

## Ketamine Suppresses Intestinal TLR4 Expression and NF- $\kappa$ B Activity in Lipopolysaccharide-treated Rats

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**Aim** To investigate whether ketamine suppresses lipopolysaccharide (LPS)-induced increase in Toll-like receptor 4 (TLR4) expression and nuclear factor-kappa B (NF- $\kappa$ B) activity in the intestines of rats.

**Methods** Six groups of rats received one of the following: normal saline control, LPS (5 mg/kg) plus saline, LPS (5 mg/kg) plus ketamine (0.5 mg/kg), LPS (5 mg/kg) plus ketamine (2.5 mg/kg), LPS (5 mg/kg) plus ketamine (10 mg/kg), or ketamine (10 mg/kg) alone. Intestinal TLR4 mRNA expression was analyzed by reverse transcription polymerase chain reaction (RT-PCR), and NF- $\kappa$ B activity was tested by electrophoretic mobility shift assay (EMSA) 1, 3, or 5 hours after the LPS injection.

**Results** Lipopolysaccharide increased TLR4 expression and NF- $\kappa$ B activity in the intestines of rats. Ketamine at the dosage of 0.5, 2.5, and 10 mg/kg suppressed the LPS-induced increase in TLR4 expression and NF- $\kappa$ B activity. Ketamine alone had no effect.

**Conclusion** The study demonstrated that ketamine inhibits NF- $\kappa$ B activation in the intestines of LPS-treated rats, possibly by suppressing TLR4 expression.

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> **Received:** August 16, 2006  
> **Accepted:** October 13, 2006

> **Croat Med J. 2006;47:825-31**

Sepsis caused by Gram-negative bacteria is today an important medical problem, which may lead to frustratingly high mortality rates, especially after the onset of shock. Lipopolysaccharide (LPS), the major structural and functional component of outer membrane of Gram-negative bacteria, is a classic and common initiator of host innate immune responses (1). Recent studies suggest that Toll-like receptor 4 (TLR4) is a primary signal transducer for LPS from gram-negative bacteria (2-4). It has

been found in monocytes/macrophages, neutrophils, dendritic cells, intestinal epithelial and endothelial cells, as well as in B and T cells (5). The engagement of TLR4 with LPS may mediate the activation of nuclear factor-kappa B (NF- $\kappa$ B) (6), which is in an inactive state, bound to I kappa B (I $\kappa$ B) in the cytoplasm, under normal cellular conditions. NF- $\kappa$ B regulates the expression of many genes in innate immune responses, including the genes encoding inflammatory cytokines (7).

The important role that the intestine plays in the inflammatory responses to sepsis and other severe illness has been increasingly recognized. It has been considered that the intestinal mucosa can produce various inflammatory cytokines (8,9) and other uncertain substances, which may influence not only the mucosa itself but other organs and tissues as well (10). Besides that, the intestinal barrier dysfunction may lead to bacterial translocation and result in endotoxemia, while the endotoxemia may promote bacterial translocation as a positive feedback (11). These events may accelerate the development of multiple organ failure (MOF) (12,13).

Ketamine has been advocated for anesthesia in patients with cardiovascular compromise induced by endotoxemia, because of its cardiovascular stimulatory effects (14,15). Besides its anesthetic and cardiostimulant properties, studies reveal that ketamine may possess anti-inflammatory effects, which are in agreement with its beneficial effects in sepsis. It has been reported that ketamine suppresses the production of LPS-induced proinflammatory cytokines in cultured human whole blood (16) and serum of carrageenan sensitized endotoxin shock mice (17,18). The mechanism which leads to the suppressive effect of ketamine on proinflammatory cytokines after LPS stimulation is not clear. Previous studies in our laboratory suggested that ketamine could inhibit the LPS-induced NF- $\kappa$ B increase in peripheral blood mononuclear cells (PBMC) in cultured rats (19), as well as in endotoxemic rats

(20). However, there is little information on the exact mechanism of the anti-inflammatory effect of ketamine.

Given that TLR4 activated by LPS may initiate intracellular NF- $\kappa$ B signaling pathway (6), which culminates in the expression of inflammatory cytokines, and that intestine is not only the injured organ in sepsis but also the accelerator of MOF (8,12,13), with TLR4 found in intestinal epithelial cells, macrophages, and lymphocytes of the lamina propria (5,21), we designed the study to investigate the influence of ketamine on TLR4 expression in the intestines (jejunums) of LPS-treated rats, and find out whether this influence is consistent with its anti-inflammatory effect.

## Material and methods

### Animals

Male Sprague-Dawley rats (250-300 g body weight) used in this experiment were obtained from Shanghai Animal Center, Shanghai, China. Food and water were provided freely. The procedure followed the Institutional Animal Care Committee guidelines.

### Experimental protocol

A total of 108 rats were randomly divided into six groups as follows: normal saline control, LPS (5 mg/kg) plus saline, LPS (5 mg/kg) plus ketamine (0.5 mg/kg), LPS (5 mg/kg) plus ketamine (2.5 mg/kg), LPS (5 mg/kg) plus ketamine (10 mg/kg), and ketamine (10 mg/kg) alone. The rats in the control group were injected with 0.9% NaCl (5 mL/kg) intraperitoneally (IP). The endotoxemia model was established by bolus injection of LPS (5 mg/kg, *Escherichia coli* O111:B4, Sigma Chemical Co., St. Louis, MO, USA) into the tail vein. Animals were then treated immediately with ketamine (0.5, 2.5, 10 mg/kg) (Ketamine Hydrochloride, Hengrui Inc., Nanjing, China) or 0.9% NaCl (5 ml/kg) (IP).

The rats of the ketamine group received only ketamine (10 mg/kg) (IP) without LPS. Six rats of each group were sacrificed at 1, 3, or 5 hours later. The tissues from the intestines (jejunums) were removed and kept in liquid nitrogen for later use.

#### **RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from each sample with TriPure Isolation Reagent (Roche Molecular Biochemicals, Basel, Switzerland), and the concentration was determined by spectrophotometric optical density measurement at 260 nm. After extraction of the RNA, reverse transcription (RT) was performed using Reverse Transcription System Kit (Promega, Madison, WI, USA) to prepare the cDNA. The primer sequences were: TLR4 (sense), TGGATACGTTTCCT-TATAAG; TLR4 (antisense), GAAATGGAG-GCACCCCTTC. Polymerase chain reaction (PCR) was performed with 100  $\mu$ L reaction mixture of 2  $\mu$ L of RT product, 1.5 mmol/L MgCl<sub>2</sub>, 2.5 U Taq DNA polymerase, 100  $\mu$ mol/L dNTP, 0.1  $\mu$ mol/L primer, and 1  $\times$  Taq DNA polymerase magnesium-free buffer (Promega). Two drops of mineral oil (Sigma) were used to overlay the reaction mixture. PCR was conducted in a thermocycler (MiniCycler PTC 150, MJ Research Inc., Watertown, MA, USA) for 38 cycles. Each PCR cycle consisted of 45 seconds at 95°C, 45 seconds at 54°C, and 60 seconds at 72°C. The last cycle was followed by a final incubation at 72°C for 3 minutes and cooled to 4°C. The polymerase chain reaction product of TLR4 was 548 bp. RT-PCR products were electrophoresed on a 1.5% ethidium bromide-stained agarose gel and saved as digital images.

#### **Electrophoretic mobility shift assay (EMSA)**

Nuclear protein of intestinal tissue was extracted and quantified as previously described (22). EMSA was performed using a commercial kit (Gel Shift Assay System, Promega). In short,

dsDNA oligonucleotide probe for NF- $\kappa$ B (5'-AGTTGAGGGGACTTTCCCAGGC-3') was used, according to our previous studies (19). The NF- $\kappa$ B oligonucleotide probe was end-labeled with [ $\gamma$ -<sup>32</sup>P] ATP (Free Biotech, Beijing, China) with T4-polynucleotide kinase. Binding reactions were performed using nuclear extract protein (80  $\mu$ g) preincubated in a binding buffer (9  $\mu$ L), containing 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L MgCl<sub>2</sub>, 50 mmol/L NaCl, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 40 mL/L glycerol, and 0.05 g/L of poly-(deoxyinosinic deoxycytidylic acid) for 15 minutes at room temperature. After addition of the 1  $\mu$ L <sup>32</sup>P-labeled oligonucleotide probe, the incubation was continued for 30 minutes at room temperature. Reaction was stopped by adding 1  $\mu$ L of gel loading buffer, and the mixture was subjected to non-denaturing 40 g/L polyacrylamide gel electrophoresis in 0.5  $\times$  TBE buffer. The gel was dried (80°C, 30 minutes) and exposed to Fuji x-ray film (Fuji Photo Film Co., Ltd, Tokyo, Japan) at -70°C. NF- $\kappa$ B activity was measured by densitometry, using Bandleader 3.0 software (Magenitec Ltd, Tel Aviv, Israel).

#### **Statistical analysis**

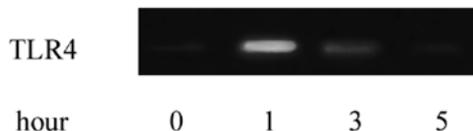
Data were presented as mean  $\pm$  standard deviation (SD). Statistical Package for the Social Sciences version 11.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Differences among groups were determined by one-way analysis of variance (ANOVA), followed by the least-significant-difference (LSD) *post hoc* test. Significance was defined as  $P < 0.05$ .

## **Results**

#### **Intestinal TLR4 expression in rats was increased by endotoxin challenge**

The intestinal TLR4 mRNA expression in rats was examined by RT-PCR, as described in the methods section. One hour after endotoxin chal-

length, the TLR4 mRNA expression in the intestine significantly increased, in comparison with the controls (Figure 1). Later on, the intestinal TLR4 mRNA expression gradually decreased. Five hours after the LPS injection, there was no difference in TLR4 mRNA expression between the LPS-treated rats and control rats (Figure 1).



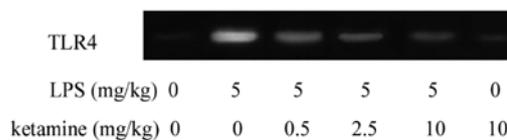
**Figure 1.** Effects of LPS on intestinal TLR4 mRNA expression of rats. The rats were sacrificed 0, 1, 3, and 5 hours after LPS challenge. Lane 1: normal saline control; Lane 2: 1 hour after LPS injection (5 mg/kg); Lane 3: 3 hours after LPS injection (5 mg/kg); Lane 4: 5 hours after LPS injection (5 mg/kg).

#### **Ketamine suppressed LPS-induced TLR4 increase in the intestines**

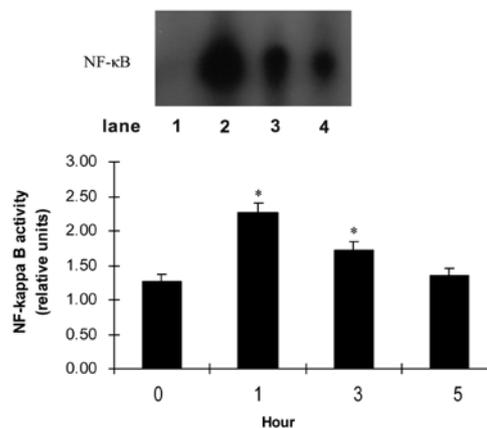
Since the TLR4 mRNA expression was most evident 1 hour after the injection of LPS, the protective effect of ketamine was observed at this time point. At three (0.5, 2.5, and 10 mg/kg) dosage levels, ketamine significantly suppressed TLR4 mRNA expression, compared with the endotoxin group, while TLR4 mRNA expression of the intestines from the ketamine-alone (10 mg/kg, IP) animals did not differ from the controls (Figure 2).

#### **LPS increased intestinal NF-κB activity of rats**

We did EMSA to determine the intestinal NF-κB activity of rats. The NF-κB activity was expressed as the ratio between the photodensity of NF-κB and the photodensity of the background. Endotoxin increased the NF-κB activation of the intestine in a time-related manner. One hour after the LPS injection, the intestinal NF-κB activity increased to  $2.26 \pm 0.15$  relative units, compared with  $1.26 \pm 0.11$  in the control group ( $P < 0.001$ ). Then the NF-κB level decreased to  $1.72 \pm 0.11$  relative units at 3 hours after endotoxin challenge. Five hours after LPS treatment,



**Figure 2.** Effects of various doses of ketamine on LPS-induced increase in TLR4 mRNA expression in intestines of rats. Lane 1: normal saline control; Lane 2: LPS (5 mg/kg) challenge; Lane 3: LPS (5 mg/kg) plus ketamine (0.5 mg/kg); Lane 4: LPS (5 mg/kg) plus ketamine (2.5 mg/kg); Lane 5: LPS (5 mg/kg) plus ketamine (10 mg/kg); Lane 6: ketamine (10 mg/kg) alone.

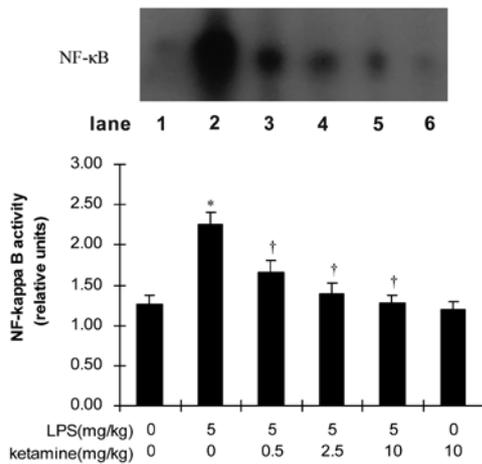


**Figure 3.** Intestinal NF-κB activity of rats after LPS challenge. Lane 1: normal saline control; Lane 2: 1 hour after LPS injection (5 mg/kg); Lane 3: 3 hours after LPS injection (5 mg/kg); Lane 4: 5 hours after LPS injection (5 mg/kg) ( $n = 6$  each group). Values are presented as mean  $\pm$  SD. Asterisk indicates  $P < 0.001$  compared with normal saline control group. LSD *post hoc* test was used for between-group differences.

there was no significant difference between the NF-κB activity of LPS-treated rats ( $1.35 \pm 0.11$ ) and control rats ( $P = 0.203$ ) (Figure 3).

#### **Ketamine inhibited LPS-induced NF-κB activation in the intestines of rats**

The protective effect of ketamine on NF-κB activity was tested 1 hour after the LPS injection, since the NF-κB activity reached the maximum at this time point. After endotoxin stimulation, ketamine at the dose levels of 0.5, 2.5, and 10 mg/kg significantly decreased the intestinal NF-κB activity to  $1.65 \pm 0.16$ ,  $1.34 \pm 0.14$ , and  $1.28 \pm 0.10$  relative units, respectively ( $P < 0.001$ ). Ketamine alone (10 mg/kg IP) did not show significant effect on the intestinal NF-κB level ( $1.20 \pm 0.09$ ), compared with the level of control group ( $P = 0.376$ ) (Figure 4).



**Figure 4.** Intestinal NF- $\kappa$ B of rats after endotoxin treatment with or without the presence of ketamine. Lane 1: normal saline control; Lane 2: LPS (5 mg/kg) challenge; Lane 3: LPS (5 mg/kg) plus ketamine (0.5 mg/kg); Lane 4: LPS (5 mg/kg) plus ketamine (2.5 mg/kg); Lane 5: LPS (5 mg/kg) plus ketamine (10 mg/kg); Lane 6: ketamine (10 mg/kg) alone. Values are presented as mean $\pm$ SD. Asterisk indicates  $P < 0.001$  compared with normal saline control group; cross indicates  $P < 0.001$  compared with LPS-challenged group. LSD *post hoc* test was used for between-group differences.

## Discussion

In this study, we demonstrated that LPS could increase the expression in intestinal TLR4 in rats in a time-related manner. Our findings also revealed that LPS could enhance NF- $\kappa$ B activity in the intestines of rats. Ketamine at the dose levels of 0.5, 2.5, and 10 mg/kg suppressed the intestinal TLR4 mRNA expression and NF- $\kappa$ B activity after LPS treatment. In addition, our results indicated that ketamine itself, without LPS stimulation, had no evident effects on either TLR4 mRNA expression or NF- $\kappa$ B activity.

TLR4 is a member of the Toll-like receptors (TLRs) family. The TLRs have been identified recently in the signal transduction induced by many pathogen-associated molecular patterns (PAMPs) (23,24). Among 10 mammalian TLRs identified, much attention has been focused on TLR4 because of its ability in signaling innate immune response to LPS from a wide range of Gram-negative bacteria (3,4). LPS binding with LPS-binding protein (LBP) may be transferred to CD14 (25,26). It has been re-

ported that TLR4 is a CD14-associated transmembrane signal transducer, which is necessary for the LPS-induced cellular responses (27,28). Recent studies suggested that the association of TLR4 with myeloid differentiation factor 88 (MyD88) results in the activation of IL-1R-associated kinase (IRAK) and TNF receptor-associated factor (TRAF)-6 (29-31). These events may lead to phosphorylation of I $\kappa$ B and subsequent activation of NF- $\kappa$ B (32). In the present experiments, we used LPS, a classic initiator of inflammation and the major ligand of TLR4, as a kind of stimulator. Our study showed that LPS enhanced the TLR4 expression in the intestines of rats. This result is similar to the results found by Song et al (33) that LPS could increase TLR4 expression in human corneal epithelial cells. Moreover, we found that the TLR4 expression of the intestine was most significant 1 hour after endotoxin challenge, and then gradually decreased. So, the effect of ketamine on LPS-induced TLR4 increase was tested 1 hour after LPS injection. Our results showed that ketamine at various dose levels could suppress the TLR4 expression after endotoxin treatment.

As mentioned above, the TLR4 signal transduction may lead to NF- $\kappa$ B activation. NF- $\kappa$ B is ubiquitously expressed and plays the essential role in regulating the expression of many genes involved in immune and inflammatory processes. We performed electrophoretic mobility shift assay to examine NF- $\kappa$ B activity in this experiment. Our study revealed that endotoxin could increase NF- $\kappa$ B activity, which reached its maximum 1 hour after LPS treatment. These results corresponded well with the reports by De Plaen et al (34,35). Previous studies from our laboratory suggested that ketamine might inhibit LPS-induced NF- $\kappa$ B activation in cultured PMBC and in endotoxemic rats (19,20). However, in the present study we tried to investigate how ketamine inhibited NF- $\kappa$ B activation. Our study demonstrated that ketamine at the doses of 0.5, 2.5, and 10 mg/kg suppresses NF- $\kappa$ B activity af-

ter LPS challenge. These results were congruent with the effect of ketamine on LPS-induced increase in TLR4 expression.

TLR4 is an essential upstream sensor for LPS, with a signal transduction pathway which may lead to NF- $\kappa$ B activation, and ketamine can suppress LPS-induced NF- $\kappa$ B activation. Therefore, we hypothesized that ketamine could exert its anti-inflammatory effect on NF- $\kappa$ B activity in sepsis through the TLR4 signal transduction pathway. In our study, we demonstrated that LPS could enhance NF- $\kappa$ B activity together with TLR4 increase. Ketamine could suppress TLR4 expression after endotoxin challenge, and this effect was congruous with the effect of ketamine on NF- $\kappa$ B activity. These results support our hypothesis. Therefore, suppressing TLR4 expression might be a possible upstream mechanism of the anti-inflammatory effect of ketamine.

Since the rats were not anesthetized at the outset of our experiment, we did not monitor their arterial pressure, pulse rate, and respiratory changes. Diarrhea was observed in most of the rats. No rats died during the experiment until they were sacrificed. Many others have used this rat endotoxemia model successfully (36).

Previous reports revealed that the production of inflammatory cytokines, such as tumor necrosis factor (TNF), reached the maximum 1 to 2 hours after LPS treatment in the sera of mice (37,38), then decreased, and were hardly detectable after 6 hours. In our study, we tested the TLR4 expression and NF- $\kappa$ B activity, which may serve as upstream elements in inflammatory processes at 1, 3, and 5 hours after endotoxin challenge, and our results corresponded with previous reports.

Sakai et al (39) reported that ketamine at the doses of 2, 5, or 10 mg/kg could inhibit LPS-induced NF- $\kappa$ B activation in intact brain cells of mice. Our results implied that ketamine at the dose levels of 0.5, 2.5, and 10 mg/kg could inhibit TLR4 expression and NF- $\kappa$ B activity in the intestines of rats in endotoxemia, which sup-

ported their findings. However, the clinic use of ketamine as a kind of anti-inflammatory medicine still needs further careful evaluation.

In conclusion, this study demonstrated that LPS could increase TLR4 expression and NF- $\kappa$ B activity in the intestines of rats. Ketamine at the doses of 0.5, 2.5, and 10 mg/kg could inhibit intestinal NF- $\kappa$ B activity of rats after endotoxin challenge. Suppressing TLR4 expression might be an upstream mechanism for this inhibitory effect of ketamine on NF- $\kappa$ B in sepsis. Better understanding of this mechanism may lead to a more effective application of ketamine, but further investigation is still required.

#### Acknowledgments

We thank Dr Genbao Feng for excellent technical assistance.

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