Mechanotransduction of stretch-induced prostanoid release by fetal lung epithelial cells

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Mechanotransduction of stretch-induced prostanoid release by fetal lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 291: L487–L495, 2006.—Mechanical ventilation is the primary supportive treatment for infants and adults suffering from severe respiratory failure. Adverse mechanical ventilation (over-distension of the lung) triggers a proinflammatory response. Along with cytokines, inflammatory mediators such as bioactive lipids are involved in the regulation of the inflammatory response. The arachidonic acid pathway is a key source of bioactive lipid mediators, including prostanoids. Although ventilation has been shown to influence the production of prostanoids in the lung, the mechanotransduction pathways are unknown. Herein, we established that cyclic stretch of fetal lung epithelial cells, but not fibroblasts, can evoke an extremely sensitive, rapid alteration in eicosanoid metabolism through a cyclooxygenase (COX)-2 dependent mechanism. Cyclic stretch significantly increased PGI2, PGF2α, PGE2, and thromboxane B2 levels in the media of epithelial cells, but did not alter leukotriene B4 or 12-hydroxyeicosatetraenoic acid levels. Inhibition of COX-2, but not COX-1, attenuated the cyclic stretch-induced PG increase in the media, suggesting that cyclic stretch primarily affected PG synthesis. Substrate (free arachidonic acid) availability for PG generation was increased because of a stretch primarily affected PG synthesis. Substrate (free arachidonic acid; phospholipase A2; calcium; mechanical ventilation regulating the injury response to adverse mechanical ventilation.

EXPERIMENTAL MODELS have left little doubt that high tidal volume ventilation triggers an inflammatory response in the adult and neonatal lung (11, 12, 48). Although cytokines have received most attention, prostanoids also have the ability to control acute inflammation. They can produce both pro- and anti-inflammatory actions, depending upon the inflammatory stimulus, the major prostanoid produced, and profile of prostanoid receptor expression (47). In contrast to cytokines, in which production is controlled at the transcriptional level, prostanoids are formed by posttranslational processes (Fig. 1). Thus altered prostanoid production and/or release can be a very early response to stress and may initiate many of the classical signs of ventilation-induced lung inflammation. Several studies have shown that mechanical stress influences the production of prostanoids in the lung. Stretch-induced prostanoid release by lung was first reported in 1969 (14). The mediator was identified as PGE2, and the authors suggested that the release of PGE2 might contribute to the hemodynamic effects of mechanical ventilation (7, 14). The release of prostanoids appears also to be altered in mechanically ventilated dogs and sheep (4, 6) as well as in rat models of ventilation-induced lung injury (52, 53). In vitro physical deformation by gentle scraping or agit-ation of cultured human pulmonary endothelial cells has been shown to increase the release of PGI2 (24). Cyclic stretching of fetal lung cells had a similar effect (42). In addition to lung cells, uterine myometrial cells, tendon fibroblasts, macrophages, and osteoblasts have all been shown to increase their prostanoid production because of increased mechanical stress (16, 25, 32, 43). Thus mechanostimulation of prostanoid production and/or release may be a conserved event in the evolu-tion of the inflammatory response triggered by mechanical forces. The mechanotransduction pathways leading to increased prostanoid formation upon physical stimulation remain to be elucidated.

Herein, we studied the intracellular signaling pathways of stretch-stimulated prostanoid formation in primary fetal lung cells. We report that cyclic stretch causes cell type-, amplitude-, and duration-dependent increases in prostanoid produc-tion that are mediated via calcium-dependent phospholipase A2 (PLA2), p44/42 mitogen-activated protein kinase (MAPK), and cyclooxygenase (COX)-2.

MATERIALS AND METHODS

Materials. Culture media, trypsin, antibiotics, and FBS were from GibCO-BRL. DNase and collagenase were from Worthington. Bioflex six-well plates (Bioflex Collagen Type 1 culture plates) were from Flexcell International. SB-203580 was from Alexis Biochemical. U-0126, AAPC05, haloenol lactone suicide substrate (HELSS), and 1,2-bis(2-aminoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) AM were from Cedarlane. Ibuprofen, Gd3+, and EGTA were from Sigma Chemical, while SC-560 and NS-398 were from Calbiochem.

Cell culture. Female timed-pregnant Wistar rats were obtained from Charles River (St. Constant, Quebec) and were housed in the Hospital for Sick Children animal facilities until used. All animal procedures were in accordance with Canadian Council of Animal Care guidelines and were approved by the Animal Care and Use Committee of the Hospital for Sick Children. Fetal timed-pregnant rats and their fetuses were killed at day 19 of gestation (term = 22 days). Rat fetal lung fibroblast and epithelial cells were isolated as described previously (10). The purity of each cell type was >90%. Within 24 h of isolation, fetal lung cells were harvested from cell culture plates with 0.25% (wt/vol) trypsin in 0.4 mM EDTA. Fibroblast and epithelial cells were inoculated separately at a density of 10⁶ cells/well on six-well type-I collagen-coated BioFlex plates. Cells were maintained for 24 h in minimal essential medium (MEM) +

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Fig. 1. Eicosanoids produced as a consequence of arachidonic acid metabolism. PLA₂, phospholipase A₂; PG, prostaglandin; TXA₂, thromboxane A₂; LT, leukotrienes; HETE, hydroxyeicosatetraenoic acid.

10% (vol/vol) FBS. Four hours before the cells being stretched, the medium was changed to MEM + 0.5% (vol/vol) FBS. After 3 h, cells were changed to fresh MEM + 0.5% (vol/vol) FBS with and without inhibitors, incubated for another hour, and then subjected to cyclic stretch or static culture.

Mechanical stretch of fetal lung cells. The Flexercell Strain Unit FX-4000 (Flexercell International) is a computer-driven instrument that simulates biological strain conditions using vacuum pressure to deform cells cultured on flexible, matrix-bonded growth surfaces. An equibiaxial strain across the surface of the membrane is achieved by applying vacuum underneath, which pulls the membrane downward to a preprogrammed level of elongation while the membrane is positioned over a stationary post. This ensures that the membrane is stretched in a single plane so that a uniform strain is generated in both the radial and circumferential direction. Thus the Flexercell system stretches the cells by changing the surface area on which the cells are attached, and the degree of stretch relates to the percent change in surface area (%ΔSA). Cyclic continuous radial elongations of 5, 10, or 17% were applied at intervals of 30 cycles/min for various durations. Based on an equation [%ΔSA = 0.0057 (%total lung capacity, TLC)² − 0.2608 (%TLC) + 4.802] describing the relationship between epithelial basement membrane surface area and lung volume in isolated rat lungs (49), these stretch regimens equate roughly to percent changes in epithelial basement membrane surface area seen in vivo at 47, 62, and 75% TLC. A 5% stretch regimen simulates the stretch amplitude of fetal breathing movements, which are intermittent movements, i.e., the fetus spends ~30% of its time making fetal breathing movements at late gestation. Thus fetal lung cells in utero will be exposed to a 5% stretch, but not continuously. Neither cell viability (trypan blue exclusion) nor cell attachment was affected by any of the applied stretch regimens or treatments. Neither cell viability (trypan blue exclusion) nor cell attachment was affected by any of the applied stretch regimens or treatments. Cell viability (trypan blue exclusion) and cell attachment were not subjected to stretch.

Mass spectral analysis of prostanoids. After exposure to the same duration of stretch or static culture, stable hydrolysis products of PGs, leukotrienes, and lipoxins were measured in the cells and culture media using an API4000 triple-quadrupole mass spectrometer (MDS SCIEX, Concord, Ontario, Canada) in the electrospray ionization negative-ion mode with Turbolon-Spray. Experimental samples were spiked with 1 ng of a mixture of deuterated analogs of the prostanoids to be measured (Cayman Chemical, Ann Arbor, MI), acidified to pH 4 with 1 N HCl, and extracted three times with ethyl acetate. The ethyl acetate layer, washed to neutrality with water, was evaporated to dryness under a stream of nitrogen and transferred to siliconized minivials for analysis by tandem mass spectrometry (MSMS). Quantitation was carried out by comparing the deuterium-to-proton ratio of the prostanoids in the sample with standard lines generated from authentic mixtures of eicosanoids. An Agilent HPLC 1100 was at the front end, equipped with a short Zorbax SB-phenyl column (3.0 × 50 mm, 3.5-μm spherical size; Chromatographic Specialties, Brockville, Ontario, Canada). The mass spectrometry (MS) source temperature was maintained at 500°C and the ion source voltage at 4,500 V. Compounds were separated on HPLC with a direct inlet into the MS source. HPLC solvents contained 4 μL propionic acid. HPLC followed the following program: 80:20 (vol/vol) water-acetonitrile at sample injection and maintained for 2 min, 75:25 (vol/vol) for 0.5 min, 50:50 (vol/vol) by 5 min, 45:55 (vol/vol) by 6.2 min, and 0:100 (vol/vol) by 11 min. The latter solvent was maintained for another 1.5 min before being replaced by 80:20 (vol/vol) water-acetonitrile for the next run. The flow rate was at 400 μL/min. MSMS parameters were established through infusion (20 μL/min) of each authentic standard separately. The Q1 spectrum was first obtained, followed by selection of the M-1 fragment ion, and recording of a Q3 spectrum after collision-induced decomposition (CID). Optimization of the parameters was carried out either manually or by running the quantitative optimization program to establish conditions for use in the analysis by the metabolic rate monitor. The CID gas was nitrogen. Authentic standards in appropriate dilutions (1 ng deuterated prostanoids of interest mixed with 50 pg-1 ng of undeuterated prostanoids) were prepared, and standard concentrations of eicosanoid were analyzed at the same time as the samples containing unknown amounts of the compound. Typically, 1 ng of deuterated standard was added to each unknown sample, and 20% (vol/vol) of the sample was injected for analysis.

RNA preparation. Two million cells (~2 wells of a 6-well bioflex plate) were placed in RLT lysis buffer, homogenized, and applied to RNA purification columns according to the manufacturer’s instructions (RNeasy; Qiagen, Mississauga, Ontario, Canada). After the columns were washed, the bound RNA was treated with DNase I, washed, and eluted.

Real-time PCR. Total RNA (2 μg) was reverse transcribed in a total volume 50 μl using random hexamers. The Sybygreen Universal Master Mix was used according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA), in which 50 ng of cDNA were amplified for COX-1 and COX-2, whereas 5 ng cDNA were amplified for 18S. COX-1 (forward primer: CCTCACCAGTCATTTCCCTGT;
One-way ANOVA was used to determine statistical significance (lung cell culture media Basal eicosanoid levels in primary fetal lung epithelial cells). Cyclic stretch of fetal lung epithelial cells did not alter the more modest than those of PGI2, PGF2 after a 30-min cyclic stretch; however, the increase was far more discernible levels of eicosanoids, with 12-hydroxyeicosatetraenoic acid (12-HETE) being the most abundant prostanooid and PGF2\(_\alpha\) the least (Table 1). A 30-min cyclic stretch (17% change in surface area) significantly increased the PG content in the media of fetal lung epithelial cells, specifically that of PGI2 (measured as 6-keto PGF1\(_\alpha\)), PGF2\(_\alpha\), PGD2, and PGE2 (Fig. 2A). Thromboxane (TX) B2 levels were also increased after a 30-min cyclic stretch; however, the increase was far more modest than those of PGI2, PGF2\(_\alpha\), PGD2, and PGE2 (Fig. 2A). A 180-min cyclic stretch revealed further increases in the media content of PGI2, PGF2\(_\alpha\), PGD2, and PGE2 (Fig. 2A). Cyclic stretch of fetal lung epithelial cells did not alter the media levels of leukotriene (LT) B4 or 12-HETE (Fig. 2A). In contrast to fetal lung epithelial cells, cyclic stretch did not alter the PG amount in the media of fetal lung fibroblasts (Fig. 2B). Cyclic stretch caused a small but significant decrease in TXB2 and 12-HETE in the media of fetal lung fibroblasts (Fig. 2B).

**RESULTS**

Stretch induces prostanoid release/production by fetal lung epithelial cells. Initially, we tested both fetal lung epithelial cells and fibroblasts for their prostanoid responses to cyclic stretch. Under static conditions, both cell types released measurable levels of eicosanoids, with 12-hydroxyeicosatetraenoic acid (12-HETE) being the most abundant prostanooid and PGF2\(_\alpha\) the least (Table 1). A 30-min cyclic stretch (17% change in surface area) significantly increased the PG content in the media of fetal lung epithelial cells, specifically that of PGI2 (measured as 6-keto PGF1\(_\alpha\)), PGF2\(_\alpha\), PGD2, and PGE2 (Fig. 2A). Thromboxane (TX) B2 levels were also increased after a 30-min cyclic stretch; however, the increase was far more modest than those of PGI2, PGF2\(_\alpha\), PGD2, and PGE2 (Fig. 2A). A 180-min cyclic stretch revealed further increases in the media content of PGI2, PGF2\(_\alpha\), PGD2, and PGE2 (Fig. 2A). Cyclic stretch of fetal lung epithelial cells did not alter the media levels of leukotriene (LT) B4 or 12-HETE (Fig. 2A). In contrast to fetal lung epithelial cells, cyclic stretch did not alter the PG amount in the media of fetal lung fibroblasts (Fig. 2B). Cyclic stretch caused a small but significant decrease in TXB2 and 12-HETE in the media of fetal lung fibroblasts (Fig. 2B).

**Graphical and statistical analysis.** All data are presented as the degree of change compared with static control cultures (medium collected at the same time as that of the stretch condition). All values are shown as means ± SE of at least three separate experiments. One-way ANOVA was used to determine statistical significance (P < 0.05) followed by post hoc analysis using Duncan’s multiple-comparison test (JMP statistical software).

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### Table 1. Basal eicosanoid levels in primary fetal lung cell culture media

<table>
<thead>
<tr>
<th>Eicosanoid</th>
<th>Epithelial Cells</th>
<th>Fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGI₂</td>
<td>34±10</td>
<td>57±9</td>
</tr>
<tr>
<td>TXB₂</td>
<td>87±14</td>
<td>18±2</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>12±2</td>
<td>25±7</td>
</tr>
<tr>
<td>PGE₂</td>
<td>64±10</td>
<td>164±9</td>
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<tr>
<td>PGD₂</td>
<td>17±2</td>
<td>24±1</td>
</tr>
<tr>
<td>LTB₄</td>
<td>38±2</td>
<td>52±9</td>
</tr>
<tr>
<td>12-HETE</td>
<td>474±27</td>
<td>520±73</td>
</tr>
</tbody>
</table>

Data are means ± SE of 5 individual experiments. Units are pg/ml. TXB₂, thromboxane B₂; LTB₄, leukotriene B₄; 12-HETE, 12-hydroxyeicosatetraenoic acid. Within 24 h of isolation, fetal lung fibroblast and epithelial cells (purity >90%) were inoculated separately at a density of 10⁶ cells/well on 6-well type-1 collagen-coated BioFlex plates and maintained for 24 h in MEM + 10% (vol/vol) FBS. The following day, the medium was changed to MEM + 0.5% (vol/vol) FBS. After 4 h, the medium was replaced with fresh MEM + 0.5% (vol/vol) FBS, and, after 3 h of incubation, the medium was collected for measurement of basal eicosanoid levels by mass spectrometry.
and in the cells (data not shown). Generally, the cell primary substrate required for PG synthesis, in the media (Fig. production. 

B: Lung epithelial cells were subjected to cyclic stretch of 5–17% elongation for 30 min, and AA acid and eicosanoid content in the media were measured. All graphs are presented as mean degree of change ± SE (n = 5 individual experiments). P < 0.05 vs. static controls (*) and vs. all other groups (#).

Inhibition of PLA2 abolishes stretch-induced PG release/production. Cyclic stretch altered the levels of free AA, the primary substrate required for PG synthesis, in the media (Fig. 3A) and in the cells (data not shown). Generally, the cell increases the levels of AA by the action of cytosolic phospholipases (cPLA2; 19, 30). To investigate the role of cPLA2 in cyclic stretch-induced AA and PG production, fetal lung epithelial cells were pretreated with inhibitors of cPLA2 activity before exposure to cyclic stretch. AACOF3 specifically inhibits the calcium-dependent cPLA2 isoform, whereas HELSS is a specific inhibitor for the calcium-independent PLA2 isoform (1, 3, 5, 26). With the exception of PGF2α, only inhibition of the calcium-dependent cPLA2 isoform attenuated the cyclic stretch-induced content of PGs in the media (Fig. 4A), implying a prominent regulatory role for calcium in stretch-induced PG production. To further clarify the role of calcium, we applied either the extracellular calcium chelator EGTA, the intracellular calcium chelator BAPTA AM, or the stretch-activated calcium channel blocker Gd3+ to the cells and subjected them to cyclic stretch for 30 min. BAPTA AM significantly reduced the stretch-induced increase of PGs in the media; however, the effect was more robust with EGTA (Fig. 4B). The removal of extracellular calcium by EGTA reduced the stretch-induced increase of PGF2α by 91%, PGE2 by 95%, PGD2 by 91%, and PGF2α by 86%, whereas chelating of intracellular calcium with BAPTA AM reduced the stretch-induced increase of PGF2α by 55%, PGE2 by 66%, PGD2 by 65%, and PGF2α by 29% (Fig. 4B). Only EGTA completely abolished the cyclic stretch-induced release of free AA (Fig. 4C). Thus it appears that an influx of extracellular calcium and subsequent activation of a calcium-dependent cPLA2 are requirements for the mechanoproduction of PG in fetal lung epithelial cells. Interestingly, the calcium influx was independent of Gd3+-sensitive stretch-activated ion channels (Fig. 4, B and C).

Effect of p42/44MAPK and p38 MAPK inhibition on stretch-induced PG release/production. Besides an increase in intracellular calcium, necessary for cPLA2 translocation to membranes, full enzymatic activation of cPLA2 also requires its phosphorylation (19, 30). MAPK, in particular p44/42MAPK, have been shown to phosphorylate and activate cPLA2 (19, 30). In addition, cyclic stretch has been reported to activate p38MAPK and p44/42MAPK in lung epithelial cells (13, 35). Using relative specific inhibitors for p44/42MAPK (U0126) and p38MAPK (SB-203580), we found that p44/42MAPK, but not p38MAPK, inhibition resulted in a significant reduction of the stretch-induced increase of PGs in the media (Fig. 5A). Inhibition of p44/42MAPK also reduced the free AA levels (Fig. 5B), in agreement with a reduction in cPLA2 activity.

Inhibition of cyclooxygenase activity blocks stretch-induced PG release/production. Once AA is released from cell membrane phospholipids, it is converted to PGG2 by the action of cyclooxygenase. There are three isoforms of cyclooxygenase, COX-1, -2, and -3 (41). Because COX-3 is only found in neuronal cells, we focused on the actions of the constitutively expressed COX-1 isoform and the inducible isofrom COX-2. Initially, using ibuprofen, a nonselective cyclooxygenase inhibitor, we determined that the stretch-induced increase of PGs in the media of fetal lung epithelial cells was indeed the result of de novo PG synthesis and not just the release of preformed PGs. Ibuprofen had a dose-dependent inhibitory effect on stretch-induced PG formation (Fig. 6A), although it did not alter AA (Fig. 6A), LTB4 (not shown), or 12-HETE (not shown) levels. Using COX-1 (SC-560)- and COX-2 (NS-398)-specific inhibitors, we found that inhibition of COX-2, but not COX-1, activity abolished the stretch-induced increase in media PGs but did not affect AA formation (Fig. 6B). In addition, we demonstrated that both COX-1 and COX-2 mRNAs are present in resting lung epithelial cells (Fig. 6C). Upon cyclic stretch, epithelial fetal lung cells responded by increasing COX-2 mRNA expression while slightly decreasing COX-1 message levels.

DISCUSSION

Recently, it has become evident that the systemic response to overwhelming infection, ischemia-reperfusion injury, or tissue damage involves an uncontrolled expression of the inflammatory response. This results in the development of the systemic inflammatory response syndrome, which can result in
Fig. 5. Inhibition of mitogen-activated protein kinase (p44/42MAPK) reduces cyclic stretch-induced PG increases in the media of fetal lung epithelial cells. Lung epithelial cells preincubated for 1 h with inhibitors specific either to p44/42MAPK (U0126) or p38MAPK (SB-203580) were subjected to cyclic stretch (17% change in surface area) for 30 min, and AA acid and eicosanoid content were measured in media by multiplex mass spectrometry. A: Preincubation with U0126 (10 μM) significantly reduced the cyclic stretch-induced increase of PG in the media, which was not observed when cells were preincubated with SB-203580 (10 μM). B: The increase in free AA levels resulting from cyclic stretch was also significantly reduced with U0126, but not with SB-203580. All graphs are presented as the degree of change ± SE (n = 4 individual experiments). P < 0.05 vs. static untreated controls (*) and vs. all other groups (#).

Fig. 4. Inhibition of calcium-dependent cPLA₂ activity abolishes cyclic stretch-induced PG increases in the media of fetal lung epithelial cells. Lung epithelial cells preincubated for 1 h with various inhibitors were subjected to cyclic stretch (17% change in surface area) for 30 min, and AA and eicosanoid content were measured in media by multiplex mass spectrometry. A: AACOF₃ (10 μM), a calcium-dependent cPLA₂ inhibitor, but not HELSS (50 μM), a calcium-independent cPLA₂ inhibitor, reduced the stretch-induced increase of PG. B: Removal of extracellular calcium using EGTA (1 mM) completely abolished the stretch-induced PG increase. Also, the intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid (BAPTA) AM (10 μM) significantly reduced the stretch-induced increase in PG, whereas Gd³⁺ (15 μM), a mechanosensitive calcium channel blocker, had no effect. C: Preincubation with EGTA completely abolished the cyclic stretch-triggered increase in free AA. BAPTA AM partially reduced the cyclic stretch increase in AA, whereas Gd³⁺ did not have any effect. All graphs are presented as the degree of change ± SE (n = 5 individual experiments). P < 0.05 vs. static untreated controls (*) and vs. all other groups (#).
multiple organ dysfunction syndrome. These syndromes involve both the activation of inflammatory cells and the production of multiple pro- and anti-inflammatory mediators. These mediators can act both locally and systemically to enhance, perpetuate, or reduce/resolve the inflammatory cascade. Among these mediators are prostanoids derived from membrane phospholipids (Fig. 1). The present study demonstrates that lung epithelial cells can significantly influence the inflammatory response when exposed to overt levels of cyclic stretch or ventilation by increasing PG and thromboxane formation. In particular, we found that the mechanotransduction machinery necessary to increase PG synthesis is present in fetal lung epithelial cells, but not fetal lung fibroblasts, and that the PG response to stretch is triggered by an increase in calcium influx from the extracellular milieu and requires the combined action of calcium-dependent cPLA2, p44/42MAPK, and COX-2 for maximal response.

Previous studies have reported that stretch increased PGI2 release in mixed fetal lung cells (42) and PGE2 levels in the whole lung (7, 14). In the present study, we used mass spectral analysis coupled with liquid chromatography to gain a better understanding of the overall effect of mechanical stretch on eicosanoid metabolism. We confirmed that cyclic stretch increased PGI2 and PGE2 formation by fetal lung epithelial cells. However, we show for the first time that cyclic stretch also increases the release of PGD2 and PGF2\(\alpha\) by fetal lung epithelial cells while not affecting 8-isoprostane, leukotriene, or 12-HETE formation. Within the lung, both PGE2 and PGI2 can act on the endothelium to promote edema formation (47), a characteristic feature of volutrauma-induced lung injury (55). However, PGI2 has also been shown to have beneficial hemodynamics (59) and anti-inflammatory effects (9). Thromboxane can also promote edema formation in the lung (40) and has the ability to increase platelet and neutrophil aggregation as well as leukocyte adhesion (44). PGD2, on the other hand, may act as a chemotactic factor for leukocytes (22), or, through its dehydration end product, PGJ2 may act as an endogenous ligand for the transcription factor peroxisome proliferator-activated receptor-\(\gamma\), thereby evoking an anti-inflammatory response (39). The exact role of PGF2\(\alpha\) in inflammation is unknown, but it may induce receptor-mediated increases in cAMP and intracellular calcium in inflammatory cells and as such trigger a proinflammatory response (47).

In addition to an increase in extracellular prostanoids, cyclic stretch of fetal lung epithelial cells also increased extracellular AA levels, which by itself can act as a second messenger and modulate a number of cellular functions independent of prostanoids (20, 29). Furthermore, we clearly demonstrate that the increase of these mediators in the media upon stretch is the result of de novo synthesis and not just the release of endogenous pools. In contrast to epithelial cells, cyclic stretch of fetal lung fibroblasts had either no effect or resulted in a small reduction of TXB2 and 12-HETE in the media. The reductions in media TXB2 and 12-HETE content may be because of either an increased release of prostanoid-catabolizing enzymes (46) or an enhanced uptake of TXB2 and 12-HETE through receptor-mediated endocytosis (21, 45). In the lung, the prostanoid mechanoresponse to cyclic stretch is cell-type dependent. Supporting this contention, several reports have found that the mechanoproduction of PGs is cell-type dependent. Gentle scraping or agitation of cultured human pulmonary endothelial

![Fig. 6. Cyclic stretch-induced increase of PGs in the media of fetal lung epithelial cells is mediated via cyclooxygenase (COX)-2.](http://ajplung.physiology.org/)
cells has been shown to increase the release of PGI₂ (24). In contrast, mechanical stimulation of human and feline airway epithelial cells resulted in a decrease in the synthesis of PGs (37). Biologically, these findings imply that there are fundamental differences in the mechanomachinery of cells from different origins. Our present data show that the lung epithelial PG response to stretch is extremely rapid and sensitive. Thus the mechanoproduction of PGs by lung epithelial cells may be a rapid initial response to altered mechanical stress, and these lipid mediators could amplify the inflammatory response associated with bronchopulmonary dysplasia and acute respiratory distress syndrome.

When the inflammatory cascade is activated, PLA₂ are often involved. Stimulation of PLA₂ activity has been demonstrated in response to inflammatory mediators like platelet-activating factor, tumor necrosis factor-α, and interleukin-1β (17). Several in vitro studies have shown that mechanical stress activates PLA₂ in a variety of cells (2, 34, 51). High tidal volume ventilation is a well-known initiator of the inflammatory cascade in the lung (11, 12, 23, 48). A recent study has shown that inhibition of PLA₂ activation reduces the high tidal volume-induced lung injury in mice (57). In the present study, we found that PLA₂ was a key regulator of stretch-induced PG synthesis in the lung epithelium. Thus we speculate that the reported protective effect on ventilator-induced lung injury by inhibition of cPLA₂ (57) is the result of a reduction in PG formation.

Mammalian cells contain structurally diverse forms of PLA₂, including secretory PLA₂, calcium-independent PLA₂, and cPLA₂. cPLA₂ is ubiquitously expressed and is regulated by micromolar concentrations of calcium and reversible phosphorylation (30). Herein, we show that stretch-induced PG synthesis in lung epithelial cells is mediated via cPLA₂ through an influx of extracellular calcium. cPLA₂ requires calcium for binding to membranes (30), and we assume that cPLA₂ translocates to membranes when intracellular calcium levels increase in response to stretch. Cyclic stretch-activated cPLA₂ through an extracellular calcium influx has also been reported for kidney epithelial cells (2). In addition, Alexander and colleagues (2) reported that stretch activation of cPLA₂ was dependent on p44/42MAPK activity. Several other studies have demonstrated that cPLA₂ activity can be regulated by MAPKs (15, 30, 58). It has been suggested that p44/42MAPK activation and an increase of intracellular calcium are both conjointly required for full cPLA₂ activation and subsequent arachidonate release (8, 30). Our observation that inhibition of calcium-dependent cPLA₂ completely abolished the stretch-induced increase in PG content, whereas inhibition of p44/42MAPK only partially reduced stretch-induced PG increases, supports the dual-activation scenario for cPLA₂. In contrast to a report suggesting that phosphorylation of cPLA₂ must precede the increase in intracellular calcium to fully activate the enzyme for AA release (38), our data are compatible with cyclic stretch promoting a rapid calcium-induced translocation of cPLA₂, resulting in enzyme activation and subsequent further activation via p44/42MAPK phosphorylation.

Once AA is released by cPLA₂, the cyclooxygenase pathway increases PGH₂ levels, which are further metabolized to PGE₂, PGI₂, PGD₂, and TXA₂ via their respective synthases. Previous studies (42) have suggested that cyclooxygenases are involved in cyclic stretch-mediated synthesis of prostacyclin in mixed fetal lung cells. The present finding of ibuprofen blocking cyclic stretch-induced increases in PG in fetal lung epithelial cells corroborates the involvement of cyclooxygenases. It also argues against the possibility that the cyclic stretch-induced increases in PG content in the media are because of the release of preformed mediators as has been shown for pulmonary surfactant (56). Both COX-1 and COX-2 have been implicated in models of acute inflammation, and it appears that the degree to which each cyclooxygenase isoform contributes depends on the inflammatory stimulus, the inflamed organ, and duration of inflammation (47). Studies using mice deficient in the expression of either COX-1 or COX-2 have identified unique roles of each cyclooxygenase isoform in various diseases. For example, COX-1 is the predominant enzyme for PG formation in AA-induced inflammation (28) and allergic airway disease (18). COX-2 predominates in inflammation models of carrageenan air pouch (27, 54) and dextran sulfate colitis (33). In the present study, we demonstrate using selective COX-1 and -2 inhibitors that COX-2 solely mediates the cyclic stretch-induced release of PG in fetal lung epithelial cells. Our observation that cyclic stretch increased COX-2 mRNA expression, while decreasing COX-1 mRNA expression, is in line with a predominant role for COX-2 in stretch-induced inflammation in the lung. In addition, COX-2 mRNA expression has been shown to be upregulated by mechanical loading in various cell types (16, 25, 32, 43). Thus it appears that COX-2 is the predominant cyclooxygenase isoform regulating the mechano-production of prostaglandins in the lung epithelium.

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