Mechanical Stress Activates Xanthine Oxidoreductase through MAP Kinase-dependent Pathways

Raja-Elie E. Abdulnour¹, Xinqi Peng¹, Jay H. Finigan¹, Eugenia J. Han¹, Emile J. Hasan¹,
Konstantin G. Birukov¹, Sekhar P. Reddy², James E. Watkins III³, Usamah S. Kayyali¹, Joe G. N. Garcia¹, Rubin M. Tuder³, and Paul M. Hassoun¹

Division of Pulmonary & Critical Care Medicine, Department of Medicine¹, Department of Environmental Health Sciences, Bloomberg School of Public Health², Division of Cardiopulmonary Pathology and Department of Pathology³, Johns Hopkins University School of Medicine, Baltimore, MD, 21224; Pulmonary and Critical Care Division, Tupper Research Institute, Tufts-New England Medical Center,
Tufts University School of Medicine⁴, Boston, MA 02111

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Address Correspondence to:
Paul M. Hassoun
Division of Pulmonary and Critical Care Medicine
5501 Hopkins Bayview Circle
Baltimore, MD 21224
Telephone: 410-550-2606
Fax: 410-550-2612
Email: phassoun@jhmi.edu.
ABSTRACT

Xanthine oxidoreductase (XOR) plays a prominent role in acute lung injury because of its ability to generate reactive oxygen species. We investigated the role of XOR in ventilator-induced lung injury (VILI). Male C57BL/6J mice were assigned to spontaneous ventilation (sham) or mechanical ventilation (MV) with low (LVT, 7ml/kg) and high tidal volume (HVT, 20ml/kg) for 2 hrs after which lung XOR activity and expression were measured and the effect of the specific XOR inhibitor allopurinol on pulmonary vascular leakage was examined. In separate experiments, rat pulmonary microvascular endothelial cells (RPMECs) were exposed to cyclic stretch (5% and 18% elongation, 20 cycles/min) for 2 hrs before intracellular XOR activity measurement. Lung XOR activity was significantly increased at 2 hrs of MV without changes in XOR expression. There was evidence of p38 MAP kinase, ERK1/2, and ERK5 phosphorylation, but no change in JNK phosphorylation. Evans Blue Dye (EBD) extravasation and bronchoalveolar lavage protein concentration were significantly increased in response to MV, changes that were significantly attenuated by pretreatment with allopurinol. Cyclic stretch of RPMECs also caused MAP kinase phosphorylation and a 1.7-fold increase in XOR activity, which was completely abrogated by pretreatment of the cells with specific MAP kinase inhibitors. We conclude that XOR enzymatic activity is significantly increased by mechanical stress via activation of p38 MAPK and ERK, and plays a critical role in the pathogenesis of pulmonary edema associated with VILI.

Key Words: Xanthine oxidoreductase; mechanical ventilation; acute lung injury
INTRODUCTION

Mechanical ventilation (MV) remains the cornerstone of treatment in the acute respiratory distress syndrome (ARDS), a devastating syndrome with high morbidity and mortality (33). However, it has become more apparent that MV, although necessary for life support, can further lung injury (9) and contribute to a systemic inflammatory response in patients with ARDS, a finding supported by animal and human studies (32). The recognition of ventilator-induced lung injury (VILI) eventually led to the ARDS Network trial which demonstrated a significant improvement in survival in patients ventilated with low tidal volume, as opposed to high tidal volume (1). However, overstretching of normal alveoli in severe ARDS cannot be completely prevented due to regional differences in lung pathology, and MV can result in VILI and enhanced edema (6, 10, 26). Therefore, a better understanding of the effects of mechanical stress on signaling pathways and downstream effectors may provide therapeutic targets aimed at minimizing VILI.

The enzyme xanthine oxidoreductase (XOR) is an important component of an integrated inflammatory response in organ dysfunction (14) and has been implicated in the pathogenesis of ARDS because of its ability to generate reactive oxygen species (ROS). We postulated that XOR might be upregulated by mechanical stress and might contribute to the development of VILI. Since XOR activation in hypoxia occurs through p38 MAP kinase activation (18), we tested activation of the different MAP kinase pathways in response to mechanical stress in vivo and in vitro, and their effects on XOR expression. Our results indicate that XOR activity is rapidly upregulated both in vivo and in vitro through activation of ERK and p38 MAP Kinase. Furthermore, allopurinol prevented pulmonary vascular leakage produced by high tidal volume MV, suggesting that XOR contributes to increased capillary permeability produced by mechanical stress in this model. Some of the results of these studies have been reported previously in the form of an abstract (29).
MATERIAL AND METHODS

Materials and Reagents - RPMI 1640 and fetal bovine serum were obtained from Hyclone (Logan, UT). Dithiothreitol, CHAPS, EDTA, PMSF, leupeptin, methylene blue, allopurinol, and SB203580 were obtained from Sigma (St. Louis, MO). PD98059 and U0126 were obtained from Calbiochem (San Diego, CA). Phospho-specific antibodies directed at p38 MAP kinase, ERK 1/2, ERK5, and JNK, along with anti-total MAP kinase antibodies, were obtained from Cell Signaling Technology (Beverly, MA). Anti-XOR antibody was obtained from NeoMarkers (Fremont, CA).

Experimental protocol and animal exposure to MV - Male C57BL/6J mice age 7-10 weeks (Jackson Laboratory, Bar Harbor, ME) were studied in a pathogen-free facility under a protocol approved by the Johns Hopkins Department of Laboratory Animal Medicine. Animals were first anesthetized with intraperitoneal ketamine (150mg/kg) and acetylpromazine (15mg/kg). A neck midline incision was then performed for exposure of the trachea to facilitate endotracheal intubation of the mouse with a 20-gauge one inch long catheter (Johnson and Johnson, New Brunswick, NJ). The animals were then subjected to MV (Harvard Apparatus, Boston, MA) with room air for 0 (sham), 0.5, 1 or 2 hrs with low tidal volume (7ml/kg, LV_T) and high tidal volume (20ml/kg, HV_T). The respiratory rate was set at 110 breaths/minute for all tidal volumes, and the dead space was adjusted to maintain arterial pH between 7.35 and 7.45. Airway pressures continuously measured during MV at 7ml/kg and 20ml/kg revealed that end-expiratory pressures remained around 0-2 cm H2O2 throughout the two hour period for both low and high tidal volumes. Mean blood pressure was continuously monitored via tail cuff using a blood pressure monitor (BP-1) and a data acquisition system (World Precision Instruments Inc., Sarasota, FL), and remained typically around 85 mm Hg. The adequacy of MV settings on gas exchange was confirmed in preliminary experiments in which arterial blood gases obtained via catheterization of a femoral artery and analyzed by automated blood gas analyzer (Instrumentation Laboratories, Lexington, MA) revealed stable levels of arterial oxygen (PaO2 of 65-80 mm Hg), carbon dioxide (PaCO2 of 30-40 mm Hg). At the end of MV the
animals were administered an intraperitoneal lethal dose of the anesthetic agent before the lungs were harvested.

*Drug delivery* - In order to assess the role of XOR on MV-induced pulmonary vascular permeability, mice received in some experiments a single dose of allopurinol (50 mg/kg) by gavage or a similar volume of vehicle (saline) 16 h before exposure to MV. *In vivo* efficiency of the drug dosage in inhibiting XOR activity was confirmed in preliminary experiments.

*Assessment of pulmonary capillary permeability* - Evans blue dye (EBD) (20ml/kg) was injected into the external jugular vein 30 minutes prior to termination of the experiment to assess vascular leak as previously described (28, 30). Briefly, at the end of the experimental protocol, a thoracotomy was performed and the lungs were perfused free of blood with PBS containing 5mM EDTA, prior to being excised *en bloc*, blotted dry, weighed, and snap-frozen in liquid nitrogen. The right lung was homogenized in phosphate-buffered saline (PBS, 1ml/100µg tissue), incubated with 2 vol. formamide (18 hrs, 60°C), centrifuged at 5000g for 30 minutes, and the optical density of the supernatant was determined by spectrophotometry at 620nm. Extravasated EBD concentration (micrograms EBD per lung) in lung homogenates was calculated against a standard curve as previously described (23).

*BAL protein concentration* - Bilateral bronchoalveolar lavage (BAL) was performed with 1ml of saline for determination of BAL fluid protein concentration as previously described(13).

*Processing of lung tissue for immunohistochemical staining* - At the end of experimental exposure, the right external jugular vein was catheterized and the lungs were flushed free of blood with ice-cold PBS containing the phosphatase inhibitor sodium orthovanadate (1µM). The lungs were inflated to 25 cm of water pressure with 0.2% of low-melting agarose, then harvested and stored in 10% formalin for about 14 hrs before embedding in paraffin. After deparaffinization, tissue sections were incubated for 1 hr at room temperature with a phospho-specific p38 antibody (1:150), followed by horseradish
peroxidase-conjugated secondary antibody. After several washes with PBS, the immunohistochemical reaction was visualized by incubation with 0.05% DAB containing 0.01% H2O2 in 50 mM Tris (pH 7.6).

**Exposure of endothelial cells to cyclic stretch** - Rat pulmonary microvascular endothelial cells (RPMECs) were cultured as previously described (8). All experiments involving cyclic stretch (CS) were performed as previously described (5), using a FX-4000T Flexercell Tension Plus system (Flexcell International, McKeesport, PA) equipped with a 25-mm BioFlex loading station designed to provide uniform radial and circumferential strain across the membrane surface of loaded Bioflex plates. All CS experiments were performed in the presence of complete culture medium containing 10% fetal bovine serum. RPMECs were seeded at standard densities (4x10^5 cells/plate) onto collagen I-coated flexible-bottomed BioFlex plates, whether for the static (control) or the stretched group, to ensure standard culture conditions. Culture medium was changed every other day until cells reached confluence. The plates were then loaded onto the Flexercell system and exposed to CS for 2 hrs at 20 cycles per minute and 18% elongation. BioFlex plates with control endothelial cells exposed to static conditions were placed in the same incubator as stretched plates. When necessary, RPMECs were preincubated with a p38 inhibitor (1µM SB203580), ERK inhibitors (PD98059 or U0126) or similar amounts of vehicle (DMSO) for 1hr prior to CS. At the end of the experiment, cells were lysed in an XOR lysis buffer consisting of 50 mM sodium phosphate (pH 7.4), 1.5 mg/mL DTT, 0.1mM EDTA, 1mg/ml CHAPS, 0.18mg/ml PMSF and 0.5µg/ml leupeptin.

**Xanthine oxidoreductase activity measurement** - The activities of xanthine dehydrogenase and xanthine oxidase in response to different treatments were assayed in cells and lung homogenates using a fluorimetric assay that measures both xanthine oxidase and xanthine dehydrogenase activities, as previously described(3). The principle of the assay involves the conversion of pterin into the fluorescent product isoxanthopterin. The rate of product formation with oxygen as the electron acceptor represents the activity of xanthine oxidase, while the combined activities of xanthine oxidase and xanthine
dehydrogenase are measured with methylene blue as the electron acceptor. In brief, cells were washed once in phosphate-buffered saline and then scraped off the plate in XOR buffer. The cells were sonicated for 5 sec and centrifuged at 10,000g for 5 min. The supernatant was collected and assayed immediately or stored at -80°C overnight. XOR activity was adjusted for µg of protein per ml of lysate, and then normalized to control XOR activity.

For determination of lung XOR activity, the left lung was harvested and homogenized in XOR lysis buffer. The homogenate was then centrifuged for 20 min at 15,000 rpm before measuring activity as described above. Since changes in xanthine oxidase and xanthine dehydrogenase occurred in parallel in response to mechanical stress, results are expressed in all experiments as total XOR activity.

**SDS-PAGE and immunoblotting** - Aliquots from cell lysates and tissue homogenates prepared as described above were assayed for protein measurement using the Bradford protein assay (12) and then diluted with Laemmli loading buffer for SDS-PAGE (13). Equal amounts of protein were then loaded in each well of 4-20% Tris/glycine gels. After electrophoresis for 90 min at 125 V constant voltage, the gel was blotted onto a PVDF membrane by electrophoretic transfer at 25 V constant voltage for one hr. The membrane was then washed, blocked with 5% blocking solution, and probed with antibodies against XOR, and MAP kinases and their phosphorylated forms. The immunoreactive bands were visualized using a secondary antibody conjugated to horseradish peroxidase and a chemiluminescent detection system (ECL, Amersham, NJ). Films were scanned using a Color Imager Scanner (Seiko Epson Corp., Tokyo, Japan) and the National Institutes of Health Image 1.44 software.

**Semi-quantitative RT-PCR** - Total RNA was isolated using a commercially available kit (Qiagen Inc., Valencia, Ca), and then quantified and loaded in a RT-PCR mix for a one step semi-quantitative reverse transcription-PCR amplification (Invitrogen, Carlsbad, CA). The PCR reaction was carried out in a 25µl solution. A pair of sense and antisense primers specific for mouse and rat XOR, designed based on
published cDNA sequences, were as follows: 5’-AGGTCGCCATAACCTGTGGGCTG-3’ (forward primer) and 5’-ATTGAGGTCAGCACTGGCAGAGG-3’ (reverse primer)(19). After thirty cycles, 15 µl of PCR products were analyzed by gel electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. To assess the adequacy of the cDNA and the efficiency of the RT-PCR system, primers for 18S ribosomal RNA were used as RT-PCR controls under the same conditions. The cDNA for 18S ribosomal RNA was amplified with the following primers: 5’ CGGCTACCACATCCAAGGAA (forward), 5’ GCTGGAATTACCGCGGCT (reverse). The steady-state mRNA levels are expressed in arbitrary units as the ratio of XOR/18S expression (25).

Statistics - Values are shown as means ± standard deviation, with n ≥ 4 for each experimental condition. Data were analyzed by a one-way ANOVA with Bonferroni correction. Significance in all cases was defined as P < 0.05.

RESULTS

Mechanical ventilation stimulates XOR activity. We investigated the effects of MV on changes in XOR expression and activity in C57/BL6 mice breathing spontaneously (sham) or exposed to ventilation with LV_T or HV_T for two hrs. Although the animals received different tidal volumes they were ventilated at the same respiratory rate with adjustment in dead space (as detailed in Methods). At the end of exposure, lungs were removed and XOR protein expression and activity were assessed as described in Methods.

As shown in Figure 1, MV with HV_T resulted in a significant increase (by ANOVA followed by Bonferroni correction) in lung XOR activity as compared to LV_T or sham animals (1.7-fold increase with HV_T, P < 0.05).
**XOR expression is not altered by MV.** In order to assess the effects of MV on XOR gene expression and protein formation, lungs from mice ventilated at HV_T for 2 hrs were homogenized, after which RNA and protein were isolated as described in Methods. Repeated semi-quantitative RT-PCRs showed no increase in XOR mRNA with MV compared to sham (Figure 2A). Moreover, immunoblotting using a XOR antibody showed no difference in XOR protein between the two groups (Figures 2B and 2C). The lack of effect of MV on XOR mRNA and protein expression is highly suggestive of post-translational modification as the underlying mechanism of XOR activation.

**Activation of the MAP Kinase pathway by MV.** We have previously demonstrated posttranslational modification of XOR by the p38 MAP kinase resulting in increased XOR activity in response to short-term (i.e., 4 hr) hypoxia (18). To check for a similar activation of the MAP kinase pathway in the current model, we exposed mice to MV for 30 min to 2 hrs and assessed activation of p38, ERK1/2, ERK5, and JNK MAP kinases as described in Methods. As shown in Figure 3A (left panel), MV with LV_T did not result in MAP kinase phosphorylation. On the other hand, there was an increase in phospho-p38 (pp38) levels which peaked at 60 min of exposure to HV_T ventilation and persisted for 120 min. There was also evidence of ERK1/2 and ERK5 phosphorylation, but no JNK phosphorylation, in response to HV_T only. Using immunohistochemistry, we confirmed p38 activation by demonstrating significant increase in pp38 immunoreactivity in both epithelial and endothelial cells in lung tissue from animals exposed to MV at HV_T for 1 hr as compared to spontaneously breathing controls (Figure 3B). The functional relationship between MAPK and XOR activation was then examined in a cellular model of mechanical stress as described below.

**Cyclic Stretch Stimulates XOR Activity in RPMEC.** Since XOR is strongly expressed in the pulmonary capillary endothelium (14) and is up-regulated by various stress factors (15, 18), we determined whether pulmonary microvascular endothelial cells could be a specific cellular source of mechanical stress-activated XOR.
Confluent RPMECs were exposed to static conditions (no stretch) or cyclic stretch (20 cycles/min and 5% or 18% elongation) for 1 and 2 hrs before measurement of intracellular XOR activity. Cyclic stretch at 5% did not alter XOR activity at 1 (Figure 4) or 2 hours (not shown). However, cyclic stretch at 18% resulted in a significant upregulation of XOR activity at one and two hrs of stretch (1.53 ± 0.4 and 1.59 ±0.11 fold increase respectively, P < 0.05) as compared to static conditions (Figure 4).

Cyclic stretch does not increase XOR gene transcription. In order to assess the effects of cyclic stretch on XOR gene expression and protein formation, RNA and protein from stretched cells were isolated as described in Methods. Repeated semi-quantitative RT-PCRs showed no increase in XOR mRNA with cyclic stretch compared to cells kept static (Figure 5). In addition, no change in XOR protein was detected by immunoblotting (results not shown), suggesting post-translational modification as the underlying mechanism of XOR activation, as suggested by the in vivo experiments.

Cyclic stretch activates the ERK and p38 MAPK pathways. RPMEC were exposed to static conditions (no stretch) and to 5, 30, 60, and 120 minutes of cyclic stretch (20 cycles/min and 18% elongation) after which cells were lysed and protein was collected. As shown in Figure 6, there was increased ERK1/2, ERK5, and p38 MAP kinase phosphorylation at 5 minutes of CS, with a return to baseline at 120 minutes. There was no evidence of JNK phosphorylation neither at baseline nor in response to cyclic stretch (not shown).

Effect of MAP kinase pathway inhibitors on CS-induced ERK phosphorylation. Pharmacologic inhibitors of the ERK pathway have been widely used in loss-of-function analysis of ERK involvement in many signaling pathways. However, studies have shown that drugs used to inhibit ERK1/2 can also inhibit ERK5 in several cell types (22). Therefore, before evaluating the role of the ERK pathway in XOR activation by CS, we sought to determine the profile of two widely used inhibitors in
RPMEC. Cells were exposed to 10 minutes of CS in the presence of increasing doses of the two ERK inhibitors, PD98059 and U0126.

As shown in Figure 7, the two drugs had somewhat opposite profiles. PD98059 inhibits ERK5 phosphorylation at all doses, but significantly prevents ERK1/2 activation only at a dose of 100μM. On the other hand, U0126 prevents ERK1/2 activation at doses as low as 0.3μM while inhibiting ERK5 phosphorylation only at the maximal dose of 10μM. Therefore, for the following experiments U0126 (1μM) and PD98095 (10μM) were used to inhibit ERK1/2 and ERK5, respectively. There was no evidence of cellular toxicity with these doses as assessed by phase contrast microscopy or trypan blue exclusion.

**ERK and p38 inhibitors block CS-induced XOR activation.** In order to examine the role of MAP kinases in XOR activation, cells were exposed to CS for 1 hr, with and without pre-treatment with the specific p38 MAPK inhibitor SB203580 (1μM, 1 hr prior to CS stimulation), the ERK1/2 inhibitor U0126 (1μM), the ERK5 inhibitor PD98095 (10μM), combination of U0126 (1μM) and PD98095 (10μM), or vehicle (DMSO) after which intracellular XOR activity was measured. As shown in Figure 8, treatment with these inhibitors did not affect basal enzymatic activity in control cells. However, there was a significant increase in XOR activity in response to cyclic stretch, which was prevented by pre-treatment with SB203580, PD98095, U0126, or the combination of PD98095 and U0126.

**Pharmacologic inhibition of XOR prevents ventilator-induced pulmonary capillary leakage.** In order to assess the effect of MV and XOR activation on the development of pulmonary capillary permeability, C57/BL6 mice were randomly assigned to spontaneous breathing (sham controls) or 2 hrs of MV at LV and HV. Pre-treatment with allopurinol or vehicle (saline) was performed as described in Methods. At the end of exposure, BAL was collected for measurement of protein concentration, while
pulmonary capillary permeability was assessed with the EBD technique in separate experiments, as described in Methods.

There was no significant change in BAL protein concentration and in EBD lung content in animals exposed to LV_T mice compared to sham control animals (data not shown). However, there was a significant increase in both BAL protein concentration (1.6 fold increase; \( P < 0.05 \), Figure 9A) and EBD extravasation (1.55 fold increase; \( P < 0.05 \), Figure 9B) in mice exposed to HV_T compared to sham controls. Allopurinol treatment significantly attenuated the increase in BAL protein and EBD accumulation in mice exposed to HV_T (Figures 9A and 9B) while almost completely inhibiting XOR activity (Figure 9C), strongly suggesting a significant role for XOR in the pathogenesis of ventilator-induced pulmonary edema.

DISCUSSION

Xanthine oxidoreductase, best known for its role in purine catabolism and as a target in gout therapy, exists as two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). The conversion of XDH to XO can be reversible, after treatment with sulphide reagents, or irreversible following proteolysis (7). In the process of oxidation of hypoxanthine to uric acid, NAD+ and molecular oxygen are the preferential electron acceptors for XDH and XO, respectively. This relates to the inability of NAD+ to bind to XO due to significant conformational changes of the enzyme. Therefore, conversion of XDH to XO by either proteolysis or post-translational modification significantly increases the amount of ROS produced by XOR (34, 38). However, reduction of molecular oxygen by either form of the enzyme yields superoxide and hydrogen peroxide, and upregulation of overall XOR activity, irrespective of XDH/XO ratios, can lead to increased ROS levels (14). It is the capacity of XOR to generate such ROS that is of major interest in clinical syndromes.
The consequences of ROS release following induction of XOR have been implicated in the pathogenesis of several diseases such as ischemia-reperfusion injury (21), multi-system organ dysfunction syndrome (12), and cardiovascular diseases (4). The contribution of XOR to ALI has also been demonstrated (11, 31, 37). Although it is generally thought that XOR is induced by cytokines, inflammatory products and hypoxia, to date, there is no evidence supporting regulation of this enzyme by mechanical forces in the lung or its contribution to VILI. However, we have recently shown in a similar murine model of VILI that MV results in peroxynitrite (ONOO) formation as evidenced by nitrotyrosine deposition in the lung (29). Because ONOO formation requires superoxide production, we sought to determine whether cyclic mechanical stress induced by MV could alter XOR activity, and whether the activation of XOR contributed to the development of capillary permeability related to VILI.

This study demonstrates for the first time that lung XOR enzymatic activity is increased by mechanical stress. Our animal model of MV demonstrates that high tidal volume ventilation causes an increase in lung XOR activity compared to spontaneous ventilation or low tidal volume MV considered clinically safe in humans (i.e., 7 ml/kg). In addition, these studies demonstrate evidence of VILI in our animal model as evidenced by increasing BAL total protein concentration and lung EBD extravasation, both reflecting alveolar-capillary barrier dysfunction. The XOR inhibitor allopurinol prevented the increase in capillary pulmonary permeability produced by MV with high tidal volumes, which strongly suggests that XOR activation by mechanical stress contributes to the ventilator-induced alveolar barrier dysfunction.

The injury in this model of VILI seems to be characterized by increased permeability of the epithelial-endothelial barrier without measurable inflammation. The efflux of protein into the alveolar space which is reflected by the increase in BAL protein indicates substantial epithelial injury, while the EBD extravasation that accompanies MV suggests injury to the endothelium. On the other hand, the evaluation of BAL inflammatory cells showed no evidence of neutrophil infiltration (data not shown).
These findings are in contrast with a recent report of a VILI model where we demonstrated evidence of neutrophil infiltration and unchanged BAL protein concentration (29). However, the two MV models differ by the respiratory rates (RR) used; we speculate that ventilating at the higher rate of 110bpm as in the current study minimizes the inflammatory reaction while maximizing alveolar barrier dysfunction. We also suspect that the more consistent endothelial and epithelial damage obtained in the current model is related to repetitive opening and closing of the alveoli at a higher RR (110bpm as opposed to 60bpm for the previous study).

Xanthine oxidoreductase is widely distributed among species and within various tissues (2), with abundant concentrations in the lung (27). Immunofluorescence and immunoperoxidase techniques have allowed the localization of XOR in the cytoplasm of endothelial cells in capillaries of several organs including the lung (17), indicating that XOR is essentially expressed in vascular endothelial cells (17). Therefore, we explored the response of cultured pulmonary microvascular endothelial cells to cyclic stretch. We opted for an 18% elongation based on the knowledge that this extent of linear distension corresponds roughly to a 35-50% increase in cell surface area, which is relevant to pathophysiological conditions produced by mechanical ventilation (5). Our in vitro model confirms activation of XOR by mechanical stress in the absence of other confounding factors, such as paracrine interactions from neighboring cells (e.g., macrophages or neutrophils). Mirroring the in vivo findings, there was no upregulation of XOR gene expression in cultured cells.

In regards to the mediators of injury, we have recently demonstrated that the pulmonary leak produced by mechanical stress in response to MV is at least partially related to oxidative/nitrosative stress. Indeed inducible nitric oxide synthase (iNOS) is activated by mechanical stress in response to MV and there is increased nitrotyrosine deposition, a footprint of peroxynitrite damage (29). Using fluorescent immunolocalization, we demonstrated that peroxynitirite formation occurs at the site of increased iNOS expression, i.e., essentially in endothelial cells. Furthermore, the lack of nitrotyrosine formation in iNOS-
deficient mice allowed us to directly incriminate iNOS upregulation in nitrosative stress. Peroxynitrite, a powerful oxidant, results from the rapid reaction between NO and superoxide. Since capillary permeability is prevented by iNOS deficiency or pharmacologic inhibition of either iNOS (29) or XOR (as in the present study), we postulate that damage to the endothelial barrier is related to endothelial XOR-derived ROS reacting locally with iNOS-derived NO to form peroxynitrite in components of the alveolar-capillary membrane.

In relation to XOR expression, the lack of protein upregulation in both our animal and cellular models suggests a post-translational modification (PTM) as a mechanism of activation. Having demonstrated the role of p38 MAP kinase in XOR PTM and activation by hypoxia (18), we sought to determine the contribution of the MAP kinases to the MV-related XOR activation. Both ERK1/2 and p38 were activated by MV and CS, respectively confirming findings from our group (5) and others (35). In addition, we now demonstrate that ERK5, a recently discovered MAP kinase with a role in many disease processes (16), is activated by mechanical stress. Furthermore, we demonstrate using pharmacologic inhibitors that both p38 and ERKs are involved in XOR activation by cyclic stretch. A potential limitation of our study lies in the general lack of specificity of pharmacologic inhibitors, which can be obviated by targeting MAP kinase effectors using gene knock-out or silencing with siRNA. These confirmatory experiments are currently being developed in our laboratory. The absence of JNK activation in our model does not preclude a role for this effector in VILI, a finding that has been suggested by other investigators (20). The absence of MAP kinase activation in response to LVT is likely related to lack of significant alveolar overdistension as this volume is closer to the murine normal tidal volume.

The mechanisms by which the addition of phosphate groups, whether in response to hypoxia (9) or mechanical stress, leads to increased XOR activity needs further investigation and are beyond the scope of the current study. However, one might speculate that the negative charges in the XOR molecule introduced by phosphorylation might affect the affinity of the enzyme for its substrates (24).
Alternatively, the phosphorylation of XOR might alter its sub-cellular localization and activity by modulating its interaction with other regulatory proteins.

In summary, this study demonstrates MAP kinase-dependent XOR activation by mechanical stress and subsequent injury in a model of VILI. The possibility of XOR as a downstream target of signaling pathways involved in inflammation and stress responses lends support to the role of XOR activation as an early cellular response to a variety of stressors (36), such as hypoxia (18), LPS (15), and mechanical stress. The availability of highly effective pharmacologic inhibitors of XOR with outstanding clinical safety profiles emphasizes the relevance of the present findings. However, the effectiveness of such inhibitors in minimizing the iatrogenic effects of MV in humans with complex medical conditions remains to be studied.
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GRANTS

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FIGURE LEGENDS

Figure 1: Mechanical Ventilation induces XOR enzymatic activity
C57BL/6 mice were randomly exposed to spontaneous breathing (sham) or to MV at low (LV_T) or high (HV_T) tidal volume for 2 hrs. The lungs were then harvested and homogenized and XOR activity was measured as described in Methods. XOR activity was significantly upregulated in response to ventilation with HV_T compared to sham or LV_T (* P < 0.01 vs Control or LV_T, n = 7-10 mice per group). There was no significant change in XOR activity in response to ventilation with LV_T.

Figure 2: XOR activation is mediated by post-translational modification
Lungs from mice ventilated with HV_T for 2 hrs were homogenized, and both RNA and protein were isolated for subsequent semiquantitative RT-PCR and immunoblotting. (2A) Repeated assays showed no increase in XOR mRNA when adjusted for the internal standard 18S rRNA. (2B) Similarly, immunoblotting using a specific XOR antibody showed no increase in protein level compared to the internal standard β-actin. (2C) Densitometric analysis of the immunoblots confirmed the absence of increased XOR protein (n = 3 for each condition).

Figure 3: Activation of MAP Kinases by MV
C57BL/6 mice were exposed to spontaneous breathing (sham) or to 30, 60, and 120 minutes of MV with low (LV_T) and high tidal volume (HV_T). The animals were then sacrificed and lungs were either homogenized in lysis buffer for immunoblotting using antibodies directed at the different phospho-MAPKs, or fixed in agarose followed by paraffin for immunohistochemistry studies. (3A) Mechanical ventilation with LV_T (left panel) does not result in MAP kinase phosphorylation. However, immunoblots from mice ventilated at HV_T demonstrates an increase in p38 MAPK, ERK1/2, and ERK5 phosphorylation as early as 60 minutes after initiation of MV, with no significant change in JNK phosphorylation. (3B) A representative immunohistochemistry slide from a sham animal shows no
phospho-p38 MAPK immunostaining (Panel A). Lung from a mouse exposed to ventilation with HV₇ shows diffuse phospho-p38 immunoreactivity, as well as nuclear distribution in both endothelial and epithelial cells (Panel B).

**Figure 4: Cyclic Stretch induces XOR enzymatic activity**

RPMECs grown on collagen I-coated BioFlex plates were kept in static conditions (Control) or subjected to cyclic stretch at 5% and 18% elongation and 20 cycles/min for one and two hrs (for 18% elongation only). Cells were then collected and XOR activity was measured. While cyclic stretch at 5% elongation did not have any effect, it significantly upregulated XOR enzymatic activity at 1 and 2 hr at 18% elongation compared to Control. * indicates $P < 0.05$ vs. control; n = 5-8 6-well plates per group.

**Figure 5: XOR activation by CS is not transcriptionally-mediated**

Semi-quantitative RT-PCR was performed on RNA isolated from stretched cells as described in Methods. Repeated runs showed no increase in XOR mRNA compared to the internal standard 18S rRNA.

**Figure 6: MAP kinase activation by CS**

RPMVECs were exposed to 0, 5, 30, 60, and 120 minutes of cyclic stretch prior to immunoblotting of cell lysates using antibodies against total and phosphorylated MAP kinases. There was increased phosphorylation of p38, ERK1/2, and ERK5 as early as 5 minutes of cyclic stretch. However, there was no evidence of JNK phosphorylation (not shown).

**Figure 7: Effect of MAP kinase inhibitors on CS-induced ERK activation**

RPMVECs were exposed to 10 minutes of cyclic stretch after 1 hr pretreatment with increasing doses of U0126 and PD98059, two MAP kinase cascade inhibitors. There was significant ERK1/2 inhibition with no effect on ERK5 activation with 0.3-3µM of U0126, while there was no effect on ERK1/2 activation but significant blunting of ERK5 phosphorylation with 10µM of PD98059.
Figure 8: Treatment with p38 and ERK inhibitors prevent XOR upregulation by cyclic stretch

RPMECs were exposed to cyclic stretch for 1 hr in the presence of the specific p38 MAPK inhibitor SB203580 (1µM, added 1 hr prior to stretch), the ERK1/2 inhibitor U0126 (1µM), the ERK5 inhibitor PD98095 (10uM), a combination of U0126 (1µM) and PD98095 (10uM), or vehicle (DMSO). There was a significant increase in XOR activity in response to cyclic stretch, which was significantly prevented by pre-treatment with SB203580, PD98095, U0126, or the combination of PD98095 and U0126. (* indicates \( P < 0.05 \) vs. static control; + indicates \( P < 0.05 \) vs. stretch with any treatment).

Figure 9: Allopurinol prevents ventilation-induced pulmonary capillary leakage

C57BL/6 mice were randomly exposed to spontaneous breathing (sham) or to MV with HV for 2 hrs, with and without allopurinol pretreatment. (9A) BAL fluid revealed significantly increased protein concentration in the ventilated group compared to sham, a finding that was prevented by allopurinol treatment. (9B) In separate experiments, EBD was administered through the right jugular vein 30 minutes prior to the termination of the experiment. Analysis of lung homogenates revealed increased EBD in ventilated lungs. (9C) Treatment with allopurinol (50 mg/kg) completely inhibited lung XOR activity (* indicates \( P < 0.05 \) vs. sham; T indicates \( P < 0.05 \) vs. allopurinol treatment in same group)
Normalized XOR Activity per lung

- Sham
- \( LV_T \)
- \( HV_T \)

Figure 1
Figure 3A

Figure 3B
Normalized XOR Activity in RPMECs

- Control
- 1hr - 5%
- 1hr - 18%
- 2hrs - 18%

* Significant difference
Figure 5
Figure 7
Figure 8
Figure 9A

[Graph showing BAL protein concentration (mg/ml) for Sham and HVT20 groups, with 'Vehicle' and 'Allopurinol' conditions indicated. The graph includes error bars and statistical significance markers (* and †).]

**Figure 9A**
Figure 9B
Figure 9C