Ketamine suppresses intestinal NF-kappa B activation and proinflammatory cytokine in endotoxic rats

Jie Sun, Xiao-Dong Wang, Hong Liu, Jian-Guo Xu

AIM: To investigate the protective effect of ketamine on the endotoxin-induced proinflammatory cytokines and NF-kappa B activation in the intestine.

METHODS: Adult male Wistar rats were randomly divided into 6 groups: (a) normal saline control, (b) challenged with endotoxin (5 mg/kg) and treated by saline, (c) challenged with endotoxin (5 mg/kg) and treated by ketamine (0.5 mg/kg), (d) challenged with endotoxin (5 mg/kg) and treated by ketamine (5 mg/kg), (e) challenged with endotoxin (5 mg/kg) and treated by ketamine (50 mg/kg), and (f) saline injected and treated by ketamine (50 mg/kg). After 1, 4 or 6 h, TNF-α and IL-6 mRNA were investigated in the tissues of the intestine (jejenum) by RT-PCR. TNF-α and IL-6 were measured by ELISA. We used electrophoretic mobility shift assay (EMSA) to investigate NF-kappa B activity in the intestine.

RESULTS: NF-kappa B activity, the expression of TNF-α and IL-6 were enhanced in the intestine by endotoxin. Ketamine at a dose of 0.5 mg/kg could suppress endotoxin-induced TNF-α mRNA and protein elevation and inhibit NF-kappa B activation in the intestine. However the least dosage of ketamine to inhibit IL-6 was 5 mg/kg in our experiment.

CONCLUSION: Ketamine can suppress endotoxin-induced production of proinflammatory cytokines such as TNF-α and IL-6 production in the intestine. This suppressive effect may act through inhibiting NF-kappa B.

Sun J, Wang XD, Liu H, Xu JG. Ketamine suppresses intestinal NF-kappa B activation and proinflammatory cytokine in endotoxic rats. World J Gastroenterol 2004; 10(7): 1028-1031

http://www.wjgnet.com/1007-9327/10/1028.asp

INTRODUCTION

Gram-negative bacteria caused sepsis remains an important cause of morbidity and mortality in septic and endotoxemic patients. Lipopolysaccharide (LPS), or endotoxin, a major component of the outer surface of Gram-negative bacteria, is a potent activator of cells of the immune and inflammatory systems, including macrophages, monocytes and endothelial cells[11,12]. The endotoxic shock syndrome is characterized by systemic inflammation, multiple organ damage, circulatory collapse and death[12,13].

The important role of the intestinal mucosa in the inflammatory and metabolic responses to sepsis, severe injury and other critical illnesses has been increasingly recognized during the last decade. Thus, there is evidence that the gut mucosa becomes the site for production of various inflammatory cytokines[5,6] and other yet unidentified substances that may influence not only the mucosa itself but also the function and integrity of remote organs and tissues[7,8]. Indeed, the gut mucosa has been proposed to be the “motor” of multiple organ failure in critical illness[9]. Besides, sepsis and severe injury are also associated with loss of mucosal integrity, resulting in increased permeability and bacterial translocation. These changes may accelerate the development of multiple organ failure[10].

Ketamine, an intravenous anesthetic, has been advocated for anesthesia in septic or severely ill patients because of its cardiovascular stimulating effects[11,12]. And several previous studies reported that ketamine could suppress LPS-induced tumor necrosis factor alpha (TNF-α) production in the serum and reduced mortality in carrageenan-sensitized endotoxin shock mice[11,14]. However, few studies were undertaken to investigate the protective effect of ketamine on the inflammatory response in the intestine during septic shock in vivo. Since local produced cytokines were regarded as the contributing factors in tissue damage during sepsis[15-17]. And nuclear factor kappa B (NF-kappa B) was verified to be an inducible transcription factor that was required for the transcription of some proinflammatory cytokines such as TNF-α, interleukin 6 and interleukin 8 (IL-6 and IL-8)[18]. Our previous study indicated that ketamine could inhibit endotoxin induced NF-kappa B and TNF-α in vitro[19]. Therefore, this study was to investigate whether ketamine could suppress endotoxin-induced NF-kappa B activation and proinflammatory cytokines in the intestine in vivo in order to define a possible mechanism of the anti-inflammatory effect of ketamine.

MATERIALS AND METHODS

Animals and treatment

Adult male Wistar rats (250-300 g body mass) used in this experiment were purchased from Shanghai Animal Center, Shanghai, China. The rats were exposed each day to 12 h of light and darkness respectively. The experimental protocol followed the institution’s criteria for the care and use of laboratory animals in research. Further, all animals received humane care in compliance with Institutional Animal Care Committee.

Experimental protocol

The Wistar rat endotoxemia model was established by injection with a dose of LPS (5 mg/kg, Escherichia coli O111: B4) (Sigma Chemical Co., USA) via the tail vein. Then all animals were immediately treated with different doses of ketamine.
and the mixture was subjected to non-denaturing 40 g/L gel was vacuum-dried and exposed to X-ray film (Fuji Reaction was stopped by adding 1 µL of [γ-32P] ATP (Free Biotech, Beijing, China) with T4-poly-nucleotide kinase. Nuclear protein (80 µg) was preincubated in 9 µL of a binding buffer, consisting of 10 mmol/L Tris-Cl, pH 7.5, 1 mmol/L MgCl2, 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 deoxyctydilic acid) for 15 min at room temperature. After addition of the 1 µL 32P-labeled oligonucleotide probe, the reaction was continued for 30 min at room temperature. Reaction was stopped by adding 1 µL of gel loading buffer, and the mixture was subjected to non-denaturing 40 g/L polyacrylamide gel electrophoresis in 0.5xTBE buffer. The gel was vacuum-dried and exposed to X-ray film (Fuji Hyperfilm) at -70 °C.

Electrophoretic mobility shift assay (EMSA) Nuclear extracts of the intestine tissue was prepared by hypotonic lysis followed by high salt extraction20-23. EMSA was performed using a commercial kit (Gel Shift Assay System; Promega, Madison, WI) as previously described. The NF-kappa B oligonucleotide probe, (5'-AGTTGAGGGGACT TTCCCAGGC-3'), was end-labeled with [γ-32P] ATP (Free Biotech, Beijing, China) with T4-poly-nucleotide kinase. Nuclear protein (80 µg) was preincubated in 9 µL of a binding buffer, consisting of 10 mmol/L Tris-Cl, pH 7.5, 1 mmol/L MgCl2, 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 deoxyctydilic acid) for 15 min at room temperature. After addition of the 1 µL 32P-labeled oligonucleotide probe, the reaction was continued for 30 min at room temperature. Reaction was stopped by adding 1 µL of gel loading buffer, and the mixture was subjected to non-denaturing 40 g/L polyacrylamide gel electrophoresis in 0.5xTBE buffer. The gel was vacuum-dried and exposed to X-ray film (Fuji Hyperfilm) at -70 °C.

Reverse-transcription polymerase chain reaction (RT-PCR) Total RNA was extracted with TriPure Isolation Reagent (Roche Molecular Biochemicals, Switzerland) and quantified by absorption at 260 nm. Reverse-transcription (RT) was implemented using Reverse Transcription System (Promega, WI, USA) according to the protocol. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as normalization control. The sequences of the primers were: TNF-α (sense) CACCCAGCTCTTCTGTCTACGTGAC, (antisense) CCGGACTCCGTATGCTCATAGTACT; IL-6 (sense) GACTGATGTTGTTGACAGCCACTGC, (antisense) TAGCC ACTCTCTTGATAGCTTACTA; GAPDH (sense) GATGGACTGGAGGAGGCAG, (antisense) GGAGGAGGAGGACTGCTGAC. A total volume of 100 µL reaction contained 2 µL of RT product, 1.5 mmol/L MgCl2, 2.5 L 5 Taq DNA polymerase, 100 µmol/LdNTP, 0.1 µmol/L primer and 1xTaq DNA polymerase magnesium-free buffer (Promega, WI, USA). Then the reaction mixture was overlaid with two drops of mineral oil (Sigma Chemical Co., USA) and incubated in thermocycler (MiniCycler PTC 150, MJ Research Inc, USA) programmed to pre-denature at 95 °C for 2 min, denatured at 95 °C for 1 min, annealed at 60 °C for 1 min and extended at 72 °C for 2 min for a total of 30 cycles. The last cycle was followed by a final incubation at 72 °C for 5 min and cooled to 4 °C. The polymerase chain reaction products were 546 bp (TNF-α), 509 bp (IL-6) and 970 bp (GAPDH) respectively. Then they were electrophoresed on a 15 g/L agarose gel stained with ethidium bromide. The gel was captured as a digital image and analyzed using Scion Image software (Maryland, USA). Values in each sample were normalized with GAPDH control.

Enzyme-linked immunoabsorbent assay (ELISA) TNF-α and IL-6 in the intestine were measured using commercially available enzyme-linked immunoassay kits (Diaclone USA for TNF-α; Biosource USA for IL-6) according to the test protocol. Values were expressed as pg per milligram protein (pg/mg prot).

Statistics and presentation of data Data were expressed as mean±SE. Statistical significance was determined by one-way ANOVA using SPSS 10.0. A value of P<0.05 was considered significant.

RESULTS
NF-kappa B activation in the intestine EMSA experiments were performed to examine the effect of ketamine on the activation of NF-kappa B induced by endotoxin. As shown in Figure 1, NF-kappa B activation in the intestine was increased after endotoxin challenge as compared with unstimulated group. The activity of NF-kappa B was in a time dependant manner after endotoxin injection. Ketamine inhibited NF-kappa B activation at three (0.5, 5, and 50 mg/kg) dosing levels (P<0.05, as compared with endotoxin group) (Figure 1).

Figure 1 Activation of NF-kappa B in the intestine. Normal saline treatment (Lane 1); 1, 4, 6 h after endotoxin challenge (Lane 2, 3, 4); endotoxin plus ketamine (0.5, 5, 50 mg·kg⁻¹) (Lane 5, 6, 7), ketamine only (50 mg·kg⁻¹) (Lane 8). *P <0.05 vs Lane 1; †P <0.01 vs Lane 1; ‡P <0.01 vs Lane 2.

Figure 2 Expression of TNF-α in the intestine. Normal saline treatment (Lane 1); 1, 4, 6 h after endotoxin challenge (Lane 2, 3, 4); endotoxin plus ketamine (0.5, 5, 50 mg·kg⁻¹) (Lane 5, 6, 7); ketamine only (50 mg·kg⁻¹) (Lane 8). *P <0.05 vs Lane 1; †P <0.01 vs Lane 1; ‡P <0.01 vs Lane 2.

TNF-α mRNA expression by endotoxin challenge and the protective effect of ketamine TNF-α sustained a baseline level in normal rats. Endotoxin caused a transient elevation of TNF-α mRNA in the intestine. This activity increased with time reaching a maximum 1 h
after sepsis. Ketamine was administered intraperitoneally soon after endotoxin challenge. TNF-α gene expression was analyzed 1 h later since TNF-α could reach the maximum level about 1 h later. Ketamine suppressed TNF-α expression in a dose dependent manner. We found that ketamine at a dose of 0.5 mg/kg could suppress TNF-α expression significantly. This dosage was far below clinical anesthetic level (Figure 2).

**IL-6 expression in intestine by endotoxin challenge and the protective effect of ketamine**

The IL-6 expression of the small intestine is shown in Figure 3. Endotoxin also enhanced IL-6 expression in the intestine. However the peak time was 4 h after endotoxin challenge. We observed the protective effect of ketamine at this peak time. Ketamine suppressed IL-6 expression in a dose dependent manner. Unlike TNF-α, the minimal dosage at which ketamine could suppress IL-6 significantly was 5 mg/kg. This was within clinical anesthetic range (Figure 3).

**Effect of ketamine on TNF-α and IL-6 production in intestine homogenates after endotoxin stimulation**

Ketamine suppressed endotoxin-induced TNF-α and IL-6 production in a dose dependent manner. Ketamine beyond the concentration of 0.5 mg/kg could inhibit TNF-α production, however the minimal dosage at which ketamine suppressed IL-6 significantly was 5 mg/kg. This was within clinical anesthetic range (Figures 4 and 5).

**DISCUSSION**

Our laboratory and others have demonstrated that ketamine could suppress endotoxin-induced some proinflammatory cytokines *in vitro*.[23] However it is to be determined in complex *in vivo* studies. We assessed the cytokines and transcriptional factor NF-kappa B in the intestine because of the important status of the intestine in sepsis or systemic inflammation reaction syndrome (SIRS). The intestine was not only the passive organs injured by sepsis but participation in the pathogenesis of SIRS.[5,6]

TNF-α is regarded as the most important proinflammatory cytokine, which is released early after an inflammatory stimulus[21]. And IL-6, which is elevated after TNF-α, contributes to both morbidity and mortality in conditions of "uncontrolled" inflammation.[25] Among the cytokines produced in the intestinal mucosa during inflammation, TNF-α and IL-6 are particularly important because of its multiple biological effects both in the intestine and in other organs and tissues. In this study, we demonstrated that ketamine suppressed both endotoxin-induced TNF-α and IL-6 expression and production in the intestine. TNF-α was the first cytokine expressed after endotoxin stimulation and later IL-6, which was consistent with several previous reports.[24,25] Studies had demonstrated that ketamine could suppress endotoxin-induced cytokines *in vitro*. However, proinflammatory cytokines just like TNF-α and IL-6 were not merely stimulated by endotoxin *in vivo*. Therefore, our experimental protocol was more physiological and closer to clinical condition.

NF-kappa B is associated in the cytoplasm with its inhibitory subunit, inhibitory kappa B (IκB), which prevents NF-kappa B from translocating into the nucleus. Endotoxin can induce the phosphorylation and degradation of IκB. Many effector genes including those encoding cytokines (TNF-α and IL-6) are in turn regulated by NF-kappa B.[26] To determine whether ketamine could inhibit NF-kappa B activation, we did EMSA to detect NF-kappa B activity in the intestine. We found a constitutive activation of NF-kappa B in intestine. Endotoxin could enhance NF-kappa B activation in the intestine and it was most significant 1 h later. Although we had previously demonstrated that ketamine could inhibit NF-kappa B activation in peripheral blood mononuclear cell (PBMC) after endotoxin challenge *in vitro*. It was to be studied whether ketamine had this effect *in vivo*. In our experiment, we found ketamine could inhibit NF-kappa B activation. However it was not in a dose dependent manner. We did not found any NF-kappa B activity changes in the group administered ketamine.
In conclusion, we demonstrated that ketamine could act through inhibiting NF-kappa B. Further study is required to elucidate the mechanism of ketamine action.

The dosage of ketamine used in this study was from 0.5 to 50 mg/kg, which covered the clinical range. Royblat et al. reported that a single dose of ketamine 0.25 mg/kg administered before cardiopulmonary bypass suppressed the increase in serum IL-6 during and after coronary artery bypass surgery. However, other studies demonstrated such a small dose of ketamine did not suppress IL-6 production. The reason was not clear. In this study, only ketamine reaching a dose of 5 mg/kg could suppress IL-6 production in the intestine. There were perhaps some differences between human being and animals or between in vitro and in vivo studies. We found 0.5 mg/kg ketamine suppressed TNF-alpha production, which was in accordance with those in vitro studies.

In conclusion, we demonstrated that ketamine could suppress endotoxin-induced TNF-alpha and IL-6 expression and production in the intestine. And this suppressive effect might act through inhibiting NF-kappa B. Further study is required to elucidate the mechanism of ketamine action.

ACKNOWLEDGEMENT

We thank Dr. Genbao Feng for his technical assistance.

REFERENCES

1. Opal SM, Cohen J. Clinical Gram-positive sepsis: does it fundamentally differ from Gram-negative bacterial sepsis? Crit Care Med 1999; 27: 1608-1616
2. Rietschel ET, Brade H, Holst O, Brade L, Muller-Loennies S, Mamut AI, Zahringer U, Beckmann F, Seydel U, Brandenburg K, Ulmer AJ, Matern T, Heine H, Schletter J, Lopnow H, Schonbeck U, Flad HD, Hauischoldt S, Schade UF, Di Padova F, Kusumoto S, Schumann RR. Bacterial endotoxin: chemical constitution, biological recognition, host response, and immunological detoxification. Curr Topics Microbiol Immunol 1996; 216: 39-81
3. Wenzel RP, Pinsky MR, Ulevitch RJ, Young L. Current understanding of sepsis. Clin Inf Dis 1995; 22: 407-412
4. Huemann D, Glauser MP, Calandra T. Molecular basis of host-pathogen interaction in septic shock. Curr Opin Immunol 1998; 1: 49-55
5. Huang L, Tan X, Crawford SE, Hsu EW. Platelet-activating factor and endotoxin induce tumor necrosis factor gene expression in rat intestine and liver. Immunology 1994; 83: 65-69
6. Meyer TA, Wang J, Tiao GM, Ogle CK, Fischer JE, Hasselgren PO. Sepsis and endotoxemia stimulate interleukin-6 production. Surgery 1995; 118: 336-342
7. Magnotti LJ, Xu DZ, Lu Q, Deitch EA. Gut-derived mesenteric lymph but not portal blood increases endothelial cell permeability and promotes lung injury after hemorrhagic shock. Ann Surg 1998; 228: 518-527
8. Sambol JT, Xu DZ, Adams CA, Magnotti LJ, Deitch EA. Mesenteric lymph duct ligation provides long term protection against hemorrhagic shock-induced lung injury. Shock 2000; 14: 416-419
9. Langkamp-Henken B, Donovan TB, Pate LM, Maull CD, Kudsk KA. Increased intestinal permeability following shock and penetrating trauma. Crit Care Med 1995; 23: 660-664
10. Swank GM, Deitch EA. Role of the gut in multiple organ failure: bacterial translocation and permeability changes. World J Surg 1996; 21: 411-417
11. Lippmann M, Appel PL, Mok MS, Shoemaker WC, Sequential cardiorespiratory patterns of anesthetic induction with ketamine in critically ill patients. Crit Care Med 1989; 11: 730-734
12. Yli-Hanka A, Kirvela M, Randell T, Lindgren L. Ketamine anesthesia in a patient with septic shock. Acta Anaesthesiol Scand 1992; 36: 483-485
13. Takenaka I, Ogata M, Koga K, Matsumoto T, Shigematsu A. Ketamine suppresses endotoxin-induced tumor necrosis factor alpha production in mice. Anesthesiology 1994; 80: 402-408
14. Koga K, Ogata M, Takenaka I, Matsumoto T, Shigematsu A. Ketamine suppresses tumor necrosis factor-alpha activity and mortality in carrageenan-sensitized endotoxin shock model. Circ Shock 1995; 44: 160-168
15. Cavailon JM, Munnuz C, Fitting C, Misset B, Carlet J. Circulating cytokines: the tip of the iceberg? Circ Shock 1992; 38: 145-152
16. Beutler BA, Milsark IW, Cerami A. Cachectin tumor necrosis factor production, distribution, and metabolic fate in vivo. J Immunol 1985; 135: 3972-3977
17. Kogel C, Fong Y, Marano MA, Seniuk S, He W, Barber A, Minei JP, Felsen D, Lowry SF, Moldawer LL. Identification of a novel tumor necrosis factor alpha cachectin from the livers of burned and infected rats. Arch Surg 1990; 125: 79-84
18. Baldwin AS Jr. The NF-xB and IxB proteins: new discoveries and insights. Ann Rev Immunol 1996; 14: 649-683
19. Yu Y, Zhou Z, Xu J, Liu Z, Wang Y. Ketamine reduces NF kappa B activity and TNF alpha production in rat mononuclear cells induced by lipopolysaccharide in vitro. Ann Clin Lab Sci 2002; 32: 292-298
20. Gong JP, Liu CA, Wu CX, Li SW, Shi YJ, Li XH. Nuclear factor xB activity in patients with acute severe cholangitis. World J Gastroenterol 2002; 8: 346-349
21. Zhou W, Jiang ZW, Tian J, Jiang J, Li N, Li JS. Role of NF-xB and cytokine in experimental cancer cachexia. World J Gastroenterol 2003; 9: 1567-1570
22. Liu Y, Yu Y, Jiang J, Li Y. Growth hormone increases lung NF kappaB activation and lung microvascular injury induced by lipopolysaccharide in rats. Ann Clin Lab Sci 2002; 32: 164-170
23. Kawasaki T, Ogata M, Kawasaki C, Ogata J, Inoue Y, Shigematsu A. Ketamine suppresses proinflammatory cytokine production in human whole blood in vitro. Anesth Analg 1999; 89: 665-669
24. Hesse DG, Tracey KJ, Fong Y, Manogue KR, Palladino MA Jr, Cerami A, Shires GT, Lowry SF. Cytokine appearance in human endotoxemia and primate bacteremia. Surg Gyneco Obstet 1988; 166: 147-153
25. Danas P, Ledoux D, Nys M, Vrints Y, Groote D, Franchimont P, Lamy M. Cytokine serum level during severe sepsis in human: IL-6 as a marker of severity. Ann Surg 1992; 215: 356-362
26. Baeuerle PA, Baltimore D. NF-kappaB: ten years after. Cell 1996; 87: 13-20
27. Arya R, Grosjean VB Jr, Weissbrodt NW, Lai M, Mailman D, Moody F. Temporal expression of tumor necrosis factor alpha and nitric oxide synthase 2 in rat small intestine after endotoxin. Dig Dis Sci 2000; 45: 744-749
28. Secchi A, Orlander JM, Schmidl W, Walther A, Gebhard MM, Martin E, Schmid H. Effects of dobutamine and doxepamine on hepatic micro- and macrocirculation during experimental endotoxemia: an intravital microscopic study in the rat. Crit Care Med 2001; 29: 597-600
29. Taniguchi T, Shibata K, Yamamoto K. Ketamine inhibits endot-oxin-induced Shock in Rats. Anesthesiology 2001; 95: 928-932
30. Royblat L, Talmor D, Rachinsky M, Greenberg L, Pekar A, Appelbaum A, Gurman GM, Shapiro Y, Duvediani A. Ketamine attenuates the interleukin-6 response after cardiopulmonary bypass. Anesth Analg 1998; 87: 266-271