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研究課題名（和文） LPS 誘発急性肺障害発症機序におけるＶＥＧＦ，ＰＡＲs とエンドセリンの関与と制御
研究課題名（英文） Time-dependent alterations of VEGF and its signaling molecules in acute lung injury in a rat model of sepsis
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研究成果の概要（和文）：リポ多糖(LPS)誘発性の急性肺障害モデルにおいて、血管内皮細胞増殖因子（VEGF)、プロテアーゼ活性化受容体(PARs)等の肺障害発生病態における関与を研究した。LPS 投与後のラット肺では、VEGF 蛋白発現の低下、VEGF 受容体発現の変化（Flt-1 増加、Flk-1 低下)、内皮 NOS(eNOS)低下が認められた。それらの変化は各種のアポトーシス関連蛋白(Caspase 3, BAX, Bcl2, pAkt)や血液凝固・炎症反応増強機序である PARs 系の発現の変化を伴って、肺組織内の微小循環動態や血管透過性の異常をもたらす可能性が示唆された。

研究成果の概要（英文）：Sepsis, one of the leading causes of morbidity and mortality, is associated with the development of acute lung injury (ALI). However, molecular mechanisms of ALI are poorly defined. Since vascular endothelial growth factor (VEGF) is a potent vascular permeability and mitogenic factor, it might contribute to the development of ALI in sepsis. Thus, using lipopolysaccharide (LPS)-induced (15mg/kg, I.P) endotoxemic rat model, we studied the time-line (1, 3, 6 and 10h) of pulmonary VEGF expression and its signaling machinery. Levels of pulmonary VEGF and its angiogenic-mediating receptor, Flk-1, were down regulated by LPS in a time-dependent manner; vascular permeability-mediating receptor of VEGF, Flt-1, in contrast, was up regulated with time. Expression of signaling, pro- and or apoptotic factors after LPS administration were as follow: phosphorylated Akt, a down stream molecule was down regulated time-dependently; endothelial nitric oxide synthase (eNOS) levels were significantly reduced; pro-apoptotic markers Caspase-3 and Bax were up regulated, whereas, levels of Bcl-2 were down regulated. The present findings show that VEGF may not play a role in increased pulmonary vascular leakage in LPS-induced ALI. Moreover, down regulation of VEGF signaling cascade may account for LPS-induced apoptosis and impaired physiological angiogenesis in lung tissues, which in turn may contribute to the development of ALI induced by LPS. In addition, blockade of Flt-1 could improve the downregulated pulmonary VEGF level and attenuate the elevated plasma and pulmonary levels of TNF-alpha.

交付決定額

（金額単位：円）

	直接経費	間接経費	合　計
２００８年度	1,600,000	480,000	2,080,000
２００９年度	1,700,000	510,000	2,210,000
年度			
総　計	3,300,000	990,000	4,290,000

研究分野：
科研費の分科・細目：
キーワード：Acute lung injury (ALI); Apoptosis; endothelial nitric oxide synthase (eNOS); Lipopolysaccharide; Vascular endothelial growth factor　(VEGF)

１．研究開始当初の背景

Sepsis in human is a disease state associated with a generalized activation and expression of inflammatory signaling pathways. However, the chronological sequence and exact mechanisms associated with other cellular and molecular events, and underlying the pathogenesis of sepsis are not yet completely understood. Acute lung injury and acute respiratory distress syndrome (ALI/ARDS), are major causes of mortality in intensive care units, and are characterized by hypoxemia, pulmonary infiltration, absence of an elevated pulmonary capillary wedge pressure, pulmonary neutrophil sequestration, intravascular coagulation, disruption of pulmonary capillary integrity leading to edema, and increased shunt fraction. The majority of these pathologic features of human ALI/ARDS have also been observed in experimental animals, in response to systemic infusions of live bacteria or endotoxin of gram-negative bacteria .

Vascular endothelial growth factor (VEGF), a potent inducer of endothelial cell growth in vitro and angiogenesis in vivo, plays a crucial role in a variety of disease conditions through the promotion of angiogenesis and by its vaso-permeability effects. It also plays a critical role, as a potent proinflammatory cytokine, in a variety of physiological and pathological immune responses. The biological effects of VEGF are largely mediated by two receptors, namely kinase domain region (KDR or Flk-1) and fms-like tyrosine kinase-1 (Flt-1). Protein kinase Akt, also referred to as protein kinase B (PKB), and nitric oxide (NO), are the most common key effector molecules mediating VEGF signaling. Thus, their levels and/or activities are, along with VEGF, likely to be altered in abnormal conditions. Since endothelial injury to large vessel and microvascular endothelium, such as development of capillary leakage, is a common occurrence in heart and lung injuries of patients with sepsis, it is likely that several sepsis-associated mediators, including lipopolysaccharide (LPS), induce VEGF production. It has been observed that VEGF inhibits LPS-induced endothelial cell apoptosis in vitro, and that it (VEGF) acts as a survival factor for endothelium. Recently, a potential role for VEGF in sepsis has been evaluated. For example, plasma VEGF levels are elevated during severe sepsis and are (VEGF plasma levels) associated with disease severity and mortality. More recently, increased plasma VEGF levels were observed during the first 48 hours of human septic shock. We have demonstrated that VEGF and its vascular permeability-mediating receptor are increased in liver tissues in LPS-induced endotoxemia in a time-dependent manner. However, the differential or organ-specific expressions of VEGF and its signaling cascade during sepsis have not been adequately investigated yet.

２．研究の目的

Since the lung is one of the primary organs affected by injury during sepsis, which subsequently critically impairs lung function, and often leading to ALI/ARDS and death, it is essential that the specific role of factors that regulate pulmonary vasculature in sepsis-associated fatalities be delineated. Thus, in the present study we used LPS-induced endotoxemia in a rat model, to study the time-line or chronological sequence of VEGF expression pattern, and its basic signaling machinery (receptors [Flk-1 and Flt-1], downstream molecules [phosphorylated Akt {pAkt} and endothelial nitric oxide synthase {eNOS}]) in lung tissues. Moreover, the extent of LPS-induced lung injury was assessed by 1) histology, 2) blood gas analysis and by 3) determining pulmonary wet to dry weight ratio and bronchoalveolar lavage fluid (BALF) albumin level. To have more insights into the alteration of VEGF in endotoxemic lung tissues, some important molecules related to apoptosis were also studied under current experimental settings. In addition, the status of endotoxemia was confirmed by blood pressure changes, plasma and pulmonary tumor necrosis factor-alpha (TNF-α) and iNOS levels as reported earlier. In the 2nd part of this study, we assessed the effects of blockade of Flt-1 on the plasma and pulmonary level of key target molecules.

３．研究の方法

Male Wistar rats (200-250 gm, 8 weeks old) were used in all experiments. Endotoxemia was induced by the intra-peritoneal (IP) administration of bacterial LPS from Escherichia coli 055: B5 (15mg/kg), dissolved in sterile saline. This dose of LPS was sufficient to induce lung injury, as well as the inflammatory cytokine. The control group received an equal volume of vehicle (sterile saline) (2 ml/body), without LPS. The different groups of animals (n =30 for each time point) were killed by Nembutal (Sodium Pentobarbital, I.P., 80 mg/kg body weight) at different time points after LPS or vehicle only (1, 3, 6, and 10 hours). The blood samples were collected by cardiac puncture for blood gas analysis, and lungs tissues were harvested gently, frozen immediately in liquid nitrogen, and stored at -80°C. For paraffin sections, the lung tissues were postfixed in 4% paraformaldehyde overnight and then processed routinely for paraffin embedding. All animals received care that was in compliance with the institutional guidelines and the experimental procedures were approved by the Animal Care and Use Committee of Hokkaido University Graduate Schools of Medicine. It should be noted that, a time course study for the control rats was also conducted in the current investigation.

In the second part of the study, in order to investigate the specific role of Flt-1 in the LPS-induced ALI, the Flt-1 blocking peptide (Flt-1 BP) was used to treat the rats in addition to LPS. For this purpose, rats were anesthetized with urethane (35 % ethyl carbamate [Wako Pure Chemical Industries, Osaka, Japan]+ 4 % alpha-chloralose [Wako] saline wt/vol, 0.4 to 0.8 g/kg, ip), and the left jugular vein was cannulated for drug administration. All drugs being tested were administered intravenously as a slow bolus injection. Based on our previous and pilot studies, LPS (15mg/kg, intravenous [iv]) was administered through the jugular vein at time 0 in different groups of rats, and then the rats were sacrificed after 6 hours. However, for the LPS+Flt-1 BP group, thirty min before LPS administration, Flt-1 BP (sc- P, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was administered intravenously as a slow bolus injection (100 µg/kg, 500µl/kg PBS) and then LPS injection was done, and after LPS administration, Flt-1 BP was continuously infused (20 µ/kg/hour, 100 µl PBS /hour) through the jugular vein with a pump for 6 hours (n=12) for each group). Non-treated rats were used as a control.

Outline of Measurements are as follows:
Wet-to-Dry Weight Ratio, BAL Fluid Collection and Albumin Measurement, Histopathology Examination, Western Blot Analysis, Immunohistochemistry, ELISA, Cell death detection ELISA, Nitric oxide colorimetric assay, RNA preparation and Real-Time quantitative polymerase chain reaction (PCR).

４．研究成果

(1) Effects of LPS on blood gas parameters, hemodynamic parameters, plasma and

pulmonary TNF-alpha and iNOS levels
Arterial PaO2 was significantly reduced from control at 1, 3, 6 and 10 h after LPS administration. Arterial PaCO2 was significantly reduced from the baseline at all time points after LPS administration. There was a significant increase in arterial pH starting from 3h to 10h after LPS administration. Base excess was markedly and progressively lowered from control throughout the experiment. Blood lactate concentrations were statistically different from control at all time points after LPS. As a quantitative measure of fluid clearance in lungs, wet-to-dry weight ratios were evaluated in lungs removed from rats killed at specified times after LPS administration. The effect of LPS on the ratios occurred in a time-dependent manner. Thus, the ratios were significantly ($P<0.05$) increased from the baseline value (4.25 ± 0.18) to a peak value (6.21 ± 0.22) after LPS administration. For assessment of changes in pulmonary vascular permeability, we also measured the BALF albumin level. Compared with the finding in control rats, a significant increase in albumin level in BALF was seen after LPS administration, which was consistent with our previous report. The peak plasma TNF-α levels and the amount of TNF-α in lung tissue were significantly elevated 1 h after LPS administration. The plasma iNOS levels, as well as the pulmonary expression level of iNOS both at protein and mRNA levels, increased markedly after LPS administration. Both the systolic and diastolic blood pressures significantly decreased at different time points after LPS administration compared to control rats, as reported in our recent study.

(2)Histopathology after LPS Administration
The lungs from the control rats showed no histologically detectable injury. In contrast, the lungs from animals treated with LPS showed congestion, neutrophil infiltration and thickening of alveolar septum after 1 hr. The same changes were also observed at 3 h and 6 h after LPS administration. At 10 h, following the administration of LPS, the features of lung injury, described above, became more evident. The lungs showed more congestion, infiltration of inflammatory cells in the alveoli and a thickening of alveolar septum, which are collectively called glanulomatous changes.

(3) Expression of VEGF and its receptors
The plasma level of VEGF was increased after LPS administration, compared to that of control rats, as revealed by ELISA. The protein expression VEGF in pulmonary tissue was down regulated in septic rats in a time-dependent manner, as demonstrated by both ELISA and immunoblotting. In contrast, protein levels of the vascular permeability-mediating receptor of VEGF, Flt-1, increased in lung tissue after LPS administration, compared to the control group, while, on the other hand, levels of the angiogenic-mediating receptor of VEGF, Flk-1, decreased after LPS administration, as revealed by immunoblot analysis and ELISA. Using Western blot analysis, VEGF, Flk-1, and Flt-1 proteins in the rat pulmonary tissues extracts were detected as single bands migrating at 45, 200, and 180 kDa, respectively. When we evaluated steady-state mRNA levels of VEGF and its receptors by real-time PCR, the mRNA level of VEGF and its receptors were also altered in LPS-treated rats in a time-dependent manner, suggesting that the alteration in pulmonary expression of VEGF and its receptor protein in septic rats likely occurred at the level of gene expression.

(4) Expression of pAkt and eNOS
We found significantly lower levels of pAkt in pulmonary tissues after LPS administration at all time points examined, as revealed by ELISA, compared to the control group. Immunoblot analysis using the antibody that specifically reacts with pAkt was clearly visualized as a major band with a molecular mass of 55 kDa in rat pulmonary tissues. Data generated from immunoblot analysis was almost consistent with that of the ELISA. Levels of eNOS protein in the pulmonary tissues after LPS administration were assessed using ELISA, showing a reduction at all time points after LPS administration compared to that of the control group, with the peak reduction occurring at 1h after LPS administration. The findings of eNOS by ELISA were confirmed by immunoblot analysis, where the molecular weight of the band was as predicted (140 kDa).

(5) Cellular localization of VEGF, Flk-1, Akt and eNOS

It should be noted that besides in the blood vessels in lung tissue, VEGF and its signaling molecules (Flk-1, pAkt and eNOS) were abundantly expressed in alveolar and bronchial structures, including epithelium and glands. In LPS-treated rats, the expression was greatly diminished. In contrast, Flt-1 expression in lung tissue after LPS administration was opposite.

(6) Cell death detection ELISA

To assess whether endotoxemia causes or is associated with apoptotic cell death in the pulmonary tissues, we performed cell death detection ELISA. The percentage of pulmonary cell death in the ELISA assay significantly ($p<0.01$) increased at 10 h after LPS administration, compared to control rat.

(7) Protein and mRNA expression of pro- and anti-apoptotic regulators

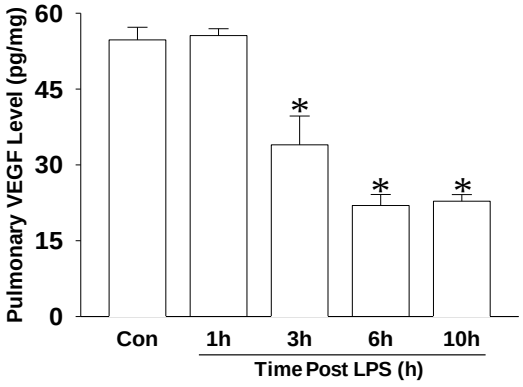
We observed that Caspase-3 activity in lung tissues increased significantly at later hours after LPS administration, compared to control rats. Consistent with the findings of Caspase-3, the protein expression of another pro-apoptotic marker, BAX was upregulated in the lungs of LPS-administered rats, compared to control rats, particularly in the later hours after LPS administration, as demonstrated by immunoblot analysis. The mRNA expression of Bax obtained from Real-Time PCR was consistent with the protein expression, suggesting that LPS likely influenced both transcription and translation. In contrast, LPS administration diminished expression of Bcl-2, an important anti-apoptotic marker, in pulmonary tissues at both protein and mRNA level.

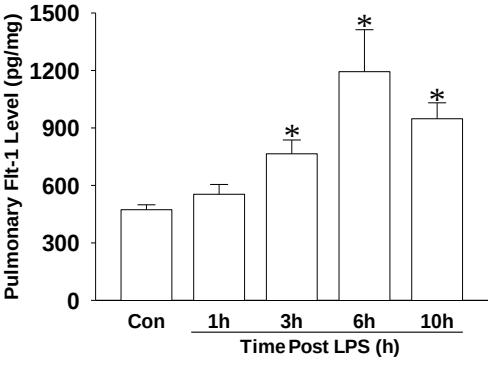
(8) Plasma Albumin Level

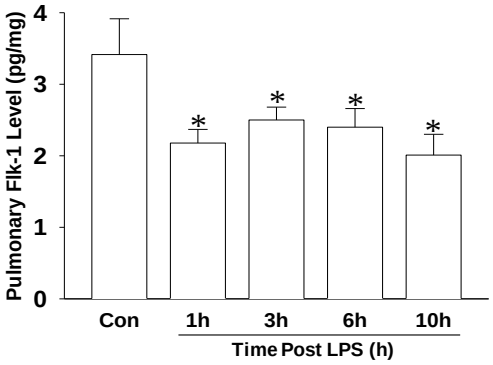
A significant decrease in the concentration of plasma albumin was observed at 3 h after LPS injection, as reported earlier, a concentration that gradually recovered. Plasma albumin levels were, in (g/dl), Con: LPS 1h: LPS 3h: LPS 6h: LPS 10h: 2.02±0.14 : 1.94±0.10 : 1.44±0.10 : 1.72±0.15 : 1.78±0.13.

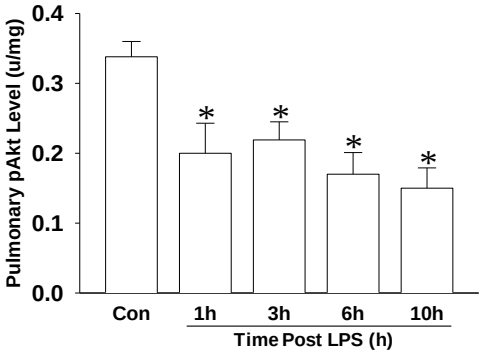
(9) Effects of Flt-1 blockade:

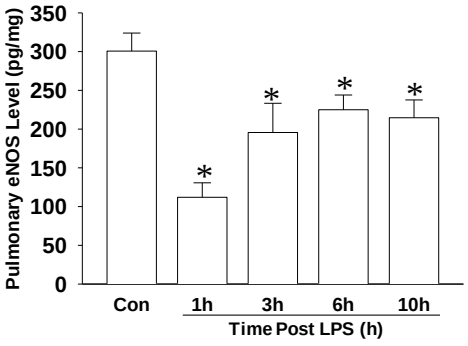
In order to explore the differences in the Flt-1 expression in relation to the time-kinetics and the patterns of expressions in the LPS-treated rats, Flt-1 BP was administered as a bolus injection followed by continuous infusion for 6h. From our detailed investigation, we found that the blockage of Flt-1 at 6h was the most beneficial in terms of reversing the different parameters related to proinflammatory cytokine, and VEGF expression. It should be noted that the Flt-1 BP dosage used in the present study was selected based on the findings of preliminary research using different doses of Flt-1 BP. The increased plasma and pulmonary TNF-alpha was significantly decreased by the blockade of Flt-1. Increased TNF-alpha in BAL fluid tended to be decreased by Flt-1 BP. The downregulated VEGF expression in lung tissues in sepsis was greatly improved with the treatment of Flt-1 BP. After the blockade of Flt-1, there was a significant improvement in some components of blood gas analysis as well as in wet-to-dry weight ratios of lungs.

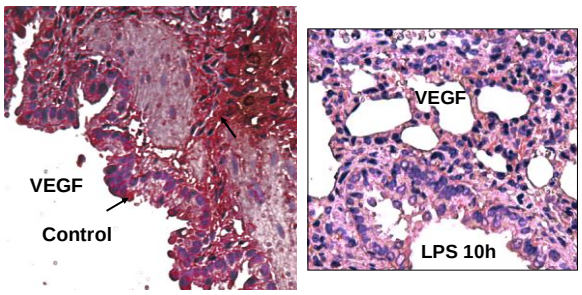


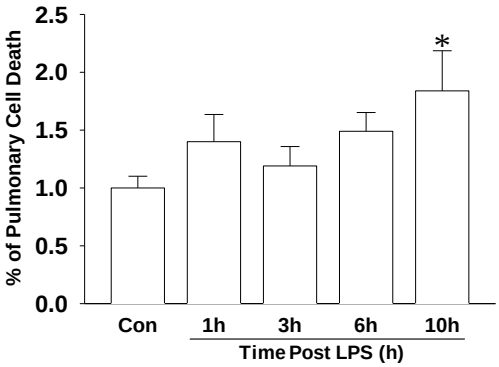


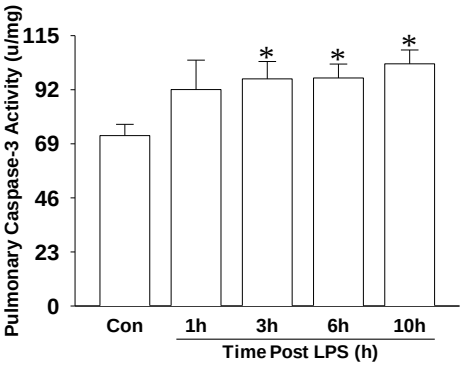


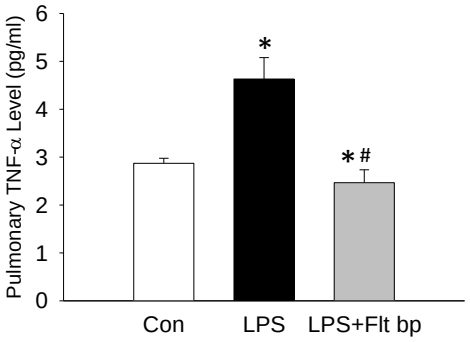


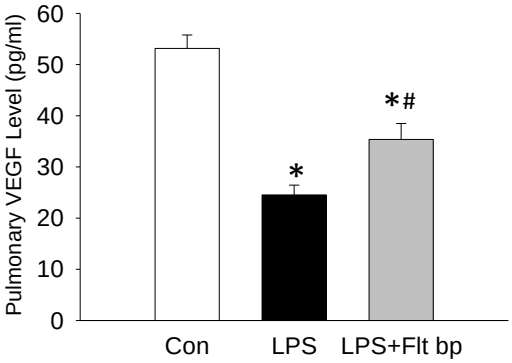












５．主な発表論文等

（研究代表者、研究分担者及び連携研究者には下線）

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〔図書〕（計 0 件）

〔産業財産権〕
○出願状況（計 0 件）

〔その他〕
ホームページ等
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