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EXPERIMENTAL

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# Inhaled milrinone attenuates experimental acute lung injury

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**Abstract** *Purpose:* To test whether inhalation of the phosphodiesterase 3 inhibitor milrinone may attenuate experimental acute lung injury (ALI). *Methods:* In rats, ALI was induced by infusion of oleic acid (OA). After 30 min, milrinone was inhaled either as single dose, or repeatedly in 30 min intervals. In mice, ALI was induced by intratracheal instillation of hydrochloric acid, followed by a single milrinone inhalation. *Results:* Four hours after OA infusion, ALI was evident as lung inflammation, protein-rich edema and hypoxemia. A single inhalation of milrinone attenuated the increase in lung wet-to-dry weight ratio and myeloperoxidase activity, and reduced protein concentration, neutrophil counts and TNF-$\alpha$ levels in bronchoalveolar lavage. This effect was further pronounced when milrinone was repeatedly inhaled. In mice with acid-induced ALI, milrinone attenuated hypoxemia and prevented the increase in lung myeloperoxidase activity. *Conclusions:* Inhalation of aerosolized milrinone may present a novel therapeutic strategy for the treatment of ALI.

**Keywords** Acute lung injury · Lung edema · Oleic acid · Inhalation · Phosphodiesterase 3 · Milrinone

## Introduction

With an incidence of 86.2 per 100,000 person-years and mortality rates of ~43%, acute lung injury (ALI) and its most severe form, the acute respiratory distress syndrome (ARDS), remain major challenges in intensive care [1, 2]. The pathological hallmarks of the disease comprise diffuse alveolo-capillary injury and increased lung permeability associated with a strong inflammatory response [3, 4]. These changes underlie the clinical presentation which is characterized by acute onset, severe hypoxemia and proteinaceous lung edema. Strikingly, many of the underlying signaling cascades are effectively counteracted by the second messenger cyclic adenosine 3′,5′-monophosphate (cAMP). Elevation of intracellular cAMP levels strengthens the microvascular barrier [5] and increases alveolar fluid clearance [6], attenuates neutrophil adhesion [7] and migration [8] and relaxes vascular smooth muscle cells [9]. Accordingly, pharmacological stimulation of cAMP synthesis, e.g. by $\beta_2$-adrenergic agonists has been proposed as treatment strategy in ALI [10]. Similarly, inhibition of cAMP

degradation by intracellular phosphodiesterase 3 (PDE3) may confer partial protection. Previous studies show that PDE3 inhibitors may attenuate inflammation [11, 12], reduce edema formation [13], improve endothelial function [14] and induce pulmonary vasodilation [15]. In a canine model of oleic acid-induced ALI, intravenous infusion of the PDE3 inhibitor milrinone reduced extravascular lung water and improved oxygenation [16]. Intravenous administration, however, lacks pulmonary specificity and thus causes undesirable side effects such as systemic hypotension [15]. Recently, we have demonstrated effective drug delivery and treatment of pulmonary hypertension by inhaled milrinone [15] which lacks systemic side effects and may be more effective in protecting lung endothelial function [14]. Based on these considerations, we hypothesized that inhalation of milrinone may present a promising strategy for the treatment of ALI and tested this concept in two experimental models.

## Materials and methods

### Animals

Experiments were performed in male Sprague–Dawley rats and Balb/c mice with body weights (bw) of 330–360 and 25–30 g, respectively. All animals were from Charles River Laboratories (Sulzfeld, Germany) and received care in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 7th edn, 1996). The study was approved by the local animal care and use committee (LAGeSo Berlin, Germany).

### Surgical preparation and hemodynamic monitoring

Animals were anesthetized by intraperitoneal injection of medetomidine (0.5 mg/kg bw, Domitor®, Dr. E. Graeub AG, Basel, Switzerland), fentanyl (0.05 mg/kg bw, JanssenCilag, Neuss, Germany) and midazolam (5 mg/kg bw, Dormicum®, Roche, Basel, Switzerland) as previously described [17]. Animals were tracheotomized and the trachea was cannulated. Rats were ventilated with a tidal volume of 6 ml/kg bw and a positive end-expiratory pressure of 3 mmHg at 80 breaths/min (Advanced Animal Respirator, TSE Systems GmbH, Bad Homburg, Germany), resulting in a peak airway pressure of 10.5 ± 1 mmHg, $P_aO_2$ and $P_aCO_2$ of 101.4 ± 2.3 and 35.5 ± 2.2 mmHg, respectively. Catheters (internal diameter 0.58 mm; Sims Portex Ltd, Hythe, UK) were introduced into the left carotid artery and the right internal jugular vein for monitoring of arterial blood pressure (AP), fluid replacement and drug delivery. After median thoracotomy, a catheter was introduced via the right ventricle into the pulmonary artery for measurement of pulmonary artery pressure (PAP). Mice were ventilated with end-inspiratory and end-expiratory pressures of 8 and 1 mmHg at 100 breaths/min, and a catheter was placed in the right carotid artery as recently described [17]. Hemodynamic pressures in rats and mice were continuously recorded by the software package DasyLab® 32 (DasyLab, Moenchengladbach, Germany).

### Experimental groups and protocol

Rats were randomly assigned to one of six experimental groups. Based on preceding biostatistic planning (SigmaStat; Jandel Scientific, San Rafael, CA) expecting a SD of residuals of 15% and a minimal detectable difference in means of 25%, a group size of ten animals was calculated. Animals in the control group received no pharmacological interventions. In the control/Mil group, aerosolized milrinone (Corotrop; Sanofi Winthrop, Paris, France) was administered in 30 min intervals over 4 h. In the oleic acid (OA) group, ALI was induced by intravenous infusion of 0.2 mg/kg OA (Sigma, Munich, Germany) in the absence of any treatment. In the OA/NaCl group, OA was infused and 0.9% NaCl was inhaled every 30 min, starting 30 min after OA administration until the end of the experiment. In the OA/singMil group, a single inhalation of milrinone was given 30 min after OA infusion, while milrinone inhalation was started 30 min after OA infusion and repeated every 30 min thereafter until the end of the experiment in the OA/repMil group. For each inhalation, a solution of 0.9% NaCl containing 1 mg/ml milrinone at a pH of 3.6 and an osmolality of 295 mosmol/kg or normal saline alone was aerosolized in room air by an ultrasonic nebulizer (Optineb®, Nebu-Tec, Elsenfeld, Germany) and inhaled for 3 min as previously described [15].

After surgical preparation and hemodynamic stabilization, baseline hemodynamics were recorded and arterial blood gases analyzed (RapidLab 348; Chiron Diagnostics GmbH, Fernwald, Germany). Arterial partial pressure of oxygen was expressed as ratio of $P_aO_2$ over the inspired oxygen fraction ($FiO_2$) which was kept at 0.21 throughout experiments. Removed blood volume was replaced by hydroxyethyl starch (6% hydroxyethyl starch 200/0,6; Fresenius, Bad Homburg, Germany). Immediately after baseline recordings, oleic acid was infused to all OA groups, while control groups received an equal volume of normal saline. In all groups, hemodynamic measurements and blood gas analyses were repeated in 60 min intervals for a total of 4 h at which time animals were euthanized by exsanguination. After in situ ligation of the right main bronchus, the right lung was excised and processed for determination of wet-to-dry

weight ratio and myeloperoxidase (MPO) activity as described below. For bronchoalveolar lavage (BAL), the left lung was washed four times with aliquots of 2.5 ml 0.9% NaCl and >90% of the applied volume was recovered.

Mice were divided into three groups of six animals each. In the ALI group and the control group, respectively, hydrochloric acid or sterile saline (2 µl/g bw each) were instilled into the trachea. In the treatment group, mice inhaled aerosolized milrinone once for 3 min immediately after acid instillation. After 2 h, arterial blood gases were analyzed, animals were euthanized, and lung wet-to-dry weight ratio and MPO activity were measured.

### Assessment of lung water and permeability

Lung water content was determined as wet-to-dry weight ratio. For assessment of lung permeability in rats, the protein concentration in the BAL fluid was measured. In brief, BAL samples from the left lung were centrifuged at 850*g* for 10 min, protein concentration in the supernatant was quantified by the Bradford assay (Bio-Rad, Richmond, VA) and compared to standard curves generated from albumin.

### Assessment of inflammatory response

For determination of inflammatory cell recruitment and cytokine production, we analyzed differential cell counts and tumor necrosis factor-α (TNF-α) levels in the BAL fluid and measured MPO activity in lung tissue. Total cell count in BAL samples from the left lung was determined by a cell counter (Coulter® Ac-T diff™; Beckman Coulter, Inc., Miami, FL). Differential cell counts were performed by light microscopic examination of 200 cells in H&E stained smears, and neutrophils were identified based on their morphology and staining characteristics. TNF-α concentration in BAL samples was determined by an enzyme-linked immunosorbent assay (Quantikine®; R&D Systems, Minneapolis, MN). MPO activity in lung homogenates was determined by a 3,3′-5,5′-tetramethylbenzidine (TMB)-based photometric assay, compared to appropriate standard curves, and expressed as units per gram lung tissue (U/g) as described [18].

### Statistical analysis

All data are presented as mean ± SEM. Data were tested for differences between groups by Kruskal–Wallis test and Student–Newman–Keuls post hoc analysis. Statistical significance was assumed at $P < 0.05$.

## Results

In rats, baseline hemodynamic measurements and blood gas analyses yielded a mean AP of 80.1 ± 1.4 mmHg, a PAP of 7.3 ± 0.2 mmHg and a $P_aO_2/FiO_2$ ratio of 476 ± 10 mmHg with no statistical differences between groups. Over the 4-h-measurement period, AP decreased in all groups to a final mean value of 59.1 ± 1.2 mmHg but did not show significant intergroup differences (data not shown). PAP increased in all groups over the 4 h protocol, but the increase was most pronounced in the groups receiving OA with or without repetitive inhalation of normal saline, resulting in higher PAP values after 4 h in these groups as compared to control. Both, single and repeated milrinone inhalation attenuated the increase in PAP, while milrinone inhalation had no effect in control rats. The $P_aO_2/FiO_2$ ratio decreased continuously in the OA and OA/NaCl groups while arterial oxygenation returned to control values when milrinone was repeatedly inhaled (Fig. 1b). When a single dose of milrinone was inhaled, the $P_aO_2/FiO_2$ ratio continued to decline in parallel with the OA group for 2 h, but then recovered almost to baseline levels after 4 h. $P_aCO_2$ remained constant between 35 and 45 mmHg in all groups over the experimental time course.

In lungs harvested 4 h after OA infusion, permeability-type lung edema was evident as increased lung wet-to-dry weight ratio (Fig. 2a) and protein-rich exudate (Fig. 2b). A single 3 min inhalation of milrinone given 30 min after induction of ALI, but not inhalation of normal saline attenuated both lung edema and barrier failure. The protective effect of milrinone was amplified when inhalations were repeated in 30 min intervals, while repetitive milrinone inhalations had no effect in controls.

Neutrophil counts in the BAL fluid were increased >8-fold in rats with OA-induced ALI as compared to controls. Single inhalation of milrinone reduced alveolar neutrophil influx by >50%, and this effect was further enhanced when milrinone was repeatedly inhaled (Fig. 3a). The anti-inflammatory properties of inhaled milrinone were further substantiated by measurements of lung MPO activity and BAL levels of the early response cytokine TNF-α. MPO activity was increased >5-fold in OA-induced ALI, and again attenuated by both single and repeated milrinone inhalation (Fig. 3b). Consistent with these data, the OA-induced increase in TNF-α levels in BAL fluid was reduced by a single inhalation of milrinone and this effect was again amplified by repeated inhalations (Fig. 3c). No attenuation of the lung inflammatory response to OA was detectable in animals inhaling normal saline instead of milrinone.

Two hours after acid instillation in mice, a marked decrease in the $P_aO_2/FiO_2$ ratio was detectable while lung water content was only slightly elevated (Fig. 4a, b). Influx of inflammatory cell was evident as a doubling of

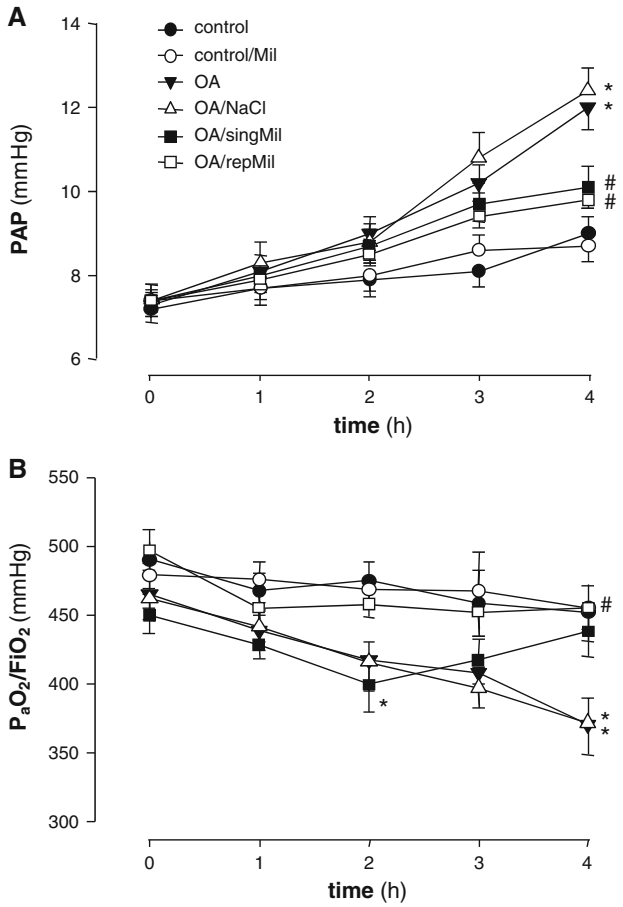


**Fig. 1** Pulmonary artery pressure and arterial oxygenation. Group data of PAP (**a**) and the $P_aO_2/FiO_2$ ratio (**b**) determined in 60 min intervals over 4 h. Following baseline measurements, OA (0.2 mg/kg) was infused intravenously in OA, OA/NaCl, OA/singMil and OA/repMil groups, while rats in control and control/Mil groups received an equivalent volume of normal saline. Milrinone (1 mg/ml) or normal saline were aerosolized and inhaled either as a single dose 30 min after OA infusion (OA/singMil) or repeatedly in 30 min intervals in OA-injured (OA/NaCl and OA/repMil) or control rats (control/Mil). All data are $n = 10$ each, $^*P < 0.05$ versus control, $\#P < 0.05$ OA/singMil or OA/repMil versus OA

lung MPO activity (Fig. 4c). A single inhalation of milrinone immediately after acid instillation attenuated hypoxemia and completely prevented the increase in lung MPO activity, thus substantiating the therapeutic potential of aerosolized milrinone in a second model of ALI.

## Discussion

In the present study, we tested the hypothesis that inhalation of the PDE3 inhibitor milrinone may be beneficial in experimental ALI. In OA-induced ALI in rats we demonstrate that a single inhalation of milrinone 30 min after ALI induction improves oxygenation and attenuates lung edema, barrier failure, and inflammation. The therapeutic benefit of PDE3 inhibition was further amplified when milrinone was repeatedly inhaled in 30 min intervals. Milrinone inhalation also attenuated lung injury in mice following acid instillation, thus validating this therapeutic concept in a second model of ALI. Under consideration of the existing clinical experiences with inhaled PDE3 inhibitors in heart failure patients or during cardiopulmonary bypass [19, 20], aerosolized milrinone may present a promising strategy in the treatment of ALI and ARDS.

### Methodological considerations

Intravenous infusion of OA causes hypoxemia, protein rich edema and infiltration of leukocytes, thus simulating the characteristic features of the acute, exudative stage of ARDS [21, 22]. OA also plays a critical role in clinical ARDS, since elevated serum concentrations and incorporation of OA into surfactant phospholipids are valid predictors for the development of the disease [23, 24]. In the present study, an experimental end-point of 4 h after OA was chosen based on previous studies reporting the peak inflammatory response to occur between 1½ and 4 h after OA infusion [25–27]. Yet, future studies are required to address the therapeutic potential of inhaled milrinone in the subsequent fibroproliferative phase of ALI. A characteristic feature of OA-induced ALI is the preservation of gas exchange by redistribution of blood flow from edematous to intact lung regions [28]. Accordingly, OA caused only moderate hypoxemia in the present model. Over the 4 h experimental period, a gradual decrease in AP and a concomitant increase in PAP were evident in all groups. These hemodynamic effects are likely attributable to the invasive surgical preparation required for cardiovascular monitoring in combination with mechanical ventilation with positive end-expiratory pressure. Statistical analyses were therefore confined to intergroup differences to eliminate systematic effects. Aerosolized milrinone or its solvent, normal saline were inhaled for 3 min either once or repeatedly every 30 min. Inhalation frequency was chosen based on previously reported half lives for the bioactive effects of inhaled milrinone which range between 20 and 60 min [15, 20, 29]. The applied milrinone concentration of 1 mg/ml was selected based on existing clinical experiences [29] as well as preceding experiments in rats in which this dose induced maximal pulmonary-selective vasodilation [15]. A 3 min nebulization of 1 mg/ml milrinone results in aerosolization of 14 µg of the phosphodiesterase 3 inhibitor. The resulting dose of $\sim 40\ \mu g\ kg^{-1}$ corresponds to reported values in human studies [29]. Effective alveolar delivery of inhaled drugs in the present model was previously demonstrated by alveolar imaging of aerosolized fluorescence tracers [15].

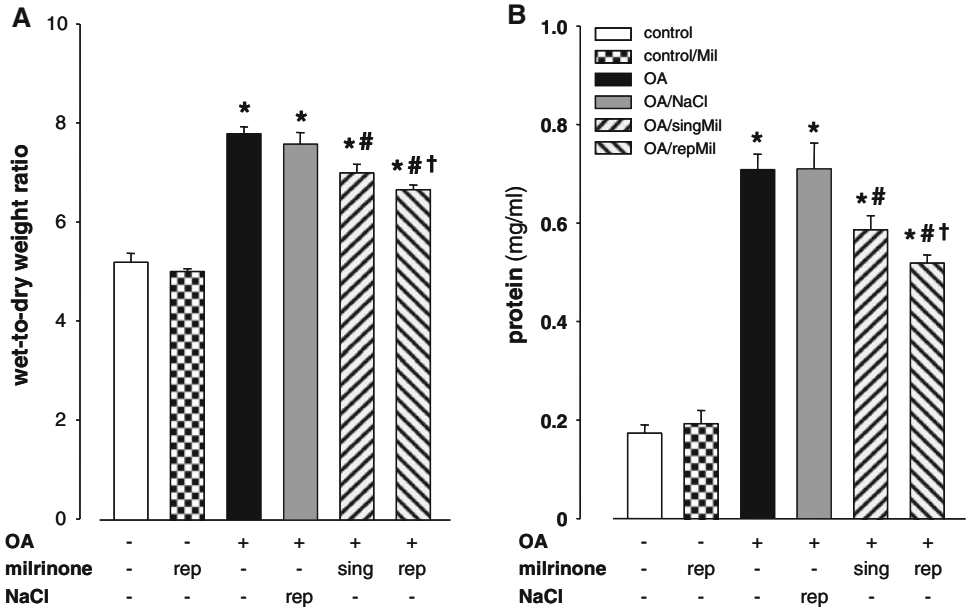


**Fig. 2** Lung water and permeability. Group data of lung wet-to-dry weight ratio (**a**) and protein concentration in the BAL fluid (**b**) determined 4 h after intravenous infusion of normal saline (control, control/Mil) or OA (OA, OA/NaCl, OA/singMil, OA/repMil) and subsequent single (OA/singMil) or repeated (control/Mil, OA/repMil) inhalation of milrinone or 0.9% NaCl (OA/NaCl), respectively. All data are $n = 10$ each, $^*P < 0.05$ versus control, $\#P < 0.05$ OA/singMil or OA/repMil versus OA, † $P < 0.05$ OA/repMil versus OA/singMil

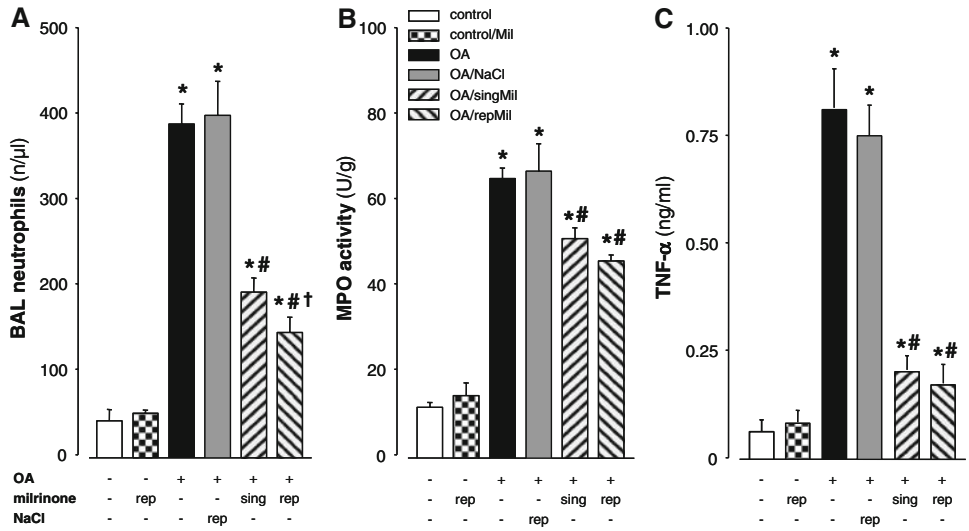


**Fig. 3** Inflammatory response. Group data of neutrophil counts in BAL fluid (**a**), lung MPO activity (**b**) and BAL concentrations of TNF-α (**c**) determined 4 h after intravenous infusion of normal saline (control, control/Mil) or OA (OA, OA/NaCl, OA/singMil, OA/repMil) and subsequent single (OA/singMil) or repeated (control/Mil, OA/repMil) inhalation of milrinone or 0.9% NaCl (OA/NaCl), respectively. All data are $n = 10$ each, $^*P < 0.05$ versus control, $\#P < 0.05$ OA/singMil or OA/repMil versus OA, † $P < 0.05$ OA/repMil versus OA/singMil

To substantiate the therapeutic concept, the effects of inhaled milrinone were further tested in a model of acid aspiration injury in mice. In contrast to oleic acid infusion which targets primarily the vascular endothelium, the acid aspiration model is predominantly characterized by injury of the airway and alveolar epithelium associated with neutrophilic infiltration, while endothelial damage and thus, edema formation may be less prominent [21]. Our finding that aerosolized milrinone also attenuated inflammation and hypoxemia in acid-injured mouse lungs provides proof-of-principle for the validity of this therapeutic strategy in different forms of ALI as well as different species.

## PDE3 inhibition as a therapeutic concept in ALI

PDE3 which catalyzes the degradation of cAMP to AMP is expressed in a variety of lung cells including alveolar epithelial [11], vascular endothelial [30] and vascular smooth muscle cells [31]. In cultured endothelial cells, PDE3 inhibition blocks $H_2O_2$-induced endothelial hyperpermeability [30], consistent with the notion that elevations in cAMP at the plasma membrane strengthen microvascular barrier function [32]. This protective mechanism likely underlies the attenuation of fluid and protein extravasation in the present study, a notion that is consistent with earlier work from Howell et al. [13]

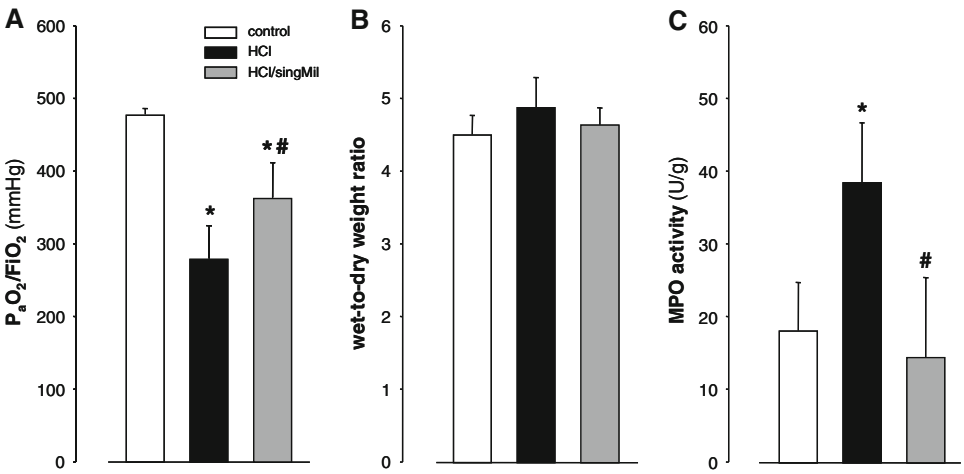


**Fig. 4** Inhaled milrinone in acid-induced lung injury in mice. Group data of the $P_aO_2/FiO_2$ ratio (**a**), lung wet-to-dry weight ratio (**b**) and MPO activity (**c**) determined 2 h after intratracheal instillation of either 0.9% NaCl (2 µl/g bw each; control) or 2 µl/g bw HCl (HCl, HCl/singMil) and subsequent single inhalation of milrinone (HCl/singMil). All data are $n = 6$ each, $*P < 0.05$ versus control, $\#P < 0.05$ versus HCl

demonstrating that pulmonary edema and protein leakage caused by LPS aerosols can be prevented by PDE3 inhibitors.

Yet in the study by Howell et al. [13] inhibition of PDE3 did not prevent the concomitant increase in lavage neutrophil counts. This lack of effect is in contrast to our present finding that inhaled milrinone attenuated the increase in BAL neutrophils and lung MPO activity following OA infusion or acid instillation, respectively. In both, the OA and the acid aspiration model, barrier failure is at least in part caused by infiltrating neutrophils [26, 33, 34], indicating that anti-inflammatory effects of PDE3 inhibition contribute critically to the protective effects of milrinone in ALI. The notion of an anti-inflammatory effect of milrinone is furthermore in agreement with recent studies demonstrating that PDE3 inhibition reduces neutrophil activation in septic rats [35] and attenuates the increase in leukocyte counts and cytokine levels in patients during cardiopulmonary bypass [36]. Since PDE3 has not been detected in significant amounts in neutrophils [37] and PDE3 inhibition does not directly attenuate the expression of adhesion molecules on human lung microvascular endothelial cells [38], the cellular mechanisms underlying the anti-inflammatory effect of inhaled milrinone remain to be elucidated.

PDE3 inhibitors cause vasodilation in resistance vessels due to the relaxation effect of cAMP in vascular smooth muscle cells. In the lung, this effect is only evident in preconstricted vessels because of the low basal tone of the pulmonary vasculature [15]. Accordingly, aerosolized milrinone had no effect on PAP in uninjured control lungs. In OA-induced ALI, the vasodilatory effect of PDE3 inhibitors may be considered as a potential explanation for the observed reduction in PAP and the concomitant improvement in gas exchange and lung edema in milrinone-treated groups. Importantly, a single milrinone inhalation 30 min after OA infusion had no effects on pulmonary hemodynamics or gas exchange for the first 90 min, but ultimately resulted in a lower PAP and a higher $P_aO_2/FiO_2$ ratio after 4 h. Given the absence of an early hemodynamic response and the biological half life of aerosolized milrinone in the range of 20–60 min [15, 20, 29], the protective effects evident after 4 h cannot be primarily attributed to a direct vasodilatory effect. The present findings rather indicate that a single inhalation of milrinone may exert long-term beneficial effects, presumably by early attenuation of signaling cascades which promote ALI in a self-propelled sequence of events [39].

## Inhalation of milrinone

While intravenous infusion of milrinone is frequently applied in cardiology and cardiac surgery, milrinone inhalation has only been studied in a few experimental and clinical trials in settings of pulmonary hypertension [15, 20, 29] and cardiopulmonary bypass [14, 19]. Inhaled milrinone may potentially provide advantages when PDE3 inhibition is primarily targeted at the lung in that the effects on lung endothelial and epithelial cells are potentiated [14] and pulmonary shunt fraction is reduced while systemic side effects such as hypotension are reduced [40].

In the present study, a single 3 min inhalation sufficed to reduce lung inflammation and improve gas exchange in two models of ALI. This finding identifies transient inhalation of milrinone as a potential therapeutic option early in the time course of the disease. Repeated delivery of milrinone provided additional benefit, as demonstrated by a further attenuation of edema and neutrophil counts in OA-injured lungs. Inhalation of milrinone has already been tested successfully in humans in a few small clinical trials without apparent negative side effects. Based on the existing experience, aerosolized milrinone may present a promising therapeutic strategy for the treatment of ALI and ARDS.

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