytoplasm extruded into the pores of the filtration membrane with 40 mmHg positive-pressure application (30). We attempted to visualize cell deformations at the membrane surface by transmission electron microscopy, but this proved inconclusive because the Transwell membrane was destroyed during processing (data not shown). No obvious cell extensions in the transmission electron or light micrographs were seen, however. Histological processing may have removed the appearance of the extensions, or the deformations into the pores were too small to detect by our methods. Deformations in the epithelial cell membranes are visible in micrographs of highly constricted airways (51). If deformation of the cell membrane is the stimulus for our response, then it is likely that this mechanism is present in both the *in vitro* and *in vivo* situations.

In highly constricted airways, there are likely to be several mechanical forces acting on the cells within the wall. Compressive stresses on the epithelial cells within the folds appear to deform the cell shape, squeezing the cells out of the fold toward the luminal space. Fluid movement within the wall and out of the wall may produce extracellular fluid shear stress and transcellular fluid pressure gradients, leading to cellular volume change and deformation. Therefore, potential transduction mechanisms in these transmembrane pressure experiments (such as deformation of the cell shape, fluid shear stress, and cellular fluid loss) are also likely to be present in constricted airways *in vivo*.

**Concluding observations.** The structure of the airway wall changes markedly in patients with asthma (20). Beneath the epithelial basement membrane is a fibrotic layer that is significantly thickened (35). It is generally believed that the increased amount of extracellular matrix material in the subepithelial collagen layer is produced by myofibroblasts (7) in response to inflammation (18, 35). Our results suggest that another potential mechanism may be at work, that the response of airway epithelial cells to mechanical stresses during prolonged smooth muscle constriction leads to elaboration of transcription factors and cytokines that could lead to airway wall remodeling. The compressive stresses studied were similar to those stresses in a constricted, buckled airway and are not present during normal lung function. The genes we selected to study have been shown to play a role in tissue remodeling. Egr-1 activates transcription of important remodeling genes such

as PDGF and TGF- $\beta$ 1 (24). ET-1 is a potent bronchoconstrictor, stimulates collagen secretion from fibroblasts (4), and is present in bronchial epithelium of symptomatic asthmatic patients (2). TGF- $\beta$ 1 plays a key role in lung fibrosis (6) and is expressed in asthmatic airways (26, 42). Thus production of these profibrotic mediators by the epithelium in response to mechanical stress could stimulate the fibroblasts to remodel the airway wall.

In conclusion, airway epithelial cell cultures stimulated by static transmembrane pressure upregulate expression of mRNAs for Egr-1, ET-1, and TGF- $\beta$ 1 in a magnitude- and time-dependent manner. Egr-1 protein is also upregulated by mechanical stress. It is unclear how transmembrane pressures stimulate our cell cultures, but we can eliminate the effects of hydrostatic pressure and membrane strain as possible mechanisms. The gene induction response may be similar to that of airway epithelium *in vivo* when severe prolonged smooth muscle constriction causes the airway wall to buckle and the epithelium folds into deep crevasses.

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