The effect of lipopolysaccharide (LPS) on the anti-coagulant factor endothelial protein C receptor (EPCR) in vivo

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Abstract

Background: The endothelial protein C receptor (EPCR) is the main member in anticoagulant system. EPCR is regulated by various molecules in its upstream. But most of studies about EPCR and its regulation mechanism are derived from in-vitro experiments.

Aims: To observe the effect of Lipopolysaccharide (LPS) on EPCR and the potential regulatory molecules in vivo.

Methods: We treated mice with LPS solution and collected the total protein of thoracic aorta, heart and lung tissues to determine the expressions of EPCR, tumor necrosis factor-α converting enzyme (ADAM17), p-ERK and protein kinase C δ (PKC δ) by immunoblotting.

Results: The results showed that, in normal mice, thoracic aorta only expressed truncated EPCR, but heart and lung expressed truncated EPCR and mature EPCR. LPS treatment caused the reduction of both of the two EPCR. Further results exhibited that LPS treatment increased the expressions of ADAM17, p-ERK and PKC δ protein in the three tissues.

Conclusion: LPS may activate PKC δ and ERK pathway to regulate the decrease or shedding of EPCR mediated by ADAM17 in vivo. These findings may help to elucidate the mechanism of coagulation disorders caused by invading pathogens in addition to therapeutic targets.

Keywords: Endothelial protein C receptor, tumor necrosis factor-α converting enzyme, protein kinase C δ

1. Introduction

Lipopolysaccharide (LPS), a component of the endotoxins released by Gram-negative bacteria, is a common and prominent activator of inflammatory response (RAETZ & WHITFIELD, [1]). It can induce large amounts of inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukins, leukotrienes, nitric oxide (NO), and prostaglandin E2 (PGE2) (CI & al. [2]), thereby contributing to Gram-negative sepsis, disseminated intravascular coagulation (DIC) and multiple organ dysfunction syndromes in vivo. Gram-negative bacteria release their endotoxin into the bloodstream, the LPS and inflammatory mediators can induce the expression of coagulation-related factors, such as tissue factor (TF), on mononuclear cells (FRANCO & al. [3]). They can also change endothelial lining of blood vessels from the anticoagulant and profibrinolytic surface into the one that is procoagulant (LIBBY & SIMON [4]).

Inflammation activates coagulation mainly through three mechanisms, including up-regulation of procoagulant pathways, down-regulation of physiological anticoagulants and inhibition of fibrinolysis (TAUBMAN & al. [5]). The protein C (PC) system is the main anticoagulant system in vivo, which consists of PC, thrombomodulin (TM), protein S (PS) and endothelial PC receptor (EPCR) (HAYASHI & SUZUKI, [6]). It also may affect other procoagulant system during the process of inflammation. PC can be activated by the thrombin–TM–EPCR complex and converted into activated PC (APC) (ESMON [7]). APC
has been known as a natural and effective anticoagulant factor, due to its activities of suppressing further thrombin formation and increasing fibrinolytic activity by neutralizing plasminogen activator inhibitor 1 (PAI-1) (VAN DE WOUWER & al. [8]). At the same time, APC has potent anti-inflammatory properties (TOLTL & al. [9]). Therefore, once PC system has been destroyed, inflammation and coagulation response will be augmented. For example, in the bacterial infection, more than 80% of patients have an APC deficiency which is closely associated with mortality (FISHER & YAN [10]).

EPCR is a type I transmembrane protein and is structurally similar to the major histocompatibility complex class 1/CDI family of proteins (FUKUDOME & ESMON [11]). EPCR binds protein C to enhance the formation of APC approximately 6-fold in cell culture and 20-fold in vivo (TAYLOR JR & al. [12]). The function of EPCR is blocked by various inflammatory mediators. The reason is that, under the inflammatory condition, the expression of total EPCR or membrane EPCR (mEPCR) have been affected. Studies in vitro have suggested that TNF-α and IL-1β decreased the levels of cell-associated EPCR protein and the transcription of EPCR mRNA in HUVEC (NAN & al. [13]), and enhanced the shedding of EPCR on cells (WANG & al. [14]). The mechanisms of decreasing EPCR expression and increasing EPCR shedding are regulated through MAPK signaling pathway and tumor necrosis factor-α converting enzyme/ADAM17 that is the most important EPCR cleavage enzyme (QU & al. [15]). However, most of these data are derived from the experiments in vitro. The expression of EPCR in vivo, especially under the LPS challenge, has not been clearly observed.

2. Materials and methods

Main Reagents

LPS from *Escherichia coli* O55:B5 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit polyclonal anti-EPCR/CD201 and mouse monoclonal anti-ADAM17 antibodies were purchased from Abcam (Cambridge, UK). PKC Isoform Antibody Sampler Kit #9960, MAPK Family Antibody Sampler Kit #9926, Phospho-MAPK Family Antibody Sampler Kit #9910 were obtained from Cell Signaling (Danvers, MA, USA). Mouse monoclonal anti-beta-actin (sc-47778), goat anti-mouse IgG-HRP (sc-2005) and donkey anti-goat IgG-HRP (c-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Mice

Specific pathogen-free male and female Kunming mice, weighing approximately 18 to 20 g, were purchased from Chengdu Dashuo experimental animal Co. Ltd (Certificate SCXK2003-0003) (Chengdu, China). The mice were maintained in a temperature- and humidity-controlled room and received food and water ad libitum. All animal experiments were performed in accordance with the Ethical Principles and Guidelines for Experiments on Animals approved by Sichuan Agricultural University.

LPS treatment

To make an endotoxemic model, mice were randomly divided into four groups (n=20 for each group) and challenged with 2.5mg/kg, 5mg/kg, 10mg/kg dosages of LPS by intraperitoneal (i.p.) injection or normal saline as a control. After LPS injection, blood and organs were collected for further analysis at 24h, 36h and 48h.

Western blot

Tissue samples of aorta, heart and lung were separated from mice challenged with LPS for 24h, 36h and 48h or control mice, respectively. These samples (average mass of <20 mg each sample) were cut into small pieces and ground broken fully in 100 μl of the lysis buffer (Beyotime, Jiangsu, China). After incubating the lysates on ice for 20 min, the mixtures were centrifuged (12000 g) for 10 min to harvest total proteins. Protein concentrations were measured by using Enhanced BCA Protein Assay Kit (Beyotime, Jiangsu, China). Equal amounts of whole proteins (40 μg/lane) were separated on 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) based on the molecular weight of target proteins.
Proteins were transferred to polyvinylidene difluoride (PVDF) membrane with wet blotting method. After blocked with 5% non-fat dry milk of TBST buffer at room temperature for 1 h, the membrane was incubated with primary antibodies at 4°C overnight and then incubated with secondary antibody conjugated to peroxidase at room temperature for 1 h. Peroxidase-bound antibodies were detected using the Western Blotting Luminol Reagent (Santa Cruz, CA, USA).

**Statistical Analysis**

Data were expressed as means ± standard deviation. Differences between mean values of data were assessed by the one-way ANOVA (Dunnett’s t-test). Statistical difference was accepted at $P<0.05$.

### 3. Results

**LPS decreases the expression of EPCR in mice**

As shown in Fig. 1A, EPCR expressions in thoracic aorta were decreased at 24 h and 36 h after LPS treatment. But at 48 h, there is no any change of EPCR expression among the different groups. After that, we detected EPCR expressions in heart and lung of LPS-stimulated mice at 24 h. As shown in Fig. 1B and C, EPCR expressions in heart and lung were different from that in thoracic aorta. There were two bands of EPCR protein both in heart and lung which represent different molecular weight of EPCR protein, while there was only one in thoracic aorta. However, LPS still decreased the EPCR expressions both in heart and lung in a dose-dependent fashion.

![Figure 1](image-url)  
*Figure 1* EPCR protein expression in thoracic aorta, heart and lung of mice. (A) Immunoblot probing for EPCR expression in thoracic aorta of mice treated with either nothing or different concentration LPS (2.5 mg/kg, 5 mg/kg and 10 mg/kg) at 24 h, 36 h, and 48 h. **$P<0.01$ versus blank group without LPS. (B, C) Immunoblot probing for EPCR expression respectively in heart and lung of mice treated with either nothing or different concentration LPS (2.5 mg/ml, 5 mg/ml and 10 mg/ml) at 24 h.
LPS increases the expression of ADAM17 and p-ERK1/2 in mice

One of the reasons for the reduction of EPCR protein is metalloproteinases such as ADAM17. Therefore, we measured the expression of ADAM17 protein in thoracic aorta, heart and lung of LPS-treated mice at 24h. As shown in Fig.2, the expression of ADAM17 protein in thoracic aorta was increased under the stimulation of LPS. The ADAM17 expression in middle and high concentrations of LPS groups were significantly raised than that in blank group. In heart and lung tissue, all of three different concentrations of LPS markedly increased ADAM17 expression.

The reason of the reduction of EPCR expression can also be attributed to the phosphorylation of ERK1/2. Then, we detected the expression of p-ERK1/2 in those three tissues of LPS-treated mice. As shown in Fig.2B, LPS significantly increased the phosphorylation of ERK1/2 in all of three tissues.

![Figure 2](image)

Figure 2 ADAM17 and p-ERK1/2 protein expression in thoracic aorta, heart and lung of LPS-treated mice. (A) Immunoblot probing for ADAM17 expression in thoracic aorta, heart and lung of mice treated with either nothing or different concentration LPS (2.5 mg/kg, 5 mg/kg and 10 mg/kg) at 24 h. (B) Immunoblot probing for p-ERK1/2 expression in thoracic aorta, heart and lung of mice treated with either nothing or different concentration LPS (2.5 mg/ml, 5 mg/ml and 10 mg/ml) at 24 h. *P<0.05, **P<0.01 versus blank group without LPS.

LPS increases the expression of PKC δ protein in mice

ADAM17 expression is mostly regulated by PKC δ. So, we firstly observed the expression of PKC δ in thoracic aorta, heart and lung of normal mice. As shown in Fig. 3A, PKC δ were expressed in all these tissues of mice. Then, we mainly determined the expression of PKC δ protein in all three tissues of LPS-treated mice at 24 h. As shown in Fig.
3B-E, LPS concentration-dependently increased the expression of PKC δ protein in thoracic aorta, heart and lung of mice.

**Figure 3** Expression of PKC δ protein in thoracic aorta, heart and lung of LPS-induced mice. (A) Immunoblot analysis of expression of PKC isoforms in thoracic aorta, heart and lung of mice without LPS treatment. (B-D) Immunoblot probing for PKC δ expression in thoracic aorta (B), heart (C) and lung (D) of mice treated with either nothing or different concentration LPS (2.5 mg/kg, 5 mg/kg and 10 mg/kg) at 24 h. (E) Densitometry quantification of PKC δ bands. The levels of PKC δ protein were comparable in all three tissues. Data are expressed as means ± SEM of three mice. ***P<0.01 versus blank group without LPS.

**4. Discussion**

It is well known that EPCR is a key factor of anticoagulant system, people therefore are interested in the mechanism of EPCR damage during various diseases, especially sepsis. Although there are published papers revealing the mechanism of EPCR damage, they are still limited in in-vitro (QU & al. [15], MENSCHIKOWSKI & al. [16]). Here, we observed the expression of EPCR in thoracic aorta, heart and lung of mice and found that the EPCR protein was differently expressed in these three tissues, and the in vivo response to LPS was to down-regulate EPCR protein levels.

Aorta, heart and lung are the central tissues of blood circle, which are firstly affected by invading pathogen in vivo. Accordingly, in our research, we investigated the effect of LPS on EPCR expression in these three tissues. X. YE & al. [17] reported that EPCR widely existed in the endothelial cells of arteries, veins, and capillaries in the lung, heart, and skin. K. FUKUDOME & al. [18] also published that the most abundant expression of EPCR was detected on the endothelial cells of the aorta. But, their findings just came from immunohistochemical test and did not relate to LPS treatment (FUKUDOME & al. [18], FAUST & al. [19]). Herein, we have used the immunoblotting to determine the expression of EPCR in thoracic aorta, heart and lung. The results showed that all of these three tissues...
expressed the 27kD EPCR protein called truncated EPCR which is the remaining transmembrane domain of whole EPCR after cut by cleavage enzyme (BIGUZZI & al. [20]). But there’s an additional EPCR protein which its molecular weight is about 50kD called mature EPCR in heart and lung tissues, except thoracic aorta. The results indicated that the EPCR protein might be regulated by different mechanisms in these normal tissues. However, our result also showed that LPS could decrease the amount of EPCR in these three tissues, both the truncated EPCR and mature EPCR, confirming that the dysfunction of PC system will happen in thoracic aorta, heart and lung when LPS or gram-negative bacteria invade into body.

We next studied the reason why the expression of EPCR was decreased. Many researchers report that surface EPCR protein can be shed from cell surface under the stimulation by inflammatory mediators such as TNF-α and IL-1β and by thrombin (ESMON [21]), which can finally contribute to the reduction of total EPCR. It has been further revealed that EPCR shedding is mediated by ADAM17 (QU & al. [15]), and increase in the expression of ADAM17 up-regulates the shedding of EPCR (MENSCHIKOWSKI & al. [22]). These points suggested that, in our results, one of the reasons of EPCR decrease was due to the raised ADAM17 in thoracic aorta, heart and lung of LPS-induced mice. Furthermore, ADAM17 is an important member of the ADAM family and is synthesized as a zymogen. Chen et al. reported that ERK activation increased the processing of proADAM17 into ADAM17 (CHEN & al. [23]). We hence detected the phosphorylation of ERK1/2 in those three tissues. The results showed that LPS challenge enhanced the expression of p-ERK1/2, which resulted in the activation of ERK pathway. According to the analysis above, it is possible that the ADAM17 expression is raised by the activation of ERK, which cause the following decrease of EPCR in our study. However, there are other members in ADAM family such as ADAM9, 10, 12, 15 (SCHÄFER & al. [24], SANDERSON & al. [25], FRIDMAN & al. [26]), which also have sheddase activity. The relationship between these members and decease of EPCR need further study.

PKC δ is a predominant isoform in all PKC family and it is involved in the regulation of multiple signaling pathways such as NF-kB (LIU & al. [27]) and Src signaling (ZHENG & al. [28]), additionally including MAPK pathway which ERK belong to (YOSHIDA & al. [29]). Therefore, we suppose that the activation of ERK in our results is probably conducted by PKC δ. This hypothesis has been proved by many other researchers. For example, the results of J.WU & al. [30] and J. WEN & al.[31] suggested that LPS induces ERK1/2 phosphorylation through up-regulating the expression of PKC δ in the astrocyte and murine microglial cells. Our studies also showed the same results which are the up-regulation of the expression of both p-ERK1/2 and PKC δ. Furthermore, it has been reported that PKC δ is related to the shedding of heparin binding-EGF (UMATA [32]), TNF-α (REDDY & al. [33]), IL-6 receptor (THABARD & al. [34]). M. KVEIBORG & al.[35] and V. SRIRAMAN & al.[36]reported that PKC δ caused the ectodomain shedding of heparin binding-EGF mediated by ERK and ADAM17. Taken together, it may be possible that PKC δ may activate ERK to regulate ADAM17-mediated EPCR shedding or decrease after LPS treatment in vivo.

In conclusion, we detected the expression of EPCR, ADAM17, p-ERK and PKC δ protein in thoracic aorta, heart and lung of LPS-induced mice using immunoblotting. LPS treatment caused the reduction of EPCR and increased the expression of ADAM17, p-ERK and PKC δ protein. These findings suggest that LPS may activate PKC δ and ERK to regulate ADAM17-mediated EPCR shedding or decrease in vivo, which can contribute to future research not only on the mechanism of coagulation disorders accompanied with infection, but also on the target screening of drug therapy.
5. Acknowledgements
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6. Competing interest
The authors declare no conflict of interest.

References