Exogenous surfactant attenuation of ischemia–reperfusion injury in the lung through alteration of inflammatory and apoptotic factors

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Objective: Lung ischemia–reperfusion injury is associated with impaired gas exchange from increased edema formation and surfactant inactivation. Surfactant replacement therapy is believed to improve gas exchange and lung function, but its effect on inflammation is less well understood. We therefore examined the effects of exogenous surfactant on inflammatory and apoptotic factors in the lung in a rat model of lung ischemia–reperfusion injury.

Methods: The left lung in rats was subjected to ischemia for 120 minutes and reperfusion for as long as 240 minutes. Sham-treated animals underwent sham surgery and mechanical ventilation for equivalent times. Rats received porcine surfactant or saline solution intratracheally either before or just after ischemia. Lungs were analyzed histopathologically and for expressions of inducible nitric oxide, cytokines, and caspase-3.

Results: Lung ischemia–reperfusion injury resulted in worse lung histopathologic characteristics than in sham-operation animals. At 2 hours of reperfusion, lung ischemia–reperfusion injury animals showed increased pulmonary caspase-3 expression. Moreover, lung ischemia–reperfusion injury resulted in inducible nitric oxide expression at all time points. Exogenous surfactant resulted in less inflammatory cell infiltration and edema in the lungs relative to saline-treated animals. Surfactant decreased activated caspase-3 expression and increased inducible nitric oxide expression relative to saline-treated animals. At 4 hours of reperfusion, surfactant increased interleukin 6 and 10 expressions in the lung.

Conclusion: This study showed a significant improvement in lung histologic characteristics after surfactant therapy, accompanied by reduced apoptosis and increased anti-inflammatory cytokine levels. Interestingly, surfactant therapy also increased pulmonary inducible nitric oxide expression.

Lung ischemia–reperfusion injury (LIRI) is a significant cause of early morbidity and mortality after lung transplantation. LIRI is characterized by acute hypoxic respiratory failure resulting from noncardiogenic pulmonary edema caused by an increased permeability of the alveolar capillary barrier. Several data from experimental studies for acute lung injury suggest that exogenous surfactant can improve lung function and outcome by decreasing edema formation, reducing protein leakage, and improving histopathologic state.^{1–6} Interestingly, surfactant also has shown strong immunomodulatory properties in vitro. For instance, surfactant proteins A and D are able to regulate toll-like receptors, resulting in altered production of inflammatory mediators.⁷ Furthermore, surfactant preparations containing surfactants

B and C reduce proinflammatory cytokine production by stimulated human alveolar macrophages and peripheral blood monocytes in vitro.^{8–10} Despite the clear effects of surfactant in vitro, data on the immunomodulatory effects of surfactant in vivo are scarce and contradictory. For instance, Vreugdenhil and colleagues¹¹ showed exogenous surfactant to restore lung function, but with no effect on proinflammatory cytokine expression in the lung. In line with these data, several groups showed in different models that exogenous surfactant had no effect on proinflammatory mediators.^{12–14} On the other hand, Stamme and coworkers¹⁵ reported increased tumor necrosis factor α and interleukin (IL) 6 production in ventilation-induced lung injury, whereas Rasaiah and colleagues¹⁶ reported decreased tumor necrosis factor α and IL-6 production in sepsis-induced lung injury. Our aim in this study was to investigate the immunomodulatory effects of surfactant on inflammatory mediators and apoptosis in a rat model of LIRI.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats, obtained from Harlan (Harlan Netherlands BV, Horst, The Netherlands), were used. Animals were treated in accordance with the Animal Welfare Act and in compliance with the Guide for the Care and Use of Laboratory Animals (www.nap.edu/catalog/5140.html). All experiments were approved by the ethical committee of the Erasmus Medical Center (Rotterdam, The Netherlands).

Abbreviations and Acronyms

IL	= interleukin
iNOS	= inducible nitric oxide
ISR	= ischemia, then surfactant, then reperfusion
LIRI	= lung ischemia–reperfusion injury
SIR	= surfactant, then ischemia, then reperfusion

Study Design

Eighty-four rats were anesthetized with a gas mixture (65% nitrous oxide, 33% oxygen, and 2% isoflurane) and subsequently intubated and mechanically ventilated (12 cm H₂O peak inspiratory pressure, 4 cm H₂O positive end-expiratory pressure, 50% inspired oxygen fraction, a frequency of 40 breaths/minute, and a ratio of inspiration to expiration time of 1:2). Anesthesia was continued with pentobarbital (60 mg/mL), and muscle relaxation was achieved with pancuronium bromide (2 mg/mL), both repeated every hour.

Except for the sham-operated group, which underwent all operative interventions but without induction of ischemia, all rats underwent 2 hours of warm ischemia of the left lung caused by clamping of the pulmonary hilum, followed by 30, 120, or 240 minutes of reperfusion (all groups $n = 7$). Rats were treated by intratracheal instillation of surfactant (natural porcine surfactant, HL-10, 200 mg/kg; LEO Pharma A/S, Ballerup, Denmark) either before (surfactant, then ischemia, then reperfusion [SIR]) or just after (ischemia, then surfactant, then reperfusion [ISR]) ischemia. Control rats received saline solution intratracheally before start of ischemia. At the end of reperfusion, the rats were killed with an overdose of pentobarbital.

Surfactant and Surfactant Administration

Surfactant was dissolved in saline solution (45 mg/mL) and administered intratracheally at a dose of 200 mg/kg, resulting in surfactant exposure of both lungs.

All SIR rats received surfactant intratracheally after they were briefly anesthetized and intubated. Three dosages (each instillation included a third of the total volume per rat) were administered to each rat during 1 hour before the start of ischemia. After each surfactant instillation, the animals recovered from anesthesia while breathing spontaneously, resulting in homogeneous distribution in both lungs. The control rats received saline solution in the same volume and according to the same protocol as the SIR rats. The ISR animals had surfactant instillation after 2 hours of ischemia. Immediately after release of the clamp at the start of reperfusion, the whole dose of 200 mg/kg was administered intratracheally in a single instillation. After recruitment, pressure-controlled ventilation was temporarily augmented to achieve homogeneous distribution of surfactant.

Western Blotting

Total lung homogenates were prepared with tissue lysis buffer and a protease inhibitor cocktail (Sigma-Aldrich Co, St Louis, Mo). From 10 to 30 μ g protein was separated by either 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed for inducible nitric oxide (iNOS) expression (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) and cleaved caspase-3 expression (Santa Cruz), respectively, by immunoblot analysis. β -Actin (Santa Cruz) was used as loading control. Immunoreactivity was detected by enhanced chemiluminescence (ECL; Amersham plc, Little Chalfont, UK).

Cytokines

IL-6 (BD Biosciences, San Jose, Calif) and IL-10 (BD Biosciences) cytokine levels were measured in total lung homogenates by enzyme-linked immunosorbent assay according to manufacturer protocol.

Lung Histologic Study

Lung tissue specimens were fixed in formalin, dehydrated, cleared, and embedded in paraffin. Specimens were cut into 8- μ m serial sections and stained with hematoxylin and eosin.

Statistical Analysis

All data are presented as mean $\pm$ SE. All parameters were analyzed by 1-way analysis of variance, followed by Bonferroni post hoc test.

RESULTS

Histologic Study

Lung specimens of the control animals were characterized by severe pathologic changes at all reperfusion time points. At 30 minutes of reperfusion, atelectasis and thickened alveolar septa that had been infiltrated by inflammatory cells were observed (Figure 1). Alveolar edema and bleeding appeared to be more prominent at 240 minutes of reperfusion.

Interestingly, both surfactant groups (SIR and ISR) only showed slightly thickened alveolar septa, slight alveolar edema, and minimal infiltration of inflammatory cells at all reperfusion time points relative to saline-treated animals (Figure 1).

Inflammatory Mediators

Western blot analysis revealed iNOS expression in the control group at all reperfusion time points, whereas iNOS was undetectable in the sham-operated group ($P < .001$; Figure 2). Surfactant treatment decreased iNOS expression at 30 minutes of reperfusion in both the SIR and ISR groups ($P < .001$). Interestingly, iNOS expression was significantly increased at 120 minutes of reperfusion in the SIR group relative to the control group ($P < .001$), and a delayed response was observed in the ISR group, resulting in higher levels of iNOS at 240 minutes of reperfusion ($P < .01$; Figure 2).

IL-6 expression in the control group remained stable at all reperfusion time points, being significantly higher at 240 minutes of reperfusion relative to the sham-operated group ($P < .05$; Figure 3). Surfactant treatment increased IL-6 expression relative to control at all time points only in the control group ($P < .05$). In the ISR group, IL-6 was only elevated relative to control at 240 minutes of reperfusion ($P < .01$; Figure 3).

IL-10 expression in the control group dropped significantly as a function of time, being significantly lower at 240 minutes of reperfusion relative to the sham-operated group ($P < .05$; Figure 4). After surfactant treatment, significantly higher IL-10 expressions were observed at 240 minutes of reperfusion in both the SIR and ISR groups ($P < .01$ SIR, $P < .001$ ISR; Figure 4).

Apoptosis

Control animals showed increased levels of the proapoptotic marker cleaved caspase-3 relative to the sham-operated group only at 120 minutes of reperfusion ($P < .001$). At 30 and 240 minutes of reperfusion, caspase-3 levels were similar

FIGURE 1. Representative paraffin sections of rat lung tissue stained with hematoxylin and eosin as function of time for sham-operated group (*sham*), control group (*saline+I+R*), and groups with surfactant administered before (*surfactant+I+R*) and after (*I+surfactant+R*) ischemia. Original magnification $\times 200$.

to those in the sham-operated group (Figure 5). Surfactant reduced cleaved caspase-3 expression at 120 minutes of reperfusion relative to control ($P < .001$ SIR and ISR). Also at 30 and 240 minutes of reperfusion, caspase-3 levels were diminished in both surfactant groups (SIR and ISR) relative to both the control and sham-operated groups ($P < .05$; Figure 5).

DISCUSSION

In this study, we determined the effects of exogenous surfactant on inflammation and apoptosis in an animal model of LRI. Although many studies have described the protective effect of exogenous surfactant on lung function and outcome in experimental models of acute lung injury,^{2,3,6} little is known about the immunomodulatory effects of surfactant in vivo. The effects of surfactant seem to depend on the experimental model, dosage, and time of administration.^{1,4,5} To address the time of administration, we treated rats either before ischemia or just after ischemia. We show here that surfactant increases IL-6 and IL-10 in the lung at 240 minutes after ischemia. IL-6 is a pleiotropic cytokine that can either induce proinflammatory mediator expression or exert cytoprotective effects, depending on the in vivo environmental circumstances.¹⁷ IL-6 is transcriptionally regulated by neutral factor κ B, but at high levels IL-6 protein in turn may limit neutral factor κ B activity in a negative feedback loop, thereby dampening the inflammatory response. We therefore suggest that the increased IL-6 response seen after surfactant treatment may be anti-inflammatory. This hypothesis is supported by data of Farivar and colleagues,¹⁸ who showed that recombinant IL-6 improved LRI. Moreover,

in a rodent model of hemorrhagic shock, exogenous IL-6 decreased lung and liver tissue injury.¹⁹

In addition to increased levels of IL-6, we also observed augmented IL-10 expression after surfactant treatment. IL-10 has a strong anti-inflammatory effect by limiting the secretion of proinflammatory cytokines by macrophages and T cells. Several studies have shown that exogenous IL-10 attenuates experimental reperfusion injury of the intestine, liver, lung, and hindlimb.²⁰⁻²³ In this study, IL-10 decreased dramatically with time in the control animals, whereas surfactant treatment normalized or even increased IL-10 expression, suggesting that surfactant induces a strong anti-inflammatory environment.

We also looked at the expression of iNOS. Baron and associates²⁴ demonstrated that expression of iNOS in lung epithelial cells is critical for the development of lung injury and furthermore mediates surfactant dysfunction. Some investigators have suggested that increased lung apoptosis could be one of the mechanisms through which iNOS causes lung injury.²⁵ We report that iNOS was specifically upregulated in the control group, whereas it was not upregulated in the sham-operated group, which supports the notion that iNOS is an important mediator during lung injury. In line with this finding, we observed increased cleaved caspase-3 expression in the control group. Interestingly, 2 and 4 hours after ischemia iNOS was strongly upregulated by surfactant either before (SIR) or after (ISR) ischemia. The enhanced expression of iNOS after surfactant treatment was not associated with enhanced apoptosis, because at all time points both surfactant treatment schedules significantly decreased

FIGURE 2. Inducible nitric oxide (*iNOS*) concentrations as function of time. Inducible nitric oxide expression levels were assessed in total cell lysates resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by Western blot. Data (mean $\pm$ SEM) were normalized to β -actin expression and depicted as percentage of control values (black bars). Insets are representative Western blots depicting immunodetectable inducible nitric oxide. ** $P < .01$; *** $P < .001$; N = 7 per group. □ sham; ■ saline + I + R; ▒ surfactant + I + R; ▤ I + surfactant + R.

apoptosis in the lung. Interestingly, there are some indications that iNOS expression may be involved in antiapoptotic pathways, depending on cell type, stimulus, and duration (as reviewed by Mehta²⁵). In our study, the early expression of iNOS, 30 minutes after ischemia, was significantly downregulated by surfactant treatment. We therefore speculate that early expression of iNOS triggers the proinflammatory response, whereas longer periods of iNOS expression trigger antiapoptotic pathways. This speculation is supported by the data of Mikawa and coworkers,²⁶ who showed that selective inhibition of iNOS with ONO-1714 improved lung oxygenation, pulmonary edema, and leukocyte sequestration when administered before or within 2 hours after induction

FIGURE 3. Interleukin 6 (*IL-6*) concentrations as function of time. Interleukin 6 levels were determined by standard enzyme-linked immunosorbent assay. * $P < .05$; ** $P < .01$; N = 7 per group. □ sham; ■ saline + I + R; ▒ surfactant + I + R; ▤ I + surfactant + R.

FIGURE 4. Interleukin 10 (*IL-10*) concentrations as function of time. Interleukin 10 levels were determined by standard enzyme-linked immunosorbent assay. * $P < .05$; ** $P < .01$; *** $P < .001$; N = 7 per group. □ sham; ■ saline + I + R; ▒ surfactant + I + R; ▤ I + surfactant + R.

of acute lung injury but not if administered 3 or 4 hours after induction.

Interestingly, comparison of the effects of surfactant administered before and after ischemia on the parameters measured showed a time-dependent relationship. The delayed response in the ISR group is explained by the time sequence of the experimental setting. It is known that the reperfusion period plays a significantly more important role in causing injury than does the ischemic period.²⁷ This might explain the equivalent responses, but with different time kinetics,

FIGURE 5. Effect of lung ischemia-reperfusion injury on cleaved caspase-3 expression as function of time. Cleaved caspase-3 expression levels were assessed in total cell lysates resolved by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by Western blot. Data (mean $\pm$ SEM) were normalized to β -actin expression and depicted as percentage of values in sham-operated group. Insets are representative Western blots depicting immunodetectable cleaved caspase-3. * $P < .05$; ** $P < .01$; *** $P < .001$; N = 7 per group. □ sham; ■ saline + I + R; ▒ surfactant + I + R; ▤ I + surfactant + R.

that we observed after both surfactant treatment schedules. From a clinical point of view, it may be interesting for future studies to analyze the effect on ischemia–reperfusion injury of surfactant administered after the onset of reperfusion. Interestingly, however, Struber and colleagues²⁸ showed that exogenous surfactant instilled in donor lungs before retrieval had protective effects on posttransplant surfactant function and on clinical outcome.²⁸

In conclusion, we have shown surfactant to have pleiotropic effects during LIRI. First, surfactant therapy efficiently improves lung architecture. Second, surfactant results in anti-inflammation, accompanied by decreased apoptosis. Finally, surfactant decreases the short-term iNOS expression but increases the long-term iNOS expression. Surfactant replacement therapy thus may provide a good approach to treat LIRI in patients, although more studies are warranted to further delineate the long-term effects of surfactant and the mechanisms involved.

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