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Pulmonary microvascular hyperpermeability and expression of vascular endothelial growth factor in smoke inhalation- and pneumonia-induced acute lung injury

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Abstract

Introduction: Acute lung injury (ALI) and sepsis are major contributors to the morbidity and mortality of critically ill patients. The current study was designed further evaluate the mechanism of pulmonary vascular hyperpermeability in sheep with these injuries.

Methods: Sheep were randomized to a sham-injured control group (n=6) or ALI/Sepsis group (n=7). The sheep in the ALI/Sepsis group received inhalation injury followed by instillation of *Pseudomonas aeruginosa* into the lungs. These groups were monitored for 24 hours. Additional sheep (n=16) received the injury and lung tissue was harvested at different time points to measure lung wet/dry weight ratio, vascular endothelial growth factor (VEGF) mRNA and protein expression as well as 3-nitrotyrosine protein expression in lung homogenates.

Results: The injury induced severe deterioration in pulmonary gas exchange, increases in lung lymph flow and protein content, and lung water content (p<0.015 each). These alterations were associated with elevated lung and plasma nitrite/nitrate concentrations, increased tracheal blood flow, and enhanced VEGF mRNA and protein expression in lung tissue as well as enhanced 3-nitrotyrosine protein expression (p<0.05 each).

Conclusions: This study describes the time course of pulmonary microvascular hyperpermeability in a clinical relevant large animal model and may improve the experimental design of future studies.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

Introduction

Severe sepsis and septic shock are major contributors to the morbidity and mortality of critically ill patients [1, 2]. The incidence of sepsis is expected to be higher in patients suffering from significant co-morbidities such as severe burn injuries. Acute lung injury (ALI) by smoke inhalation is frequently complicated by superimposed pneumonia and sepsis [3] and markedly increases the mortality of burn patients [4]. Previous studies have demonstrated that ALI and sepsis lead to endothelial dysfunction and pulmonary microvascular hyperpermeability to fluids and protein [5, 6]. Sepsis-induced lung injury and pulmonary fluid accumulation are frequent entities and related to impaired oxygenation, multiple organ failure, and finally death. It has been suspected that excessive formation of nitric oxide (NO) by NO synthases and enhanced expression of vascular endothelial growth factor (VEGF) play major roles during the injury process. Excessive NO may contribute to lung tissue edema formation by two means. First, it can increase the hydrostatic pressure gradient by vasodilation and augmentation of airway blood flow. Second, high concentrations of NO may increase the permeability of the capillary wall by inducing oxidative/nitrosative stress and the expression of VEGF [7]. It has been shown that ALI and sepsis are associated with significant increases in NO production, oxidative/nitrosative stress markers, and VEGF expression in the lung [5, 6], but the time changes of these tissue markers have not been evaluated yet. However, when considering potential treatment strategies, the identification of the time course of the above mentioned pathogenetic factors may be crucial.

The current study was designed to evaluate the tissue changes of important determinants of nitrosative stress and vascular permeability in a relevant large animal model of ALI and pneumonia-induced 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.

Surgical preparation and injury

The model of ovine ALI by smoke inhalation and pneumonia-induced sepsis by instillation of *Pseudomonas aeruginosa* into the lungs has been previously described in detail [6, 8, 9]. A total of 29 adult female sheep (mean body weight, 31 ± 1 kg) were surgically prepared for chronic study. The right femoral artery was cannulated with a polyvinylchloride catheter (Intracath, 16-G, 24 inches, Becton Dickinson Vascular Access; Sandy, UT). A thermodilution catheter (model 93A-131-7F, Edwards Critical Care Division; Irvine, CA) was inserted into the right external jugular vein through an introducer sheath (Edwards Lifescience; Irvine, CA) and advanced into the common pulmonary artery. Through the left fifth intercostal space, a Silastic catheter (0.062 inch inner diameter (ID) and 0.125 inch outer diameter (OD), Dow Corning; Midland, MI) was positioned in the left atrium. Through the right fifth intercostal space, a Silastic catheter (0.025 inch ID and 0.047 inch OD) was placed into an efferent lymphatic vessel from the caudal mediastinal lymph node. Ligation of the tail of the caudal mediastinal lymph node and cauterization of the systemic diaphragmatic lymph vessels were performed to remove the systemic lymph contribution. After a recovery period of 5-7 days, a baseline measurement was performed in spontaneously breathing sheep. Thereafter, the animals were anesthetized using intravenous ketamine 5 mg/kg. Tracheotomy was performed and anesthesia was maintained with a halothane (1.1-2.0 Vol%)-oxygen mixture.

Inhalation injury was then induced. Cotton cloth (40 g) was ignited in a modified bee smoker connected to the endotracheal tube, and the smoke was delivered to the animal via the bellows of the smoker. The bellows of the bee smoker delivers ~15 mL/kg tidal volume. This was performed consistently with each animal. A thermistor was attached to the endotracheal tube to prevent the temperature of the smoke insufflated into the sheep from exceeding 40°C. Twelve breaths of smoke were delivered in this fashion, and the process was repeated four times so that the animals received a total of 48 breaths of smoke [9]. Afterward, a stock solution of live *Pseudomonas aeruginosa* (2.5×10^{11} colony-forming units from a burn patient at Brooke Army Medical Center, San Antonio, TX) suspended in 30 ml of 0.9 % NaCl solution was instilled into the right middle and lower lobe and left lower lobe of the lung (10 ml each) using a bronchoscope. Anesthesia was then discontinued, and the sheep were allowed to awaken.

Experimental protocol

Thirteen animals were allocated to the following groups that were studied for a 24-hour period:

- 1) Injured animals that received acute lung injury (ALI) by smoke inhalation and instillation of bacterial suspension into the lungs (ALI/sepsis group; $n = 7$)
 - 2) Sham-injured animals that received inhalation of room air instead of smoke and instillation of normal saline instead of bacterial suspension into the lungs (control group; $n = 6$)
- Additional 16 sheep were injured and euthanized after 3, 6, 12, and 18 hours ($n = 4$ each) to obtain lung tissue at the respective time points postinjury. 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. The respiratory rate was initially set at 20 breaths per minute and adjusted according to blood gas analysis. All animals were fluid resuscitated, initially started with an infusion rate of 2 ml·g⁻¹·h⁻¹ lactated Ringer's solution and adjusted to maintain the hematocrit at baseline values ± 3 %. During the study period, the animals had free access to dry food, but not water.

Measurements

Systemic and pulmonary hemodynamic variables were determined from the femoral and pulmonary artery catheters using pressure transducers (Baxter-Edwards Critical Care, Irvine, CA) and recorded on a hemodynamic monitor (monitor V24C, Philips Medicine System Bollinger, Bollinger, Germany). Cardiac output was measured in triplicate with the thermodilution technique (Monitor 9530, Baxter-Edwards Critical Care). Blood gases were measured using a blood gas analyzer (Synthesis 15, Instrumentation Laboratories; Lexington, MA). PaO₂/FiO₂ ratio, pulmonary shunt fraction (Qs/Qt), and pulmonary capillary pressure were calculated using standard equations [10]. Lung lymph flow (Q_L) was determined by collecting lymph fluids for an hour and measuring the amount with graduated test tubes. Plasma (Π_P) and lung lymph (Π_L) colloid oncotic pressures (COP) were determined with a colloid osmometer (model 4420, Wescor; Logan, UT). The plasma (C_P) and lung lymph (C_L) protein concentrations were measured with a refractometer (National Instrument; Baltimore, MD). For estimation of pulmonary microvascular permeability to protein, the lung lymph-to-plasma protein ratio (C_L/C_P) and lung lymph-to-plasma COP ratio (Π_L/Π_P) were calculated [6]. Lung permeability index was calculated using the formula $Q_L \times (C_P \text{ lymph} / C_P \text{ plasma})$. Nitric oxide levels in the plasma and lymph were evaluated by measuring the intermediate and end products (NO_x), using a nitrate/nitrite colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI, USA) [8]. The lung lymph NO_x production per hour was calculated as the product of lymph NO_x concentration (in μmol/l) and lymph flow (in ml/h).

To harvest lung tissue at different time points, the animals were euthanized under deep anesthesia and the right lung was removed. A 1-cm thick section was taken from the lower lobe of the right lung and immediately frozen in liquid nitrogen. Vascular endothelial growth factor (VEGF) and 3-nitrotyrosine protein expressions were measured using a western blot protocol as described previously [7]. Blots were quantified by NIH IMAGE J scanning densitometry, and normalized to total actin expression. For the determination of VEGF mRNA, 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 [9]. The copy numbers were normalized between samples using glyceraldehyde 3-phosphate dehydrogenase (GADPH) copy numbers determined with an external standard constructed from the *v-erb* gene. All results were expressed as copy numbers per microgram of total RNA. From the remaining part of the right lower lobe, bloodless lung wet-to-dry weight ratio was calculated as an index of lung water content [11]. Microvascular blood flow of the distal trachea was measured at indicated time points by injection of colored microspheres (Interactive Medical Technologies, Los Angeles, CA) [12].

Statistical analysis

All values are expressed as means $\pm$ standard error of the mean. Results were compared by a two-way analysis of variance (ANOVA) for repeated measurements with appropriate Student-Newman-Keuls *post hoc* comparisons to compare differences within and between groups. One-way ANOVA was used to compare groups when measurements were made at only one time point; and the Newman-Keuls procedure was used for *post hoc* pairwise comparisons. The statistical analysis of tissue markers was performed using the one-way 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

None of the measured variables was significantly different between the control and ALI/sepsis group at baseline.

Pulmonary function and systemic hemodynamics

After induction of ALI and sepsis, all sheep developed acute respiratory dysfunction as evidenced by an early decrease in $\text{PaO}_2/\text{FiO}_2$ ratio and a concomitant increase in pulmonary shunt fraction. Calculated pulmonary capillary pressure increased in both groups and tended to be higher in injured animals. Furthermore, the injury induced a hypotensive-hyperdynamic circulation with significant decreases in systemic vascular resistance index and mean arterial pressure associated with a significant elevation of cardiac index (Table 1).

Microvascular permeability, edema formation, and blood flow

The injury was also linked to progressive increases in lung lymph flow (Fig. 1A) and lung permeability index (Fig. 1B). Furthermore, transient increases in C_L/C_P (Fig. 1C) and Π_L/Π_P (Fig. 1D) were observed. Bloodless lung wet/dry weight ratio started to increase early and was significantly higher than in the control group at 18 and 24 hours postinjury (Fig. 2). In addition, the regional microvascular blood flow of the trachea increased significantly post-injury (Fig 3).

Nitric oxide production, VEGF expression, and nitrosative stress

The injury was associated with increases in both plasma NOx contents (Fig. 4A) and lung lymph NOx contents (Fig. 4B). VEGF mRNA in lung tissue homogenate was significantly

increased at the 3-hour time point and returned to values of control animals afterward (Fig. 5A). The overexpression of VEGF mRNA was followed by a transient increase in VEGF protein expression in lung tissue from 6-18 hours postinjury (Fig. 5B). Protein expression of 3-nitrotyrosine in lung tissue was significantly increased towards the control group from 12-24 hours after the double hit injury (Fig. 6).

Discussion

In the current study, the development of ALI by smoke inhalation and pneumonia in sheep was associated with progressive increases in pulmonary transvascular fluid flux and lung edema formation and a transient increase in pulmonary microvascular hyperpermeability to proteins. This was related to excessive NO formation and a significant elevation in airway (tracheal) blood flow, representing potential mechanisms of microvascular hyperpermeability in the lung. Furthermore, overexpression of nitrosative stress markers and VEGF in lung tissue occurred, both potentially contributing to increased transvascular fluid and protein flux.

Fluid accumulation in the lung secondary to pulmonary microvascular hyperpermeability represents a major cause of sepsis-induced lung injury and acute respiratory distress syndrome. It leads to impaired oxygenation and often requires incremental increases in the inspired oxygen fraction and ventilatory pressures. Both high concentrations of inspired oxygen and high inflation pressures are known to negatively influence the degree of lung injury by fostering the development of atelectasis and exposing the endothelial and epithelial barriers to excessive stress, respectively. Thus, novel therapeutic strategies to interrupt this vicious circle of septic lung injury, pulmonary microvascular hyperpermeability, and ventilator-associated lung injury are of great clinical interest. In this regard, experimental inhibition of different isoforms of NO synthases delivered promising results [7, 13]. However, when considering specific treatment strategies, the identification of the time course of pathogenetic key factors of sepsis resulting from ALI and pneumonia appears to be crucial. Therefore, the current investigation was designed to evaluate the time changes of important pathogenetic factors of vascular hyperpermeability following ALI and sepsis, including the co-upregulation of NO and VEGF.

Changes in transvascular fluid flux are mainly determined by: (a) the hydrostatic pressure gradient, (b) the oncotic pressure gradient, (c) the permeability of the capillary wall to water and protein, and (d) the capillary surface area. We were not able to directly measure the hydrostatic pressure in the lung vessels, but the calculated pulmonary capillary pressure, as an indicator of hydrostatic pressure, tended to be higher in injured sheep. Moreover, the pulmonary hydrostatic pressure is known to be directly related to airway blood flow. The regional microvascular blood flow of the trachea, as a marker of airway blood flow, significantly increased in sheep with ALI and sepsis (Fig. 4), probably due to excessive NO production. In this regard, previous studies showed that the increases in airway blood flow and transvascular fluid flux following ALI were associated with significant increases in NO_x production [14, 15]. Blockade of different isoforms of NO synthase inhibited the increases in airway blood flow, pulmonary transvascular fluid flux, and lung water content [14, 15]. In the current investigation, the NO-induced increase in airway blood flow, in turn, probably caused the progressive elevations in transvascular fluid flux and lung water content.

Alterations in vascular permeability can further be evaluated by the determination of the ratio of native proteins in the lymph and plasma [6, 16]. In contrast to the linear increases in transvascular fluid flux and lung water content, the markers of permeability of the capillary wall to proteins (C_L/C_P) increased only transiently with a peak at 12 hours postinjury.

Interestingly, this peak concurred with the expression of VEGF protein in lung tissue. VEGF is a potent permeability factor that is known to be a significant contributor to vascular hyperpermeability and edema formation in the lung during ALI and acute respiratory distress syndrome [17, 18]. The expression of VEGF may be induced by nitrosative stress and the formation of NO. Previous experiments using the same animal model demonstrated that the injury-related expression of VEGF in lung tissue can be suppressed by pharmacological inhibition of NO synthases [7]. Thus, it is conceivable that high levels of NO may induce pulmonary vascular hyperpermeability in this model of sepsis via promotion of VEGF expression. This hypothesis should be tested in future experiments using non-specific NOS inhibitors.

Although the current study was not designed to definitely prove causative mechanisms, it demonstrates the time changes of pulmonary vascular hyperpermeability and describes different potentially contributing pathomechanisms, such as the co-upregulation of NO and VEGF. Enhanced understanding of the time course of these crucial factors may ameliorate the timing and allow for the development of novel treatment strategies in future experiments. Those descriptive data may, therefore, help to overcome relevant weaknesses of previous studies [7, 13, 19].

It is important to note that 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) [20]. Thus, the applied tidal volumes of 12-15 ml·kg⁻¹ in the current study do not induce lung injury in sheep. In preliminary studies, we tested various tidal volumes (8, 10, 12, and 15 ml·kg⁻¹) in the same animal model. We found that the degree of lung injury was similar between groups, except for a higher incidence of atelectasis in the groups with lower tidal volumes (data unpublished). This result confirms the necessity of applying higher tidal volumes to sheep than those recommended for the ventilation of patients with lung injury. Because the current experiment evaluated sheep with ALI secondary to both smoke inhalation and pneumonia, the effects of the solitary injuries cannot be differentiated. However, patients with smoke inhalation-induced ALI frequently suffer from superimposed bacterial infection and sepsis [3]. In addition, it cannot be excluded that the pulmonary reaction to ALI of other causes may be different, but it has previously been shown that the bacterial infection mainly influences the pathological alterations in different sheep models of smoke inhalation and pulmonary sepsis [9, 21].

Conclusions

The present study demonstrates the time changes of pulmonary vascular hyperpermeability to fluids and proteins secondary to ALI by smoke inhalation and bacterial infection in sheep. Pulmonary failure and hyperpermeability to fluid and protein were related to excessive NO production and elevated airway blood flow as well as enhanced expressions of VEGF and markers of nitrosative stress in lung tissue. This novel information enhances the pathophysiological understanding of patients with these injuries and may improve the experimental design of future studies.

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Fig. 1. Time changes in (A) lung lymph flow, (B) lung permeability index, (C) lung lymph-to-plasma protein ratio (C_L/C_P), and (D) lung lymph-to-plasma colloid oncotic pressure ratio (π_L/π_P) of animals in the control and acute lung injury (ALI)/sepsis group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ between groups.

Fig. 2.

Bloodless lung wet/dry weight ratio, an index of lung water content, of animals in the control and acute lung injury (ALI)/sepsis group at different time points postinjury. * $P < 0.05$, ** $P < 0.01$ between groups.

Fig. 3.

Time changes in regional microvascular blood flow of the trachea of animals in the control and acute lung injury (ALI)/sepsis group. ** $P < 0.01$, *** $P < 0.001$ between groups.

Fig. 4. Time changes in (A) plasma and (B) lung lymph contents of nitrite/nitrate (NOx) of animals in the control and acute lung injury (ALI)/sepsis group. * $P<0.05$, ** $P<0.01$ between groups.

Fig. 5. Expression of (A) vascular endothelial growth factor (VEGF) mRNA and (B) VEGF protein in lung tissue homogenates of animals in the control and acute lung injury (ALI)/sepsis group at different time points postinjury. * $P<0.05$, ** $P<0.01$ between groups.

Fig. 6. Protein expression of 3-nitrotyrosine, an index of protein nitration, in lung tissue homogenates of animals in the control and acute lung injury (ALI)/sepsis group at different time points postinjury. * $P < 0.05$ between groups.

Table 1

Time changes in pulmonary oxygenation and pulmonary and systemic hemodynamic variables of animals in the control and acute lung injury (ALI)/sepsis group.

	Time postinjury					
	0 h	3 h	6 h	12 h	18 h	24 h
PaO ₂ /FiO ₂ ratio, mmHg						
Control	510 ± 16	482 ± 35	519 ± 16	501 ± 20	532 ± 32	521 ± 12
ALI/Sepsis	488 ± 12	174 ± 30 ^c	244 ± 46 ^c	114 ± 9 ^c	121 ± 18 ^c	120 ± 23 ^c
Pulmonary shunt, Qs/Qt						
Control	0.15 ± 0.01	0.19 ± 0.02	0.17 ± 0.02	0.21 ± 0.03	0.18 ± 0.02	0.18 ± 0.01
ALI/Sepsis	0.17 ± 0.01	0.37 ± 0.02 ^c	0.31 ± 0.03 ^a	0.48 ± 0.03 ^c	0.49 ± 0.04 ^c	0.42 ± 0.05 ^c
PCP, mmHg						
Control	13 ± 1	17 ± 1	18 ± 1	17 ± 2	16 ± 1	17 ± 1
ALI/Sepsis	12 ± 1	15 ± 1	16 ± 1	18 ± 1	19 ± 1 ^a	19 ± 1
MPAP, mmHg						
Control	20 ± 1	23 ± 1	24 ± 1	27 ± 1	28 ± 1	27 ± 1
ALI/Sepsis	20 ± 1	25 ± 1	27 ± 1	26 ± 1	26 ± 1	25 ± 1
PVRI, dyne·s·cm ⁻⁵ ·m ²						
Control	129 ± 10	160 ± 21	168 ± 14	177 ± 28	167 ± 30	198 ± 24
ALI/Sepsis	119 ± 8	144 ± 16	131 ± 15	167 ± 17	135 ± 11	147 ± 17
MAP, mmHg						
Control	96 ± 2	104 ± 3	109 ± 4	105 ± 4	102 ± 3	101 ± 4
ALI/Sepsis	96 ± 2	93 ± 2	94 ± 3	83 ± 5 ^b	79 ± 5 ^b	80 ± 6 ^b
SVRI, dyne·s·cm ⁻⁵ ·m ²						
Control	1277 ± 47	1358 ± 76	1445 ± 120	1457 ± 153	1422 ± 114	1477 ± 139
ALI/Sepsis	1277 ± 61	1071 ± 82	970 ± 53 ^b	819 ± 57 ^c	634 ± 65 ^c	705 ± 101 ^c
CI, l·min ⁻¹ ·m ⁻²						
Control	5.6 ± 0.2	5.5 ± 0.2	5.6 ± 0.4	5.5 ± 0.7	5.3 ± 0.4	5.2 ± 0.5
ALI/Sepsis	5.7 ± 0.2	6.6 ± 0.5	7.1 ± 0.3	7.2 ± 0.2	8.6 ± 0.4 ^c	8.3 ± 0.7 ^c

PaO₂, arterial partial oxygen pressure; FiO₂, inspired oxygen fraction; PCP, pulmonary capillary pressure; MPAP, mean pulmonary arterial pressure; PVRI, pulmonary vascular resistance index; MAP, mean arterial pressure; SVRI, systemic vascular resistance index; CI, cardiac index.

^aP<0.05,

^bP<0.01,

^cP<0.001 between groups.