EFFECTS OF PROPOFOL ON TOLL-LIKE RECEPTOR 4 SIGNAL PATHWAY IN ALVEOLAR MACROPHAGES INDUCED BY LIPOPOLYSACCHARIDE

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ABSTRACT

Aim: To investigate the effects of propofol on toll-like receptor 4 (TLR4) signaling pathway induced by lipopolysaccharide (LPS) in alveolar macrophages (AMs).

Methods: AMs isolated from 10 patients were cultured for 1 hour. These cells were pooled and randomly divided into five groups. The final concentrations of LPS and propofol were 1μg/mL and 30μM, respectively. AMs were continually cultured for 24 hours after LPS addition. RT-PCR and real-time RT-PCR analyses were performed to quantify the cellular expression of TLR4 mRNA; activities of cellular NF-κB were detected; and TNF-α and IL-6 in culture supernatant were measured.

Results: After AMs were stimulated by LPS, the expression of TLR4 mRNA and NF-κB activation were significantly increased, and the production of TNF-α and IL-6 enhanced drastically. Co-treatment of propofol and LPS, or treatment of propofol 1 hour before LPS, significantly inhibited the expression of TLR4 mRNA and the activation of NF-κB, and decreased the production of TNF-α and IL-6.

Conclusions: Early administration of propofol at a therapeutic concentration inhibited TLR4 signal pathway induced by LPS in AMs in vitro.

Key words: Propofol, toll like receptor 4, lipopolysaccharide, alveolar macrophage.

Introduction

Sepsis and septic shock are the leading causes of death in critically ill patients. U.S. and European surveys have estimated that severe sepsis accounts for 2-11% of all admissions to hospital or intensive care units. Hospital mortality from severe sepsis and septic shock (30% and 60%, respectively) has not changed much over recent decades[1, 3]. Lipopolysaccharide (LPS) is an important component of the outer membrane of Gram-negative bacteria, and has a pivotal role in inducing sepsis. In the inflammation induced by LPS, alveolar macrophages (AMs) are activated to produce and release large amounts of inflammatory mediators associated with the initiation and progression of sepsis[3].

Propofol is commonly used as an intravenous anesthetic in clinical anesthesia or a sedative in intensive care unit, often used for sedative purpose in patients with lung injury or sepsis. Previous research suggested that propofol might inhibit inflammatory reaction induced by LPS[4, 10]. LPS inflammatory signal is transferred by toll like receptor 4 (TLR4) signal pathway[2]. TLR4 is a type I transmembrane protein with an extracellular leucine rich repeat domain and an intracellular domain homologous to interleukin 1 receptor. TLR4 extracellular domain first recognizes LPS and transmits LPS signals into TLR4 intracellular domain. The TLR4 intracellular domain interacts with an intracellular adaptor protein MyD88, and eventually leads to translocation of the NF-κB to the nucleus, with
consequent up regulation proinflammatory media-
tors (such as TNF-α and IL-6). However, it is not
clear whether propofol inhibits inflammation by
influencing TLR4 signal pathway. The current
investigation was motivated by the clinical goal of
exploring the mechanism by which propofol inhib-
its inflammation. Thus, we were to investigate the
effects of propofol on TLR4 signal pathway indu-
ced by LPS in AMs.

Materials and methods

Ethics

The Human Ethics Committee of the Second
Artillery General Hospital approved the protocol of
this study. Informed consents were obtained from
all subjects.

Collection, Purification, and Culture of AMs

AMs are isolated from a total of ten patients (6
men and 4 women, with an average age of
67.2±11.2 years, no diabetes, no tuberculosis).
These patients have undergone lobectomy or pneu-
monectomy for small peripheral carcinoma.

Bronchoalveolar lavage (BAL) was performed
under sedation and local anesthesia before operati-
on by instilling a total volume of 200 mL (4×50
mL) sodium chloride into the right middle lobe,
with immediate aspiration by gentle suction after
each aliquot. The retrieved BAL fluid was filtered
through two layers of sterile gauze and subse-
quently centrifuged at 500 g for 10 min at 4°C. Cell
viability as determined by trypan blue exclusion
was higher than 90%. The cell pellet was washed
three times with phosphate-buffered saline solu-
tion. The cells were subsequently suspended to a con-
centration of 1×10^6/mL in RPMI 1640 medium
(Gibco), supplemented with 10% heat-inactivated
fetal calf serum, 2 mmol/L L-glutamine, 200 U/mL
penicillin, and 200 μg /mL streptomycin. The cell
was continually cultured for 24 hour after LPS addition. At the end
of the incubation period, the total RNA and nuclear
protein in cells were extracted, and the culture
supernatants were harvested by centrifugation.
They were stored at -80°C until further analysis
was performed. Cell viability after incubation was
always over 90%.

Reverse transcription-PCR (RT-PCR) analy-
sis for TLR4 mRNA

Total RNA was prepared from AMs with the
RNA extraction kit (Roche) following the manufac-
turer’s directions. The RNA samples were stored at
-80°C until used. All RNA samples with a ratio of
A260 to A280 between 1.8 and 2.1 were used for
experiments. RT-PCR was carried out using a RT-
PCR kit (TaKaRa, Dalian, China). In brief, 100-ng
aliquots of total RNA were reverse-transcribed at
42°C for 15 min. The resulting cDNA were stored
at -20 °C until amplification. The PCR primers used
in the experiments are shown in Table 1.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Product size</th>
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<tbody>
<tr>
<td>TLR4 (ACCESSION</td>
<td>499bp</td>
</tr>
<tr>
<td>NM_138554) Left: 5’-AGCCCTGCGAGCCCTTTTCG-3’</td>
<td>Right: 5’-CAAATGGACAGGCCCTCTTAG-3’</td>
</tr>
<tr>
<td>GAPDH (ACCESSION</td>
<td>294bp</td>
</tr>
<tr>
<td>NM_002046) Left: 5’-GTGACCTCAACAGCAATTCACATC-3’</td>
<td>Right: 5’-GTTATGGGGTTCTGAGTTGTTG-3’</td>
</tr>
</tbody>
</table>

Table 1: Primer used for RT-PCR amplification of TLR4 and GAPDH gene.

For GAPDH housekeeping gene, PCR was
performed with 25 cycles of 1 minute each at 95°C,
60°C, and 72°C, respectively, in a microprocessor-
driven thermal cycles. For TLR4 amplification,
PCR was performed with 35 cycles of 1 minute
each at 95°C, 57°C, and 72°C, respectively. The
PCR products were electrophoresed on agarose gels
containing ethidium bromide, and subsequently video-photographed on an ultraviolet transilluminator. The results of TLR4 were normalized with GAPDH. The mRNA quantity was determined by digital imaging densitometry with UTHSCSA Image Tool system (University of Texas Health Science Center, San Antonio, USA).

**Real-time RT-PCR**

To verify the results of RT-PCR, a quantitative real-time RT-PCR was performed. The TLR4 mRNA levels were measured using TaqMan primers designed using Beacon Designer 5.0. The primers and probes are shown in table 2. PCR was carried out in ABI 7500 Sequence Detector (PE Applied Biosystems, Foster City, CA). Each 25-µl reaction contained 2 µl of first-strand cDNA, 1× PCR master mix, 300 nmol/L of each forward and reverse primer, and 75 nmol/L taqman probe. All reactions were carried out using the following cycling parameters: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Positive controls (plasmids) and negative controls (no adding samples) were performed simultaneously.

**Determination of NF-kB activity**

NF-kB activity was determined with TransAM kit (Active Motif, Carlsbad, CA), according to manufacturer’s instruction. Nuclear extracts of cells in each group were prepared with a Nuclear Extract kit (Active Motif, Carlsbad, CA). Briefly, diluted standard protein and nuclear extracts were added into plates and then were pre-coated with oligonucleotide containing NF-kB consensus sequence (5’-GGGACCTTTCC-3’). Subsequently, the reaction system was incubated at room temperature for 1 h to facilitate conjugation. Primary antibody, which only conjugates with activated NF-kB/p50, was added into each well and then incubated at room temperature for 1 h. Then, horseradish peroxidase conjugated anti-IgG antibody was added and then incubated at room temperature for 1 h. Finally, developer and fixing bath were added. The absorbance was read at 450 nm by an automated micro plate reader (Synergy HT, Bio-Tec instrument, USA). This assay is specific for NF-kB/p50 activation and more sensitive than electrophoretic mobility shift assay.

**Enzyme Linked Immunosorbent Assay (ELISA)**

The amounts of TNF-α and IL-6 in the culture medium of AMs in each group were determined following the standard protocol of the ELISA kits purchased from Sigma.

**Statistical Analysis**

All data were presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 12.0 software package. The differences between groups were analyzed using analysis of variance (ANOVA). The test was considered significant if P < 0.05.

**Results**

**Expression of TLR4 mRNA in AMs**

After AMs were stimulated by LPS, the expression of TLR4 mRNA significantly increased.

<table>
<thead>
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<th>Amplicon</th>
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<tr>
<td><strong>TLR4 (ACCESSION NM_138554)</strong></td>
<td>Left: 5’-AAAGCC-GAAAGGT-GATTGTTGTG-3’ Right: 5’-TGAAGATGATAC-CAGCAGACTG-3’ Probe: 5’-TCCCAG-CACTCCATCCA-GAGCCGC-3’</td>
</tr>
<tr>
<td><strong>GAPDH (ACCESSION NM_002046)</strong></td>
<td>Left: 5’-GGACCT-GACCTGCCGTCTAG-3’ Right: 5’-TAGCCCCAG-GATGCCCTTGGAG-3’ Probe: 5’-CCTCC-GAGCCTGCTTACACACTC-3’</td>
</tr>
</tbody>
</table>

Table 2: Real-time RT-PCR primer and probe sequences.
Propofol influenced the expression of TLR4 mRNA induced by LPS. When propofol was added 1 hour before LPS stimulation, or when propofol and LPS were simultaneously used for AMs, the expression of TLR4 mRNA was notably inhibited. When propofol was administered 1 hour after LPS stimulation, the expression of TLR4 mRNA was not significantly inhibited (Fig 1 and 2).

Variation of NF-κB activation
TransAM NF-κB kit analyses revealed that activities of NF-κB were low, but detectable, in untreated AMs. Administration of LPS significantly enhanced activation of NF-κB. However, co-treatment of propofol and LPS, or treatment of propofol 1 hour before LPS, significantly decreased activation of NF-κB. Exposure to propofol 1 hour after LPS did not significantly inhibit activation of NF-κB (Fig 3).

Variation of TNF-α and IL-6 production
ELISA analyses showed that the contents of TNF-α and IL-6 in supernatant of untreated AMs were low but detectable. After stimulated by LPS, AMs produced a large amount of TNF-α and IL-6. However, co-treatment of propofol and LPS, or treatment of propofol 1 hour before LPS, significantly inhibited production of TNF-α and IL-6. Exposure to propofol 1 hour after LPS had no significant effect on TNF-α and IL-6 production (Fig 4).

Discussion
AMs play a pivotal role in the lung inflammation induced by LPS(3). LPS inflammatory signals are transmitted by transmembrane receptor TLR4 present on the membrane of cells. LPS first interacts with a serum protein, the LPS-binding protein, which transfers LPS to the membrane-anchored protein CD14. CD14 lacks a transmembrane and cytoplasmic domain for signal coupling, but appears to use TLR4 as a coreceptor. Another protein, MD-2, associates constitutively with TLR4 and confers enhanced responsiveness to LPS. During intracellular signal transduction, the TLR4 intracel-
lular domain interacts with an intracellular adaptor protein MyD88. A phosphorylation cascade involving IL-1 receptor associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) ensues, and this signaling activates transcription factor NF-κB. Activated NF-κB combines specified κB sequence in gene, and regulates expression of many inflammation mediators (such as TNF-α, IL-1, IL-6). These signals cumulate in inflammatory reactions\(^3,4\). The present research displays a notable increase of the expression of TLR4 mRNA after AMs was stimulated by LPS. Activation of NF-κB was also increased, as well as the production of TNF-α and IL-6. These are consistent with the changes of TLR4 expression.

**Fig. 3:** Effects of propofol on NF-κB activation in AMs induced by LPS (n=8)
The result of TransAM NF-κB kit analysis. (C, L, P1, P2 and P3 stand for control, LPS, propofol 1 h after LPS, propofol +LPS, propofol 1 h before LPS, respectively.). *P<0.01 vs Control; #P<0.05 vs LPS.

**Fig. 4:** Fig. 4. Effect of propofol on TNFα and IL-6 in AMs induced by LPS (n = 8) (P1, P2 and P3 stand for propofol 1 h after LPS, propofol +LPS, and propofol 1 h before LPS, respectively). *P<0.001 vs Control; #P<0.01 vs LPS; \(\Delta P<0.05\) vs P2.

TNF-α and IL-6 are two most important cytokines induced by LPS and regulated by NF-κB, which play an important role in the development of septic shock. Increased systemic TNF-α concentrations have been correlated with mortality rates for septic shock in both adults and children\(^{16,17}\). TNF-α can induce hypotension, tissue injury, and death in animals, and is considered to be the major mediator of endotoxin-induced shock. IL-6 is both a marker and a mediator of sepsis. High IL-6 levels are associated with endotoxemia\(^{18}\). Numerous studies have shown an association between mean plasma IL-6 concentrations over time and mortality rate. Plasma IL-6 concentration have been found to be 69% higher in non-survivors compared with survivors with sepsis\(^{19}\). For this reason, we chose the two cytokines as research objects. The results of the present study demonstrated that AMs produced a large amount of TNF-α and IL-6 after LPS stimulation.

Propofol is a widely used as an intravenous anesthetic agent in clinical anesthesia, and is often administered to critically ill patients as a sedative or anesthetic. Some studies\(^4-11\) suggested that propofol inhibit inflammation, especially LPS-induced inflammatory reaction, reduce production of cytokines (such as TNF-α and IL-6 et al) induced by LPS, and decrease mortality rate. Effects of propofol on TLR4 and NF-κB have not been reported. Concentrations of LPS used in in vitro studies were about 1 μg/mL\(^{12}\). Bysani et al\(^{20}\) reported that the plasma concentration of LPS in a patient with fatal Klebsiella pneumonia sepsis was 25 ng/ml. In the present study, we have demonstrated that 1μg/mL LPS could induce AMs to produce large amounts of TNF-α and IL-6. In addition, propofol may significantly reduce TNF-α and IL-6 production of AMs induced by LPS. This inhibitory action was more significant when applying propofol before LPS stimulation. Concentration of propofol used in this study was 30 μM, corresponding to a clinical plasma concentration\(^{13}\). Hence, therapeutic concentration of propofol inhibited activation of AMs induced by LPS. Propofol inhibited expression of TLR4 mRNA and NF-κB activation induced by LPS, which was consistent with the observation that propofol inhibited production of TNF-α and IL-6. This was because LPS inflammatory signals were mediated by TLR4 signal pathway. In addition, the effects of propofol were significant only when co-treatment of propofol and LPS, or treatment of propofol before LPS stimulation, was administered. However, the effects were not significant when applying propofol after LPS stimulation.

The mechanism responsible for the fact that propofol inhibited TLR4 signal pathway in AMs induced by LPS remains unknown. These effects may result from a direct action of propofol on the
TLR4 protein itself. TLR4 is a transmembrane protein, and propofol is so lipophilic that it might disrupt the plasma membrane and results in decrease of NF-κB activation and inflammatory cytokines production mediated by TLR4 in AMs. Perhaps propofol-preconditioning stimulus first triggers organism to produce some endogenous activators of TLR4, leading not only to inflammation but also to simultaneous up regulation of feedback inhibitors of inflammation. These inhibitors, such as signaling inhibitors, decoy receptor, and anti-inflammatory cytokines, reduce the subsequent inflammatory response induced by LPS. If these reasons are proven to be true, we may understand that when severe inflammatory reactions have taken place after the organism is attacked by LPS, applying propofol cannot inhibit inflammatory response induced by LPS.

This study reveals that LPS stimulation results in activation of TLR4 inflammatory signal pathway in AMs, and that AMs produce large amounts of cytokines such as TNF-α and IL-6. In addition, propofol inhibits activation of TLR4 and NF-κB, and reduces production of TNF-α and IL-6. Thus, propofol inhibits TLR4 signaling pathway induced by LPS in AMs. The present preliminary study is based on a limited sample. Future studies need to evaluate response curve of propofol effects on TLR4 signal pathway induced by LPS in AMs based on a large dose range. Further, whether propofol inhibits receptor binding or whether propofol maybe bind LPS should be investigated.

However, there’s some drawback of present study. Due to Alpha the limited number of AMs, the experimental groups is also limited, so we only selected the most commonly used dose of propofol, without selecting a series of dose range, this needs further investigations.

Conclusions

Early administration of propofol at a therapeutic concentration inhibited TLR4 signal pathway induced by LPS in AMs in vitro.

References

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