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Tezosentan-induced attenuation of lung injury in endotoxemic sheep is associated with reduced activation of protein kinase C

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Abstract

Introduction Studies *in vitro* reveal that endothelin-1 (ET-1) activates the α isoform of protein kinase C (PKC-α) in cultures of endothelial cells, thereby deranging cellular integrity. Sepsis and endotoxemia are associated with increased plasma concentrations of ET-1 that induce acute lung injury (ALI). We recently reported that non-selective ET-1 receptor blockade attenuates ALI in sheep by reducing the endotoxin-induced increase in extravascular lung water index (EVLWI). The aim of this study was to find out whether this attenuation is associated with reduced translocation of PKC-α from the cytosolic to the membrane fraction of lung tissue homogenate.

Methods Seventeen awake, instrumented sheep were randomly assigned to a sham-operated group (n = 3), a lipopolysaccharide (LPS) group (n = 7) receiving an intravenous infusion of Escherichia coli 15 ng/kg per min for 24 hours, and a tezosentan group (n = 7) subjected to LPS and, from 4 hours, an intravenous injection of tezosentan 3 mg/kg followed by infusion at 1 mg/kg per hour for the reminder of the experiment. Pulmonary micro-occlusion pressure (Pmo), EVLWI, plasma concentrations of ET-1, tumor necrosis factor-a (TNF-a), and interleukin-8 (IL-8) were determined every 4 hours. Western blotting was used to assess PKC-α.

Results In non-treated sheep a positive correlation was found between the plasma concentration of ET-1 and Pmo in the late phase of endotoxemia (12 to 24 hours). A positive correlation was also noticed between Pmo and EVLWI in the LPS and the LPS plus tezosentan groups, although the latter was significantly reduced in comparison with LPS alone. In both endotoxemic groups, plasma concentrations of ET-1, TNF-α, and IL-8 increased. In the LPS group, the cytosolic fraction of PKC-α decreased by 75% whereas the membrane fraction increased by 40% in comparison with the sham-operated animals. Tezosentan completely prevented the changes in PKC-α in both the cytosolic and the membrane fractions, concomitantly causing a further increase in the plasma concentrations of ET-1, TNF-α, and IL-8.

Conclusion In endotoxemic sheep, ET-1 receptor blockade alleviates lung injury as assessed by a decrease in EVLWI paralleled by a reduction in Pmo and the prevention of activation of PKC-α.

Introduction

Endothelin-1 (ET-1) has been identified as the most potent vasoconstrictor peptide known so far [1,2]. Locally produced ET-1 acts on three types of G-protein-coupled receptor: ET_A, ET_{B1}, and ET_{B2} [3]. The ET_A and ET_{B2} receptors are expressed in vascular smooth muscle cells, whereas ET_{B1} is localized mainly in the endothelium. Binding of ET-1 to ET_A and ET_{B2} leads to vascular constriction, whereas ET_{B1} induces relaxation by releasing nitric oxide and prostacyclin [4]. In the lowest concentration range, ET-1 mainly acts on the ET_{B1} receptor [5].

ALI = acute lung injury; ET-1 = endothelin-1; EVLWI = extravascular lung water index; IL = interleukin; LPS = lipopolysaccharide; PKC = protein kinase C; Pmo = pulmonary capillary micro-occlusion pressure; TNF = tumor necrosis factor.

In sepsis, endotoxin and other microbial products that are released into the bloodstream trigger endothelial cells to the enhanced generation of ET-1 causing local vasoconstriction [6-9]. The effect of ET-1 is most prominent in the pulmonary circulation where the ET_A and ET_B receptors are widely distributed [10,11]. Previous investigators have noticed that intravenously infused ET-1 results in increased pulmonary artery pressure and lung edema [12,13]. Moreover, in isolated rat lungs in which the vasculature has been paralyzed, ET-1 enhances microvascular permeability, but the mechanisms involved have not yet been settled [14].

Studies *in vitro* have shown that the binding of ET-1 to its receptor might induce the activation of protein kinase C (PKC) [15,16]. Activation of the α isoform of PKC (PKC-α) might cause disturbances in the shape of the cells as well as of the intercellular junctions. The latter changes might promote acute lung injury (ALI) [17-19]. However, we are unable to determine whether any study *in vivo* has tested whether PKC-α is activated in endotoxin-induced ALI.

In sheep subjected to continuous infusion of endotoxin, we recently found that the dual ET_A and ET_B receptor blocker tezosentan precludes ALI as evaluated by improved gas exchange and a partial reversal of the increases in pulmonary vascular pressures and extravascular lung water index (EVLWI) [9]. However, the mechanisms involved in the tezosentan-induced reduction of EVLWI still remain obscure. We speculate whether non-selective blockade of ET-1 receptor by tezosentan alleviates ALI by dampening the activation of PKC-α and modulating inflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8).

The aim of the present study was twofold: first, to investigate in sheep subjected to endotoxin-induced lung injury whether a relationship exists between the plasma concentration of ET-1 and characteristics of ALI such as the increases in lung microvascular pressure and extravascular lung water content, with or without tezosentan; and second, to assess the effects of tezosentan on the activation of PKC-α in lung tissue in parallel with changes in the plasma concentrations of TNF-α and IL-8.

Methods

The present investigation is based partly on data from a previously published study from our group [9] that was approved by the Norwegian Experimental Animal Board.

In brief, 17 yearling sheep were instrumented with a pulmonary artery thermal dilution catheter introduced via an introducer in the left external jugular vein, a thermo-dye dilution catheter introduced via an introducer in the ipsilateral common carotid artery, and a catheter in the left atrium, as described previously [9].

Experimental protocol

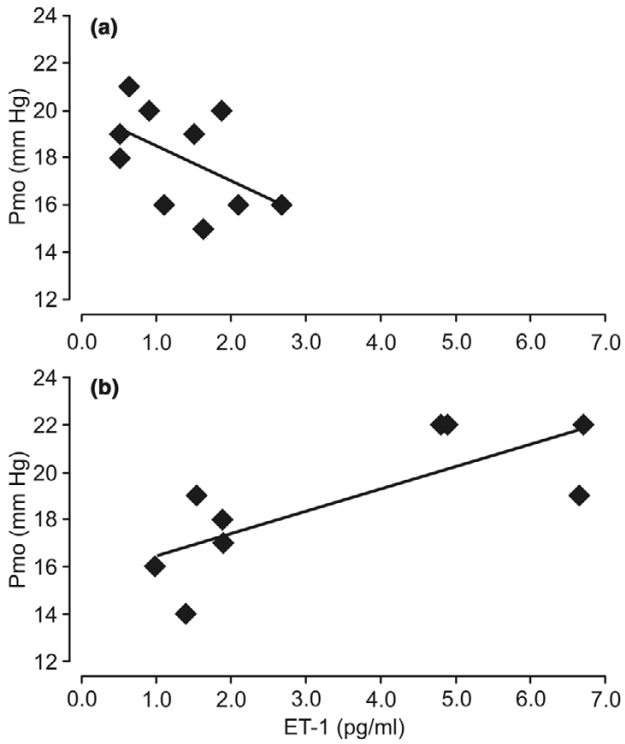
The animals were randomly assigned to a sham-operated group ($n = 3$), a group ($n = 7$) receiving an intravenous infusion of *Escherichia coli* lipopolysaccharide (LPS) 15 ng/kg per min for 24 hours (LPS group), and a group ($n = 7$) subjected to LPS and, from 4 hours, an intravenous injection of tezosentan 3 mg/kg followed by infusion at 1 mg/kg per hour for the reminder of the 24-hour experiment (LPS plus tezosentan group). During the experiment, sheep had free access to food and water.

EVLWI was assessed by the thermal-dye dilution method (Cold Z-021; Pulsion Medical Systems, Munich, Germany). Pulmonary micro-occlusion pressure (Pmo) was determined every 4 hours, as described previously [20]. In brief, Pmo was determined by advancing the Swan-Ganz catheter into the occlusion position in a distal pulmonary artery with the balloon deflated. The criteria for attainment of the micro-occlusion position included: first, easy retrograde aspiration of blood from the catheter; second, a pH, partial pressure of oxygen (PO_2) and carbon dioxide (PCO_2) of aspirated blood consistent with occlusion position, that is, partial pressure of oxygen in occlusion position higher than arterial partial pressure of oxygen ($PmoO_2 > PaO_2$) and partial pressure of carbon dioxide in occlusion position higher than arterial partial pressure of carbon dioxide ($PmoCO_2 < PaCO_2$); third, micro-occlusion pressure greater than proximal occlusion pressure; and fourth, micro-occlusion pressure greater than left atrial pressure, with true zero confirmed by connecting the left atrial catheter and the Swan-Ganz catheter sequentially to the same fixed transducer. Blood for biochemical analysis was sampled at 0, 4, 12, and 24 hours. After the sheep had been killed, lung samples were taken and kept in liquid nitrogen for further analyses.

Western blotting

The activation of PKC-α was assessed by translocation of kinase from cytosolic and/or membrane fractions of lung tissue extracts. In brief, lung tissue samples were homogenized (Polytron homogenizer, blade rotation speed 5,000 r.p.m.) in 1 ml of ice-cold extraction buffer consisting of (in mmol/l): 250 sucrose, 1 EDTA, 1 EGTA, 20 Tris-HCl pH 7.5, 10 2-mercaptoethanol, 20 dithiothreitol and 1 tablet of Complete™ EDTA-free protease inhibitor cocktail per 10 ml. Crude extracts were centrifuged at 200*g* to remove debris, followed by 100,000*g* for 60 min at 4°C. The supernatant represented the cytosolic fraction. The pellet was resuspended by sonication in 200 ml of a similar buffer supplemented with 1% Triton X-100 and centrifuged at 25,000*g* for 15 min at 4°C. The supernatant was collected as the Triton X-100-soluble membrane fraction. For SDS-PAGE, 10% polyacrylamide gels were loaded with 10 mg of protein per lane. After the end of electrophoresis, proteins were electroblotted to nitrocellulose membranes. Membranes were probed overnight with anti-PKC-α primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C and for 1 hour with sheep anti-rabbit horseradish

Figure 1



Relationship between plasma concentration of endothelin-1 (ET-1) and pulmonary micro-occlusion pressure (Pmo) in sheep. **(a)** From 0 to 12 hours of LPS infusion ($r = -0.51$, $P = 0.12$, $n = 10$); **(b)** from 12 to 24 hours of LPS infusion ($r = 0.75$, $P < 0.01$, $n = 9$).

peroxidase-conjugated secondary antibodies (Zymed, San Francisco, CA, USA) at 22°C. Blots were incubated with ChemiLucent detection kit (Chemicon, Temecula, CA, USA). Immunopositive bands of PKC-α were detected with a Kodak Image Station 1000 (Kodak, Rochester, NY, USA) and densitometry readings were taken for statistical analysis.

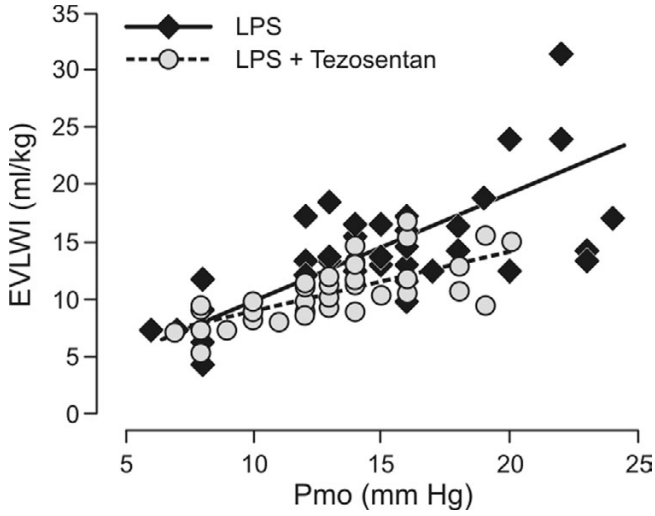
Biochemical measurements

The ET-1 plasma levels were measured by chemiluminescent enzyme immunoassay (QuantiGlo QET00; R&D Systems, Minneapolis, MN, USA). Plasma levels of TNF-α and of IL-8 were determined with an Immulite instrument (Diagnostic Products Corporation, Los Angeles, CA, USA).

Statistical analysis

Data were checked for normal distribution by the Kolmogorov–Smirnov test. The relationship between ET-1, Pmo, and EVLWI was evaluated by regression analysis with the Pearson correlation coefficient. Equality of regression lines between the LPS and the LPS plus tezosentan groups was tested by single multiple regression [21]. The detected relative amounts of PKC-α in the groups and tissue fractions were compared by one-way analysis of variance. Plasma concentrations of ET-1, TNF-α, and IL-8 were analyzed by analysis of variance for repeated measurements. If F was statistically significant, Scheffe's test was used for *post hoc* intergroup analysis. To evaluate differences within groups towards the baseline value (time 0 hours), we used test of contrasts. We regarded $P < 0.05$ as statistically significant.

Figure 2



Relationship between extravascular lung water index (EVLWI) and pulmonary micro-occlusion pressure (Pmo) in endotoxemic sheep. LPS alone ($r = 0.73$, $P < 0.01$, $n = 42$). LPS with tezosentan ($r = 0.67$, $P < 0.0001$, $n = 42$).

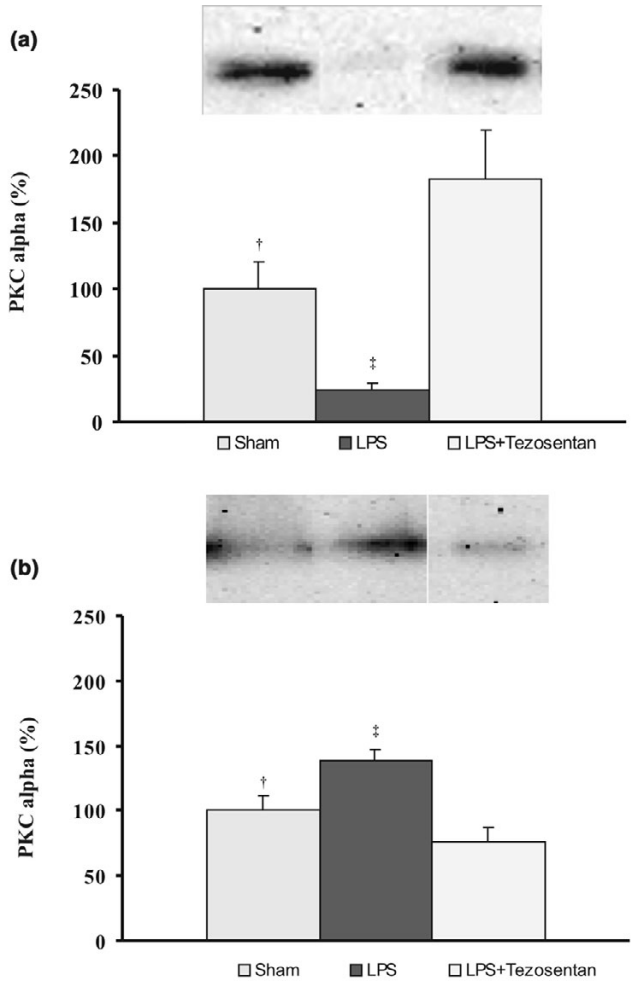
Results

In sham-operated sheep, all variables remained unchanged throughout the 24-hour experiments. During the first 12 hours we found no significant correlation between the plasma concentration of ET-1 and Pmo in sheep subjected to LPS (Fig. 1a). In contrast, we found a positive correlation between these variables beyond 12 hours ($P < 0.01$; Fig. 1b). A positive correlation also existed between Pmo and EVLWI in the LPS and the LPS plus tezosentan groups, as depicted in Fig. 2. However, tezosentan reduced the slope of the regression line compared with LPS alone ($P < 0.05$; Fig. 2).

As shown in Fig. 3, extracts of lung tissue from sheep exposed to LPS displayed a 75% reduction of the cytosolic fraction of PKC-α in comparison with samples from sham-operated animals ($P < 0.05$). The membrane fraction of PKC-α simultaneously increased by 40% in the LPS group compared with sham-operated sheep. Administration of tezosentan completely prevented the translocation of PKC-α from the cytosolic to the membrane fractions.

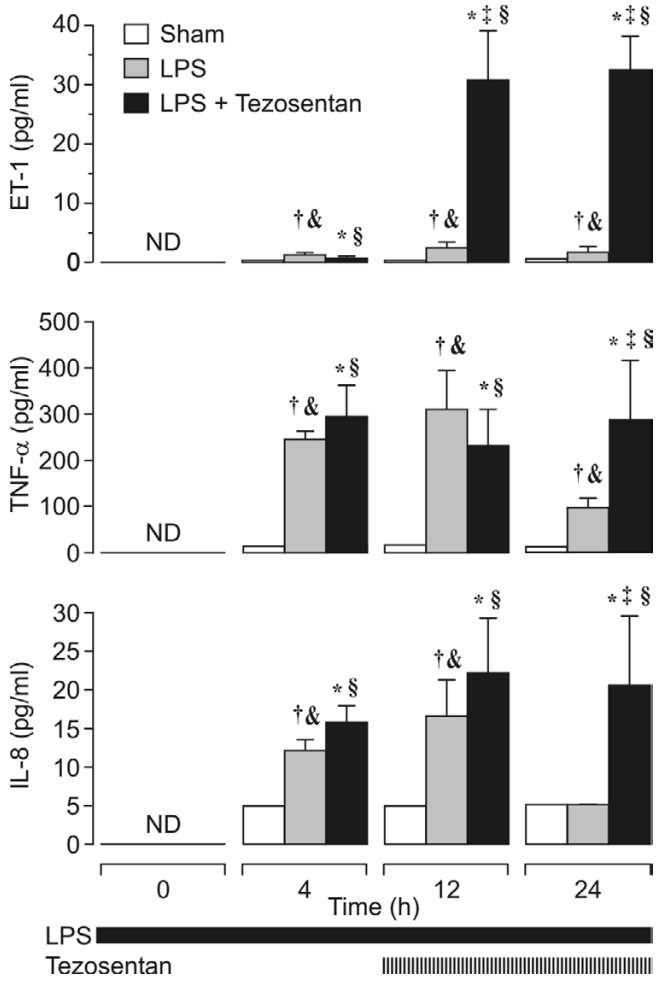
Figure 4 shows that after 4 hours of exposure to LPS, in parallel with the rise in ET-1, the plasma concentrations of TNF-α and IL-8 increased compared with intragroup baseline and sham-operated animals ($P < 0.05$). Notably, on cessation of the experiment, plasma concentrations of ET-1, TNF-α, and IL-

Figure 3



Protein kinase Cα (PKC-α) in sheep lung tissue homogenates detected by Western blotting. **(a)** In the cytosolic fraction; **(b)** in the membrane fraction. Results are means ± SEM. Groups were as follows: sham-operated group ($n = 3$); lipopolysaccharide group (LPS; $n = 4$); LPS plus tezosentan group ($n = 4$). $^{\dagger}P < 0.05$ between sham-operated and LPS groups; $^{\ddagger}P < 0.05$ between LPS and LPS plus tezosentan groups.

Figure 4



Plasma concentration of endothelin-1 (ET-1), tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8). Results are means ± SEM. Groups were as follows: sham-operated group ($n = 3$); lipopolysaccharide group (LPS; $n = 7$); LPS plus tezosentan group ($n = 7$). ND, not detectable. $^{\dagger}P < 0.05$ between sham-operated and LPS groups; $^{\ddagger}P < 0.05$ between LPS and LPS plus tezosentan groups; $^{*}P < 0.05$ between sham-operated and LPS plus tezosentan groups; $^{\&}P < 0.05$ from $t = 0$ hours in the LPS group; $^{\S}P < 0.05$ from $t = 0$ hours in the tezosentan group.

8 were significantly higher in the LPS plus tezosentan group than with LPS alone ($P < 0.05$).

Discussion

The present study shows that during the late phase of endotoxemia in sheep (12 to 24 hours), the plasma concentration of ET-1 is significantly correlated with the microvascular pressure, whereas no such correlation was found during the early phase. Moreover, we observed a significant and positive correlation throughout the experiment between microvascular pressure and EVLWI in both endotoxemic groups. Interestingly, the regression line had a significantly lower slope in animals receiving tezosentan. To our knowledge, this is the first study demonstrating that blockade of ET-1 receptors precludes endotoxin-induced changes in PKC-α in cytosolic and membrane fractions of lung tissue.

Pulmonary microvascular pressure and permeability are important determinants of lung edema [22]. As reported previously by our group and others, increases in EVLWI in animals exposed to infusion of LPS are associated with enhanced pulmonary microvascular pressure [9,23,24]. However, none of these investigators have focused on the relationship between the plasma concentration of ET-1 and the pulmonary microvascular pressure. There is therefore no general agreement about where in the course of illness, or how, ET-1 exerts its action. One previous report suggests that ET-1 contributes

directly to the severity of ALI by increasing the pulmonary microvascular pressure from the first hours of endotoxemia [25]. However, at variance with these results, we found that a fairly strong correlation between the plasma concentration of ET-1 and Pmo exists only in the late phase of endotoxemia. This is also in accordance with investigators who argue that thromboxane A_2 is the dominating mediator of vasoconstriction during the first hours of endotoxemia, whereas ET-1 is responsible for vasoconstriction in the late phase [26-29].

The significantly positive correlation between Pmo and EVLWI in endotoxemia, and the decrease in the relationship after treatment with tezosentan, agrees fully with a recent investigation in endotoxemic pigs [30]. However, the beneficial effects of ET-1 blockade cannot be explained solely by attenuation of the endotoxin-induced increase in pulmonary artery pressure. The declining slope of the regression line between Pmo and EVLWI in tezosentan-treated animals indicates that additional factors affecting lung fluid filtration might be active. A few years ago, investigators found that ET-1 increases fluid filtration in isolated blood-perfused rat lungs pretreated with papaverine to deprive the lungs of any vascular tone [14]. Because the ability of the lung microvascular pressure to increase was precluded, the authors interpreted their findings as a result of increased permeability. However, the exact mechanisms involved still remain obscure.

PKC consists of a set of different isoenzymes: classical (α, β, γ), novel (ε, δ, θ, η), and atypical (ξ, λ), of which only the classical isoforms are sensitive to changes in intracellular Ca^{2+} concentration [31]. Recent studies have shown that ET-1 stimulates the release of Ca^{2+} from the endoplasmic reticulum and activates PKC-α in the cell membrane [15-19]. After being activated, PKC-α has been shown to mediate the disruption of vascular endothelial cadherin junctions [32]. Moreover, PKC-α activates myosin light chain kinase, which is involved in endothelial cell gap formation and barrier dysfunction [33]. In the lung vasculature, PKC-α-induced disruption might derange the endothelial integrity [19]. We therefore speculate that the increase in vascular permeability and the evolution of ALI might be due to ET-1-induced activation of PKC-α in the cell membrane. We believe that blockade of ET-1 receptors, resulting in a combination of reduced microvascular pressure and decreased activation of PKC-α, is one of the main reasons for the amelioration of ALI in the present study.

It is well established that infusion of LPS stimulates a release of inflammatory mediators such as TNF-α, IL-8, and ET-1 [34-36]. In contrast, ET-1 stimulates monocytes and macrophages to release TNF-α and IL-8 in its own right [37,38]. In the present study, enhanced plasma concentrations of TNF-α, IL-8, and ET-1 were found after 4 hours in both endotoxemic groups. However, at the end of the experiments the plasma concentrations of all three mediators were significantly higher in tezosentan-treated animals than in animals given LPS alone. The increases in ET-1 and TNF-α are consistent with a previous investigation employing the endothelin receptor antagonist bosentan [39], but in contrast to that short-term study, we exposed sheep to 24 hours of endotoxemia. According to another recent study, ET_B receptors in the lungs are involved in the clearance of ET-1 from the circulation [40]. Consequently, ET-1 receptor blockade prolongs ET-1 half-life in the plasma and reportedly shifts tissue uptake from the lungs to other organs [41]. In the present study, tezosentan increased the plasma concentration of ET-1 to an extent that might have enhanced the release of TNF-α and IL-8 from the monocytes and macrophages.

The present endotoxin-induced lung injury model in sheep is not ideal for elucidating the effects of ET-1 receptor blockade on permeability because microvascular pressure cannot be deliberately changed. Further studies of ET-1 receptor blockade on permeability are therefore required in a more complex experimental setting on intact animals or in isolated perfused lungs.

Conclusion

In endotoxemic sheep, ET-1 plasma concentration is significantly correlated with Pmo in the late phase. Moreover, Pmo and extravascular lung water content demonstrate a positive correlation from the first hours of endotoxin infusion. Blockade of ET-1 receptors attenuates ALI by reducing the pulmonary microvascular pressure and most probably also by decreasing permeability secondary to reducing the activation of PKC-α. However, further studies are needed to explain the exact mechanisms behind the decrease in extravascular lung water and the prevention of activation of PKC-α after ET-1 receptor blockade.

Key messages

- In endotoxemic sheep, extravascular lung water content correlates positively with pulmonary microvascular pressure.
- Non-selective endothelin-1 receptor blockade attenuates ovine endotoxin-induced lung injury by reducing pulmonary microvascular pressure and probably also by decreasing microvascular permeability secondary to reduced activation of PKCα.

Competing interests

This study was supported by Helse Nord (Norwegian governmental funds), project number 4001.721.132 and departmental funds of the Departments of Anesthesiology, Physiology and Clinical Chemistry, University of Tromsø, Norway.

Authors' contributions

VK participated in the design of the study, analyzed the data, and drafted the manuscript. MK, MS, TA, OCI and KY contributed to the biochemical analysis and participated in the design

of the study. LB administered the study, participated in the design of the study and suggested improvements to the manuscript. All authors read and approved the final manuscript.

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