The Effect of Matrix Metalloproteinase 3 Deficiency on Pulmonary Surfactant in a Mouse Model of Acute Lung Injury

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The Effect of Matrix Metalloproteinase 3 Deficiency on Pulmonary Surfactant in a Mouse Model of Acute Lung Injury.


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Abstract

The acute respiratory distress syndrome (ARDS) is characterized by arterial hypoxemia accompanied by severe inflammation and alterations to the pulmonary surfactant system. Published data has demonstrated a protective effect of matrix metalloproteinase-3 (Mmp3) deficiency against the inflammatory response associated with ARDS, however, the effect of Mmp3 on physiologic parameters and alterations to surfactant have not been previously studied. It was hypothesized that Mmp3 deficient mice (Mmp3−/−) would be protected against lung dysfunction associated with ARDS and maintain a functional pulmonary surfactant system. Wild type (WT) and Mmp3−/− were subjected to acid-aspiration followed by mechanical ventilation. Mmp3−/− maintained higher arterial oxygenation compared to WT at the completion of ventilation. Significant increase in functional large aggregate surfactant forms were observed in Mmp3−/− compared to WT. These findings further support a role of Mmp3 as an attractive therapeutic target for drug development in the setting of ARDS.

Word count = 144

Key words
matrix metalloproteinase-3; acute respiratory distress syndrome; acute lung injury; pulmonary surfactant; mechanical ventilation; acid aspiration; hypoxemia
Introduction

The acute respiratory distress syndrome (ARDS) is characterized by severe lung dysfunction defined by a decrease in the partial pressure of arterial of O\textsubscript{2} (PO\textsubscript{2}) to the fractional inspired O\textsubscript{2} (FiO\textsubscript{2}) ratio after an acute pulmonary insult (Ranieri et al. 2012). ARDS can occur as a result of a direct insult such as gastric aspiration, while the effects of mechanical ventilation that are necessary to support the subsequent lung dysfunction, have been shown to secondarily exacerbate and accelerate the disease process (Livingston et al. 1995; Slutsky et al. 2000). Importantly, there are no proven pharmacological therapies while the mortality associated with ARDS continues to exceed 30% (Bosma et al. 2010). Further studies on molecular targets that influence the progression of ARDS are required in order to develop potential new pharmacological therapies.

Despite a physiologic clinical definition, the underlying pathophysiologic processes involved in ARDS are complex and include the development of overwhelming pulmonary inflammation, leakage of protein-rich edema into the distal airspaces and alterations to the pulmonary surfactant system (Ware et al. 2000). The changes to the pulmonary surfactant system have been of therapeutic interest as the known biophysical properties of surfactant enhance lung compliance and promote arterial oxygenation (Goerke 1998). Impairment of the surfactant system has been well documented in the setting of ARDS and includes a reduction in the relative amount of functional large aggregate (LA), compared to the inactive small aggregates (SA) forms, as well as an impairment in the ability of LAs to reduce or lower surface tension (Frerking et al. 2001).

One of the recent molecular targets identified in the pathogenesis of ARDS in animal models is matrix metalloproteinase 3 (Mmp3). This matrix metalloproteinase belongs to a family of zinc-dependent proteases that has important roles in innate immunity and inflammation (Nissinen et al. 2014; Senior et al. 1992). For example, it has been observed that mice lacking Mmp3 were protected against the development the pulmonary inflammation in two distinct models of ARDS. (Nerusu et al.
From a clinical perspective, elevated levels of Mmp3 in lavage fluid of patients with ARDS have been shown to correlate directly with mortality and the development of multi-organ failure (Fligiel et al. 2006). To our knowledge, the effects of Mmp3 on physiologic changes observed in ARDS and its effects on the host pulmonary surfactant system have not been reported. We hypothesized that mice deficient in Mmp3 would develop a less severe lung injury which would be associated with a preservation of a more functional surfactant system.

Methods

**Animals:** All experiments were performed in concordance with Canadian Council of Animal Care and approved by the Animal Use Sub-Committee of Western University. Ten to sixteen week old C57BL/6 wild type (WT) and Mmp3 deficient (Mmp3<sup>-/-</sup>) (Taconic, Hudson, NY, USA) female mice weighing between 20-25g were used for all experiments. Mice were bred and housed in our laboratory and had unlimited access to food and water.

**Injury Model:** Details of this model have been previously reported (Yamashita et al. 2014). Briefly, WT and Mmp3<sup>-/-</sup> mice were anesthetized and instrumented with vascular lines and connected to a ventilator (Harvard Apparatus, MA, USA,) via an endotracheal tube and ventilated with the following parameters: respiratory rate = 150 breaths/ minute; tidal volume = 10mL/kg; positive end expiratory pressure (PEEP) = 3cm H₂O; and FiO₂ = 1.0 and baseline physiological values were recorded. Subsequently, animals were randomized to receive an intra-tracheal bolus of air or 50µL of 0.05M hydrochloric acid (HCl, pH 1.10), resulting in 4 experimental groups: 1) WT Air, 2) Mmp3<sup>-/-</sup> Air, 3) WT Acid, and 4) Mmp3<sup>-/-</sup> Acid. Animals were ventilated for 240 minutes. Physiological measurements were taken at 120 and 240 minutes. Following mechanical ventilation, mice were sacrificed (sodium pentobarbital, 110mg/kg) and whole lung lavage was performed.

**Lung lavage and surfactant analysis:** Whole lung lavage and surfactant isolation was performed as previously described (Yamashita et al. 2014). To determine surfactant pool sizes, samples were
extracted using the Bligh and Dyer method and quantified using the Duck-Chong phosphorous assay (Bligh et al. 1959; Duck-Chong 1979; Yamashita et al. 2014). Surfactant activity was measured using a constrained sessile drop surfactometer (CDS; BioSurface Instruments, HI, USA) (Valle, Wu, and Zuo 2015). Briefly, a nine microliter drop of LA sample at 1mg phospholipid/ml was placed on the instrument pedestal. The drop was cyclically compressed-expanded for 25 cycles at a rate of 1 cycle/second, and a compression of 22+/-2% using a computer controlled external stepper motor. Images were recorded at a rate of ten images per second, and were used to determine the surface tension in conjunction with axisymmetric drop shape analysis. Minimum surface tension at cycles 1, 5 and 25 was used as an indication of biophysical function.

Statistical Analysis: All data are expressed as mean ± SEM. Statistical analysis was performed using statistical software package GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A two-way ANOVA to explore any interactive effects followed by a one-way ANOVA with Tukey’s post hoc test for pairwise comparison of groups. P-values less than 0.05 were considered statistically significant.

Results

The arterial oxygenation over 240 minutes of mechanical ventilation of WT and Mmp3^-/- mice exposed to acid aspiration and baseline controls is shown in Figure 1. At baseline, no significant differences were observed in arterial oxygenation among the 4 experimental groups. Statistical comparison shows that after 120 and 240 minutes of mechanical ventilation, PaO2/FiO2 ratios were significantly lower than those observed at baseline for all experimental groups. At 120 minutes, the oxygenation values were not significantly different among the 4 groups. However, at the completion of mechanical ventilation, WT mice randomized to acid aspiration had a significantly lower PaO2/FiO2 ratio compared to WT mice receiving a control air bolus at the same time point. In contrast, Mmp3^-/- mice randomized to acid aspiration did not show a significant difference in the PaO2/FiO2 ratio.
compared to air-instilled animals. Furthermore, comparison of WT and Mmp3\(^{-/-}\) revealed a significant difference in PaO\(_2\)/FiO\(_2\) between acid-instilled WT and Mmp3\(^{-/-}\) at 240 minutes.

Values of other physiological parameters measured during mechanical ventilation are shown in Table 1. Overall, values of PCO\(_2\), PIP and heart rate were not significantly different among the four groups. The PCO\(_2\) values increased during ventilation in all groups and similarly, PIP increased during ventilation in all experimental groups. A trend toward higher PIP values was observed in animals receiving acid-instillation, however these differences did not reach statistical significance. Significant difference were observed in PIP values measured at earlier time points (i.e. 90 minutes) in animals receiving acid aspiration compared to air-controls, however, these differences did not persist for the remainder of the mechanical ventilation protocol (data not shown).

Results from the analysis of surfactant pools and biophysical activity are depicted in Figure 2 and Table 2 respectively. The total amount of surfactant measured in the lavage fluid was not significantly different among the four groups (Figure 2A). Significant differences in the amount of LA were observed between groups (Figure 2B). Specifically, WT mice randomized to acid aspiration had lower amounts of LA compared to WT air-instilled mice at the completion of the mechanical ventilation while no differences in LA pools were observed between acid-instilled and air-instilled Mmp3\(^{-/-}\) animals. The amount of small aggregate was elevated in the WT acid group, did not reach statistical significance (Figure 2C). Expression of the aggregate pool sizes as a percentage of LA (Figure 2D) also revealed a significant decrease in WT acid versus WT air-instilled mice, with no significant differences observed between the two Mmp3\(^{-/-}\) groups. Analysis of the surface activity, expressed as minimum surface tension of the LA fractions during dynamic compression cycles showed low surface tension values for all samples with no significant differences among groups (Table 2).

Discussion
In the current study, it was hypothesized that mice deficient in Mmp3 would be protected against the development of the physiologic dysfunction associated severe lung injury and this would be associated with a more functional surfactant system. In general, our data supported this hypothesis as Mmp3^-/- mice exposed to acid injury followed by ventilation maintained higher PO_2/FiO_2 values compared to Mmp3^+/+. In addition, WT mice demonstrated a decrease in the relative percentage of LA in the lavage typically seen in the setting of ARDS, whereas this change was not observed in the Mmp3 deficient mice. We conclude that the deficiency in Mmp3 is protective against the physiologic changes observed in lung injury, and this is, in part, due to maintenance of functional surfactant system.

Whereas previous studies have focused on the inflammatory effects of Mmp3 deficiency in the context of ARDS (Nerusu et al. 2007; Warner et al. 2001), the current study addressed the potential effects of Mmp3 deficiency on the pulmonary surfactant system in a relevant animal model of lung injury. The focus of the current study was based on the two relevant observations. Firstly, in the context of ARDS, alteration to surfactant represents one of the fundamental deleterious processes contributing to decreased oxygenation (Frerking et al. 2001), and secondly, protease activity has been shown to alter surfactant metabolism and function (Gross 1995; Malloy et al. 2005). For example, Gross and colleagues have shown that that a specific protease, convertase, could enhance the conversion of LA into SA surfactant sub-fractions in vitro (Gross 1995). Furthermore, our lab has shown that other proteases could also propagate this LA to SA conversion and that this process was mediated by the degradation of either surfactant-associated protein A (SP-A), or SP-B (Veldhuizen et al. 1994). Preliminary experiments in our lab indicated that purified Mmp3 could also degrade purified SP-A, however this effect was mitigated when the SP-A was incubated together with surfactant lipids (data not shown). It is therefore notable in that Mmp3 deficient mice exhibited maintenance of LA pool sizes compared to wild type mice when exposed to lung injury resulting from acid aspiration with mechanical ventilation. This maintenance of LA pool sizes was consistent with preservation of arterial
oxygenation at the completion of the study. It is tempting to speculate that the maintenance of LA pool sizes can be attributable to the lack of Mmp3, however due to the complex biological pathways of surfactant metabolism and synthesis, other mechanisms which could contribute to this observation would require further studies.

In addition to pool sizes, we also analyzed surfactant function using a constrained sessile drop surfactometer (Zuo et al. 2008). This technique represents an ideal method for analysis of mouse samples as it allows for rapid determination of surfactant function with small sample volumes. The low surface tension achievable in each of the experimental groups, indicative of preserved surfactant function, would appear contrary to the assessment of surfactant function described previously in patients with ARDS or experimental animal models of ARDS (Frerking et al. 2001). The model used in the current study, although representative of ARDS via acid aspiration followed by mechanical ventilation, represented a relatively minor and early stage of lung injury based on measurements of several physiological parameters. Since surfactant function is dependent on both its inherent surface activity as well as its pool size, the data suggest that a decrease in pool sizes may be the main mechanism by which alteration of surfactant could influence the lung.

Overall, several lines of published evidence support a role of Mmp3 in contributing toward the pathogenesis of ARDS (Fligiel et al. 2006; Nerusu et al. 2007; Warner et al. 2001). For example, previous studies have demonstrated a protective effect of Mmp3 deficiency against inflammatory responses in several experimental models of acute lung injury (Nerusu et al. 2007; Warner et al. 2001). In addition, increased levels of Mmp3 levels in lung lavage material from patient with ARDS correlated with disease severity, incidence of multi-organ failure, and mortality (Fligiel et al. 2006). The current study adds to the current state of the literature by providing evidence for the protective effect of Mmp3 deficiency in a clinically relevant model of lung injury through maintenance of functional surfactant pool sizes. Despite some limitations of our study, such as 1) potential indirect
effects of Mmp3 deficiency on alterations in other MMPs or TIMP activity 2) the clinical relevance of short-term experimental models of ARDS or 3) uniform, weight-independent volumes of acid delivered, the combination of our results with current literature provides a strong rationale for further studies examining the potential role of Mmp3 as a therapeutic target in ARDS.

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References


Figure legends:

Figure 1: Oxygenation values over time. Arterial blood gas samples were analyzed to determine the PO$_2$/FiO$_2$ before randomization (Baseline) and after 120 and 240 min of mechanical ventilation. $n=6/7$ animals/group. * = $p<0.05$ versus WT air, # = $p<0.05$ versus WT acid.

Figure 2: Values of total surfactant (A), Large Aggregates (B), Small Aggregates (C) and Percent Large aggregates (D) as recovered from lung lavage in the 4 experimental groups. $n=5/6$ group, * = $p<0.05$ versus WT air.
Figure 1

The figure shows a bar chart comparing 

- WT Air
- WT Acid
- Mmp3\(^{-/-}\) Air
- Mmp3\(^{-/-}\) Acid

The chart measures PaO\(_2\)/FiO\(_2\) (mmHg) against time (min) with the following time points:

- Baseline
- 120 min
- 240 min

Statistical significance is indicated by:
- * for a significant difference
- # for a significant difference

The chart illustrates changes in PaO\(_2\)/FiO\(_2\) over time for each condition.
Figure 2

A. Total surfactant

B. LA

C. SA

D. Percent LA

Phospholipid (µg/g of Body Weight)

Phospholipid (µg/g of Body Weight)

Phospholipid (µg/g of Body Weight)

Percentage LA (%)

WT  MMP3 -/-  WT  MMP3 -/-  WT  MMP3 -/-  WT  MMP3 -/-
Table 1 - Arterial partial pressure of carbon dioxide (PCO₂), peak inspired pressure (PIP) and heart rate (HR) values at different time points during mechanical ventilation for WT and Mmp3⁻/⁻ mice randomized to an intra-tracheal bolus of air or acid followed by 240 minutes of mechanical ventilation. Values are expressed as means +/- SEM, n=4-6.

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
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<th>Acid</th>
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<tr>
<td></td>
<td>WT</td>
<td>Mmp3⁻/⁻</td>
<td>WT</td>
<td>Mmp3⁻/⁻</td>
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<tr>
<td><strong>PCO₂ (mmHg)</strong></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>39.4 ± 2.7</td>
<td>45.2 ± 3.1</td>
<td>41.2 ± 1.4</td>
<td>46.3 ± 4.8</td>
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<tr>
<td>120 min</td>
<td>51.0 ± 2.8</td>
<td>54.8 ± 3.2</td>
<td>59.5 ± 2.8</td>
<td>64.9 ± 6.1</td>
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<tr>
<td>240 min</td>
<td>53.9 ± 3.6</td>
<td>48.3 ± 3.2</td>
<td>60.4 ± 4.9</td>
<td>62.4 ± 6.7</td>
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<tr>
<td><strong>PIP (cmH₂O)</strong></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>8.4 ± 0.2</td>
<td>9.6 ± 0.5</td>
<td>8.0 ± 0.3</td>
<td>8.3 ± 0.2</td>
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<tr>
<td>120 min</td>
<td>11.7 ± 0.2</td>
<td>12.6 ± 0.6</td>
<td>13.5 ± 0.2</td>
<td>13.1 ± 0.5</td>
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<td>240 min</td>
<td>12.7 ± 0.4</td>
<td>12.9 ± 0.8</td>
<td>14.2 ± 0.2</td>
<td>13.6 ± 0.5</td>
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<td><strong>HR (beats/min)</strong></td>
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<tr>
<td>Baseline</td>
<td>285 ± 29</td>
<td>298 ± 26</td>
<td>263 ± 30</td>
<td>288 ± 31</td>
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<tr>
<td>120 min</td>
<td>239 ± 35</td>
<td>262 ± 26</td>
<td>244 ± 18</td>
<td>235 ± 24</td>
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<tr>
<td>240 min</td>
<td>263 ± 30</td>
<td>263 ± 33</td>
<td>242 ± 25</td>
<td>274 ± 30</td>
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Table 2 - Minimum surface tension of isolated surfactant large aggregates over various dynamic compression-expansion cycles. Values are expressed as means +/- SEM, n=4-5. There were no significant differences among groups.

<table>
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<th>Minimum surface Tension (mN/m)</th>
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<tr>
<td>WT</td>
<td>Mmp3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>WT</td>
</tr>
<tr>
<td>Cycle 1</td>
<td>3.9 ± 0.6</td>
<td>6.1 ± 0.9</td>
</tr>
<tr>
<td>Cycle 5</td>
<td>4.0 ± 0.3</td>
<td>4.5 ± 0.4</td>
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<tr>
<td>Cycle 25</td>
<td>3.8 ± 0.5</td>
<td>3.2 ± 0.2</td>
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