Effect of Ketamine on NF-kappa B Activity and TNF-alpha Production in Endotoxin-Treated Rats

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Abstract. The effects of ketamine on endotoxin-induced NF-κB activation and TNF-α expression were studied in vivo. A sepsis model was created by bolus injection of endotoxin (5 mg/kg) into the tail vein of adult Wistar rats. The rats were immediately treated with various doses of ketamine (0.5, 5, or 50 mg/kg) or 0.9% NaCl (10 ml/kg) ip. At 1, 4, or 6 hr post-treatment, NF-κB and TNF-α were assayed in the intestine, liver, and lung by electrophoretic mobility shift assay (EMSA) and reverse-transcription polymerase chain reaction (RT-PCR), respectively. Serum TNF-α was analyzed by ELISA. Endotoxin enhanced NF-κB activity and TNF-α expression in the intestine, liver, and lung and it increased TNF-α concentration in serum. Ketamine dosages 0.5 mg/kg suppressed the endotoxin-induced NF-κB activation and TNF-α expression in the intestine. The lowest dose to inhibit NF-κB activity and TNF-α expression in the lung was 5 mg/kg. Ketamine did not inhibit endotoxin-induced NF-κB activity or TNF-α expression in the liver; ketamine itself at a dose of 50 mg/kg enhanced NF-κB activity and TNF-α expression in the liver. Ketamine dosage 0.5 mg/kg inhibited endotoxin-induced TNF-α elevation in the serum. In conclusion, ketamine can suppress the induction of NF-κB and TNF-α by endotoxin in vivo. Subanesthetic dosages of ketamine have an anti-inflammatory effect, but large dosages may be harmful. (received 3 November 2003; accepted 24 December 2003)

Keywords: endotoxin, NF-κB, ketamine, TNF-α, sepsis

Introduction

Gram-negative sepsis is an important cause of morbidity and mortality in endotoxemia. Lipopolysaccharide (LPS), or endotoxin, is a major component of the outer surface of Gram-negative bacteria, and a poten activator of the immune and inflammatory systems, including macrophages, monocytes, and endothelial cells. It contributes to the systemic changes seen in septic shock. The endotoxic shock syndrome is characterized by systemic inflammation, multiple organ damage, circulatory collapse, and death [1].

The iv anesthetic ketamine has been advocated for use in endotoxemic or severely ill patients because of its stimulatory effects on the cardiovascular system [2,3]. It also suppresses the appearance of LPS-induced tumor necrosis factor alpha (TNF-α) in serum, and reduces mortality in carrageenan-sensitized endotoxin shock mice [4,5]. Few studies have tested for an in vivo protective effect of ketamine on the inflammatory response in tissues during septic shock, since locally produced cytokines such as TNF-α, interleukin 6 and interleukin 8 (IL-6 and IL-8) are believed to be responsible for tissue damage during sepsis [6-8]. Nuclear factor kappa B (NF-κB) is an inducible transcription factor, required for the transcription of those proinflammatory cytokines [9]. Since we previously reported that ketamine can inhibit endotoxin-induced NF-κB and TNF-α in vitro [10], the present study tested...
whether ketamine suppresses endotoxin-induced NF-κB activation and TNF-α expression in vivo in vital organs and whether ketamine inhibits LPS-induced elevation of serum TNF-α concentration.

Materials and Methods

Animals and experimental procedures. Male Wistar rats (n = 48, 250 to 300 g body wt) were purchased from Shanghai Animal Center, Shanghai, China. The rats were exposed daily to 12 hr of light and 12 hr of darkness. Food and water were provided freely. There were 6 rats in each group. The protocol followed institutional criteria for the care and use of laboratory animals in research.

Sepsis model. The sepsis model was established by bolus injection with LPS (5 mg/kg, Escherichia coli O111:B4, Sigma Chemical Co., St. Louis, MO, USA) into the tail vein of rats. The rats were treated immediately with ketamine (0.5, 5, or 50 mg/kg, ip) (ketamine hydrochloride, Hengrui, Inc., Nanjing, China) or NaCl solution (0.9%, ip). At 1, 4, or 6 hr after LPS, the rats were anesthetized with diethylether and exsanguinated by cardiac puncture. Serum was separated and stored at -70°C. The lungs, liver, and intestine were removed and samples of these tissues were stored in liquid nitrogen.

Nuclear protein extraction. Nuclear extracts of the tissues were prepared by hypotonic lysis followed by high salt extraction [11]. In brief, −0.1 g of frozen tissue was homogenized in 0.8 ml of ice-cold buffer A, composed of 10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1.0 mM dithiothreitol (DTT), and 0.5 mM phenylmenthysulfonylfluoride (PMSF) (all from Sigma Co.). The homogenate was incubated on ice for 20 min, after which 50 µl of 10% Nonidet P-40 solution was added (Sigma Co.); the mixture was vortexed for 30 sec and centrifuged for 1 min at 5000 x g at 4°C. The crude nuclear pellet was resuspended in 200 µl of buffer B, containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 25% (v/v) glycerol, and was incubated on ice for 30 min with intermittent mixing. The suspension was centrifuged at 12,000 x g at 4°C for 15 min. The supernatant containing the nuclear proteins was collected and kept at -70°C. Protein concentration was assayed by the Bradford procedure, based on the observation that the absorbance peak of an acidic solution of coomassie brilliant blue G-250 (Sigma Co.) shifts from 465 to 595 nm when the dye binds to protein.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed using a kit (Gel Shift Assay System, Promega, Madison, WI, USA) as previously described [10]. The NF-κB oligonucleotide probe, 5'-AGTTGAGGGGACTTTCCCAGGC-3', was end-labeled with [γ-32P] ATP (Free Biotech, Beijing, China) with T4-polynucleotide kinase. Nuclear protein (80 µg) was preincubated in 9 µl of a binding buffer, consisting of 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol, and 0.05 g/L of poly(deoxyinosinic deoxyctydylid acid) for 15 min at room temperature. After addition of 1 µl 32P-labeled oligonucleotide probe, the incubation was continued for 30 min at room temperature. The reaction was stopped by adding 1 µl of gel loading buffer, and the mixture was subjected to non-denaturing 4% polyacrylamide gel electrophoresis in 0.5x TBE buffer. The gel was dried (80 °C, 30 min) and exposed to X-ray film (Fuji Hyperfilm) at -70°C. NF-κB activity was measured by densito-metry (Bandlead 3.0 software, Magnitec Ltd., Israel). The density of the NF-κB band was normalized for the background density of the X-ray film.

Reverse-transcription polymerase chain reaction (RT-PCR). RNA was extracted with TriPure Isolation Reagent (Roche Molecular Biochemicals, Switzerland) and quantified at 260 nm. Reverse-transcription (RT) was performed with Reverse Transcription System (Promega, WI, USA) according to the manufacturer’s protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. The primer sequences were:

TNF-α (sense):
CACCCAGCTCCTCTGCTACTGAAC

TNF-α (antisense):
CCGGACTCCGTGATGCTAAGTACT

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A total reaction volume of 100 µl contained 2 µl of RT product, 1.5 mM MgCl₂, 2.5 U Taq DNA polymerase, 100 µM dNTP, 0.1 µM primer, and 1x Taq DNA polymerase magnesium-free buffer (Promega, WI, USA). The reaction mixture was overlaid with two drops of mineral oil (Sigma Co.) and incubated in a thermocycler (MiniCycler PTC 150, MJ Research, Inc., USA) programmed to predenature at 95°C for 2 min, denature at 95°C for 1 min, anneal at 60°C for 1 min, and extend at 72°C for 2 min, for a total of 30 cycles. The last cycle was followed by incubation at 72°C for 5 min and cooling to 4°C. The polymerase chain reaction products were 546bp (TNF-α) and 970bp (GAPDH). They were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. The gel was saved as a digital image and analyzed using Scion Image software (Scion Corp., USA). TNF-α values for each sample were normalized by reference to the GAPDH control [12].

**Enzyme-linked immunoassorbent assay.** Serum TNF-α was measured by enzyme-linked immunosorbent assay (ELISA) using an enzyme-linked immunoassay kit (rat TNF-α ELISA kit, Diaclone, Besançon, France) according to the manufacturer’s protocol. In brief, 96-well plates containing anti-TNF-α monoclonal antibody were incubated with serum samples or TNF-α standards for 2 hr at room temperature with shaking. A standard curve was established using amounts of TNF-α from 0 to 2000 pg/ml. Plates were washed 5 times followed by 2 hr of incubation at room temperature with anti-rat TNF-α antibody. After 5 washes with buffer and 30 min incubation at room temperature with streptavidin-HRP, the plates were incubated with ready-to-use TMB substrate for 10 min at room temperature. When the samples became blue, the reaction was stopped by adding 100 µl of 1 N H₂SO₄ to each well and the absorbance was read at 450 nm using a plate reader. TNF-α content was expressed as pg/ml.

**Statistics.** Data analysis was performed by a computer program, Statistical Product for the Social Sciences–10.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. Differences among groups were tested by a one-way analysis of variance (ANOVA). Significant values were verified by the Student-Newman-Kuels post-hoc test.

**Results**

**EMSA for NF-κB in tissues** (Table 1). EMSA experiments examined the effect of ketamine on the activation of NF-κB induced by endotoxin. Activity of NF-κB in nuclear extracts from the intestine was enhanced in a time-dependent manner after endotoxin challenge, compared to controls. Since the activity of NF-κB was most significant at 1 hr

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intestine NF-κB activity (densitometer units, mean±SD, n =6)</th>
<th>Liver NF-κB activity (densitometer units, mean±SD, n =6)</th>
<th>Lung NF-κB activity (densitometer units, mean±SD, n =6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle controls</td>
<td>1.16±0.10 (1 hr)</td>
<td>2.12±0.23 (1 hr)</td>
<td>1.06±0.17 (1 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg, iv)</td>
<td>2.09±0.22** (1 hr)</td>
<td>2.84±0.27** (1 hr)</td>
<td>1.45±0.14* (1 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg, iv) + ketamine (0.5 mg/kg, ip)</td>
<td>1.02±0.23† (1 hr)</td>
<td>2.58±0.27 (1 hr)</td>
<td>1.45±0.18 (4 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg, iv) + ketamine (5 mg/kg, ip)</td>
<td>1.16±0.22† (1 hr)</td>
<td>2.60±0.19 (1 hr)</td>
<td>1.19±0.12† (4 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg, iv) + ketamine (50 mg/kg, ip)</td>
<td>1.07±0.20† (1 hr)</td>
<td>2.84±0.32 (1 hr)</td>
<td>0.99±0.16† (4 hr)</td>
</tr>
<tr>
<td>ketamine (50 mg/kg, ip)</td>
<td>1.12±0.24 (1 hr)</td>
<td>2.86±0.25** (1 hr)</td>
<td>0.89±0.21 (1 hr)</td>
</tr>
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</table>

* p <0.05 and ** p <0.01 vs vehicle controls; † p<0.01 vs corresponding LPS alone group.
after endotoxin, the protective effect of ketamine was tested at this time point. At all dosage levels (0.5, 5, and 50 mg/kg, ip), ketamine significantly inhibited the NF-κB activation in the nuclear extracts from the intestine. Ketamine, administered alone (50 mg/kg, ip, 1 hr before sacrifice) without endotoxin, did not affect the NF-κB level in nuclear extracts from the intestine.

Activity of NF-κB in the nuclear extracts from the liver was enhanced significantly after endotoxin stimulation, compared to controls, but unlike the intestine, the activity of NF-κB did not decline with time. At the 0.5, 5, or 5.0 mg/kg dosage levels, ketamine did not inhibit NF-κB activation in the liver. Ketamine, administered alone (50 mg/kg, ip, 1 hr before sacrifice) without endotoxin, caused a significant increase of NF-κB activity in nuclear extract from the liver.

Activity of NF-κB in nuclear extracts from the lungs was enhanced significantly after endotoxin administration, compared to controls. The increased activity of NF-κB in the lung was most prominent 4 hr after LPS injection. Therefore the effect of ketamine was tested at this time point. Ketamine inhibited the NF-κB activation at 2 dosages (5, 50 mg/kg, ip), but not at the lowest dose (0.5 mg/kg, ip). Ketamine, administered alone (50 mg/kg, ip, 0.5 hr before sacrifice), did not significantly affect the NF-κB activity in nuclear extract from the lungs.

**RT-PCR analysis for TNF-α mRNA** (Table 2). Endotoxin caused a transient elevation of TNF-α mRNA in the intestine. The TNF-α mRNA reached a maximum at 1 hr after endotoxin stimulation. Ketamine suppressed the LPS-induced increase of TNF-α expression in a dose-dependent manner. At the lowest dosage of 0.5 mg/kg (ie, below the clinical anesthetic level), ketamine significantly suppressed TNF-α expression in the intestine, measured at 1 hr after LPS injection. Ketamine alone (50 mg/kg, ip, 1 hr before sacrifice), did not significantly affect TNF-α expression in the intestine.

In the liver, TNF-α mRNA level at 1, 4, and 6 hr after endotoxin stimulation was increased significantly when compared with unstimulated controls. Administration of ketamine (0.5, 5, and 50 mg/kg, ip) did not prevent LPS-stimulated TNF-α expression. Instead, ketamine alone (50 mg/kg, ip, 1 hr before sacrifice) significantly increased TNF-α expression in the liver.

In the lung, TNF-α mRNA at 1, 4, and 6 hr after endotoxin stimulation was enhanced significantly when compared with unstimulated negative controls. At the 2 higher dosage levels (5, 50 mg/kg, ip), ketamine inhibited TNF-α expression at 1 hr post-LPS administration. Ketamine alone (50 mg/kg, ip, 1 hr before sacrifice) did not affect TNF-α expression in the lung.

**Serum TNF-α level.** As shown in Table 3, serum TNF-α concentration increased from a baseline of 3.4±0.5 pg/ml in control rats to a maximum of 1,499±175 pg/ml at 1 hr after endotoxin administration. In a dose-dependent manner, ketamine
suppressed the LPS-induced elevation of serum TNF-α level. Injection of ketamine alone (50 mg/kg, ip, 1 hr before sacrifice) did not significantly affect the serum TNF-α level.

Discussion

In this study, endotoxin provoked a transient elevation of TNF-α concentration in the serum and enhanced NF-κB activity and TNF-α expression in rat intestine, lung, and liver, which was in accordance with the literature [13,14]. However, the peak time was not the same in different organs, perhaps because of organ-specific reactions to endotoxin. Administration of ketamine inhibited the endotoxin-induced elevations of TNF-α and NF-κB in intestine and lung, but not in liver. Previous studies showed that ketamine can suppress some proinflammatory cytokines in vitro [15].

NF-κB is associated in the cytoplasm with its inhibitory subunit, inhibitory kappa B (IκB), which prevents NF-κB from translocating to the nucleus. Multiple stimuli, including endotoxin, induce the phosphorylation and degradation of IκB [16]. This process frees NF-κB from the NF-κB/IκB complex and enables NF-κB to translocate to the nucleus where it regulates gene transcription. Genes that encode cytokines (TNF-α, IL-1, IL-6, IL-8, and IL-12) are regulated by NF-κB [16]. A previous study demonstrated that ketamine suppresses LPS-induced TNF-α, IL-6, and IL-8 elevations in human whole blood, and reduces the systemic production and release of a proinflammatory cytokine, IL-6, during and following cardiopulmonary bypass (CPB) [17]. This prompted us to hypothesize that ketamine suppresses proinflammatory cytokines by inhibiting NF-κB activation. Our laboratory has previously demonstrated that ketamine suppresses endotoxin-induced NF-κB activation in peripheral blood mononuclear cells (PBMC) [10]. We also showed that it can inhibit NF-κB activation in vivo, although not in a dose-dependent manner.

The doses of ketamine used in this study were 0.5, 5, and 50 mg/kg, ip, which include the clinical range. The minimal dose that inhibited NF-κB and TNF-α expression in the intestine was 0.5 mg/kg. At this dose, ketamine did not inhibit NF-κB or TNF-α expression in the lung. Two reasons may be involved. First, since ketamine was administered ip, the effective concentration of ketamine in the lung may be decreased by the “first-pass” effect of the liver. Second, endotoxin provokes more significant inflammation in the lung [18], and consequently more ketamine may be needed to inhibit this inflammation.

In the intestine and lung, we did not find any significant changes of NF-κB activity and TNF-α expression in rats that received ketamine alone (50 mg/kg, ip, 1 hr before sacrifice), However in the liver, ketamine (50 mg/kg, ip, 1 hr before sacrifice) enhanced NF-κB activation and TNF-α expression, which indicates that such a high dose of ketamine may harm the liver. This is consistent with the observation that ketamine administration (10 mg/kg, im) to cats and dogs caused reversible damage to the liver and kidneys and increased the activity of reticuloendothelial cells [19].

We measured TNF-α concentrations in rat serum, since proinflammatory cytokines, such as TNF-α, IL-1 and IL-6, are down-stream products of NF-κB. Endotoxin caused a transient elevation of serum TNF-α, and administration of ketamine (0.5, 5, and 50 mg/kg, ip) inhibited this elevation. A previous study reported that ketamine (>73 µM) inhibited endotoxin-induced TNF-α expression in vitro [15]. Li et al [20] showed that >10 µM ketamine inhibited nitric oxide production in LPS-treated rat alveolar macrophages. Ketamine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum TNF-α (pg/ml)</th>
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<tbody>
<tr>
<td>Vehicle controls</td>
<td>3.4±0.05 (1 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg)</td>
<td>1449±175* (1 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg)</td>
<td>985±119* (4 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg)</td>
<td>201±19* (6 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg) + ketamine (0.5 mg/kg)</td>
<td>870±55† (1 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg) + ketamine (5 mg/kg)</td>
<td>740±68† (1 hr)</td>
</tr>
<tr>
<td>LPS (5 mg/kg) + ketamine (50 mg/kg)</td>
<td>680±86† (1 hr)</td>
</tr>
<tr>
<td>ketamine (50 mg/kg)</td>
<td>4.6±0.08 (1 hr)</td>
</tr>
</tbody>
</table>

* p <0.01 vs vehicle controls
† p <0.01 vs corresponding LPS alone group.
concentration in human plasma can reach 110 µM after an injection of ketamine (2 to 2.2 mg/kg, iv) [21]. Roytblat et al [17] reported that a single dose of ketamine (0.25 mg/kg) suppressed the increase of serum IL-6 during and after coronary artery bypass surgery. These previous reports and the present study all indicate that a subanesthetic dose of ketamine can inhibit endotoxin-induced expression of TNF-α and other proinflammatory mediators.

In summary, ketamine at subanesthetic dosage can suppress NF-κB activation and TNF-α mRNA induction by endotoxin in vivo. However, caution is advised and further study is needed, since a high dose of ketamine may be toxic.

Acknowledgement

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References