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Time profile of oxidative stress and neutrophil activation in ovine acute lung injury and sepsis

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Abstract

The formation of oxidative stress in the lung and activation of neutrophils are major determinants in the development of respiratory failure following acute lung injury (ALI) and sepsis. However, the time changes of these pathogenic factors have not been sufficiently described. Twenty-four chronically instrumented sheep were subjected to cotton smoke inhalation injury and instillation of live *Pseudomonas aeruginosa* into both lungs. The sheep were euthanized at 4, 8, 12, 18, and 24 hours postinjury. Additional sheep received sham injury and were euthanized after 24 hours. Pulmonary function was assessed by determination of oxygenation index and pulmonary shunt fraction. In addition, lung tissue was harvested at the respective time points for the measurement of malondialdehyde, interleukin-6 (IL-6), poly(ADP ribose) (PAR), myeloperoxidase, and alveolar polymorphonuclear neutrophil score. The injury induced severe respiratory failure which was associated with an early increase in lipid peroxidation and IL-6 expression. The injury further led to an increase in PAR activity that reached its peak at 12 hours postinjury and declined afterward. In addition, progressive increases in markers of neutrophil accumulation in the lung were observed. The peak of neutrophil accumulation in the lung was associated with a severe depletion of circulating neutrophils. The results from our model may enhance the understanding of the pathophysiological alterations following ALI and sepsis and thus be useful in exploring therapeutic interventions directed at modifying the expression or activation of inflammatory mediators.

Keywords

lipid peroxidation; malondialdehyde; sheep; smoke inhalation

Introduction

Acute lung injury (ALI) following inhalation injury is frequently associated with secondary opportunistic pulmonary infections and the development of severe sepsis and septic shock (1, 2). Despite the introduction of international guidelines for the management of these patients (3), severe sepsis and septic shock are still associated with unacceptably high mortality rates (4, 5). Thus, the establishment of novel and innovative treatment strategies is of significant clinical interest.

Results from previous experimental studies revealed important pathophysiological pathways in pneumonia-induced sepsis, including neutrophil activation, excessive nitric oxide (NO) production, and poly(ADP ribose) polymerase (PARP) activation, representing potential therapeutic targets (6, 7). Although promising results were reported from experimental approaches targeting various pathways (7, 8), these treatments failed in clinical studies and even increased the mortality of patients with septic shock (6). The failure of translating the knowledge from experimental studies into clinical practice may be explained by the lack of information on the time course of the expression of pathogenic factors that are involved in the septic process (9).

Previously, we have established an ovine model of sepsis that mimics the clinical setting by smoke inhalation-induced ALI and subsequent instillation of live *Pseudomonas aeruginosa* bacteria into the lung (10, 11). However, the time course of crucial indicators of oxidative stress and neutrophil activation has not yet been sufficiently characterized. Therefore, the current study was designed to describe the time profile of oxidative stress and neutrophil activation in our clinically relevant ovine model of sepsis.

Materials and Methods

This study was approved by the Animal Care and Use Committee of the University of Texas Medical Branch and conducted in compliance with the guidelines of the National Institutes of Health and the American Physiological Society for the care and use of laboratory animals.

Animal model

The ovine model of ALI and sepsis induced by smoke inhalation and instillation of *Pseudomonas aeruginosa* into the lungs has been previously described in detail (11, 12). In brief, 24 adult female sheep (body weight, 33 ± 2 kg) were surgically prepared for chronic study with a femoral artery catheter, a pulmonary artery thermodilution catheter, and a left atrial catheter. After a recovery period of 5-7 days, the animals received tracheostomy followed by inhalation injury with 48 breaths of cotton smoke ($<40^{\circ}\text{C}$) using a modified bee smoker. Afterward, a stock solution of live *Pseudomonas aeruginosa* ($2-5 \times 10^{11}$ colony-forming units, from a burn patient at Brooke Army Medical Center; San Antonio, TX) suspended in 30 ml of 0.9% saline solution was instilled into the right middle and lower lobe and left lower lobe of the lung (10 ml each). Anesthesia was then discontinued, and the sheep were allowed to awaken.

Experimental protocol

The study protocol was similar to previously published protocols using the same animal model (13). The animals were randomly allocated to the study groups and euthanized at 4, 8, 12, 18, and 24 hours after the injury ($n = 4$ each time point). Four additional sheep received sham injury (inhalation of room air and instillation of normal saline into the lungs) and were euthanized after 24 hours to serve as sham-injured control group. All sheep were mechanically ventilated (Servo Ventilator 900C, Siemens; Elema, Sweden) with a tidal volume of 12-15 mL·kg⁻¹ and a positive end expiratory pressure of 5 cmH₂O. Of note, sheep require higher tidal volumes than humans because the physiological ovine lung compliance is higher and the ovine dead space/tidal volume ratio is larger (0.57 in sheep versus 0.30 in humans) (14). Thus, higher tidal volumes are necessary for the ventilation of sheep than those recommended for the ventilation of patients with lung injury. The FiO₂ is set at 1.0 for the first three hours postinjury and then adjusted stepwise to maintain sufficient oxygenation (SaO₂ > 95%, PaO₂ > 90 mmHg) whenever possible. The respiratory rate was initially set at 20 breaths per minute and then adjusted to maintain the PaCO₂ within 5 mmHg of the baseline value. All animals were fluid resuscitated, initially started with an infusion rate of 2 mL·kg⁻¹·h⁻¹ lactated Ringer's solution and adjusted to maintain hematocrit ($\pm 3\%$) and cardiac filling pressures at baseline values. During the study period, all animals had free access to food, but not to water. Additional four sheep were euthanized without prior instrumentation and without any injury to serve as uninstrumented and uninjured control group (no injury). From these animals only lung tissue samples were obtained. After completion of the experiment, the animals were deeply anesthetized with ketamine and xylazine and euthanized by intravenous injection of saturated potassium chloride.

Measurements

Arterial and venous pressures were measured using pressure transducers (model P×3×3, Baxter Edwards Critical Care Division) which were connected to a hemodynamic monitor (model 7830A, Hewlett Packard; Santa Clara, CA). Cardiac output was determined with the thermodilution method (Monitor 9530, Baxter-Edwards Critical Care). Blood gases were measured three-hourly and immediately before euthanization using a blood gas analyzer (Synthesis 15, Instrumentation Laboratories; Lexington, MA). Pulmonary shunt fraction (Qs/Qt) and oxygenation index (PaO₂/FiO₂) were calculated using standard equations (15). Neutrophil count was performed with a Hemavet 850 (Drew Scientific, Oxford, CT) adjusted for sheep blood.

Tissue analyses

Immediately after death, the right lung was removed. To determine alveolar polymorphonuclear (PMN) scores, a 1-cm thick section was taken from the lower lobe of the right lung, inflated with 10% formalin, embedded in paraffin, sectioned into 4- μ m pieces and stained with hematoxylin eosin. Four blocks from each animal were examined by a pathologist who graded the amount of PMN in the alveolar space on a scale of 0-4 (0: normal; 1: focal PMN; 2: multiple foci, < 50% of section area; 3: multiple foci, 50-80% of section area; 4: PMN > 80% of section area). In addition, aliquots from adjacent lung tissue were taken and snap frozen in liquid nitrogen. The activity of myeloperoxidase (MPO), an

indicator of neutrophil accumulation, was evaluated in homogenized tissue with a commercially available assay (CytoStore, Calgary, AB, Canada). One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance at 450 nm for 10 minutes. MPO activity was normalized to lung wet/dry weight ratio and reported as U/g dry tissue. Malondialdehyde formation was utilized to quantify lipid peroxidation in lung tissue and measured as thiobarbituric acid reactive material. Malondialdehyde levels were quantified with a commercially available assay (Northwest Life Science Specialties, Vancouver, WA, Canada). The level of lipid peroxidation is expressed as mmol per mg protein.

For the immunohistochemical detection of PAR, monoclonal anti-PAR antibody (Calbiochem, San Diego, CA, USA; 1:1000, overnight, 4°C) was used after antigen retrieval. Secondary labeling was achieved by using biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) (30 min, room temperature). Horseradish peroxidase-conjugated avidin (30 min, room temperature) and brown-colored diaminobenzidine (6 min, room temperature) were used to visualize the labeling (Vector Laboratories). The sections were counterstained with hematoxylin (blue color). The intensity of PAR staining of individual sections was determined by a blinded experimenter according to a semiquantitative PAR positivity score of the bronchial airway epithelium, bronchial glands (glandulae bronchiales), and lung alveoli from 1 to 10. (1=no staining, 2=light cytoplasmic staining, 3=few positive nuclei, 4=light nuclear staining in approximately 10% of cells, 5=light nuclear staining in approximately 25% of cells, 6=light nuclear staining in approximately 50% of cells, 7=strong nuclear staining in approximately 50% of cells, 8=approximately 75% of the nuclei are positive, 9=approximately 90% of the nuclei are positive, 10=few negative cells) (16).

For the measurement of interleukin 6 mRNA in lung tissue (RT-PCR), total RNA was obtained using a commercially available total RNA purification kit (Purescript; Gentra Systems, Inc., Minneapolis, MN, USA). Quantitative PCR was performed as described previously (11). The copy numbers were normalized between samples using GAPDH copy numbers determined with an external standard constructed from the v-erb gene. The results are expressed as copy numbers per microgram of total RNA.

Statistical analysis

All values are expressed as means $\pm$ SEM. The statistical analysis was performed using the one-way analysis of variance (ANOVA) followed by a *post hoc* Dunnett's test as the multiple comparison method. A value of $P < 0.05$ was regarded as statistically significant.

Results

Cardiopulmonary function

Injured sheep developed signs of severe respiratory impairment as evidenced by a significant decrease in oxygenation index ($\text{PaO}_2/\text{FiO}_2$ ratio) and an increase in pulmonary shunt fraction (Qs/Qt) toward the sham-injured group ($P < 0.01$ both). In addition, all injured

animals exerted a hypotensive-hyperdynamic circulation with a significant fall in mean arterial pressure and an elevation of cardiac index toward sham ($P < 0.01$ both, Table 1).

Lipid peroxidation in lung tissue

Malondialdehyde content, an index of lipid peroxidation, in lung tissue homogenates increased early and was highest at four hours postinjury ($P < 0.01$ vs. sham and no injury). Afterward, malondialdehyde decreased over time, but was still significantly elevated at 24 hours ($P < 0.05$ vs. sham, Fig. 1).

Interleukin-6 mRNA in lung tissue

Interleukin (IL)-6 mRNA was not detectable in lung tissue homogenates of the sham-injured group, but started to increase in lung tissue of injured animals at four hours, and was significantly increased toward sham and no injury at eight hours postinjury ($P < 0.05$). Afterward, pulmonary IL-6 mRNA expression declined almost to values of the sham and no injury group (Fig. 2).

PARP activation in lung tissue

Immunohistochemical staining revealed marked increases in poly(ADP ribose) (PAR) in lung tissue of injured sheep. The semiquantitative PAR positivity scores of bronchial airway epithelium ($P < 0.05$), bronchial glands ($P < 0.05$), and lung alveoli ($P < 0.05$) were significantly increased versus the sham and no injury group at the 12-hour time point (Fig. 3A-C).

Neutrophil activation in lung tissue

After injury, both alveolar polymorphonuclear (PMN) histopathology score and myeloperoxidase activity in lung tissue similarly increased over time. The increases in PMN score and myeloperoxidase activity were significant at 18 and 24 hours compared to sham injury and no injury ($P < 0.05$ both, Fig. 4). Inversely, the neutrophil blood count progressively decreased over time (Table 1).

Discussion

The main findings of the current study using our well-established ovine model of ALI and sepsis are that the injury-related respiratory failure was associated with early increases in lipid peroxidation and IL-6 expression in lung tissue. These parameters exhibited a time-dependent decrease during the remainder of the 24-hour time course. In addition, the injury led to an increase in PAR activity that reached its peak at 12 hours postinjury and declined afterward. Finally, we have observed progressive increases in the markers of neutrophil accumulation in the lung over time. The peak of neutrophil accumulation in the lung was associated with severe depletion of circulating neutrophils.

The development of ALI after inhalation injury and bacterial infection is triggered by the activation of a complex orchestra of inflammatory mediators (13, 17). The burst of inflammatory cytokines such as IL-6 and tumor necrosis factor alpha (TNF-alpha) by activated neutrophils may trigger increased expression of NO synthase (18). Reactions

between excessive amounts of NO and superoxide radicals from activated neutrophils can exert cytotoxic effects by yielding reactive oxygen species and peroxynitrite that, in turn, may induce excessive activation of the nuclear repair enzyme PARP, which may cause ATP depletion and cellular injury (19). Altogether, these processes can trigger the development of respiratory failure and acute respiratory distress syndrome (20, 21). These pathways represent potential treatment options to modulate the inflammatory response at the molecular level. Although the involvement of all these pathogenetic factors in the development of ALI- and pneumonia- induced pulmonary failure are established and promising preliminary results by interrupting these pathways were reported (7, 8, 20, 22, 23), these therapeutic approaches failed in clinical studies or even increased the mortality of patients with septic shock (6, 20). Substantial evidence suggests that this failure may be explained by a time dependency of mediator activation during ALI and sepsis (9, 13, 24). When considering treatment strategies at the molecular level, it is crucial to identify the respective time course of mediator activation/expression to be able to intervene when they are actually activated or expressed. However, the time course of neutrophil activation and the oxidative stress reaction in our model has not been sufficiently defined.

The present data revealed that the occurrence of oxidative stress in lung tissue, as evidenced by malondialdehyde contents, was an early event after the double-hit injury. Malondialdehyde concentrations peaked as early as four hours postinjury and showed a linear trend to decrease until the 24-hour time point. It has previously been shown that the inhalation of cotton smoke causes a local burst of oxidation in the lung (25, 26). In the current study, it appears that the oxidative stress reaction led to the observed increase in IL-6 expression in lung tissue. The documented time course further suggests that the early burst of oxidative stress and IL-6 expression probably came from other sources than activated neutrophils, such as NADH oxidation. This early increase in lipid peroxidation in the ovine lung represents a novel and unexpected finding. Increased oxidative stress (Fig. 1) and expression of cytokines, such as IL-6 (Fig. 2), may have triggered enhanced peroxynitrite formation and time-delayed PARP activation in small airways, airway glands, and alveoli with a peak at the 12-hour time point (Fig. 3). PARP activation is known to upregulate multiple pathways of pro-inflammatory signaling (27). PARP-induced pro-inflammatory or signaling events may have fostered the observed accumulation of neutrophils in the lung (Fig. 2), which appeared as relatively late event in this experimental setting. These findings indicate that therapeutic interventions directed at modifying the oxidative stress reaction in our model should best be started early after the injury because the present data suggest that otherwise the induced chain of events, including IL expression and subsequent activation of PARP will lead to cellular injury and deterioration of pulmonary function which was evidence by the early decline in $\text{PaO}_2/\text{FiO}_2$ ratio below 200 mmHg. If a potential therapeutic anti-oxidant treatment were to start later than the 12-hour time point, the PARP-induced cell death and associated decrease in pulmonary function were probably irreversible.

It may be regarded as a limitation that the study design only included six time points of tissue sample collection. It is possible that short-term increases in markers of neutrophil activation or oxidative stress were not detected. In addition, ALI and sepsis may result from various incidents and the combination of inhalation trauma and pulmonary infection with

Pseudomonas aeruginosa represents only one possible scenario. Thus, the results from the current experiment are not necessarily transmittable to acute lung injury from other etiologies. However, we chose this double-hit injury because it represents a frequent clinical setting in burn patients with smoke inhalation injury (2) and it provides the possibility of relating the current findings to several previous studies using the same animal model (7, 8, 10-13, 28). Moreover, we did not compare the contributions of the solitary injuries in the current experiment, but we have previously shown that the pulmonary response to the combination of both smoke inhalation and bacterial infection is different and more severe than with either injury alone (11). We could demonstrate that not only the cardiopulmonary deteriorations, but also the histological damage is significantly more pronounced when the injuries are combined and concluded that the bacterial infection mainly influences the pathological alterations in ovine smoke inhalation injury and pneumonia-induced sepsis (11, 29). It is a further limitation that the current study was not designed to definitely prove causative mechanisms, but despite its descriptive character, it delivers valuable information on the time changes of key markers of ALI.

In conclusion, the current study describes the time course of important markers of neutrophil activation and oxidative stress in lung tissue of sheep subjected to ALI by smoke inhalation and pneumonia. The results from our model may enhance the understanding of the pathophysiological alterations after this type of injury and thus be useful in exploring therapeutic interventions directed at modifying the expression or activation of inflammatory mediators.

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Fig. 1.

Time profile of malondialdehyde content, an index of lipid peroxidation, in lung tissue of animals at different time points after induction of acute lung injury (ALI) + sepsis.

Uninstrumented, uninjured animals (no injury) and animals that were observed for 24 hours after sham injury served as control groups. Each group includes four animals. †P < 0.05, ‡P < 0.01 vs. sham group; *P < 0.05, **P < 0.01 vs. no injury.

Fig. 2.

Time profile of interleukin (IL)-6 mRNA in lung tissue of animals at different time points after induction of acute lung injury (ALI) + sepsis. Uninstrumented, uninjured animals (no injury) and animals that were observed for 24 hours after sham injury served as control groups. Each group includes four animals. †P < 0.05 vs. sham group; *P < 0.05 vs. no injury.

Fig. 3.

Time profile of poly(adenosine diphosphate ribose) (PAR) polymerase activity in lung tissue of animals at different time points after induction of acute lung injury (ALI) + sepsis.

Uninstrumented, uninjured animals (no injury) and animals that were observed for 24 hours after sham injury served as control groups. Each group includes four animals. †P < 0.05, ‡P < 0.01 vs. sham group; *P < 0.05 vs. no injury.

Fig. 4.

Time profile of (A) alveolar polymorphonuclear neutrophil (PMN) scores and (B) myeloperoxidase activity, an index of neutrophil activation, in lung tissue of animals at different time points after induction of acute lung injury (ALI) + sepsis. Uninstrumented, uninjured animals (no injury) and animals that were observed for 24 hours after sham injury served as control groups. Each group includes four animals. †P < 0.05 vs. sham group; *P < 0.05, **P < 0.01 vs. no injury.

Table 1

Time changes in pulmonary gas exchange, systemic hemodynamic variables, and neutrophil blood count

	Injury (ALI + sepsis)				Sham injury	
	4 h	8 h	12 h	18 h	24 h	24 h
PaO ₂ /FiO ₂ , mmHg	337 ± 47 ^b	157 ± 58 ^b	118 ± 52 ^b	115 ± 34 ^b	82 ± 24 ^b	527 ± 15
Qs/Qt	0.38 ± 0.03	0.43 ± 0.05	0.58 ± 0.09 ^b	0.49 ± 0.11 ^a	0.58 ± 0.08 ^b	0.20 ± 0.01
MAP, mmHg	96 ± 1 ^b	88 ± 3 ^b	71 ± 4 ^b	60 ± 3 ^b	57 ± 3 ^b	103 ± 3
CI, l·min ⁻¹ ·m ⁻²	6.5 ± 0.8	6.8 ± 0.5	6.7 ± 0.8	8.4 ± 1.3 ^a	8.5 ± 1.0 ^a	4.6 ± 0.3
Neutrophils, K/ μ L	1.6 ± 0.6 ^a	0.9 ± 0.4 ^b	0.8 ± 0.1 ^b	0.7 ± 0.4 ^b	0.3 ± 0.2 ^b	4.4 ± 1.3

Values recorded at different time points after induction of acute lung injury (ALI) + sepsis and 24 hours after sham injury. Each group includes four animals. CI: cardiac index, MAP: mean arterial pressure, Qs/Qt: pulmonary shunt fraction.

^a $P < 0.05$,

^b $P < 0.01$ vs. sham group.