Prevention of Ventilator-Induced Lung Edema by Inhalation of Nanoparticles Releasing Ruthenium Red

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Abstract

The acute respiratory distress syndrome (ARDS), a devastating lung disease that has no cure, is exacerbated by life-supportive mechanical ventilation that worsens lung edema and inflammation through the syndrome of ventilator-induced lung injury. Recently, the membrane ion channel transient receptor potential vanilloid 4 (TRPV4) on alveolar macrophages was shown to mediate murine lung vascular permeability induced by high-pressure mechanical ventilation. The objective of this study was to determine whether inhalation of nanoparticles (NPs) containing the TRPV4 inhibitor ruthenium red (RR) prevents ventilator-induced lung edema in mice. Poly-lactic-co-glycolic acid NPs containing RR were evaluated *in vitro* for their ability to block TRPV4-mediated calcium signaling in alveolar macrophages and capillary endothelial cells. Lungs from adult C57BL6 mice treated with nebulized NPs were then used in *ex vivo* ventilation perfusion experiments to assess the ability of the NPs to prevent high-pressure mechanical ventilation-induced lung edema. Poly-lactic-co-glycolic acid NPs (300 nm) released RR for 150 hours *in vitro*, and blocked TRPV4-mediated calcium signaling in cells up to 7 days after phagocytosis. Inhaled NPs deposited in alveoli of spontaneously breathing mice were rapidly phagocytosed by alveolar macrophages, and blocked increased vascular permeability from high-pressure mechanical ventilation for 72 hours in *ex vivo* ventilation perfusion experiments. These data offer proof of principle

that inhalation of NPs containing a TRPV4 inhibitor prevents ventilator damage for several days, and imply that this novel drug delivery strategy could be used to target alveolar macrophages in patients at risk of ventilator-induced lung injury before initiating mechanical ventilation.

Keywords: acute respiratory distress syndrome; ventilator-induced lung injury; TRPV4; *ex vivo* ventilation perfusion; inhalation

Clinical Relevance

This study provides a proof of principle for the effectiveness of inhaled nanotherapy in preventing ventilator injury. Considering that there is no cure for acute respiratory distress syndrome (ARDS) and that mortality remains high, this novel prophylactic drug delivery strategy, if proven safe and effective in humans, would greatly impact the outcome of patients with impending respiratory failure, and would represent a paradigm shift in the management of patients with ARDS, moving treatment from after to before initiation of mechanical ventilation and diagnosis of ventilator-induced lung injury.

Acute respiratory distress syndrome (ARDS) is a devastating lung disease characterized by increased pulmonary vascular permeability and inflammation, and high mortality (1–4). Mechanical

ventilation, required as life support in ARDS, exerts pathological mechanical forces on lung cells that are transduced into biochemical signals that worsen edema and inflammation, contributing to

mortality through ventilator-induced lung injury (VILI) (5). The molecular basis of VILI is not well understood, and there are no medicines to reduce mortality from either ARDS or VILI. A protective

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ventilation strategy that minimizes ventilator volumes and pressures is recommended (3).

Recently, the force-sensitive transient receptor potential vanilloid 4 (TRPV4) ion channel in the alveolar macrophage cell membrane was shown to mediate murine lung vascular permeability from high-pressure mechanical ventilation (HPMV) (6, 7). TRPV4 is a Ca^{2+} -permeable cation channel gated by numerous stimuli, including integrin- and osmosis-dependent mechanical forces (8–10), heat (11, 12), and endogenous and synthetic compounds (12–15). It is expressed in many tissues (16, 17), including pulmonary bronchiolar and alveolar epithelia (13), alveolar macrophages, and extra-alveolar vessel smooth muscle cells and endothelium (6, 13). *In vitro* data suggest that TRPV4 can be activated within 5 milliseconds of stimulation by mechanical forces (8). Consequently, similarly near-instantaneous activation of TRPV4 by mechanical force from the ventilator could represent the initial upstream signaling event in the induction of ventilator-induced pulmonary edema *in vivo*. We therefore hypothesized that treating lung cells with inhibitors of TRPV4 before initiating mechanical ventilation would mitigate the development of VILI.

To test this hypothesis, we developed a nanoparticulate formulation containing the TRPV4 inhibitor ruthenium red (RR) (6, 9, 18) for inhalational delivery in the mouse. We focus on the inhaled route because inhalational drug delivery would generate high pulmonary drug levels relative to systemic delivery, thereby minimizing systemic side effects (19, 20), which is important considering the ubiquitous nature of TRPV4 (21). In addition, the initiation of mechanical ventilation in humans at risk of or with ARDS is universally preceded by a period during which oxygen is delivered via facemask, so inhaled nanotherapy could be given to all patients during this time to prevent VILI. Use of sustained release technology using nanoparticles (NPs) would further enhance the therapeutic index and prolong duration of effect.

In this study we used *in vitro* calcium imaging, whole-animal nebulization, an *ex vivo* mouse lung ventilation perfusion model, and pharmacokinetic analyses to show that inhaled poly-lactic-co-glycolic acid (PLGA) NPs containing RR block HPMV-induced lung edema for up to 3 days. These data suggest that this novel nanotherapy could be tailored for use

in humans in the early management of patients at risk for ARDS and VILI, two devastating syndromes that have no cure, and of those at risk for other diseases, who also might benefit from targeted and sustained drug delivery to the lungs.

Materials and Methods

Ex Vivo Lung Ventilation Perfusion

Male C57BL/6 mice (8–12 wk old; The Jackson Laboratory, Bar Harbor, ME) were prepared for *ex vivo* lung ventilation and perfusion experiments, as we have done in the past (22). Briefly, mechanical ventilation was initiated through a tracheal tube placed via surgical tracheotomy, and the lungs perfused (0.5 ml/min) using a recirculating system volume of 6 ml. The lung and heart were excised *en bloc* and hung by attaching the vascular cannulae and tracheal tube to a circular adapter suspended from a force transducer (FT03C; Grass, Quincy, MA). The venous pressure was set at 5 cm H_2O . Perfusate and lung temperatures were maintained during the experiment at 37°C by housing the entire *ex vivo* ventilation perfusion system inside a standard humidified (90–95%) cell incubator without CO_2 (Forma Scientific, Marietta, OH). Vascular and airway pressures, pulmonary airflow, and lung weight were recorded using Polyview16 software (Grass Technologies, West Warwick, RI). To remove any pooled liquid on the surface of the hanging heart lung bloc during the experiment, a thin strip of Kimwipe paper was suspended proximally from diaphragm tissue on the infero-posterior edge of the lung and hung distally into a container of distilled water, thus serving as a “wick” (personal communication, S. Vogel, University of Chicago). The FT03 force transducer was calibrated during a 30-minute period of observation before each experiment.

Control and HPMV Protocols

All perfused *ex vivo* lungs were similarly ventilated during the initial 30-minute observation period and then divided into control ventilation or HPMV protocols, as described in Figure 1A.

Capillary Filtration Coefficient Measurement

After a 30-minute equilibration period and attainment of an isogravimetric state,

the pulmonary vein pressure (Ppv) was increased by 6 cm H_2O (from 5 to 11 cm H_2O) for 10 minutes. The change in capillary pressure was determined by double occlusion before and after the Ppv increase. Capillary filtration coefficient (Kf; in ml/min/cm H_2O /100 g) was calculated as the rate of lung weight gain between 7 and 10 minutes divided by the change in capillary pressure. All Kf values were normalized to 100-g predicted lung weight on the basis of the ratio of lung:body weight (BW) according to predicted lung weight ($0.00452 \pm 0.003 \times \text{BW}$) (23).

Nebulization Experiments

Animals were placed in a whole-animal nebulization chamber (14 cm $\times$ 5 cm $\times$ 8 cm) and allowed to spontaneously inhale nebulized RR (1 ml of 1 mM RR in 3 ml 0.9% normal saline; Proneb Ultra II Nebulizer, Pari Respiratory Equipment, Midlothian, VA) or normal saline as control for indicated time periods. For inhaled nanotherapy, mice inhaled nebulized fluorescent-labeled (as indicated) blank or RR NPs (0.1% suspension in 3 ml 0.9% normal saline) for 30 minutes. Animals were anesthetized at indicated time points after nebulization and their lungs were prepared for histology, or for *ex vivo* lung ventilation perfusion experiments.

Statistical Analyses

For three or more groups, differences among the means were tested for significance in all experiments using ANOVA with the Dunnett's *post hoc* test to compare differences between the control and experimental groups. For two groups, differences among the means were tested for significance using Student's unpaired *t* test. Significance was reached at a *P* value less than 0.05. Data are presented as means ($\pm$ SEM); *n* is indicated for each set of data.

Results

We used an *ex vivo* lung ventilation perfusion model that has been used to study lung vascular function in many animal types, including mice (24–30). The time courses of airway and venous pressures used in our control and HPMV injury protocols are shown in Figure 1A. The overall experimental approach for this study was to expose healthy mice to

Figure 1. *Ex vivo* ventilation perfusion and ruthenium red (RR) pharmacokinetics. (A) Experimental *ex vivo* lung ventilation perfusion scheme. Time course of peak inspiratory pressure (PIP; *top*) in control ventilation (*thin dashed line*) and high-pressure mechanical ventilation (HPMV; *solid line*) protocols, and pulmonary venous pressure (Ppv; *bottom, thick dashed line*) in both ventilation protocols. Perfused *ex vivo* lungs were mechanically ventilated during an initial 30-minute observation period, with a PIP of 10 cm H₂O, a positive end-expiratory pressure (PEEP) of 3 cm H₂O, and a rate of 60 breaths/min. The baseline capillary filtration coefficient (Kf₁) was measured in all lungs between 30 and 40 minutes by increasing the Ppv from 5 to 11 cm H₂O (see MATERIALS AND METHODS). At 40 minutes, the Ppv was returned to baseline, and lungs in the HPMV group were ventilated for 20 minutes at a rate of 60 breaths/min with a PIP of 35 cm H₂O and a PEEP of 3 cm H₂O. Control lungs were ventilated at a rate of 60 breaths/min, with a PIP of 10 cm H₂O and a PEEP of 3 cm H₂O for the entire experiment. A second Kf measurement (Kf₂) was made in all lungs between 60 and 70 minutes by raising the Ppv from 5 to 11 cm H₂O. Changes in vascular permeability induced by HPMV or control ventilation were reflected in an increase in Kf₂ relative to Kf₁. (B) Intravenous RR inhibits HPMV-induced increases in lung vascular permeability. Wet:dry lung weight ratio of lungs exposed to control ventilation (–) or HPMV (+) *ex vivo* protocols in the presence of 0, 20, or 35 μM RR delivered intravenously (IV) into the perfusate. Data are means ± SEM. $F = 4.369$; $^*P < 0.015$ using ANOVA and Dunnett's *post hoc* test; $n =$ at least 4 in each group. (C–D) Inhaled RR is retained in the lung during *ex vivo* perfusion. (C) Lung RR content (μg) after 30 minutes *ex vivo* perfusion in the presence of 35 μM RR in the perfusate, or after 5, 30, or 60 minutes of inhalation of nebulized RR (1 mM in 0.9% saline) in a whole-animal nebulization chamber. Data are means ± SEM. $F = 55.96$; $^*P < 0.0001$ using ANOVA and Dunnett's *post hoc* test; $n = 3$. (D) Lung RR content (μg) after 30 minutes of inhalation of nebulized RR in the whole-animal nebulization chamber followed by indicated duration of *ex vivo* perfusion.

inhalation therapy (free RR or RR NPs), and to then harvest their lungs for *ex vivo* ventilation perfusion studies (see MATERIALS AND METHODS) to test the efficacy of inhalation therapy in mitigating HPMV-induced increases in lung vascular permeability.

Before using NPs, we tested whether inhalation of free drug prevented HPMV-induced lung edema in our system. These results would allow a comparison of the efficacy of inhaled soluble drug relative to inhaled NPs containing the same drug.

Published studies have shown that treatment of *ex vivo* perfused mouse lungs with RR or other inhibitors of TRPV4 delivered directly into recirculating perfusate blocked ventilator-induced increases in lung vascular permeability

Figure 2. Inhaled RR inhibits HPMV-induced increases in lung vascular permeability. (A) K_f s measured at baseline (K_{f1}) and during experimental state (K_{f2} ; see Figure 1) in *ex vivo* lungs exposed to control ventilation or HPMV protocols at 5, 60, or 120 minutes after inhalation of nebulized (neb), normal saline (NS), or RR in a whole-animal nebulization chamber. Data are means $\pm$ SEM. * $P < 0.013$ versus K_{f1} in same group using Student's *t* test; $n = 9$. ** $P < 0.001$ versus K_{f1} in same group using Student's *t* test; $n = 8$. (B and C) Wet lung weight:body weight (BW) ratio (B), and wet:dry lung weight ratio (C) of *ex vivo* lungs from groups described in (A). Data are means $\pm$ SEM, $F = 5.054$, * $P < 0.002$ in (B); $F = 2.712$, * $P < 0.043$ in (C), using ANOVA and Dunnett's *post hoc* test, $n =$ at least 8 in each group. (D–G) Representative micrographs of hematoxylin and eosin (H&E)-stained sections of lung from mice treated with nebulized NS (D and E) or RR (F and G) and exposed 5 (D–F) or 60 (G) minutes later to control ventilation (D) or HPMV *ex vivo* protocols (E–G). Br, bronchus; PA, pulmonary artery. Arrow shows large perivascular cuff. Scale bar, 100 μ m. (H) Quantification of lung edema (perivascular cuff volume as a fraction of total wall volume [V_c/V_w]) from histological data (D–G) using a modification of a point-counting strategy (see MATERIALS AND METHODS). Data are means $\pm$ SEM. $F = 9.697$, * $P < 0.0001$ using ANOVA and Dunnett's *post hoc* test, $n > 4$, and at least 39 vessels examined in each group. (I) Inhaled RR blocks effects of HPMV (noted above) despite low perfusate RR concentration. Steady-state perfusate RR concentration during *ex vivo* perfusion (control ventilation protocol) of lungs harvested from mice treated with nebulized free RR or RR nanoparticles (NPs) 2, 4, or 24 hours before lung isolation.

(6, 7). We hypothesized that for inhaled RR to be effective here, the content of inhaled drug remaining in lungs at steady-state *ex vivo* perfusion should at least equal the steady-state drug content generated during *ex vivo* experiments in which intravenously delivered RR blocked lung edema. To test this hypothesis, and to establish dosing protocols for inhalation

therapy, we determined the minimum intravenous RR dose required to block HPMV-induced lung edema in our model, and then measured the *ex vivo* lung drug content that resulted using this intravenous dosing. In these initial studies, we measured the lung wet:dry weight ratio of *ex vivo* lungs exposed to control or HPMV protocols in the absence or presence of

increasing concentrations of RR in the perfusate. A perfusate concentration of 20 μ M RR did not significantly block the effects of HPMV on wet:dry lung weight ratio, whereas a concentration of 35 μ M did (Figure 1B). The RR content in lungs perfused at 35 μ M was approximately 2 μ g (Figure 1C). Inhalation of nebulized RR (5, 30, or 60 min, 1 mM in 0.9% saline)

Figure 3. RR NPs inhibit transient receptor potential vanilloid 4 (TRPV4)-mediated calcium signaling in alveolar macrophages *in vitro*. (A) Relative change in cytosolic calcium (F/F_0) in response to 4- α -PDD (arrow shows time of application) in Fluo-4-loaded alveolar macrophages cultured for 2 hours on fibronectin-coated glass petri dishes and pretreated for 5 minutes with increasing concentrations of soluble RR. (B) Representative Western blotting results confirming knockdown of TRPV4 protein levels in alveolar macrophages treated 48 hours earlier with control or TRPV4 small interfering RNA (siRNA). (C) Relative change in cytosolic calcium in Fluo-4-loaded alveolar macrophages, treated 48 hours earlier with indicated siRNA, in response to 4- α -PDD (arrow). (D) Cumulative RR release over time from 10 mg poly-lactic-co-glycolic acid (PLGA) NPs under infinite sink conditions. (E) Relative change in cytosolic calcium in response to 4- α -PDD (arrow) in Fluo-4-loaded alveolar macrophages 24 hours after exposure to 100 μ g/ml blank or RR NPs.

in a whole-animal nebulization chamber deposited almost five to eight times this target drug level in the lung (Figure 1C). Importantly, *ex vivo* perfusion of these lungs rapidly removed inhaled drug from the lung that led to steady-state lung drug levels within 30 minutes that were nevertheless similar to those found in *ex vivo* lungs perfused with 35 μ M RR (i.e., 2 μ g; Figure 1D). This is important, because baseline capillary filtration coefficients are measured after 30 minutes of *ex vivo* perfusion (see Figure 1A), and experimental lung injury is induced (if indicated) at 40 minutes of perfusion. These pharmacokinetic results therefore supported the use of our *ex vivo* model to test whether inhaled RR pretreatment could abrogate ventilator-induced changes in lung vascular permeability.

Inhaled Free RR Blocks HPMV Edema

To determine whether inhaled RR blocks HPMV-induced lung edema, mice were

pretreated with nebulized normal saline or RR, and their lungs exposed 5, 60, or 120 minutes later to control or HPMV *ex vivo* protocols, and Kf values (Kf_1 and Kf_2), wet lung:BW ratio, and wet:dry lung weight ratio were measured. Increases in Kf_2 seen after HPMV were prevented when inhaled RR was delivered 5 or 60, but not 120 minutes, before lung isolation (Figure 2A). Increases in wet lung:BW and wet:dry lung weight ratios seen in lungs exposed to HPMV were similarly inhibited when inhaled RR was delivered 5 or 60, but not 120 minutes, before isolation (Figures 2B and 2C). Representative micrographs from hematoxylin and eosin-stained lung sections from the above experimental groups are shown in Figures 2D–2G, and confirm that inhaled RR prevented HPMV-induced lung edema for up to 60 minutes after treatment. Perivascular fluid collections (perivascular cuffs) seen in the HPMV group treated with inhaled normal saline were diminished if lungs were pretreated with inhaled RR 5 and

60 minutes before isolation (Figures 2D–2H). Of note, the steady-state concentration of inhaled RR in the perfusate for these experiments was less than 1 μ M (Figure 2I).

RR NPs Inhibit Calcium Signaling *In Vitro*

We next fabricated 300 nm PLGA NPs with or without RR for inhalation therapy. We used PLGA NPs because they are biodegradable, protect drugs against degradation, and stably transfer into aerosols (20, 31, 32). Before testing on animals, we confirmed the ability of the NPs to block TRPV4-mediated calcium signaling *in vitro* (i.e., biological effect) using alveolar macrophages harvested from bronchoalveolar lavage (BAL) fluid, and bovine capillary endothelial (BCE) cells. We used alveolar macrophages because these cells scavenge airborne debris deposited in the lungs, and would thus be among the first lung cells exposed to inhaled nanotherapy (33). In addition, alveolar macrophages have

Figure 4. Inhaled nebulized PLGA NPs (300 nm) deposit in the mouse alveoli. (A–D) Representative 5× (A; scale bar, 200 μm), 10× (B; scale bar, 100 μm) and 60× (C and D; scale bar, 20 μm) magnification fluorescent micrographs of 50 μm thick lung sections (stained with DAPI to show cell nuclei) from mice treated 5 minutes earlier with nebulized Cy5-labeled NPs show inhaled NPs (red lining of airways in [A] and [B], arrows in [C] and [D]). (C) A sagittal view of a bronchiole with air lumen (black) at the right (see corresponding Video E1). (D) A cross-section of a bronchiole. (E and F) The 63× magnification phase-contrast (E) and fluorescent (F) micrographs of lung sections treated as described above showing NPs (arrows in [F]) on the surface of an alveolus (see corresponding Videos E2 and E3). Scale bar, 20 μm. (G–J) Representative fluorescent micrographs of z-stack of images from bottom (G) through to the top (J) of an alveolar macrophage harvested from the lungs of a mouse treated with nebulized Cy5-labeled PLGA NPs. Macrophages were plated on glass coverslips, fixed with paraformaldehyde (at 60 min), and stained with 4',6-diamidino-2-phenylindole (nuclei [blue]). Endogenous endosome, lysosome, and phagosome autofluorescence (green). Arrows indicate CY5-labeled (red) NPs in the cytoplasm (see corresponding Video E4). Scale bar, 5 μm.

been shown to be critical for the permeability response and resulting lung edema induced by HPMV (6, 7), and thus may be responsible for initiating this form of lung injury. BCE cells were also selected for TRPV4 cell signaling studies, because they express TRPV4 (9), display characteristic transient calcium signal responses to the TRPV4 agonist 4- α -PDD, and are more easily maintained long term in cell culture, enabling an assessment of the longer-term effect of RR NPs on TRPV4 signaling (i.e., 1 wk; see subsequent description). We chose 300-nm-diameter particles based on preliminary inhalation experiments with live mice, which showed that only

particles 300 nm or less deposited in the alveoli (data not shown).

Activation of TRPV4 by 4- α -PDD results in calcium influx, with varying kinetics dependent on cell type (6, 9). To determine whether RR NPs inhibit TRPV4-mediated calcium signaling in alveolar macrophages, cells were harvested from BAL fluid and adhered to fibronectin-coated glass-bottom Petri dishes, then loaded with the calcium reporter dye, Fluo-4, treated with 4- α -PDD, and calcium influx was measured using microfluorimetry (34). Exposure of alveolar macrophages to 4- α -PDD in the absence of RR induced a steady rise in

intracellular calcium, and this response could be almost completely abolished by treatment with soluble RR (Figure 3A). To confirm that this calcium response was mediated by TRPV4 in these cells, we knocked down the expression of TRPV4 using specific TRPV4 small interfering RNA (siRNA); siRNA was used as control (Figure 3B), and was found to result in a significant reduction in the 4- α -PDD-induced calcium response as well (Figure 3C). To determine whether RR NPs inhibit TRPV4 calcium signaling in alveolar macrophages, we first confirmed that these cells rapidly phagocytose the NPs. We treated live mice with Cy5-labeled NPs (300 nm)

nebulized into a whole-animal nebulization chamber, and determined that the NPs: (1) deposited deep into the lungs by documenting their presence in bronchioles and alveoli in lung sections prepared 5 minutes after inhalation (Figures 4A–4F and *see* Videos E1–E3 in the online supplement); and (2) appeared inside alveolar macrophages harvested from BAL fluid from similarly treated mice (Figures 4G–4J and *see* Video E4). These fluorescence-labeled NPs similarly appeared inside macrophages within 5 minutes of exposure *in vitro* (data not shown). Treatment of macrophages with RR NPs, which displayed 150 hours of sustained RR release in infinite sink conditions (Figure 3D) also inhibited the calcium response to 4- α -PDD (Figure 3E). We found it difficult to maintain robust calcium signaling in the macrophages beyond 48 hours in culture, and so turned to BCE cells to assess the ability of RR NPs to suppress TRPV4-mediated calcium signaling longer term. Indeed, RR NPs were also rapidly phagocytosed by BCE

cells (Figures E1A–E1D), which inhibited calcium signaling by 4- α -PDD (Figures 5A–5C) for up to 7 days.

To determine whether the observed inhibition of TRPV4 signaling in cells treated with RR NPs is mediated by RR released by the NPs into the cell cytoplasm or into the cell culture media, we cocultured BCE cells treated separately and before coculture with either Cy5-labeled blank (no RR) NPs or with unlabeled RR NPs, and measured the calcium signaling response to 4- α -PDD in both groups of cells while in the same dish at 24 hours of coculture. Cells containing blank NPs (identified by the presence of Cy5 signal) displayed typical TRPV4-mediated calcium transients, whereas cells containing RR NPs (identified by the absence of Cy5 signal) did not (Figure 5D). Taken together, our data suggest that phagocytosis of RR NPs results in sustained inhibition of TRPV4 in treated cells only, presumably due to sustained intracellular release of RR (*see* DISCUSSION).

Because RR is a general transient receptor potential (TRP) inhibitor, and also interacts with many non-TRP proteins, including Ca^{2+} -ATPase (35), mitochondrial Ca^{2+} uniporter (36), tubulin (37), myosin light-chain phosphatase (38), and Ca^{2+} -binding proteins, such as calmodulin (39), RR toxicity using RR NPs is expected (21). We therefore screened for RR toxicity from exposure to RR NPs in both alveolar macrophages and BCE cells. Soluble RR inhibited the calcium response in alveolar macrophages to both TRPV2 and TRPV4 agonists (lipopolysaccharide [LPS, (40)] and hydrogen peroxide [H_2O_2 , (41)], respectively) whereas treatment with RR NPs (4 h) did not (Figure 6). Interestingly, cocultured BCE cells (described in Figure 5D) containing RR NPs, and which had diminished responses to the TRPV4 agonist 4- α -PDD, displayed higher calcium transients in response to soluble ATP (delivered 15 min after the 4- α -PDD) than

Figure 5. RR NPs inhibit TRPV4-mediated calcium signaling in bovine capillary endothelial (BCE) cells *in vitro*. (A) Relative change in cytosolic calcium (F/F_0) in response to 4- α -PDD (arrow shows time of application) in Fluo-4-loaded BCE cells pretreated for 5 minutes with increasing concentrations of soluble RR. (B) Relative change in cytosolic calcium in response to 4- α -PDD (arrow) in Fluo-4-loaded BCE cells 24 hours after exposure to 100 $\mu\text{g/mL}$ blank NPs or increasing doses of RR NPs. (C) Averages of peak increases in cytosolic calcium in response to 4- α -PDD in Fluo-4-loaded BCE cells at 1, 3, or 7 days after exposure to 50 $\mu\text{g/mL}$ blank or RR NPs. Data are means $\pm$ SEM, and are normalized relative to averages in blank NP treated cells at each time point. * $P < 0.05$ versus RR NP at same time point using Student's *t* test; $n =$ at least 45, 70, or 62 cells in each group at 1, 3, or 7 days, respectively. (D) Relative change in cytosolic calcium in response to 4- α -PDD (arrow) in Fluo-4-loaded BCE cells treated 1 day earlier with either 50 $\mu\text{g/mL}$ blank or RR NPs and cocultured for 24 hours in the same dish. (E) Relative change in cytosolic calcium in cells described in (D) in response to soluble ATP (5 μM) delivered 15 minutes after the 4- α -PDD dose.

cells in the same dish containing blank NPs (Figure 5E).

RR NPs Block HPMV-Induced Lung Injury

To determine whether inhaled RR nanotherapy provides sustained inhibition of HPMV-induced edema beyond that which we documented from inhaled free RR, mice were pretreated with inhaled blank or RR NPs, and their lungs prepared for *ex vivo* ventilation studies at 4 hours or 1, 3, or 7 days after inhalation. Increases in K_f seen after HPMV were prevented if RR NPs were delivered via inhalation 4 hours or 1 or 3 days, but not 7 days before lung isolation (Figure 7A). In addition, increases in wet lung BW and wet:dry lung weight ratios induced by HPMV were similarly inhibited by inhaled RR NPs if delivered up to 3 days before lung isolation (Figures 7B and 7C). Quantitative analysis of histological sections from the above groups confirmed these findings. Perivascular fluid collections from HPMV were reduced in RR NP-treated groups for up to 3 days after inhaled nanotherapy compared with lungs from the HPMV groups treated

with blank NPs (Figures 7D–7H).

Importantly, the steady-state concentration of RR in the perfusate during perfusion of lungs harvested from mice 4 and 24 hours after inhaled nanotherapy was less than $1\ \mu\text{M}$ (Figure 2I). As with inhaled RR, this perfusate RR concentration was significantly lower than that which was required to block HPMV-induced lung edema using intravenous drug delivery. Together, these results indicate that inhalation pretreatment of live mice with RR NPs provides sustained inhibition of HPMV-induced lung edema, despite negligible drug levels in the *ex vivo* lung vasculature.

Discussion

To address the urgent need to provide therapies to prevent VILI and lower mortality from ARDS, we developed a nanoparticulate formulation to be delivered by inhalation before instituting mechanical ventilation. The NPs were designed to release RR, an inhibitor of the force-sensitive membrane ion channel TRPV4 to block lung cells involved in

early mechanical signaling leading to VILI (6, 7, 42). We used a whole-animal nebulization chamber, an *ex vivo* murine lung ventilation perfusion model, and quantitative analyses of lung permeability and lung edema development to demonstrate that inhaled free RR prevented HPMV-induced lung edema for 60 minutes, and that inhaled RR NPs deposited in the mouse alveoli were rapidly phagocytosed by alveolar macrophages, and prevented HPMV-induced lung edema for 3 days. This study therefore provides a proof of principle for the effectiveness of inhaled nanotherapy in preventing ventilator injury. Considering that there is no cure for ARDS, and that mortality remains high (43, 44), this novel *prophylactic* drug delivery strategy, if proven safe and effective in humans, would greatly impact the outcome of patients with impending respiratory failure, and would represent a paradigm shift in the management of patients with ARDS, moving treatment from after to before initiation of mechanical ventilation and diagnosis of VILI.

Despite a significant lowering of inhaled lung drug content by *ex vivo*

Figure 6. Effects of RR NPs on non-TRPV4-mediated calcium signaling in alveolar macrophages. (A and B) Relative change in cytosolic calcium (F/F_0) in response to LPS (400 $\mu\text{g/ml}$; arrow shows time of application) in Fluo-4-loaded alveolar macrophages pretreated for 5 minutes with 0 or 25 μM RR (A) or for 24 hours with 100 $\mu\text{g/ml}$ blank or RR NPs (B). (C and D) Relative change in cytosolic calcium (F/F_0) in response to H_2O_2 (600 μM ; arrow shows time of application) in Fluo-4-loaded alveolar macrophages pretreated for 5 minutes with 0 or 25 μM RR (C) or for 24 hours with 100 $\mu\text{g/ml}$ blank or RR NPs (D).

Figure 7. Inhaled RR NPs provide sustained inhibition of HPMV-induced increases in lung vascular permeability. (A) K_{f1} and K_{f2} in *ex vivo* lungs exposed to control ventilation or HPMV protocols 4, 24, or 72 hours, or 7 days after inhalation of nebulized (neb) blank (–) or RR NPs in a whole-animal nebulization chamber. Data are means $\pm$ SEM. * $P < 0.004$ versus K_{f1} in the same group using Student's *t* test, $n > 5$; $^{\#}P = 0.0027$ versus K_{f1} in the same group using Student's *t* test, $n = 5$; $^{\wedge}P = 0.007$ versus K_{f2} Blank NP + HPMV at 4 hours; $^{\#}P < 0.05$ versus K_{f2} Blank NP + HPMV at 1 day. (B and C) Wet lung weight:BW ratio (B), and wet:dry lung weight ratio (C) of *ex vivo* lungs from groups described in (A) Data are means $\pm$ SEM. $n > 5$; $F = 3.019$; * $P < 0.011$ in (B) using ANOVA and Dunnett's *post hoc* test. (D–G) Representative micrographs of H&E-stained lung sections from mice treated with inhaled RR (D, F, and G) or blank (E) NPs then exposed 24 hours (D and E), 72 hours (F), or 7 days (G) later to control ventilation (D) or HPMV (E–G) *ex vivo* protocols. Br, bronchus; PA, pulmonary artery. Arrows show large perivascular cuffs. Scale bar, 100 μ m. (H) Quantification of lung edema (V_c/N_w) from histological data (D–G) using a modification of a point-counting strategy (see MATERIALS AND METHODS). Data are means $\pm$ SEM; $F = 39.563$; * $P < 0.0001$, using ANOVA and Dunnett's *post hoc* test; $n > 3$, and at least 79 vessels examined in each group.

perfusion that nevertheless results in negligible inhaled drug levels in *ex vivo* perfusate, inhaled therapies with RR or RR NPs both blocked HPMV-induced lung edema. These low lung and perfusate drug levels confirm that inhalation therapies can generate high pulmonary drug levels relative to systemic therapy (19, 20), and our results with RR NPs reinforce the notion that sustained release

NP technology can enhance the duration of therapeutic effect. However, low lung drug and perfusate levels may not accurately reflect drug concentrations in the alveolar milieu or specific lung cell compartment where this therapy is effective. Although we show that inhaled RR NPs are rapidly phagocytosed by alveolar macrophages, the lung cells shown to be critical for the permeability response and the resulting lung

edema induced by HPMV (6), and that these NPs block TRPV4 signaling in these cells, the mechanism by which inhaled RR nanotherapy blocks TRPV4 and VILI signaling in whole lungs remains unclear.

It is important to note that lung vascular permeability and lung edema are related terms, but are not synonymous. Lung edema, defined as abnormal accumulation of fluid in the extravascular space of the lungs (45),

results from increased lung vascular permeability, but accumulates to variable degrees depending on pathophysiological conditions. It develops in patients due to increased pulmonary capillary permeability (such as in ARDS), increased pulmonary capillary hydrostatic pressure (hydrostatic or cardiogenic pulmonary edema), or both (2, 46). The gravimetric K_f that we used to estimate lung vascular permeability is defined as the product of the hydraulic conductivity and filtration surface area (and see MATERIALS AND METHODS), and measures the lung vascular endothelial barrier permeability to convective water transport in isolated ventilated perfused lungs (47). This measure is extremely consistent between species when normalized to lung weight or BW (48). The amount of lung edema that develops during *ex vivo* lung ventilation perfusion, however, is dependent on several experimental variables, including the timing of induction of lung injury, the timing, number, and length of permeability measurement periods, and the step increase in hydrostatic pressure used during these periods. Importantly, we only observed weight gain in our *ex vivo* lung preparations during the two brief 10-minute K_f measurement periods. This explains why the protective effects of inhaled RR and RR nanotherapy appear to be more pronounced on permeability (i.e., K_f) than on lung edema development (see Figures 2 and 7). Furthermore, perhaps the most important clinical implication of our findings is that the effect on lung vascular permeability from inhaled RR nanotherapy would likely be more sustained *in vivo* compared with *ex vivo*, as inhaled lung drug content would not be significantly lowered (as it is by *ex vivo* perfusion) before injurious mechanical ventilation (see Figure 1).

Our studies revealed that TRPV4-mediated calcium signaling remained intact in untreated cells cultured in the same dish as cells treated with RR NPs (Figure 4F). These results suggest that RR was released from phagocytosed NPs and blocked TRPV4 from the cytoplasmic side of the cell membrane. This is in contrast to a study using patch clamp that showed that RR inhibited TRPV4 from the extracellular side of patched plasma membrane by binding to a site in the channel pore, but had little to no effect on TRPV4 activity when applied to the intracellular face of the patched plasma membrane (18). In our studies, cells were washed and fresh medium was exchanged before calcium microfluorimetry: it is therefore unlikely that RR in the media was responsible for TRPV4 inhibition. Alternatively, it is possible that RR entered the channel pore from inside the cell after channel activation, or leaked out of treated cells and bound TRPV4 on these same cells from the extracellular side. However, because RR is bulky and charged, and considered to be membrane impermeant and known to impact calcium handling in cells via effects on other TRP channels (40, 41, 49), and non-TRP proteins (35, 37–39), including a mitochondrial calcium uniporter (36), it remains possible that the effect of the RR NPs on ventilator-induced edema in our studies was not directly via TRPV4 *per se*, but via modulation of downstream intracellular calcium handling. Although we found in RR toxicity screening studies using macrophages that RR NPs did not affect TRPV2- or TRPM-mediated calcium signaling (induced by LPS or H_2O_2 , respectively), *in vivo* studies are indicated to confirm these findings, to screen for local and pulmonary inflammation from the NPs (50, 51), as well as pulmonary and systemic toxicity from TRPV4 inhibition

(21), and to elucidate the mechanism of action of RR NPs in blocking ventilator-induced edema. It is important to note that we used RR, because its perfusate and lung concentrations could be easily monitored, which aided in the design and interpretation of our results. Because RR toxicity is likely if used in humans, the successful future development of this novel inhaled cell-targeted drug delivery strategy for VILI should focus on discovery and encapsulation of safe inhibitors specific to force induced TRPV4 activation so as to minimize local and systemic toxicity.

In summary, our unique inhaled nanotherapy and *ex vivo* mouse lung ventilation perfusion models revealed three major findings: (1) inhalation of soluble RR provided prophylaxis against HPMV lung edema for 60 minutes; (2) phagocytosed RR NPs blocked TRPV4-mediated calcium signaling *in vitro* in targeted cells for up to 1 week; and (3) inhalation of nebulized suspensions of these same NPs deposited in mouse alveoli were phagocytosed by alveolar macrophages, and blocked HPMV-induced lung edema for 3 days. This proof of principle study suggests that inhalation of NPs targeting TRPV4 can potentially be used in patients with impending respiratory failure to prevent VILI before instituting mechanical ventilation, and to alter the outcome of ARDS, a devastating disease that has no cure. Use of a similar inhaled prophylactic nanotherapy approach may also benefit patients with other pulmonary diseases such as asthma, chronic obstructive pulmonary disease, or pulmonary hypertension. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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