TLR4 Inhibitor Alleviates Sepsis-Induced Organ Failure by Inhibiting Platelet Mtros Production, Autophagy, and Gpiib/Iiia Expression

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Research Article

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Abstract

Thrombocytopenia and impaired platelet aggregation are associated with sepsis-induced organ failure. Many studies have shown that mitochondrial ROS, autophagy is related to organ injury in sepsis. However, the relationship between them is unknown. Here, we explored whether Toll Like Receptor 4 inhibitor alleviates sepsis organ failure by inhibiting platelet mitochondrial ROS production, autophagy, and GPIIb/IIIa expression. We found Toll Like Receptor 4 inhibitor TAK242 alleviated LPS-induced acute kidney and lung injury and decrease platelet activation in a mouse model of sepsis mice. In vitro study, inhibiting Toll Like Receptor 4 reduced inward flow of Ca^{2+} and decreased endogenous mitochondrial ROS production in platelets treated with LPS, and TAK242 inhibited autophagy and NOX expression in LPS treated platelets. Thus, we supposed that Toll Like Receptor 4 inhibitor effectively alleviate lung and kidney injury in a mouse model of sepsis induced by LPS and its effects are related to the inhibition of mouse platelets GPIIb/IIIa, and also can reduce LPS-induced mitochondrial ROS generation related to Ca^{2+} influx, thus reducing platelet activation. LPS can induce platelet autophagy by generating mitochondrial ROS, which may be the pathophysiological mechanism of organ injury in sepsis.

1 Introduction

Sepsis is a critical illness with extremely high mortality in intensive care units (ICUs), with an incidence of 6–30% [1]. The mortality from sepsis is as high as 28%-50% because of multiple organ failure such as sepsis-induced lung injury and kidney failure[2]. A study from over 40 years ago found that sepsis is closely related to thrombocytopenia[3]. Platelet activation and consumption due to inflammatory storm and a platelet count less than 50,000/ μL are indicators of poor prognosis in sepsis patients[4, 5]. Thrombocytopenia and impaired platelet aggregation are associated with sepsis-related mortality and sepsis-induced organ failure. Disorders of the coagulation mechanism, microthrombosis, vascular obstruction, and insufficient perfusion of tissues and organs caused by abnormal platelet function are the pathophysiological basis of organ dysfunction. Increased activation of the platelet surface marker sP-selectin is related to acute lung injury[6–11] and platelet aggregation leads to acute kidney injury by promoting microvesicle concentration [12]

Intracellular Reactive Oxygen Species (ROS) induced by bacterial liposaccharide (LPS) through Toll Like Receptor (TLR4) can induce mitochondrial damage and activate platelets. However, the source of intracellular ROS and related molecular mechanisms have not been shown[13]. Mitochondria are involved in pathophysiological processes such as energy production, intracellular signal transduction and cell death regulation, and play a key role in cell metabolism, survival and homeostasis. Many studies have shown that mitochondrial disorders are related to impaired organ function in sepsis.

ROS mainly come from the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) pathway and the mitochondrial electron transporter pathway. Organ failure in sepsis is mainly related to the increase of mtROS. Reducing the production of mtROS can alleviate lung injury in sepsis[14]. Moreover, autophagy may be upstream of the production of mtROS, and is related to organ injury in sepsis. LPS can induce

autophagy and mtROS production in macrophages to induce acute liver injury[15]. Inhibiting autophagy in macrophages can inhibit the generation of mtROS and reduce inflammation[16]. Autophagy is related to septic kidney injury induced by LPS via the PI3K/AKT/mTOR pathway, and inhibition of autophagy can reduce septic kidney injury [17].

Despite lacking a nucleus, platelets have rich internal organelles such as mitochondria and lysosomes, may be a key target for the regulation of micro-thrombosis and inflammation in sepsis. According to current research, LPS causes the release of mtROS through TLR4, leading to impaired platelet function, which is significant for organ dysfunction in sepsis. However, the signal pathways through which platelet TLR4 is activated to mediate organ injury in sepsis are still unclear. In previous in vivo studies, our team found that the use of TAK242 to inhibit the expression of platelet TLR4 can reverse LPS-mediated septic organ injury in mice. Here, we explored whether TLR4 inhibitor alleviates sepsis organ failure by inhibiting platelet mtROS production, autophagy, and GPIIb/IIIa expression.

2 Methods And Materials

2.1 Preparation of platelets

Peripheral venous blood of healthy volunteers and Blood collected from mice heart treated with anti-coagulant then the blood was centrifuged for 10 min at 150 g, and then the PRPs was collected. PRPs were washed with Ca^{2+} free-Tyrodes HEPES buffer and then centrifuged for 10 min at 850 g. The supernatant was discarded and then washed platelets was suspended in Ca^{2+} Free-Tyrodes HEPES buffer.

2.2 Materials

LPS was purchased from Sigma Chemicals (St. Louis, MO, USA). TAK242 (TLR4 receptor inhibitor) was purchased from MedChem Express (New Jersey, USA). H_2O_2 was purchased from Sinopharm Chemical Reagent Company (Shanghai, China). N-acetyl-L-cysteine (NAC) and Calcium Detection were bought from Byotime (Shanghai, China). GAPDH, RIPA buffer, PMSF, protease, and phosphatase inhibitor cocktails were obtained from Cell Signaling Technology (Beverly, MA, USA). MitoSOX™ Red mitochondrial superoxide indicator (MitoSOX™) was obtained from Thermo Fisher (USA).

2.3 Flow cytometric analysis

PRPs ($2 \times 10^7/\text{mL}$) were separately treated with LPS (10 $\mu\text{g}/\text{mL}$), LPS (10 $\mu\text{g}/\text{mL}$) and TAK242 (132 nM), NAC (5 mM), H_2O_2 (100 μM), or vehicle at 37°C for 30 min. We diluted the 5 mM MitoSOX™ reagent stock solution in HBSS/Ca/Mg to make a 5 μM MitoSOX™ reagent working solution. We applied 1.0–2.0 mL of 5 μM MitoSOX™ reagent working solution to cover PRPs adhering to coverslip(s). We then incubated cells for 10 min at 37°C, protected from light. We incubated the calcium probe with PRPs for 15 minutes in a thermostatic dark box at 37°C and then analysis the fluorescence intensity by a flow cytometer (BD Calibur, USA).

2.4 Western blot analysis

Washed platelet lysates (10^9 cells/mL) were incubated with LPS(10 $\mu\text{g}/\text{ml}$), LPS(10 $\mu\text{g}/\text{ml}$) combined with TAK242(132 nM) or vehicle for 5 min at 37°C. Processed samples were lysed and equal-amounts of protein were subjected to 12% SDS-PAGE and transferred onto PVDF for subsequent probing (LC3B or human NOX2).

2.5 Sepsis organ injury and the effect of TLR4 inhibitor in vivo.

Male BALB/c mice (20–25 g) were purchased from SLAC Laboratory Animal Central (Changsha, China). Animals are bred in an air-conditioned room with temperature of 25°C and relative humidity of 55%, and acclimated for 7 days under a regular light-dark cycle. Mice in the LPS group received dorsal subcutaneous injections of LPS (0.18 $\mu\text{g}/\text{ml}$) from day 2 to day 3. Mice in the TLR4 inhibited group received dorsal subcutaneous injections of TAK242 (5 mg/kg) from the first day to the third day, and LPS (0.18 $\mu\text{g}/\text{ml}$) was used on day 2 to day 3. Mice in the vehicle group received subcutaneous injection of saline solution with equivalent volume on days 1-3. After sacrificed, mice lungs and kidneys were harvested for HE staining and photographed at 200 $\times$ and 400 $\times$, respectively. PRPs was separated from heart whole blood and was analyzed by flow cytometric analysis.

3 Results

3.1 TLR4 inhibitor alleviates LPS-induced acute kidney and lung injury and decreases platelet activation in a mouse model of sepsis mice.

To investigate the effect of LPS and TLR4 inhibitor on mice, histopathological studies were performed. H&E staining showed that LPS injection caused acute kidney and lung injury. LPS induced edema of renal tubular epithelial cells and hemorrhage (Fig. 1A). LPS induced increased pulmonary vascular permeability and pulmonary hemorrhage compared with the control group (Fig. 1B). TAK242 (20 mg/kg) efficiently prevented both kidney and lung injury and inflammatory cell infiltration into kidney and lung tissue, as shown by H&E staining compared with LPS group.

Blood samples were collected from the three groups of mice and the Platelet Rich Plasma (PRPs) were separated. The proportion of CD41(+)CD62P(-) platelets in mice treated with LPS was increased compared with that of the blank control group ($p < 0.05$). The proportions of CD41(-) CD62P(+) and CD41(+)CD62P(+) platelets were significantly higher than that of the blank control group ($p < 0.001$), and the proportion of CD41(-)CD62P(-) platelets was slightly lower than that of the blank control group ($p < 0.01$). The proportion of CD41(+) CD62P(-) platelets in the TAK242 intervention group was significantly lower than that in the LPS group ($p < 0.01$), and there was no statistical difference compared with the blank group ($p > 0.05$). The proportion of CD41(-) CD62P(+) and CD41(+) CD62P(+) platelets was

significantly lower in the TAK242 intervention group than that of the LPS control group, and the proportion of CD41(-) CD62P(-) platelets was slightly higher than that of the LPS group and not statistically different than that of the blank group ($p>0.05$) (Fig. 1C).

3.2 Inhibiting TLR4 reduces inward flow of Ca^{2+} in platelets treated with LPS

In previous study, we found that LPS induced an increase in the expression of P-selectin on the surface of platelets and an increase in the maximum platelet aggregation rate via releasing endogenous ROS and ultimately leading to platelet activation[13]. Platelet activation is related to the influx of calcium ions[18]. Here, we used the calcium ion probe Fluo-3 AM ester which penetrates the platelet cell membrane and combines with intracellular calcium ions to generate strong fluorescence. We found that the calcium ion concentration in platelets treated with LPS increased significantly, (Fig. 2, LPS v.s. NC, $P<0.001$), but found no difference between the LPS and H_2O_2 groups (Fig. 2, LPS v.s. H_2O_2 , $P>0.05$). The TLR4 inhibitor TAK242 decreased the intraplatelet calcium ion concentration after intervention by LPS (Fig. 2, LPS+TAK242 v.s. LPS, $P<0.001$) to levels that were indistinguishable from those of the blank group (Fig. 2, LPS+TAK242 v.s. NC, $P>0.05$). No statistically significant difference was found between the effects of TAK242 and the antioxidant drug NAC (Fig. 2, LPS+TAK242 v.s. LPS+NAC, $P>0.05$).

3.3 TLR4 inhibitors decreased endogenous mtROS production in LPS-induced platelets

In previous study, we found that LPS promoted the release of intracellular ROS through platelet TLR4 to activate platelets. In this study, we found that LPS can induce platelets to produce large amounts of mtROS. (Fig. 3, LPS v.s. NC, $P<0.0001$), TLR4 inhibitors can decrease mtROS production by LPS-induced platelets (Fig. 3, LPS+TAK242 v.s. LPS, $P<0.001$), and the inhibitory effect of TLR4 inhibitor on platelet mtROS is no different from that of the oxygen free radical scavenger NAC (Fig. 3, LPS+NAC v.s. LPS+TAK242, $P>0.05$). The effect of H_2O_2 on platelet aggregation in the positive control group was not different from that of the LPS group (Fig. 3, H_2O_2 v.s. LPS, $P>0.05$).

3.4 TAK242 inhibited autophagy and NOX expression in LPS treated platelets

LC3B is widely used as an indicator of autophagy. The ratio of LC3BII and LC3BI can be used to estimate autophagic flux. In this study, we found that LPS induces autophagic flux in platelets using Western blot analysis of LC3BII and LC3BI (Fig. 4, LPS v.s. NC, $P<0.01$). We also found that TLR4 inhibitors reduce LPS-induced autophagy (Fig. 4, LPS+TAK242 v.s. LPS, $P<0.01$).

Nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase (NOX) isoforms are the main sources of ROS in platelets, followed by cyclooxygenase (COX), xanthine oxidase (XO), and mitochondrial respiration[19]. We found that LPS induced a significant increase in platelet NOX expression (Fig. 4, LPS

v.s. NC, $P < 0.01$) and that the TLR4 inhibitor TAK242 decreased the expression of NOX in LPS-treated platelets (Fig. 4, LPS+TAK242 v.s. LPS, $P < 0.05$). However, the expression of NOX in LPS+TAK242 group was still slightly higher than that in the blank group (Fig. 4, LPS+TAK242 v.s. NC, $P < 0.05$).

4 Discussion

Platelets are particles shed from the cytoplasm during the maturation of megakaryocytes. They are rich in organelles and contents but with no nucleus. The process of platelet activation is mainly completed by these organelles and their contents. Platelet activation is accompanied by the expression of many specific surface markers, such as platelet granule membrane glycoprotein P-selectin (CD62P) and platelet plasma membrane glycoprotein (CD41 or GPIIb)[20]. During the resting period, the platelet membrane surface contains 800-1120 CD62P molecules per platelet on average. When platelets are activated by thrombin, the alpha granules in the platelet fuse with the plasma membrane through the Open Canalicular System (OCS), increasing the number to $1.0-1.3 \times 10^3$ CD62P molecules on the plasma membrane.

Many bacterial proteins directly promote the expression of the GPIIb/IIIa complex via binding to the arginine-glycine-aspartic acid (RGD) sequence on the platelet surface, and thus activate the platelet [21]. Therefore, in this study, we used the platelet activation markers CD41, representing GPIIb/IIIa, and CD62P, representing PS ectopion. We successfully constructed a mouse model of sepsis by subcutaneous injection of LPS. We observed sepsis-induced pulmonary hemorrhage and kidney injury in mice by HE staining of pathological sections. The platelet-rich plasma of septic mice showed an increase in the expression of platelet-activated surface antibodies. The expression of CD41 increased more than the expression of CD62P. This might be related to the fact that flow cytometry results cannot accurately measure the precise number of specific glycoproteins on the surface of each platelet. Our results are consistent with prior work[21].

LPS binds to its receptor TLR4 on the surface of platelets to mediate micro thrombosis and organ injury due to insufficient perfusion. Furthermore, in response to *Escherichia coli* infection, platelet TLR4 contributes to bacterial trapping by supporting NET formation[22]. Platelets promote leukocyte recruitment in LPS-induced lung injury. In contrast, low platelet counts are associated with increased secondary hemostasis, kidney damage, and exacerbated bacteremia and systemic bacterial dissemination in bacteria induced sepsis [23–25]. There are many ways to produce intracellular ROS in cells. Bacterial invasion transfers the TNF receptor associated factor 6 (TRAF6) to the outer mitochondrial membrane through TLR4, and regulates the process of oxidative phosphorylation via Evolutionarily Conserved Signaling Intermediate in Toll Pathways (ECSIT), which ultimately leads to the ubiquitination of TRAF6-dependent ECSIT and the generation of mtROS [26]. In several model systems, Ca^{2+} concentration in blood is significantly lower when sepsis occurs, while Ca^{2+} overload appears in mitochondria [27–29]. Ca^{2+} overload acts as a signal to open Mitochondrial permeability transition pore (MPTP) in sepsis internal environment simulation experiments[30].

Based on our previous research, we further found that LPS stimulated Ca^{2+} influx in platelets. The effect of LPS on Ca^{2+} influx in platelets is consistent with the effect of adding exogenous reactive oxygen species (i.e. H_2O_2). The inhibitory effect of TLR4 inhibitor TAK242 on LPS-induced Ca^{2+} in platelets is consistent with the effects of the ROS scavenger NAC. This phenomenon is analogous to the effect of LPS on platelet-induced intracellular ROS production described in our previous study.

Mitochondria-targeted hydroethidine (Mito-SOX) is a redox probe in which the HE molecule is conjugated to a triphenyl phosphonium group (TPPb) via an alkyl side chain. Mito-SOX is targeted to mitochondria because of the presence of the TPPb moiety[31]. It has excitation/emission maxima of approximately 510/580 nm. We therefore used the fluorescence intensity detected in the PE channel of the flow cytometer to determine mtROS levels in platelets.

Exogenous H_2O_2 can induce endothelial cell mitochondria to produce mtROS (32). We therefore used H_2O_2 as a positive control[32]. We found that H_2O_2 in mitochondria induces the production of a large amount of mtROS. LPS induced production of a large amount of mtROS on platelets, exhibiting the same effect as H_2O_2 . The inhibitory effect of TAK242 of mtROS production is not significantly different from that of the LPS combined with NAC group, suggesting that TAK242 and NAC have the same effect on mtROS production induced by LPS. Meanwhile, in vivo experiments showed that TAK242 inhibited the aggregation of platelets in mice and reduced lung and kidney injury in septic mice. We speculate that TLR4 inhibitors can reduce platelet activation by inhibiting platelet mtROS to alleviate organ injury in sepsis. We further conclude that intracellular ROS of platelets activated by LPS in our previous study were likely mtROS.

mtROS are incorporated into signaling pathways, including those regulating immune responses and autophagy. NOX isoforms are the main sources of ROS in platelets, followed by cyclooxygenase (COX), xanthine oxidase (XO), and mitochondrial respiration[19]. mtROS and NOX-dependent ROS come from different pathways but are closely connected. In neutrophils and endothelial cells, as the concentration of mtROS and NOX-derived ROS in the cytoplasm increases, activation of PKC and cSrc by mtROS upon its translocation to the cytosol leads to ROS production by NOX2[33, 34]. In our previous study, we found that LPS decreases p-PKC expression in platelets, but in this study, we found that LPS increases NOX2 expression in platelets and a concomitant increase in mtROS. The inhibition of PKC expression may be related to the increase of endogenous mtROS[35].

mtROS are closely related to autophagy. In physiological conditions, autophagy helps cells remove damaged mitochondria and peroxidized proteins. In most cases, autophagy supports survival. Intracellular ROS cause mitochondrial damage, thereby initiating autophagy to clear damaged mitochondria[13]. Therefore, autophagy has a protective effect against organ damage in sepsis. Blocking liver autophagy in a murine model of sepsis accelerates hepatocyte apoptosis and mitochondrial damage and decreases survival[36]. Interestingly, it was proposed in 2015 that platelet activation can induce autophagy in platelets and is closely related to thrombocytopenia and thrombosis [37]. Consistent

with this hypothesis, we found that LPS induces platelet aggregation through release of mtROS, accompanied by platelet autophagy.

5 Conclusion

5.1 TLR4 inhibitors effectively alleviate lung and kidney injury in a mouse model of sepsis induced by LPS and its effects are related to the inhibition of mouse platelets GPIIb/IIIa.

5.2 TLR4 inhibitors can reduce LPS-induced mtROS generation related to Ca^{2+} influx, thus reducing platelet activation.

5.3 LPS can induce platelet autophagy by generating mtROS, which may be the pathophysiological mechanism of organ injury in sepsis.

Declarations

Ethics approval and consent to participate

Volunteers were aware of this study and signed an informed consent form. All procedures were conducted in accordance with the protocol approved by the ethical committee of the Laboratory Animals Center of Central South University and all the procedures were under the guideline of the Third Xiangya Hospital of Central South University Human Research Committee.

Author contributions

Guo Feng designed this study. Guo Feng and Ying Li performed the research, analyzed data, and wrote the paper. All authors read and approved the final version of this manuscript.

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Data Availability

The datasets generated during and analysed during the current study are not publicly available due to privacy of volunteers are involved but are available from the corresponding author on reasonable request.

Informed Consent

The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and Figure(s)2-4.

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Figures

Figure 1

TAK242 improves LPS-mediated sepsis-induced lung and kidney injury. A. Under an optical microscope, H&E-stained mouse kidney tissue was visualized at 200× and 400× magnification after treatment with saline, LPS, or LPS combined with TAK242, n = 3 in each group. B. Under an optical microscope, H&E-stained mouse lung tissue was visualized at 200× and 400× magnification after treatment with saline, LPS, or LPS combined with TAK242, n = 3 in each group. C. CD41 and CD62p levels on the surface of

platelets measured by flow cytometry. Measurement data are represented as mean $\pm$ SEM, * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.)

Figure 2

TAK242 reduced calcium influx induced by LPS in platelets. The intercellular Ca²⁺ concentration ([Ca²⁺]_i) production was assayed by flow cytometry, $n = 3$ in each group. The histogram displayed the MFI of [Ca²⁺]_i. Measurement data are represented as mean $\pm$ SEM, ** $p < .01$, *** $p < .001$.

Figure 3

TAK242 decreased mtROS production induced by LPS in platelets. mtROS production was assayed by flow cytometry, n = 3 in each group. The histogram displays the MFI of mtROS. Measurement data are represented as mean $\pm$ SEM, ***p < .001, ****p < .0001.

Figure 4

TAK242 decreased autophagy and NOX2 expression induced by LPS in platelets. The expression of LC3B and NOX2 were detected by Western blotting. Representative blotting images (left) and densitometric analysis (right) are shown, n = 3 in each group. Measurement data are represented as mean $\pm$ SEM, *p < .05, **p < .01.