Mechanical stretch stimulates macrophage inflammatory protein-2 secretion from fetal rat lung cells

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Received 4 January 2000; accepted in final form 3 May 2000

Mechanical stretch stimulates macrophage inflammatory protein-2 secretion from fetal rat lung cells. Am J Physiol Lung Cell Mol Physiol 279: L699–L706, 2000.—Ventilation-induced lung injury has been related to cytokine production. Immaturity and barotrauma are important contributors to the development of bronchopulmonary dysplasia in infants. In the present study, stretch of organotypic cultured fetal rat lung cells was used to simulate ventilation of preterm newborns. Cells were stimulated with lipopolysaccharide (LPS; 100 ng/ml) and/or mechanical stretch. After 4 h, stretch enhanced LPS-induced macrophage inflammatory protein (MIP)-2 production in a force- and frequency-dependent manner. The maximal effect of stretch was seen with 5% elongation at 40 cycles/min. In contrast, after 1 h of stimulation, stretch alone significantly increased MIP-2 production, which was not blocked by cycloheximide, an inhibitor of protein synthesis. At both the 1- and 4-h time points, only LPS increased MIP-2 mRNA levels. Stretch-induced MIP-2 release was associated with cell injury as measured by lactate dehydrogenase release and was not inhibited by gadolinium, a stretch-activated ion channel blocker. Taken together, these results suggest that the major effect of stretch on MIP-2 production from fetal rat lung cells is to stimulate its secretion.

ventilation; acute lung injury; physical force; cytokine; chemokine

Recent studies have demonstrated that ventilation-induced lung injury may be mediated through proinflammatory cytokines and other inflammatory mediators (5, 35) that may cause or exacerbate lung injury by an inflammatory response (15). For example, in a surfactant-depletion rabbit model, conventional ventilation resulted in progressive hypoxemia, decreased pulmonary compliance, hyaline membrane formation, and neutrophil accumulation in the lung (10). The number of polymorphonuclear leukocytes and the levels of inflammatory chemical mediators were significantly increased in bronchoalveolar lavage (BAL) fluid (10), and mRNA of tumor necrosis factor (TNF)-α, a proinflammatory cytokine, was increased in the cells isolated from BAL fluid after conventional ventilation (31). Blockade of the function of proinflammatory cytokines such as interleukin (IL)-1 (20) and TNF-α (9) attenuated the severity of ventilation-induced lung injury in surfactant-depleted rabbits.

To study the cellular and molecular mechanisms of ventilation-induced cytokine production from the lung, several groups (34, 38) have used isolated lungs as models and demonstrated that ventilation could increase cytokine production. Others have employed cell culture models to study mechanical stretch-induced production of IL-8, a C-X-C chemokine important for neutrophil recruitment and activation (1–3), in a human lung carcinoma cell line, A549 cells (23, 36, 37). In these studies (23, 36, 37), cells were cultured as a confluent monolayer on flexible membranes that could be stretched with negative pressure or other mechanisms. Stretch alone or in combination with other stimuli increased IL-8 production (23, 36, 37).

The preterm lung is highly susceptible to injury during resuscitation or mechanical ventilation because of the small gas volumes per kilogram of body weight (11). Excessive ventilator settings, oxygen, and prematurity have been suggested to be the most important factors for bronchopulmonary dysplasia (BPD) in preterm infants (32, 33), especially in very low birth weight (VLBW) infants (11). It has been reported that ventilation of the VLBW infant is associated with increases in proinflammatory mediators in air space samples (8).

We hypothesized that mechanical stretch may induce cytokine production from preterm lung cells, which could play a role in ventilation-induced lung injury and contribute to the development of BPD. In contrast to previous studies (23, 36, 37), we applied mechanical stretch to primary cultured fetal rat lung cells in an organotypic culture to simulate the effects of ventilation on premature lungs. The three-dimensional culture system allowed fetal lung cells to form alveolar-like structures. In addition, lipopolysaccharide (LPS),
the major component of endotoxin, was used to determine whether there was a synergistic effect on cytokine release in combination with mechanical stretch. Macrophage inflammatory protein (MIP)-2 is a rodent homologue of human IL-8 and an important mediator in inflammatory reactions (6, 18, 30). Xavier et al. and Zhang et al. have recently found that primary cultured adult rat pneumocytes (40) and lung explants (42) produced MIP-2 in response to LPS stimulation. Therefore, this C-X-C chemokine was chosen as a representative cytokine for study. Our data suggest that the major effect of mechanical stretch on MIP-2 production from fetal lung cells is to stimulate its secretion.

**METHODS**

Materials. Cell culture media, fetal bovine serum, and trypsin were obtained from Gibco (Burlington, ON). DNase and collagenase were from Worthington (Freehold, NJ). Gel-foam sponges were from Upjohn (Toronto, ON). All other chemicals were from Sigma (St. Louis, MO).

Mechanical strain of fetal lung cells in organotypic culture. Female timed-pregnant Wistar rats (200–250 g) were purchased from Charles River (St. Constant, PQ). Pregnant rats were killed by an excess of halothane on day 19 of gestation (term = 22 days). Fetal lungs were pooled from two litters for every cell isolation procedure. Organotypic cultures of fetal lung cells were established as previously described (14). Briefly, fetal rat lungs were dissected out, minced, and resuspended in Hanks’ balanced salt solution. The minced lung tissue was trypsinized (0.125% wt/vol) trypsin and 0.4 mg/ml of DNase), filtered, and centrifuged. The cells were inoculated on 2 × 2 × 0.25-cm Gelfoam sponges at a density of 3 × 10^6 cells/sponge and incubated overnight in MEM plus 10% (vol/vol) fetal bovine serum. The sponges were washed two times with serum-free MEM, and the medium was replaced by MEM and changed daily. Three days after cell isolation, the cells were challenged by mechanical stretch and/or LPS.

Bio-Stretch, a mechanical strain device (ICCT, Markham, ON) used in these studies, has been described in detail elsewhere (13). It consists of a programmable Bio-Stretch controller, a personal computer, and three sets of solenoids. Each set contains five solenoids and can be programmed individually. A culture dish with a Gelfoam sponge was placed in front of each solenoid. One end of the sponge was fixed to the bottom of the dish, and the other end was attached to a movable metal bar that was wrapped and sealed in sterile plastic tubing. A magnetic force generated through the solenoids acted on the metal bar to apply strain to the organotypic cultures. The sponges were subjected to various degrees of elongation from their original length at a defined frequency for various time periods. The recoil properties of the sponge allowed it to return to its original length. At any time, all data were collected without saturation or missing bands. The background of the OD reading for each band was subtracted locally. Each experiment was conducted at least two times to ensure reproducibility.

Statistical analysis. All experiments were carried out with materials collected from at least two to three separate cell cultures in triplicate or more. All data are expressed as means ± SE from separate measurements and were analyzed by one-way ANOVA followed by Student-Newman-Keuls test (29), with significance defined as P < 0.05.

**RESULTS**

Mechanical stretch enhanced LPS-induced MIP-2 production from fetal rat lung cells. Xavier et al. (40) have recently shown that primary cultured adult rat lung epithelial cells responded to LPS stimulation by producing MIP-2. The optimal concentration of LPS was 10 μg/ml, and the peak MIP-2 production was at 4 h (40). To determine whether fetal rat lung cells could produce MIP-2 in response to LPS, cells isolated from day 19 fetal lungs (cannicular stage of lung develop-
ment) were cultured in Gelfoam sponges and subjected to LPS stimulation (0.1–1,000 ng/ml) for 4 h. MIP-2 concentration in the culture medium increased from 660 to 1,800 pg/ml in an LPS dose-dependent manner (data not shown).

To study the effects of mechanical stretch and/or LPS on MIP-2 production, a dose of LPS (100 ng/ml) that induced a submaximal increase in MIP-2 concentration in the culture medium after a 4-h incubation was chosen. The cells were stimulated with and without LPS for 4 h and continuously subjected to either 2 or 5% stretch at 40 cycles/min. In the presence of LPS treatment, mechanical stretch significantly increased MIP-2 production in a force-dependent fashion ($P < 0.05$; Fig. 1).

To determine the influence of stretch frequency on MIP-2 release, the cells were incubated with and without LPS (100 ng/ml) and subjected to 5% elongation at 20, 40, or 80 cycles/min for 4 h. In the absence of LPS, as stretch frequency increased from 20 to 80 cycles/min, MIP-2 levels increased from 400 to 700 pg/ml. However, this increase was not significant (Fig. 2). In the presence of LPS, the shape of the MIP-2 versus frequency curve was altered, with a plateau in MIP-2 released at 40 cycles/min (Fig. 2). In the presence of LPS, both 40 and 80 cycles/min of stretch significantly increased MIP-2 production ($P < 0.05$) compared with that in cells stretched at 20 or 40 cycles/min without LPS (Fig. 2). Because 40 cycles/min is a commonly used ventilation frequency for preterm newborns, this stretch regimen (5% elongation, 40 cycles/min, 100 ng/ml of LPS) was chosen for further experimentation.

Using these settings, we performed several experiments to ensure the reproducibility of the results. We noted that the basal MIP-2 levels in the control group varied among different experiments. In some experiments, it was $<200$ pg/ml (Figs. 1 and 3A), whereas in others, it could be $>1,000$ pg/ml (Fig. 3B). The magnitude of stretch- and/or LPS-induced MIP-2 in the culture medium also changed accordingly (Fig. 3). These alterations may be due to the variations in primary cell...
culture conditions (see DISCUSSION). In general, after a 4-h incubation, stretch alone did not induce a significant increase in MIP-2 (Fig. 3). However, in the presence of LPS, stretch always induced a significant increase in MIP-2 production ($P < 0.05$; Fig. 3), which was consistent from experiment to experiment (Figs. 1–3).

Mechanical stretch induced immediate release of MIP-2 and cell injury. Using the selected stretch regimen, we then studied the time course of LPS- and/or stretch-induced MIP-2 production. The cells were stimulated for three different time periods: 1, 2, and 4 h. Interestingly, at 1 or 2 h of incubation, LPS treatment did not increase MIP-2 levels, whereas mechanical stretch induced a significant elevation in MIP-2 ($P < 0.05$; Fig. 4). The combined effect of LPS and stretch on MIP-2 production was mainly due to stretch (Fig. 4). After 4 h of incubation, the results (Fig. 4) looked very similar to the results described in Mechanical stretch enhanced LPS-induced MIP-2 production from fetal rat lung cells (Figs. 1–3); that is, LPS-induced MIP-2 was enhanced by mechanical stretch. We used the 1- and 4-h incubation periods for further studies.

To determine whether stretch and/or LPS stimulation induced cell damage, LDH concentrations in the culture medium were measured. After 1 h of stretch, LDH levels increased ~60% ($P < 0.05$; Fig. 5). After 4 h of incubation, the LDH level in the culture medium was elevated, which was further increased by mechanical stretch ($P < 0.05$; Fig. 5). Similar to data we obtained from adult rat lung epithelial cells (40) and rat lung explants (42), LPS alone had no such effect (Fig. 5). When the cells were subjected to both stretch and LPS stimulation, the LDH levels were the same as those with stretch alone. Therefore, cell injury was mainly due to mechanical stretch.

The effect of stretch on MIP-2 release is to stimulate its secretion. To determine whether stretch-induced MIP-2 production requires increased gene expression of MIP-2, cells were treated for 1 or 4 h as described in Mechanical stretch induced immediate release of MIP-2 and cell injury. Total RNA was extracted, and steady-state mRNA levels of MIP-2 and b-actin were analyzed with a semiquantitative RT-PCR as previously described (40). The steady-state mRNA of b-actin was not altered by LPS and/or stretch (Fig. 6); thus the ratio of MIP-2 to b-actin mRNA was used to present the alteration in MIP-2 gene expression. As shown in Fig. 6, at both time periods, it was LPS treatment that increased mRNA levels of MIP-2 ($P < 0.05$), whereas mechanical stretch had no such effect in either the absence or presence of LPS treatment (Fig. 6). This suggested that the effect of stretch on MIP-2 production from fetal lung cells is at the posttranscriptional level.

To determine whether stretch induced immediate secretion of MIP-2, we pretreated cells with cycloheximide (8 lM) for 30 min to block protein synthesis. The cells were then stimulated with LPS and/or stretch (Fig. 6). Conversely, after a 4-h incubation, cycloheximide reduced MIP-2 levels in all groups (Fig. 7B).

Stretch-induced MIP-2 release was not blocked by the stretch-activated ion channel inhibitor gadolinium. Liu et al. and Xu et al. have shown that a stretch-activated ion channel blocker, gadolinium, inhibited intermittent mechanical stretch-induced fetal rat lung cell proliferation (17) as well as intermittent stretch-induced release of glycosaminoglycan from these cells (41). Recently, it has been shown that gadolinium ameliorated high airway pressure-induced permeability increases in isolated rat lungs (22). To determine whether stretch-induced MIP-2 release is related to stretch-activated ion channels, the cells were treated with...
gadolinium (10 μM) and subjected to LPS and/or stretch. Figure 8 shows that gadolinium did not block the stretch-induced immediate release of MIP-2 at 1 h (Fig. 8A). Interestingly, it reduced the MIP-2 production induced by LPS alone or in combination with stretch at 4 h (*P < 0.05; Fig. 8B).

**DISCUSSION**

Stretch-induced cytokine from the lung and lung cells. Using an ex vivo rat lung ventilation model, Tremblay et al. (34) demonstrated that injurious ventilation regimens increased BAL fluid concentrations of several cytokines including TNF-α, IL-1β, IL-6, IL-10, MIP-2, and interferon-γ. With an isolated perfused mouse lung model, it has been shown that hyperventilation induced release of TNF-α and IL-6 into the perfusate (38). These experiments demonstrated that ventilation-induced cytokines are from the lung, and cytokines induced from the lung can be secreted into the alveolar space and released into the pulmonary circulation. Interestingly, although ventilation increased IL-10 in BAL fluid (34), anti-inflammatory cytokines such as IL-10 and soluble TNF-α receptors in the perfusate were either not measurable or not altered by the ventilation (38). Thus the imbalance between pro- and anti-inflammatory cytokines released from the lung into the circulation may contribute to the development of multiple-organ dysfunction syndrome (4, 28).

Pugin et al. (23) have shown that human macrophages produced IL-8 in response to stretch for 8–32 h at 20 cycles/min. This stretch regimen also enhanced LPS-induced TNF-α and IL-6 production from macrophages (23). Ventilation-derived mechanical stretch is primarily applied to pulmonary cells, especially alveolar epithelial cells. It has recently become clear that these cells can also produce various cytokines and chemokines (21, 25). When Pugin et al. (23) applied the same stretch regimen to human lung epithelial A549 cells, however, the increase in IL-8 at 24 h was minimal. Vlahakis et al. (37) also studied the effect of stretch on IL-8 production from A549 cells. They reported that 30% (but not 20%) stretch at 20 or 40 cycles/min increased IL-8 in A549 cells after 12–48 h. The stretch-induced increase in IL-8 production varied from experiment to experiment, but the trends were consistent (37). Using different stretch apparatus, Tsuda et al. (36) reported that after 8 h in culture, IL-8 production was not affected by stretch alone. However, in the presence of glass fibers or crocidolite asbestos, stretch significantly increased IL-8 production from A549 cells (36). These studies provided evidence that mechanical stretch plays an important role in cytokine
There are few inflammatory cells in the fetal lung; thus the MIP-2 measured in our experiments should be primarily from lung parenchymal cells. Because mixed cells were used, we could not identify the specific source of MIP-2. Primary cultured rat alveolar epithelial cells produce large amounts of MIP-2 (40). MIP-2 gene expression is increased in a rat fibroblast cell line (RFL-6) on TNF-α stimulation (7). Thus fetal rat lung epithelial cells and fibroblasts may also be able to produce MIP-2. The response of fetal lung epithelial cells and fibroblasts to mechanical stretch should be studied further. Interexperimental variations of MIP-2 levels observed in our studies were likely due to a number of factors: the number of fetuses from each litter, the size and degree of maturation of fetuses from experiment to experiment, and the cell isolation procedure. Nonetheless, the response of cells to stretch and/or LPS stimulation was consistent in all our experiments. The fetal lung cell stretch model could be used to further study the mechanisms of ventilation-induced cytokine production from preterm lungs and the possible roles of cytokines in the development of BPD.

Stimulation of stretch on MIP-2 secretion. There are several lines of evidence to suggest that the major effect of stretch on MIP-2 production from fetal rat lung cells is to stimulate its secretion. The mRNA levels of MIP-2 were elevated only by LPS and not by stretch. A rapid release of MIP-2 was observed as soon as 1 h after the initiation of stretch. This suggests that stretch induced release of premade MIP-2. Furthermore, when we used cycloheximide to inhibit protein synthesis, this stretch-induced immediate release of MIP-2 was still observed. In the studies of IL-8 production from human lung A549 cells (23, 36, 37), stretch was conducted for at least 8 h; thus the early effect of stretch on cytokine production was not described. In the ex vivo rat lung model, hyperventilation induced significant increases in cytokine production in the BAL fluid within 2 h (34). With the mouse isolated perfused lungs, hyperventilation-induced cytokines in the perfusate also increased significantly by 30 min (38).

An additive effect between a submaximal concentration of LPS and mechanical stretch on MIP-2 production was observed after 4 h. A similar result was obtained by Vlahakis et al. (37), who found that stretch increased the effect of TNF-α on IL-8 production when A549 cells were stimulated with lower doses (0.01 and 0.1 ng/ml) of TNF-α. Stretch also increased IL-8 production when A549 cells were stimulated with fibrous particles (36). It is possible that LPS, TNF-α, fibers, and other pathogens primarily control the synthesis of cytokines, whereas the major effect of stretch is to stimulate their secretion. Clinically, when ventilation is applied to patients with sepsis, bacterial infection, or other complications, this combined effect may enhance the production of cytokines.

The pathways by which stretch regulates the secretion of cytokines from lung cells merit further investigations. It is known that mechanical stretch can stimulate secretion of pulmonary surfactant from rat epithelial cells and fibroblasts.
alveolar epithelial cells (24, 39). Mourgeon et al. (19) have recently shown that mechanical stretch can stimulate secretion of fibronectin from fetal rat lung cells, which was independent from fibronectin mRNA expression and synthesis. Stretch also stimulated both constitutive- and regulated-secretion of glycosaminoglycans from fetal rat lung cells, which was mediated through G protein- and stretch-activated ion channels, respectively (41). Gadolinium, a stretch-activated ion channel blocker, however, did not affect stretch-induced MIP-2 release in this study. Interestingly, after 4 h of incubation, this agent inhibited LPS-induced MIP-2 production. Because gadolinium is a nonspecific channel blocker, other ions may be involved in LPS-induced MIP-2 production.

**Mechanical stretch-induced cell injury.** With LDH release as a marker, we have demonstrated that the selected stretch regimen used in most of our experiments induced cell injury. The amplitude of stretch we used (5% of elongation) was the same as our laboratory used previously for cell proliferation (14, 16, 17) and differentiation studies (19, 41). The major difference is that a continuous cyclic stretch was used in the present study. Liu et al. (16) and Tanswell et al. (33) have found that an intermittent stretch regimen (5% elongation, 60 cycles/min, 15 min/h) stimulated cell proliferation, whereas a continuous stretch with the same amplitude and frequency induced cell injury as determined by [14C]adenine release. The continuous cyclic stretching could be a nonphysiological stress to fetal lung cells because fetal breathing movements are irregular and intermittent. Jobe and Ikegami (11) suggested that in VLBW infants, BPD could represent the maldevelopment sequence from interference or interruption of normal development (11). Continuous mechanical ventilation might be considered as interference by fetal lung cells that are normally stretched by intermittent fetal breathing movements. On the other hand, the clinical relevance of 5% elongation is not clear because the surface area-volume relationship of premature lungs is unknown. The absolute stretch amplitude of fetal rat lung cells in vitro should not be extrapolated directly to that of mechanical ventilation of the human infant lung in vivo. However, these experimental data may provide clues for clinical studies.

It is not clear whether stretch-induced MIP-2 secretion is related to cell injury. Because stretch increased release of LDH at both the 1- and 4-h time points but stretch alone only induced MIP-2 release at 1 h, we suspect that its effect on MIP-2 secretion is not a direct consequence of cell injury.

In summary, in this study, primary cultured fetal rat lung cells were used as a model to simulate the effect of mechanical ventilation on premature lungs at the cellular level. LPS-induced production of MIP-2 was enhanced by mechanical stretch in a force- and frequency-dependent manner. Although the amplitude of stretch was the same as that used for physiological studies, changing from an intermittent to a continuous stretch regimen is sufficient to induce cytokine production and cell injury. This could be one of the mechanisms by which mechanical ventilation induces cytokine production and cell injury in premature lungs. Furthermore, in the present study, we demonstrated that the major effect of stretch on cytokine production is to stimulate secretion, not gene expression.

We are grateful to Xiao-hui Bai for technical assistance.

This work was supported by operating grants from the Medical Research Council of Canada (MT-13270), Canadian Cystic Fibrosis Foundation, the National Sanitarium Association of Canada, Ontario Thoracic Society, and James H. Cumming’s Foundation.

E. Mourgeon was a recipient of Fellowships from the Société Française d'Anesthésie et de Réanimation (French Anesthesiology and Critical Care Society) and the Faculty of Medicine (University of Toronto, Toronto, Ontario, Canada). N. Isowa was a recipient of a Fellowship from the Department of Surgery and Dean’s Office, Faculty of Medicine (University of Toronto). M. Liu is a Scholar of the Medical Research Council of Canada.

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