Mechanical Ventilation Alters Airway Nucleotides and Purinoceptors in Lung and Extrapulmonary Organs

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Extracellular nucleotides are stress-responsive ligands that mediate a variety of cellular processes via purinoceptors. We hypothesized that mechanical ventilation (MV) would alter the extracellular adenyl-nucleotide profile and purinoceptor expression in lung and extrapulmonary tissues. Twenty-eight rats were randomized to: (*i*) unventilated control animals; (*ii*) tidal volume (V_T; 6 ml/kg); (*iii*) V_T (6 ml/kg) and positive end-expiratory pressure (PEEP; 5 cm H_2O); (*iv*) V_T (12 ml/kg); or (*v*) V_T (12 ml/kg) and PEEP (5 cm H_2O). Bronchoalveolar lavage (BAL) was analyzed for adenyl-nucleotides. Pulmonary, hepatic, and renal tissues were assessed for P_2Y_4, P_2Y_6, P_2X_7, A_3, and A_{2b} receptor expression by real-time reverse transcriptase–polymerase chain reaction and Fas/Fas ligand mRNA was quantified in the lung. MV produced volume-dependent changes in BAL nucleotides; AMP and adenosine increased, whereas ATP and ADP proportions decreased. Large-volume MV increased A_{2b} mRNA and decreased P_2X_7 in the lung; mRNA changes in lung Fas ligand paralleled P_2X_7. PEEP normalized BAL nucleotide profiles and A_{2b} expression. Injurious MV reduced hepatic and renal P_2X_7 mRNA; PEEP normalized these levels in both tissues. Large-volume MV also decreased renal A_{2b} mRNA. MV alters the BAL adenyl-nucleotide profile and purinoceptor patterns in lung, liver, and kidney. PEEP normalizes the BAL nucleotide profile and receptor patterns in lung and extrapulmonary tissues.

Keywords: mechanical ventilation; nucleotide; purinoceptor; ventilator-induced lung injury

Clinical studies support basic scientific observations that mechanical ventilation (MV), particularly with large tidal volumes (V_Ts), can be injurious (1). Although it is known that large-volume ventilation can produce histologic lung injury, pulmonary edema, capillary stress failure, local and systemic inflammation, and, ultimately, increased mortality, the mechanisms responsible for these events are unknown (1). Although transpulmonary shear-stress and alveolar overdistension can result in direct physical disruption of anatomic structures at extremes, it is clear that distension of a lesser magnitude can produce lung injury and induce ventilation-associated pathologic responses in extrapulmonary tissues (2). Consequently, it is important to identify a system of mechanotransduction capable of translating airway stress to intracellular signaling pathways at the molecular level.

Extracellular nucleotides are stress-responsive molecules that mediate a host of physiologic responses via ubiquitous membrane-bound purinoceptors (3, 4). It is known that shear-stress at the cellular level is a potent physical stimulus for the regulated release of nucleotides in a host of cell types, including pulmonary epithelium and vascular endothelium (5, 6). A variety of extracellular and cell surface–associated enzymes tightly regulate the extracellular nucleotide profile and provide a complex environment for differential stimulation of the purinergic receptor superfamilies (3, 7).

Through changes in transmembrane ionic conductance or G-protein–linked intracellular signaling pathways, purinergic stimulation produces diverse responses in cell culture–based *in vitro* assays (3, 4, 8, 9). In smooth muscle cells, inflammatory cells, and lung epithelia, extracellular nucleotides regulate vascular tone and alter transmembrane ionic conductance and fluid dynamics (3, 4). Furthermore, purinergic stimulation can modulate cytokine release, induce apoptosis, and coordinate a variety of proximal nuclear transcriptional signals (10, 11). Many of these processes have been mechanistically implicated in the pathogenesis of ventilator-associated lung injury.

We have previously demonstrated with luminometric assays that MV generates an increase in luminal ATP levels and that pharmacologic receptor stimulation *in vivo* can induce changes in the lung commonly observed with ventilator-associated lung injury (12, 13). We hypothesized that MV would alter the total adenyl nucleotide profile within the airspaces and produce changes in purinergic expression patterns, both within the lung and select extrapulmonary tissues, thereby modifying the purinergic receptor–ligand milieu.

MATERIALS AND METHODS

Animals

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. A total of 28 male Sprague-Dawley rats (body weight: 481 ± 6.8 g) were obtained from Harlan (Indianapolis, IN). Animals were block-randomized into five groups: (*i*) control (Ctrl, no MV, $n = 6$) or MV with: (*ii*) small V_T with positive end-expiratory pressure (PEEP) (V6P, V_T = 6 ml/kg, PEEP = 5 cm H_2O, $n = 5$); (*iii*) small V_T with zero end-expiratory pressure (ZEEP) (V6Z, V_T = 6 ml/kg, PEEP = 0 cm H_2O, n = 5); (*iv*) large V_T with ZEEP (V12Z, V_T = 12 ml/kg, PEEP = 0 cm H_2O, $n = 6$); and (*v*) large V_T with PEEP (V12P, V_T = 12 ml/kg, PEEP = 5 cm H_2O, $n = 6$).

Animals were anesthetized with pentobarbital (USP, 50 mg/kg intraperitoneal; Abbott Laboratories, North Chicago, IL) and the cervical trachea was cannulated via midline cervical incision. Control animals breathed spontaneously through the cannula. After confirming a surgical plane of anesthesia, MV animals were chemically paralyzed (pancuronium bromide 0.4 mg/kg, intravenous; Abbott Laboratories) and connected to a small-animal ventilator (Inspira ASV; Harvard Instruments, Holliston, MA), which defined time 0. MV animals were ventilated according to protocol randomization, with room air, at a respiratory rate of 60 breaths per minute (bpm) and an inspiratory:expiratory ratio of 1:1. After 2 h, blood was sampled from the jugular vein. Bronchoalveolar lavage (BAL) was performed by slowly instilling 2 ml of sterile solution (0.9% NaCl containing 10.5 mM buffered sodium citrate, pH = 7.3) intratracheally, followed by 2 ml of air. Lung fluid was drained by gravity and gentle abdominal massage, and specimens were collected

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TABLE 1. OLIGONUCLEOTIDE PRIMERS USED TO DETECT RECEPTOR SUBTYPES BY REAL-TIME REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

Gene	Forward	Reverse
A_{2b}	TTTCACAGCTGCCTCTTCCT	GGTGGCACGGTCTTTACTGT
A_3	GTCTTCACCCATGCTTCCAT	GGGTGGAGCTGTTTTGAGAG
P_2Y_4	CTGGGCTGGACTAAGGAAGC	CATGTAGGTGGCTGTTGCAT
P_2Y_6	GTGGAATTCAGGCTGAGGAC	GCCTGCTTTTCCTATGCTTG
P_2X_7	GCGTTTTGACATCCTGGTTT	AAGAAGTCCGTCTGGGGTCT
Fas	GGGCATGGTTTAGAAGTGGA	GGATTCCAGATTCAGGGTCA
Fas ligand	AACCAAAAAGCCAAGGAGTGT	TCTTCTCCTCCATTAGCACCA
GAPDH	AGGTGACCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

Definition of abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

on ice. Animals then received a lethal intravenous injection of pentobarbital, and tissue samples from lungs, liver, and kidney were harvested and immediately preserved in RNAlater solution (Qiagen, Valencia, CA).

Urea-Based Epithelial Lining Fluid Volume Determination

BAL and serum urea concentrations were determined using a commercially available kit (Sigma, St. Louis, MO). The simultaneous determination of urea concentrations in paired serum and BAL samples ($Urea_{serum}$ and $Urea_{BAL}$, respectively) permitted the calculation of the volume of epithelial lining fluid as described by the equation: epithelial lining fluid = $(Urea_{BAL}/Urea_{serum}) \times Vol_{BAL}$, where Vol_{BAL} is the volume of BAL collected (14).

Protein

Total BAL protein content was measured spectrophotometrically by the commercially available bicinchoninic acid protein assay (Pierce, Rockford, IL).

Nucleotide Analysis

Immediately after iced collection, BAL samples were centrifuged at 4°C, and supernatants boiled for 2 min. Nucleotides were derivatized into fluorescent 1,N^6-ethenopurines for quantification by high-pressure liquid chromatography (HPLC), according to the technique previously described by Lazarowski and colleagues (15, 16). Briefly, samples were incubated at 72°C with 1M chloroacetaldehyde, 25 mM Na_2HPO_4, then alkalinized with NH_4HCO_3 before analysis by HPLC (Waters, Milford, MA) using a PRP-X100 anion exchange column (Hamilton, Reno, NV). HPLC fluorescence was integrated and analyzed using Millennium software (Waters). Data from test samples were compared against known concentrations of ATP, ADP, AMP, and adenosine (ADO), which were derivatized and analyzed in parallel; these control samples were injected at the beginning and again at the end of each HPLC injection set to determine within-run precision (< 3%).

Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Tissue RNA was extracted using Qiagen RNeasy kit. RNA quality was assessed spectrophotometrically and by denaturating agarose gel electrophoresis. cDNAs were prepared using random primers and moloney murine leukemia virus reverse transcriptase (SuperScriptII RNase H^- RT; Invitrogen, Carlsbad, CA), as per manufacturer's instructions. For real-time polymerase chain reaction (PCR), primer sets, described in Table 1, were designed using the NCBI website, Primer3 (Whitehead Institute, Cambridge, MA) and Genosys (Sigma) software. Primers were synthesized by MWG Biotech Inc. (Charlotte, NC). Real-time PCR was performed on a LightCycler Instrument (Roche Diagnostics, Indianapolis, IN), using LightCycler FastStart DNA Master SYBR Green I (Roche), following the manufacturer's instructions. The crossing point (CP) for each sample was determined by LightCycler LCDA version 3.5.28 Software (Roche). Each sample was amplified at least in duplicate, for which the CP SD was < 0.5. For each run, a negative control was performed, in which the cDNA template was replaced by water. Melting curves facilitated discrimination between potential primer dimers and specific amplified products, and controlled for the homogeneity of a single amplified sequence. Serial dilutions of a control template permitted the establishment of a standard curve. The slope of the linear regression of the CP versus the log cDNA concentration was used to calculate amplification efficiency (E), as $E = 10^{(1/slope)}$. The relative quantification of gene mRNA levels was calculated as a ratio (R), in comparison to a reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The equation for R, as described by Pfaffl (17), was:

$$R = E_{target}^{(CP_{target}\ control - CP_{target}\ sample)} / E_{ref}^{(CP_{ref}\ control - CP_{ref}\ sample)}$$

where E_{target} and E_{ref} are, respectively, the efficiency of target gene and reference gene (GAPDH); CP_{target} control is the average of crossing points of control animals cDNA for the target gene; CP_{target} sample is the crossing point of the sample for the target gene; CP_{ref} control is the average of crossing points of control animals cDNA for the reference gene; CP_{ref} sample is the crossing point of the sample for the reference gene.

Statistical Analysis

Data are presented as mean ± SEM. Data were analyzed by ANOVA and Fisher's PLSD tests where appropriate. Significance was defined as $P < 0.05$.

RESULTS

Airway Pressures

All animals survived the 2-h experimental period of MV. Airway pressures were monitored during MV and are reported in Table 2. The delivery of larger VTs significantly increased peak inspiratory pressure ($P < 0.0001$ V12Z versus V6Z; $P < 0.0001$ V12P versus V6P), and mean airway pressure (MPaw; $P = 0.001$ V12Z versus V6Z; $P = 0.02$ V6P versus V12P). Due to the volume-controlled ventilatory mode, the addition of PEEP increased MPaw and peak inspiratory pressure (MPaw; $P < 0.0001$ V6P versus V6Z; $P < 0.0001$ V12P versus V12Z; peak inspiratory pressure: $P = 0.0007$ V6P versus V6Z; $P = 0.0001$ V12P versus V12Z). Small levels of auto-PEEP were detected in the V12Z group. End-expiratory pressure could not be reliably measured due to the relatively high respiratory rates in all groups.

TABLE 2. TIDAL VOLUMES AND AIRWAY PRESSURES IN EXPERIMENTAL GROUPS

Groups	V_T (*ml/kg*)	PIP (*cm H_2O*)	Observed PEEP (*cm H_2O*)	MPaw (*cm H_2O*)
Control animals	Spontaneous	N/A	N/A	N/A
V6P	6	14.8 ± 1.3	6.2 ± 1.0	8.5 ± 1.0
V6Z	6	10.0 ± 0.0	0.0 ± 0.0	3.6 ± 0.1
V12Z	12	18.2 ± 0.6	1.2 ± 0.2	6.4 ± 0.3
V12P	12	23.2 ± 0.4	5.3 ± 0.2	10.2 ± 0.2

Definition of abbreviation: MPaw, mean airway pressure; PEEP, positive end-expiratory pressure; PIP, peak inspiratory pressure; V_T, tidal volume.

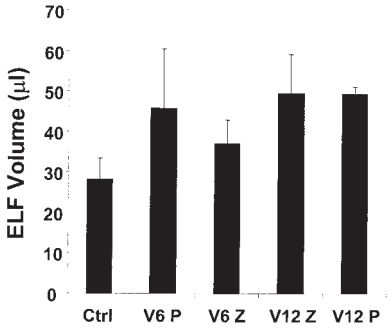

Figure 1. The volume of epithelial lining fluid in ventilated animals remained comparable to that of nonventilated control animals.

Epithelial Lining Fluid and BAL Protein

Regardless of ventilation protocol, the urea-based volume of epithelial lining fluid remained comparable to that of unventilated control animals (Figure 1). MV without PEEP, regardless of VT administered, increased the concentration of BAL protein ($P = 0.038$ V6Z versus Ctrl; $P = 0.008$ V12Z versus Ctrl). The addition of PEEP to either tidal volume protocol normalized BAL protein levels (P = NS V12P versus Ctrl; $P = 0.05$ V12P versus V12Z; Figure 2).

BAL Nucleotides

The adenine-based nucleotides were measured in BAL by a highly sensitive fluorescence-based HPLC analysis. Compared with control animals, the total adenine nucleotide concentration was unchanged after small-volume ventilation, regardless of end-expiratory pressure. Total nucleotide levels significantly increased (95%) after 2 h of ventilation with a large VT ($P = 0.006$; Figure 3). The addition of PEEP to the large-volume strategy reduced nucleotides to a level similar to that of control animals. Analysis of individual nucleotide species demonstrated an increase in the levels of ADO and AMP in the V12Z group (ADO: $P < 0.0001$ V12Z versus Ctrl; AMP: $P = 0.0065$ V12Z versus Ctrl), which was normalized by the addition of PEEP (ADO: $P = 0.004$ V12Z versus V12P; P = NS V12P versus Ctrl; AMP: P = NS V12P versus Ctrl; Figure 4).

The proportion of each nucleotide species expressed as a percentage of total nucleotides is presented in Figure 5. Ventilation with ZEEP, regardless of VT, decreased the relative proportion of ADP ($P = 0.022$ V6Z versus Ctrl; $P = 0.008$ V12Z versus Ctrl); the addition of PEEP to either VT returned ADP levels to control values (P = NS V6P versus Ctrl; P = NS V12P versus Ctrl). Similarly, the ATP reduction observed after 6 ml/kg and 12 ml/kg volume ventilation ($P = 0.049$ V12Z versus Ctrl) was not detected when PEEP was administered. Compared with unventilated control animals, the relative proportion of ADO

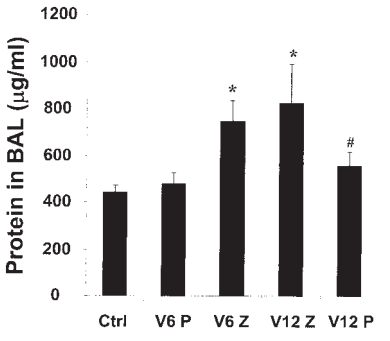

Figure 2. MV with ZEEP (*V6Z* and *V12Z*) significantly increased the BAL protein concentration when compared with nonventilated control animals (*Ctrl*). The addition of 5 cm H2O of PEEP (*V6P* and *V12P*) normalized the BAL protein concentration. $*P < 0.05$ versus Ctrl; $\#P < 0.05$ versus V12Z.

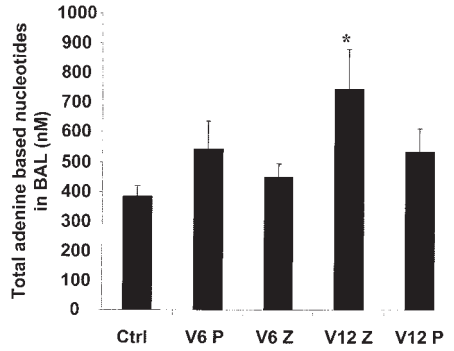

Figure 3. MV with large Vt and ZEEP (*V12Z*) significantly increased the concentration of adenine-based nucleotides in the BAL when compared with nonventilated control animals (*Ctrl*). Ventilation with small VT (*V6Z* and *V6P*) or the addition of 5 cm H_2O of PEEP (*V12P*) normalized the BAL adenyl-nucleotide concentration. $*P < 0.05$ versus Ctrl.

was increased in all groups except V6P ($P = 0.033$ V6Z versus Ctrl; $P = 0.0001$ V12Z versus Ctrl; $P = 0.010$ V12P versus Ctrl).

Pulmonary Purine Receptor mRNA Levels

Purine receptor mRNA was quantified by real-time PCR and expressed as a ratio of GAPDH expression. All tested receptors (P_2Y_4, P_2Y_6, P_2X_7, A_{2b}, and A_3) were detected in lung, kidney, and liver tissue. MV, regardless of strategy, did not significantly alter the mRNA levels of P_2Y_4, P_2Y_6 or A_3 in the lung (Figure 6). Large-volume ventilation without PEEP significantly increased the amount of A_{2b} mRNA ($P = 0.049$ V12Z versus Ctrl). Adding PEEP normalized the A_{2b} mRNA level to that of control animals (P = NS, V12P versus Ctrl). MV with the V6P, V12Z, and V12P protocols significantly decreased lung P_2X_7 mRNA amounts ($P = 0.0288$ Ctrl versus V6P; $P = 0.0043$ Ctrl versus V12Z; $P = 0.0005$ Ctrl versus V12P).

Extrapulmonary Purine Receptor mRNA Levels

In renal tissue, P_2Y_4, P_2Y_6, and A_3 receptors were constitutively present and expression ratios remained unchanged by MV (Figure 7A). Renal A_{2b} mRNA levels were reduced in V6Z,

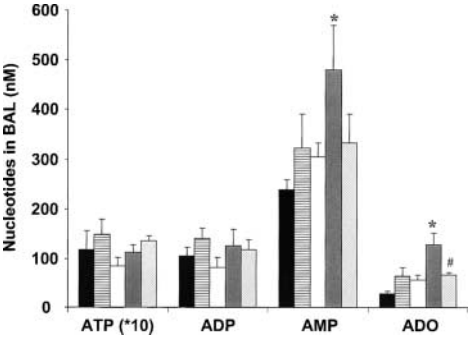

Figure 4. MV with large Vt and ZEEP (*V12Z*) significantly increased the concentrations of AMP and ADO in the BAL when compared with nonventilated control animals (*Ctrl*). The addition of 5 cm H2O of PEEP (*V12P*) normalized the BAL nucleotide profile. *ATP (*10)*: in this graph, the concentrations of ATP have been multiplied by 10 to allow visualization. $*P < 0.05$ versus Ctrl. $\#P < 0.05$ versus V12Z. *Black bars*, control animals; *horizontal hatched bars*, V6P; *white bars*, V6Z; *gray bars*, V12Z; *diagonally hatched bars*, V12P.

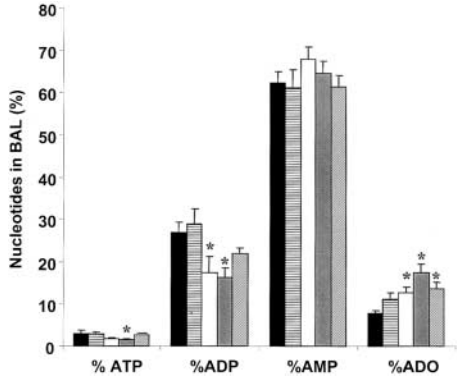

Figure 5. MV altered the relative proportions of adenine based nucleotides (*$P < 0.05$ versus Ctrl). *Black bars*, control animals; *horizontal hatched bars*, V6P; *white bars*, V6Z; *gray bars*, V12Z; *diagonally hatched bars*, V12P.

V12Z, and V12P ($P = 0.032$ V6Z versus Ctrl; $P = 0.059$ V12Z versus Ctrl; $P = 0.040$ V12Z versus Ctrl), but were similar to control in V6P (P = NS V6P versus Ctrl). Renal P_2X_7 receptor mRNA quantity was not altered by MV. We detected a ZEEP-associated decrease in renal P_2X_7 receptor mRNA levels ($P = 0.029$ both groups with ZEEP versus Ctrl), which was normalized in animals ventilated with PEEP (P = NS both groups with PEEP versus Ctrl; $P = 0.028$ PEEP groups versus ZEEP groups).

In the liver, P_1 (A_{2b} and A_3) and P_2Y expression was unchanged by MV. Large-volume MV without PEEP significantly decreased hepatic P_2X_7 receptor mRNA levels ($P = 0.023$ V12Z versus Ctrl). The addition of PEEP returned the hepatic P_2X_7 levels to control values (P = NS V12P versus Ctrl; Figure 7B).

Pulmonary Fas and Fas Ligand mRNA Levels

Pulmonary Fas and Fas ligand (FasL) mRNA levels were quantified by real-time PCR (Figure 8). MV with the V6P, V12Z, and V12P strategies resulted in a significant reduction of FasL mRNA levels ($P = 0.003$ V6P versus Ctrl; $P = 0.017$ V12Z versus Ctrl; $P = 0.022$ V12P versus Ctrl). Similar decreases, although not statistically significant, were observed in the pulmonary mRNA levels of Fas.

DISCUSSION

Evidence is mounting that MV, particularly with large VTs, may be injurious (1). Data published by the ARDSnet consortium revealed that VT limitation during MV yielded a profound reduction in acute respiratory distress syndrome (ARDS)–associated mortality (18). Protective ventilatory strategies have been advocated to reduce ventilator-induced lung injury (VILI) in an effort to improve outcome from pulmonary failure (19).

It has been hypothesized that MV with high end-inspiratory and low end-expiratory pressures is injurious to the lung because both result in the delivery of significant stress forces to pulmonary surfaces (1). It is clear that at very high pressures and large volumes, MV can produce a variety of physical derangements at the alveolar–capillary level. Although shear-associated mechanical failure may account for some of the derangements observed during MV, additional factors likely play a role (e.g., activation of stretch-induced epithelial cation channels [20]). In fact, lung injury and increased capillary permeability can occur even with moderate airway pressures that do not produce overt mechanical stress failures (20, 21). These data suggest that there are molecular systems of mechanotransduction that link airway stress to the cellular events causing VILI.

In 1929, Drury and Szent-Gyorgyi demonstrated that nucleotides can independently exert such profound physiologic effects as negative cardiac chronotropy and coronary vasodilatation (22). It has subsequently become clear that nucleotides play an important role as extracellular messengers and orchestrate a broad range of physiologic responses via binding to ubiquitous membrane-bound nucleotide receptors. Purinergic receptors can be broadly divided into two large superfamilies: the ADO-binding P_1 receptors (A_1, A_{2a}, $A_{2b,}$ and A_3), and the larger P_2 nucleotide-binding group. The latter can be subdivided into ionotropic (P_2X) ligand-gated cation channels and G-protein–coupled (P_2Y) purinoceptors, which signal via inositol-triphosphate and Ca^{2+}. Purinoceptors are historically characterized by the rank-order potency of natural and synthetic agonists and antagonists (3, 4).

Extracellular nucleotides have been implicated in many cellular processes that may be mechanistically pivotal in VILI. In animal microglial, dendritic and mesangial cells, ATP or its breakdown product, ADO, act via P_2Y or A_{2a} receptors to mediate proinflammatory effects, such as stimulating interleukin (IL)-6 expression and release (8, 9, 23). These data suggest that extracellular nucleotides may have complex effects mediated by a variety of metabolic products. In human cell lines, P_2Y-mediated proinflammatory stimulation has been demonstrated in monocytes (IL-8) and astrocytes (IL-1β) (10, 24). In contrast, prolonged P_2X_7 stimulation leads to apoptosis in a variety of cell cultures (11, 25, 26).

Large amounts of ATP are released in response to mechanical stimulation by a host of cell types, including nasal epithelial, human bronchial epithelial, endothelial, smooth muscle, and fibroblasts (5, 6). It is known that ATP is released *in vitro* from polarized airway epithelia by luminal mechanical stimulation and stimulates purinoceptors on both the apical and basolateral membranes (27). We have previously demonstrated that MV, with either very large VTs (40 ml/kg) or pressures (40 cm H_2O), increases *in vivo* BAL levels of ATP in a VT/inspiratory pressure–dependent manner (12, 13).

In the current study, we examined the effect of comparatively smaller VTs (6 ml/kg and 12 ml/kg) on the levels of airway ATP and downstream metabolic products by derivatizing purine nucleotides into ethenopurines and analyzing the product profile by HPLC. We observed that MV, even at 12 ml/kg, produced an increase in total airway adenyl nucleotides and altered the downstream metabolic nucleotide profile. The ventilatory parameters applied were selected based on previous animal data demonstrating that these conditions provide preserved oxygenation over the duration of our experiments (28–30). Although it is known that changes in the concentration of inspired oxygen

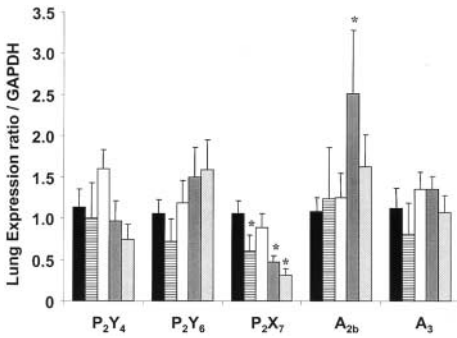

Figure 6. MV altered the pulmonary mRNA levels of P_2X_7 and A_{2b} purinoceptors, but not that of P_2Y_4, P_2Y_6, or A_3 (*$P < 0.05$ versus Ctrl). *Black bars*, control animals; *horizontal hatched bars*, V6P; *white bars*, V6Z; *gray bars*, V12Z; *diagonally hatched bars*, V12P.

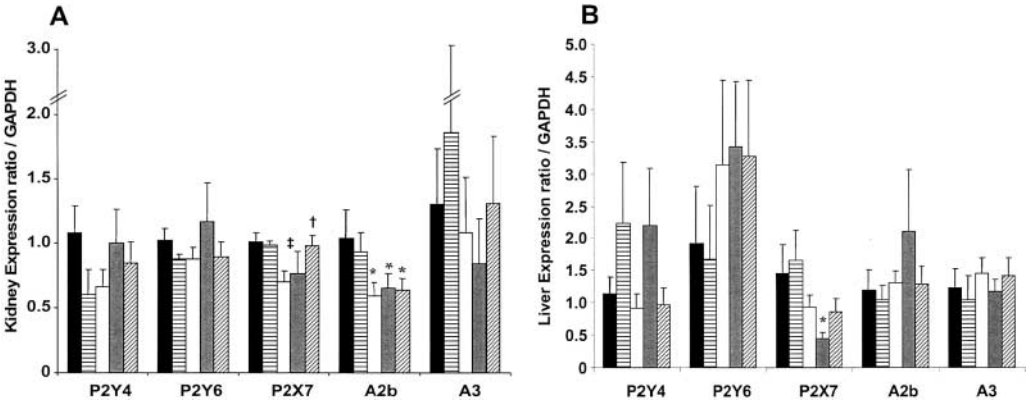

Figure 7. MV altered the mRNA levels of P_2X_7 and A_{2b} purinoceptors in the kidney (*A*), and that of P_2X_7 in the liver (*B*). P_2Y_4, P_2Y_6, and A_3 expression remained unchanged in both tissues (*$P < 0.05$ versus Ctrl; ‡$P < 0.05$ ZEEP versus Ctrl; †$P < 0.05$ ZEEP versus PEEP). *Black bars*, control animals; *horizontal hatched bars*, V6P; *white bars*, V6Z; *gray bars*, V12Z; *diagonally hatched bars*, V12P.

can impact lung injury, it was not the goal of the current study to evaluate these effects on the purinergic system.

Because a spectrum of purinoceptors are often regionally expressed, and their activities vary depending on relative agonist potency, the local extracellular nucleotide milieu has great impact on cellular responses (3, 4). The relative concentrations of extracellular nucleotide species are regulated by numerous enzymes, such as 5′-nucleoside triphosphatase, ecto-nucleotide pyrophasphatase, adenylate kinase, nucleoside diphosphokinase, ecto-apyrase CD39, and ecto-5′-nucleotidase CD73 (7). In our experimental system, ATP and its metabolic products can be measured in nanomolar concentrations by etheno-derivatization and HPLC. The increase in total nucleotides, AMP and ADO observed with large-volume MV, combined with a proportionate decrease in ATP and ADP, can be interpreted at least in part as an enzymatic conversion of the precursors ATP and ADP toward the less phosphorylated metabolites AMP and ADO. Labeling studies, difficult in *in vivo* systems, will be required to definitively state whether the AMP and ADO rise is due to relatively increased metabolic conversion of ATP/ADP or is a result of release of these nucleotides into the airspaces. Regardless of source, these MV-associated changes could be expected to produce increased purinergic stimulation via the P_1 (A_{2b}) pathway (3).

The application of PEEP, generally regarded as a protective strategy during large-volume MV, returned the total adenyl nucleotide levels toward that of unventilated control animals. Similarly, exposure to PEEP normalized the complete etheno-generated metabolic profile so that its appearance was similar to that of control animals. These normalizing effects occurred despite comparatively higher peak and MPaws.

Localization studies have demonstrated that purinergic receptors are expressed in the majority of the cell and tissue types assayed. By real-time reverse transcriptase–PCR, we confirmed that the purinergic receptors P_2Y_4, P_2Y_6, P_2X_7, A_3, and A_{2b} are constitutively expressed in the unventilated rat lung. Furthermore, our data supports that after only 2 h of positive-pressure MV, P_1 and P_2X mRNA concentration patterns can be significantly modified both by applied VT and PEEP.

Exposure to MV resulted in a quantitative decrease in the mRNA of pulmonary P_2X_7 in all groups except V6Z, which was the group exposed to the lowest peak and MPaws. This observation suggests that lung P_2X_7 mRNA levels are airway pressure–sensitive. It is known that prolonged cell surface P_2X_7 stimulation generates a conformational change in receptor transmembrane protein structure, which creates a nonselective transmembrane pore and can initiate pro-apoptotic cellular signals (11, 25, 26). ATP is a natural agonist for the P_2X_7 receptor. Our observation that MV with 12 ml/kg VTs resulted in a proportional decrease in airway ATP and a decrease in P_2X_7 mRNA levels suggests a ligand–receptor environment conducive to decreased P_2X_7 activation. It is intriguing to speculate that apoptotic changes observed by others in association with VILI and ARDS may relate to P_2X_7-mediated apoptosis and a consequent decrease in total P_2X_7 mRNA in the lung.

We demonstrated that ventilation-associated pulmonary changes in Fas and FasL mRNA levels, measured by real-time PCR, paralleled the changes observed in P_2X_7 mRNA levels. It is well established that stimulation of the Fas/FasL system is associated with induction of apoptosis. It is known that Fas and FasL are expressed on pulmonary epithelia and inflammatory cells, and that their expression can be altered in the setting of lung injury (31–34). Although most observations have documented associated changes after prolonged durations (hours to days), it is re-

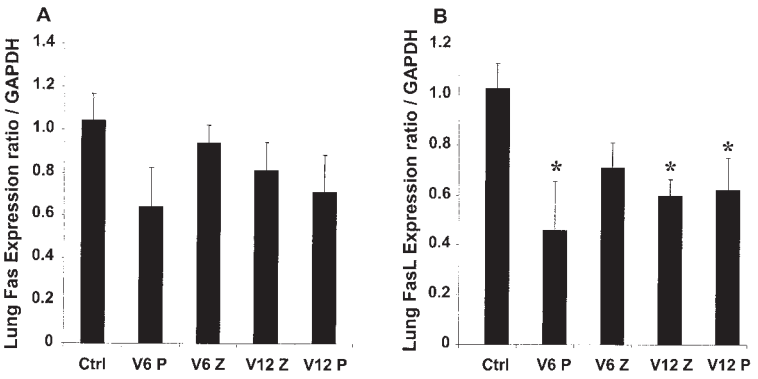

Figure 8. MV altered the pulmonary mRNA levels of Fas (*A*) and FasL (*B*) (*$P < 0.05$ versus Ctrl).

markable that we observed decreases in this system over a period of 2 h. It is unclear if these observations result from changes in expression or ventilation-associated changes in the recruitment and residence of pulmonary inflammatory cells. Regardless of the mechanism, it is plausible that either event could have significant implications regarding lung injury.

mRNA levels of the P_1 purinergic receptor A_{2b} were similarly responsive to large-volume MV. Whereas ventilation with 6 ml/kg volumes had no discernable effect on A_{2b} mRNA quantity, regardless of the applied PEEP, mRNA levels were significantly increased upon 2 h of exposure to 12 ml/kg volumes. Comparable to the effect demonstrated on airway extracellular nucleotide levels, the addition of PEEP to the large-volume MV strategy reduced A_{2b} mRNA to levels not significantly different from unventilated control animals. The large volume MV-associated and PEEP-sensitive increase in both A_{2b} and its ligand ADO provides a milieu conducive to a comparatively increased stimulation of this receptor.

Others and we have previously shown that MV can be associated with the locoregional release of cytokines (35, 36). In bronchial smooth muscle cell cultures, A_{2b} stimulation has been demonstrated to induce the release of the proinflammatory cytokines IL-6 and monocyte chemoattractant protein–1 (37). Furthermore, ADO stimulation of P_1 receptors has been observed to mediate IL-13–induced inflammation and remodeling in the murine lung and regulate IL-8 production in human mast cell–1 cells (38, 39). Given these data, we speculate that the MV-associated increase in ADO and A_{2b} receptor mRNA levels may result in activation of the P_1 system and could be a mechanistic candidate for the proinflammatory state observed with MV and the syndrome of VILI.

ARDS-related mortality is often associated with the development of multiple system organ dysfunction rather than discrete physiologic failure of the lung (40). Observational studies support the concept that extrapulmonary organ dysfunction may occur in a V_T-dependent manner, and that MV can lead to distant organ injury, cytokine elaboration, and even cellular apoptosis (2). We examined the effect of MV, as a sole stimulus, on the tissue mRNA levels of purinergic receptors on organs frequently involved in ARDS-associated multiple system organ dysfunction (i.e., the liver and kidney).

Large-volume MV decreased the mRNA levels of P_2X_7 in both the liver and kidney. Both effects were reversed by the application of PEEP. Because these changes appeared after only 2 h of ventilation, it is possible that the changes in mRNA levels may not represent an actual change of expression, but rather a rapid death or loss of resident cells (either epithelial or inflammatory) or recruitment into the organ of immune/inflammatory cells that do not express P_2X_7. Because of V_T-dependent increases in intrathoracic pressure and potential pentobarbital-associated decreases in cardiac output, another explanation is simply an MV-dependent alteration in intraorgan blood volume. However, this hypothesis is not supported by the observation that PEEP reversed the process, despite higher MPaw and Paw values, an effect that could be expected to induce even greater limitations in preload and cardiac output. Unfortunately, the fact that vascular manipulations caused by the placement of indwelling monitoring catheters can impact nucleotide release precluded direct measurement of vascular pressures.

Others have observed that MV induces apoptotic changes in extrapulmonary organs, such as the kidney (2), but the cellular populations responsible for this event were undefined. We describe here MV-associated changes in pulmonary, renal, and hepatic purinoceptor and Fas/FasL mRNA levels, but we did not seek to identify specific apoptotic cell types nor did we determine the physiologic consequences of these alterations.

MV produced a significant decrease in the quantity of renal A_{2b} mRNA, an effect not reversed by the application of PEEP. It is known that A_{2b} receptors are abundantly expressed within the renal preglomerular microcirculation, and their stimulation has been implicated in the regulation of renal blood flow dynamics (41, 42). Furthermore, ADO-stimulated P_1 agonism has been associated with increased renal erythropoetin (EPO) production, and ADO deficiency with an EPO-depleted state (43). Further studies are required to determine if our observations account for the gross derangements in renal blood flow distribution and EPO-deficient anemia states often associated with MV and the syndrome of VILI.

In summary, our results demonstrate that MV alters the mass and spectrum of extracellular nucleotides in airspaces and modifies purinoceptor mRNA patterns in the lung. These ligand receptor changes could result in the V_T-dependent stimulation of distinctive purinergic receptor subtypes, mediating a variety of ventilation-associated cellular responses. Furthermore, we observed that positive-pressure ventilation of the lung, as a sole stimulus, can modify purinoceptor mRNA levels in extrapulmonary tissues, such as the liver and kidney. The application of PEEP as a protective strategy during large-volume ventilation normalized many of the V_T-dependent nucleotide and purinoceptor alterations observed with injurious ventilation.

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