Reactive oxidant and p42/44 MAP kinase signaling is necessary for mechanical strain-induced proliferation in pulmonary epithelial cells

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MECHANICAL VENTILATION is frequently used as a life-saving intervention in respiratory failure. Infants born prematurely often receive prolonged pulmonary support resulting in potentially fatal lung injury (33). Adults with trauma, aspiration, or pneumonia resulting in respiratory failure often progress to acute respiratory distress syndrome, with a mortality of 40–70% (42). The interventions initiated to support patients with respiratory failure in acute respiratory distress syndrome, including mechanical ventilation, have been implicated in initiating a systemic response contributing to this mortality (14, 16, 29, 30). Understanding the signaling cascades initiated by mechanical strain could help optimize beneficial strain responses while mitigating detrimental effects of this therapy.

Mechanical strain is known to induce proliferation in lung cells (13, 26). The signaling molecule p42/44 mitogen-activated protein (MAP) kinase, activated by mechanical strain in lung epithelial cells (13), is a key effector in signaling proliferative responses in other cell types (10, 38). Strain has also been found to initiate an inflammatory response in isolated mouse lungs (43). Reactive oxygen species (ROS) are known to be signaling molecules involved in proliferation in a variety of cell types (3, 5, 15, 21, 37). ROS also play a role in signaling inflammatory cytokines (24) and have been linked to p42/44 MAP kinase signaling (17). Our laboratory has demonstrated a strain-induced oxidant response in pulmonary epithelial cells (12).

The present work investigates the hypothesis that enhanced ROS production is involved in strain-induced signaling in lung epithelial cells and that this ROS production mediates strain-induced p42/44 MAP kinase activation and ultimately strain-induced proliferation. Although cell-cell interactions can be studied more effectively in whole animal models, in vitro studies allow investigation of specific cell types in complex signal transduction cascades. Experiments in this paper focus on in vitro studies that address pulmonary epithelial cell mechanical strain-induced proliferative signaling.

MATERIALS AND METHODS

Cell culture. Human pulmonary adenocarcinoma H441 cells and MLE15 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were plated in RPMI 1640 or DMEM/F12, respectively (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) per 0.1% 10 mg/ml gentamicin (GIBCO BRL, Grand Island, NY), at 2 × 10^5 cells/well on flexible bottom, type I collagen-coated, silicone elastomer six-well Bioflex culture plates (Flexcell, McKeensport, PA) for 24 h, serum starved for 24 h, and then treated as described. Cell number was determined after cells were removed from the wells by trypsin digestion followed by hemocytometer cell count.

Inhibitor studies. Inhibitor concentrations were determined on the basis of literature review, toxicity as determined by Trypan blue dye exclusion assessment, and activity determination. For N-acetylcyeste-
ine (NAC) experiments, cells were pretreated with 20 μM NAC (Sigma-Aldrich, St. Louis, MO) while serum starving for 24 h before strain. For other inhibitor experiments, cells were pretreated for 2 h before strain. The following concentrations were used: 10 μM PD98059 (Calbiochem-Novabiochem, La Jolla, CA), 10 μM UO126 (Calbiochem-Novabiochem), 10 μM cyclohexamide (Sigma-Aldrich), 100 μM gadolinium chloride (Sigma-Aldrich), 10 μM genistein (Calbiochem-Novabiochem), and 500 μM allopurinol (Sigma-Aldrich). GsMtx-4, a blocker of nonselective cation mechanosensitive channels isolated from tarantula toxin (4), was added at a concentration of 5 μM just before initiation of strain.

Strain application. Equal biaxial strain was applied by use of a computer-driven Flexercell strain unit with bioflex posts (Flexcell). Cultured cells were strained at 14 kPa (20% elongation) at 60 cycles per minute for the specified time. Visual inspection confirmed that the membrane fully relaxed during each cycle. Control cells were also plated on Bioflex plates to avoid variations on the basis of tissue culture plates.

ROS activity. After treatment for the time specified, wells were rinsed twice with serum-free medium without phenol red. Cells were incubated with 3 μM 6-carboxy 2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl ester) (DCFDA; Molecular Probes, Eugene, OR) and 2 μM Hoechst 33342 (Sigma) in media without phenol red for 15 min at 37°C, 5% CO₂ in darkness. For experiments using cells transfect with dominant-negative mitogen-activated protein kinase kinase-1 (MEK1) (see MEK1 dominant-negative transfection), chloromethyl-DCFDA (Molecular Probes) was used. The chloromethyl group reacts with intracellular thiols aiding in cell retention of dye. Cells were rinsed five times with media without phenol red in subdued light. Cells were covered with media without phenol to maintain cell viability, then with no. 1 coverslips (24 × 50 mm) (Electron Microscopy Sciences, Fort Washington, PA). Samples were illuminated by a 100-W mercury lamp and viewed with a FITC followed by a 4,6-diamidino-2-phenylindole filter on a Nikon E800 microscope (Nikon, Melville, NY) to view DCFDA and Hoechst fluorescence, respectively. Fields were viewed at ×20 at a constant exposure time within each experiment to minimize variation due to photobleaching. Images were captured with a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI). Relative fluorescence per unit area and number of fluorescing cells per unit area were determined utilizing Metamorph software, using Hoechst staining of nuclei to normalize to total number of cells per unit area.

Western blot analysis. Cells were cultured as previously described, grown to confluence, and changed to serum-free media for 24 h. After treatment, cells were collected in Laemmli sample buffer with 100 μM Na vanadate and 30 mM Na pyrophosphate and boiled for 5 min. Protein quantification was performed by use of bicinchoninic acid analysis (Pierce Chemical, Rockford, IL). Twenty micrograms of protein were loaded per well on an SDS polyacrylamide gel (National Diagnostics, Manville, NJ). After electrophoresis, proteins were transblotted...
was assessed by using Metamorph quantification as described above under ROS activity. Statistical analysis. ANOVA with Scheffé’s post hoc analysis was used for analysis. A value of the null hypothesis ($P < 0.05$) was considered statistically significant. Data are shown as means ± SE.

RESULTS

Pulmonary epithelial H441 cells mechanically strained showed an increase in ROS as measured by DCFDA fluorescence, which peaked by 30 min (Fig. 1). Pretreatment with GsMtx-4, a blocker of nonselective cation mechanosensitive channels (Fig. 1), or gadolinium, a mechanosensitive calcium channel blocker, or PD98059 or UO126, p42/44 MAP kinase inhibitors, blocked strain-induced increase in DCFDA fluorescence (Fig. 2). The xanthine oxidase inhibitor allopurinol, the tyrosine kinase inhibitor genistein, or pretreatment with cyclohexamide to inhibit new protein synthesis did not inhibit strain-induced increase in DCFDA fluorescence, suggesting that these pathways are not involved in strain-induced ROS production (Fig. 2). There was a trend to increased ROS production after 30 min of strain plus genistein or cyclohexamide that did not reach statistical significance. ROS quantification is presented as total number of fluorescing cells (Fig. 1) and relative fluorescent intensity (Fig. 2) to demonstrate comparable trends in ROS after strain in both methods of quantification. To determine whether strain-induced increase in ROS production is necessary for strain-induced proliferation, cells were pretreated for 24 h in NAC and then exposed to unstrained or strained conditions for 24 h. NAC pretreatment blocked strain-induced increase in cell number, suggesting that ROS production is necessary for strain-induced proliferation in pulmonary epithelial cells (Fig. 3).

To determine whether ROS production is necessary for strain-induced p42/44 MAP kinase signaling, H441 cells were pretreated with the antioxidant NAC before strain. Western blotting demonstrated that NAC pretreatment inhibited strain-induced MAP kinase phosphorylation (Fig. 4). This, together with the previous results that demonstrated that inhibition of p42/44 MAP kinase signaling inhibits strain-induced ROS production, suggests a feedback signaling mechanism between ROS production and p42/44 MAP kinase signaling. Gadolinium also inhibited strain-induced p42/44 MAP kinase phos-
phosphorylation (Fig. 4). Pretreatment with genistein did not inhibit strain-induced p42/44 MAP kinase phosphorylation (data not shown). Initial experiments were performed using H441 cells, because of their relatively long doubling time and more robust increase in cell number in response to strain compared with MLE15 cells (data not shown). H441 cells could not be effectively transfected with the MEK1 dominant-negative DNA, so MLE15 cells were used for subsequent studies investigating strain-induced p42/44 MAP kinase signaling. Transfection efficiency, assessed by percentage of cells staining positive for HA by immunohistochemistry, was 80%. MLE15 cells were strained for 5 min in the presence and absence of PD98059 or UO126, p42/44 MAP kinase inhibitors, followed by Western blotting for phospho-p42/44 MAP kinase. PD98059 or UO126 at 10 μM concentration blocked strain-induced p42/44 MAP kinase phosphorylation (Fig. 5). PD98059 or UO126 also blocked strain-induced increase in cell number (data not shown). Because pharmacological inhibitors can have unknown unrelated effects, transiently transfected MEK1 dominant-negative MLE15 cells were studied. FBS is known to cause phosphorylation of p42/44 MAP kinase, so this stimulation was used as a positive control to assess p42/44 MAP kinase signaling. Nontransfected MLE15 cells demonstrated phosphorylation of p42/44 MAP kinase after 5 min exposure to FBS. MEK1 dominant-negative MLE15 cells exposed to FBS for 5 min did not demonstrate FBS-induced phosphorylation of p42/44 MAP kinase, demonstrating inhibition of the p42/44 MAP kinase signaling pathway (Fig. 6). To determine whether p42/44 MAP kinase signaling is necessary for strain-induced proliferation, MEK1 dominant-negative MLE15 cells were exposed to unstrained or strained conditions for 24 h. MEK1 dominant-negative MLE15 cells did not demonstrate strain-induced increase in cell number, providing further evidence to suggest p42/44 MAP kinase signaling is necessary for strain-induced proliferation in pulmonary epithelial cells (Fig. 7). MLE15 cells transiently transfected with dominant-negative MEK1 also demonstrate inhibition of strain-induced ROS production as measured by chloromethyl-DCFDA (Fig. 8).

A high-molecular-weight protein, ~135 kDa (strain protein 135 or SP135), determined by immunoprecipitation to not be FAK 125 or pp130, has been found to be tyrosine phosphorylated in response to mechanical strain (13). To determine whether ion channel activation or ROS production is necessary for strain-induced SP135 tyrosine phosphorylation, H441 cells were strained in the presence and absence of NAC or gadolinium. Neither NAC nor gadolinium pretreatment blocked SP135 phosphorylation (Figs. 9 and 10). There was no increase in cell death as measured by Trypan blue dye exclusion under any of the above conditions (data not shown). Table 1 summarizes the signaling pathways elucidated with the present work, demonstrating the common features of ROS/p42/44 MAP kinase signaling.
 signaling. It also outlines the divergent pathway of strain-induced tyrosine kinase signaling. The present work provides evidence to support the critical role of ROS production and p42/44 MAP kinase activation in strain-induced proliferation in pulmonary epithelial cells.

**DISCUSSION**

The lungs are constantly exposed to a variety of environmental stimuli. Normal physiological lung development requires fetal breathing movements. Without this stretching potentially fatal pulmonary hypoplasia develops, as seen in neurological anomalies that inhibit fetal breathing, prolonged premature rupture of membranes or congenital diaphragmatic hernia, where herniation of abdominal contents displace the lung, blocking lung distension (36). Although mechanical strain is critical for normal lung growth and development, abnormal strain as seen in mechanical ventilation can lead to lung damage and death (7). Understanding the mechanisms regulating strain-induced growth and injury can help to optimize beneficial strain effects and leads to the potential for pharmacological modification of strain-induced changes in the lung, while mitigating damaging sequelae.

We have recently demonstrated that pulmonary epithelial cells exhibit a strain-induced oxidant response (12), but the role of ROS in p42/44 MAP kinase signaling and strain-induced proliferation in the lung has not been elucidated.

Pulmonary epithelial A549 cells exposed to 15% strain for 4 h demonstrated an increase in IL-6 and IL-8. These strain-induced increases were inhibited by NAC pretreatment and exacerbated by depleted intracellular glutathione with buthionine-sulfoximine, demonstrating a role for ROS production in strain-induced inflammation (19). Primary rat alveolar epithelial cells exposed to hyperoxia generate ROS and demonstrate activation of Bax and cell death, all of which are inhibited by the superoxide dismutase and catalase mimetic EUK-134, linking ROS production with hyperoxic lung damage (8, 9).

ROS signaling in response to mechanical strain has been studied in nonpulmonary cell types, most extensively in endothelial cells. Strain is thought to play a major role in atherosclerotic disease, with redox signaling in endothelial cells leading to injury. Strain induces H2O2 production in porcine aortic endothelial cells (18). Endothelial cell NAD(P)H oxidase activity is enhanced by cyclic strain, as is mobilization of the transcription factor NF-κB, an effect that is blocked by a pharmacological inhibitor of NAD(P)H (28). Pretreatment of cardiac myocytes with NAC inhibits strain-induced activation of p38 MAP kinase (1). Antioxidants inhibit mechanical strain-induced tenasin-C upregulation in cardiac myocytes (46). Endothelial cells pretreated with NAC abolish strain-induced ROS generation as well as strain-induced plasminogen activator inhibitor-1 release (11). Strain-induced increase in MCP-1 mRNA levels in endothelial cells could be inhibited with catalase or NAC (45). Stretch-induced increases of phosphatidylcholine biosynthesis in astrocytes were significantly reduced by pretreating cells with superoxide dismutase or catalase (23). Our findings that strain induces ROS production in pulmonary epithelial cells demonstrate a similar strain-induced ROS signaling response compared with these other cell types.

In addition to initiating injury, ROS production signals proliferation. Proliferation of airway smooth muscle cells in

**Table 1. Summary of modifier and inhibitor effects on signaling pathways**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Inhibitor</th>
<th>NAC</th>
<th>Genistein</th>
<th>PD98059/UO126</th>
<th>Gadolinium</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS</td>
<td>↓</td>
<td>No effect</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>p42/44 MAP kinase</td>
<td>↓</td>
<td>No effect*</td>
<td>No effect</td>
<td>No effect*</td>
<td>No effect</td>
</tr>
<tr>
<td>Phosphotyrosine</td>
<td>No effect</td>
<td></td>
<td>↓</td>
<td>↓</td>
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</tr>
</tbody>
</table>

↓. Inhibition of signaling pathway; ROS, reactive oxygen species; MAP, mitogen-activated protein. *Data not shown; †data in Ref. 13.
culture is inhibited by catalase and NAC (5). Treatment of cultured hamster lung fibroblasts with oxidants or after partial inhibition of superoxide dismutase or glutathione peroxidase increased cell proliferation by ~50%. The upregulation of cell proliferation was suppressed by pretreatment with hydroxyl radical scavengers and iron-chelating agents (21). The present study further demonstrates that ROS signaling is necessary for strain-induced proliferation in pulmonary epithelial cells.

The role of p42/44 MAP kinase as a downstream effector of ROS production has been described in other systems. Antioxidant treatment inhibits strain-induced ROS production and p42/44 MAP kinase activation in vascular endothelial cells (20). Strain-induced p42/44 MAP kinase activation in cardiac myocytes is ROS dependent (35). Isolated rat extensor digitorum longus muscle exposed to concentric contractions induced a fivefold increase in p42/44 MAPK phosphorylation, which was blocked by application of NAC or diithiothreitol phosphorylation (44). Pulsatile flow strain in vascular endothelial cells induces phosphorylation of p42/44 MAP kinase, which was blocked by NAC (2). Polyethylene glycol-superoxide dismutase abolished p42/44 MAP kinase activation by pulsatility in isolated rabbit aortas (25). Stretch can also induce alveolar epithelial cell damage, which can be mitigated by FGF-10-induced MAPK activation (41). Our data, which demonstrate that inhibition of ROS signaling by NAC pretreatment blocks strain-induced p42/44 MAP kinase phosphorylation, support a feedback mechanism between the two. This role of MAP kinase signaling as a moderator of ROS signaling has also been found in lung endothelial cells, where hyperoxia-induced p42/44 MAP kinase activation was necessary for hyperoxia-induced NADPH oxidase activation (34).

ROS signaling can be initiated by a number of pathways. ROS signaling in phagocytic cells is primarily via NADPH oxidase. Nonphagocytic cells can also produce ROS via NADPH oxidase activation or as by-products of electron transfer reactions in the mitochondria, endoplasmic reticulum, or nuclear membranes. Cytokine receptors, such as the receptors for TNF-α and IL-1, and growth factor receptors that bind receptor tyrosine kinases, such as PDGF and EGF, generate intracellular ROS (40). ROS signaling can also occur via calcium mobilization (37). Stimulated human lymphocytes incubated with calcium and ROS-sensitive dyes demonstrate that trans-plasma membrane calcium mobilization is necessary for phytohemagglutinin-induced generation of ROS (32). Human cervical cancer cells exposed to hypotonic swelling activated the cytosol. J Cell Biol 160: 1115–1127, 2003.


