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Neutral sulfate berberine modulates cytokine secretion and increases survival in endotoxemic mice[1]

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Key words

lipopolysaccharide; berberine; tumor necrosis factor-α; interleukin-10; interleukin-12; interferon-γ; nitric oxide; mice

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Abstract

Aim: Berberine is thought to be an immunomodulator, so the present study aimed to investigate the effect of berberine on mortality, lung and intestine injury in endotoxemic mice, and the mechanism of its action. **Methods:** Mice were challenged with lipopolysaccharide (LPS, 28 mg/kg, ip), and neutral sulfate berberine was administrated intragastrically. Mortality was monitored every 12 h, and histology of the lungs and intestine as well as the plasma tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-12 (IL-12), IL-10, and nitric oxide (NO) levels were examined. **Results:** Pretreatment with 50 mg/kg neutral sulfate berberine once a day for 5 days significantly decreased the mortality rate and attenuated tissue injury of the lungs and small intestine in mice challenged with LPS. LPS stimulated a marked increase in plasma levels of TNF-α, IFN-γ, IL-12, IL-10, and NO. The administration of berberine significantly reduced plasma TNF-α, IFN-γ, and NO levels, but did not suppress plasma IL-12 levels in mice exposed to LPS. Furthermore, pretreatment with neutral sulfate berberine augmented IL-10 secretion stimulated by LPS in mice. **Conclusion:** Pretreatment with neutral sulfate berberine attenuates tissue injury and improves survival in endotoxemic mice, which may be mediated, at least in part, by the inhibition of pro-inflammatory mediator production and upregulation of IL-10 release. These findings might provide a new strategy for the treatment of endotoxemia.

Introduction

Mortality of patients with septic shock is still 40%–60% in spite of rapid progress in developing antibiotics and other therapeutic methods in clinical practice[1–3]. One of the major reasons is that endotoxin or lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, induces the disturbance of immune and inflammatory responses and causes extensive tissue damage[4]. LPS activates toll-like receptor 4-MD-2 complex on host cells, in particular on monocytes and macrophages, and initiates systemic inflammatory response that accompanies sepsis, characterized by the release of pro- inflammatory cytokines and other inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-12, interferons (IFN) and nitric oxide (NO)[4,5]. The progressive production of these inflammatory mediators may result in severe tissue damage and septic shock. In order to prevent tissue injury caused by excessive inflammatory mediators, some anti-inflammatory mediators, such as IL-10 and glucocorticoids, increase *in vivo* during endotoxemia[6,7]. Although excessive anti-inflammatory reaction will lead to compensatory anti-inflammatory response syndrome (CARS), which results in immunosuppression and an increase in the sensitivity of host to infection, proper anti-inflammatory response is beneficial for preventing tissue injury challenged by LPS. The administration of recombinant IL-10 inhibits inflammatory cytokine production and improves survival in experimental endotoxemia[8]. Based on these findings, clinical studies that block the action of LPS and inflammatory cytokines have been performed, including anti-LPS strategies and anti-cytokine strategies. However, these therapeutic strategies do not offer consistent success in decreasing the mortality of septic patients[7]. Therefore, it is very important to develop new therapeutic strategies that

improve survival in septic patients.

Berberine is an alkaloid initially isolated from some Chinese medicinal herbs, such as *Cortex phellodendri* (Huangbai) and *Rhizoma coptidis* (Huanglian). This compound has been known to have many pharmacological activities, including anti-microbial and anti-inflammatory activities[9,10]. Kuo *et al* found berberine could inhibit cyclooxygenase-2 expression and prostaglandins E_2 (PGE_2) production through regulating transcription factor activator protein 1[10]. In addition, Kang *et al*[11] and Kim *et al*[12] reported that berberine induced IL-12 p40 production via the activation of p38 mitogen-activated protein kinase (p38 MAPK) and α_2-adrenergic receptor in mouse macrophages, and deviates $CD4^+$ T cell from a Th2 to a Th1 response. More recently, our study has shown that berberine can inhibit LPS-stimulated myocardial TNF-α secretion and improves LPS-induced contractile dysfunction in the intact heart[13]. These findings suggest that berberine may have beneficial effects against endotoxemia. However, there is no direct evidence to determine whether berberine improves survival in endotoxemia. In the present study, we observed the effect of berberine on mortality and lung and intestine histological changes in endotoxemic mice and further investigated the mechanisms of its action.

Materials and methods

Mice All experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by US National Institute of Health. Male Kunming strain mice (Grade II, Certificate No 2004A019), weighing 20 to 23 g, were purchased from the Guangdong Province Center for Laboratory Animals. The mice were housed in microisolator cages and received food and water *ad libitum*. Laboratory temperature was 24±1 °C and relative humidity was 40%–80%. Before experimentation, the mice were left to adapt to the experimental environment for 2–3 d.

Survival study The survival study included two parts. In the first part, mice were administered intragastrically with distilled water (0.01 mL/g) or 50 mg/kg neutral sulfate berberine (0.01 mL/g, Sigma, St Louis, USA) once a day for 1, 3 or 5 d and injected intraperitoneally with normal saline or 28 mg/kg LPS from *Escherichia coli* (serotype 055:B5, Sigma) 1 h after the last gavage. In the second part, mice were treated intragastrically with distilled water (0.01 mL/g) or neutral sulfate berberine (Sigma) at doses of 25, 50, or 100 mg/kg every day for 5 d prior to intraperitoneal injection of LPS (28 mg/kg) or saline. After intraperitoneal injection of LPS or saline, the survival of mice in each group was assessed every 12 h for 7 d.

Histopathological examination and plasma preparation The mice were divided randomly into control, LPS, berberine alone and berberine+LPS group, and administered intragastrically with distilled water (0.01 mL/g) or 50 mg/kg neutral sulfate berberine (0.01 mL/g) once a day for 5 d. One hour after berberine treatment on d 5, LPS (28 mg/kg) or normal saline was injected intraperitoneally. Survival mice were killed at 24 h after intraperitoneal injection of LPS or saline. The lungs and intestine were harvested, fixed in 10% formaldehyde solution and embedded in paraffin. The evaluation of hematoxylin and eosin-stained sections was performed. The degree of lung injury was scored according to inflammatory cell infiltration and hemorrhage, the severity of injury was graded by the following criteria: 0 for no injury; 1 for injury to 25% of the field; 2 for injury to 50% of the field; 3 for injury to 75% of the field; and 4 for diffuse injury[14]. The severity of intestinal mucosal injury was scored as follows: 0 for no injury; 1 for surface epithelium damaged; 2 for less than 50% mucosa damaged; 3 for more than 50% mucosa damaged; and 4 for entire mucosa damaged[15]. All samples were analyzed according to the above histological scoring system by a pathologist. Previous studies reported that plasma cytokine and NO levels increased markedly or peaked at different time points in LPS-treated mice[16,23], in another experiment, blood samples were collected and the plasma was prepared for cytokine and nitric oxide determination at these indicated time points after LPS challenge.

Analysis of plasma cytokines and NO Levels of plasma TNF-α, IL-12, IFN-γ, and IL-10 were determined by enzyme-linked immunoabsorbent assay (ELISA) according to the manufacturer's instructions. ELISA kits for mouse TNF-α, IFN-γ, and IL-10 were purchased from R&D Systems (Minneapolis, MN, USA), and ELISA kits for mouse IL-12 (p70) was from Bender Medsystems (San Bruno, California, USA). The concentration of plasma NO was determined with the technique of nitrate reductase.

Statistical analysis Differences in survival rate were assessed with χ^2 test. Survival data were analyzed with the Kaplan-Meier test. Other data were expressed as mean±SD. The significance of the differences between individual groups was determined by using one-way ANOVA (Dunnett's *t*-test) and Student's *t*-test. Statistical difference was accepted at $P<0.05$.

Results

Effects of neutral sulfate berberine on the survival of mice challenged with LPS First, we investigated the effects of neutral sulfate berberine at different administration times on the survival rate of mice challenged with LPS. As shown

in Table 1, the survival rate of mice in the LPS group was lower than the control 24 h after LPS challenge, but pretreatment with neutral sulfate berberine (50 mg/kg) for 3–5 d significantly enhanced the survival rate of mice treated with LPS at 24 h. There were 79% of mice survived in the 5-day berberine treatment group 7 d after LPS challenge. In contrast, only 22% of mice in the LPS group were alive. Moreover, pretreatment with neutral sulfate berberine (50 mg/kg) at 1 h before LPS exposure also reduced the mortality rate of mice. After treatment with neutral sulfate berberine at a dose of 50 mg/kg for 5 d alone, no mice died during the experiment (data not shown).

Second, we further observed the effects of neutral sulfate berberine at different doses on the survival of mice challenged with LPS. As shown in Figure 1, the administration of 28 mg/kg LPS to mice resulted in a survival rate of 22% after 48 h. In contrast, when mice were given neutral sulfate berberine at doses of 25 mg/kg, 50 mg/kg, 100 mg/kg once a day for 5 d before LPS challenge, 76%, 79%, and 57% of them survived at 48 h, respectively, which was significantly better than that of mice exposed to LPS (P<0.01 or 0.05).

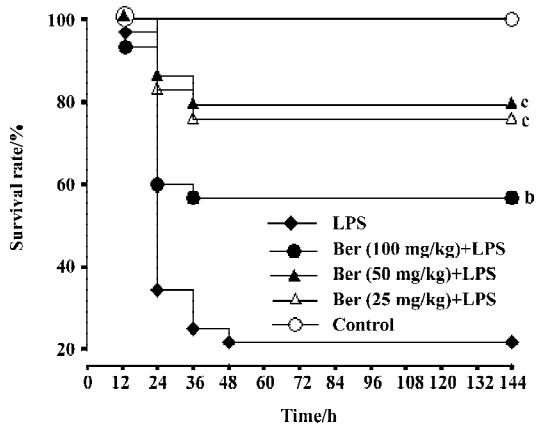

Figure 1. Time-course of survival rates of mice challenged with LPS (28 mg/kg, ip). Mice were divided into control (n=10), LPS (n=32) and berberine treatment groups (n=30 for each subgroup). Mice in berberine treatment group were treated intragastrically with 25 mg/kg, 50 mg/kg and 100 mg/kg neutral sulfate berberine (Ber) once a day for 5 d. Mice in control and LPS groups were given water; 5 d later, normal saline (control) or LPS was injected intraperitoneally. The survival was assessed every 12 h throughout the experiment. ^{b}P<0.05, ^{c}P<0.01 *vs* LPS group.

Effect of neutral sulfate berberine on histological changes of the lung and intestine in mice challenged with LPS As the lung is the primary target organ of endotoxemia, we firstly assessed lung injury by histological examination. Marked lung congestion, edema, alveolar septal thickening, hemorrhage and influx of inflammatory cells were observed at 24 h after LPS administration. Pretreatment with berberine remarkably relieved the above histological changes at 24 h after LPS administration. The inflammatory cell infiltration score (1.62±0.74) was reduced significantly in berberine+LPS group (n=8) compared to the LPS group (n=5, 2.80±0.84, P<0.05), and the hemorrhage score in berberine+LPS group (n=8, 1.25±0.46) was also decreased compared to the LPS group (n=5, 2.20±0.48, P<0.05). There was no abnormal change in histological architecture of control and berberine alone group (Figure 2). We further examined intestine structure of mice challenged with LPS, as shown in Figure 3, there was marked congestion, edema, and influx of inflammatory cells in intestinal villus at 24 h after LPS administration, and mass inflammatory cells were observed in the intestine cavity. Pretreatment with berberine remarkably relieved the above histological changes at 24 h after LPS administration. The intestine mucosal injury score was significantly reduced in the berberine+LPS group (n=8, 1.80 ± 0.71) compared to the LPS group (n=5, 2.60±0.55, P<0.05).

Effect of neutral sulfate berberine on plasma TNF-α, IL-12, IFN-γ, NO, and IL-10 contents in LPS-treated mice To investigate the effect of berberine pretreatment on the production of cytokines and inflammatory mediators induced by LPS in mice, plasma TNF-α, IL-12, IFN-γ, NO, and IL-10

Table 1. Effect of neutral sulfate berberine (Ber, 50 mg/kg) on survival rate (%) of mice challenged with LPS (28 mg/kg). ^{c}P<0.01 *vs* control. ^{e}P<0.05, ^{f}P<0.01 *vs* LPS group.

Group	n	Time after LPS injection/h											
		12	24	36	48	60	72	84	96	108	120	132	144
Ber(5 d)+LPS	29	100	86^{f}	79^{f}	79^{f}	79	79	79	79	79	79	79	79
Ber(3 d)+LPS	29	97	83^{f}	76^{f}	76^{f}	76	76	76	76	76	76	76	76
Ber(1 d)+LPS	30	97	70^{f}	53^{e}	53^{e}	53	53	53	53	53	53	53	53
LPS	32	97	34^{c}	25^{c}	22^{c}	22	22	22	22	22	22	22	22
Control	20	100	100	100	100	100	100	100	100	100	100	100	100

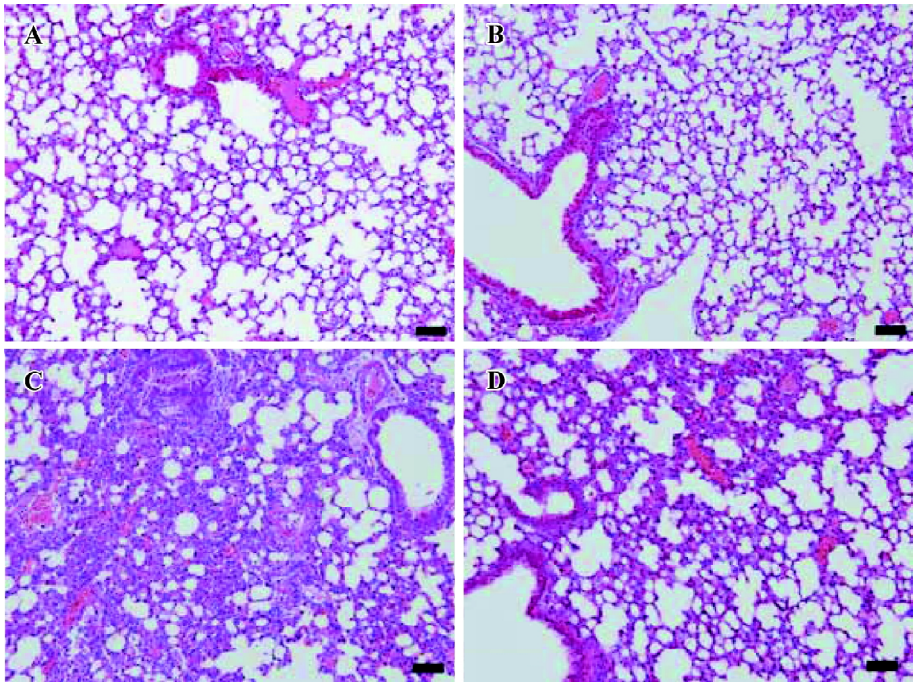

Figure 2. Histological changes in lung of mice 24 h after LPS or normal saline injection (HE staining, original magnification ×200, scale bar 100 μm). A, control; B, berberine alone group; C, LPS group; D, berberine+LPS group. Sections shown are representatives of three sections of lung per mouse, from five (in control, LPS and berberine alone group) or eight (berberine+LPS group) mice.

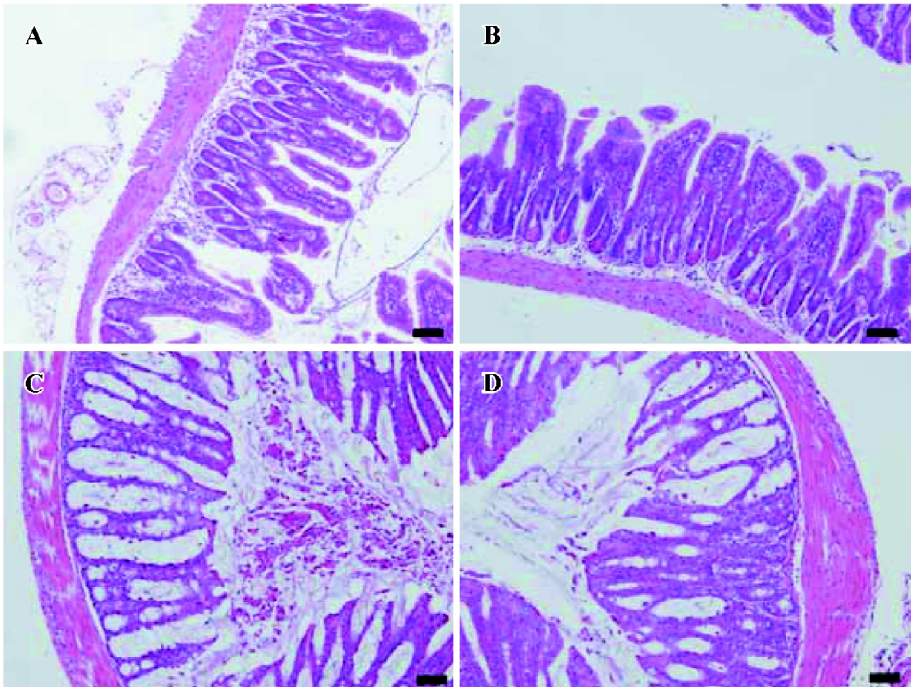

Figure 3. Histological changes in the intestine of mice 24 h after LPS or normal saline injection (HE staining, original magnification ×200, scale bar 100 μm). (A) Control; (B) Berberine alone group; (C) LPS group; (D) Berberine+ LPS group. Sections shown are representatives of three sections of lung per mouse, from five (in control, LPS and berberine alone group) or eight (berberine+LPS group) mice.

contents were determined at different time points after LPS injection. Plasma TNF-α, IFN-γ(191.94±131.76 ng/L, 4 h after LPS challenge) levels in the LPS group were higher than the control, pretreatment with 50 mg/kg neutral sulfate berberine once a day for 5 d significantly suppressed the LPS-induced production of TNF-α (Figure 4) and IFN-γ(45.04±57.06 ng/L, n=6, P<0.05) in mice. Plasma TNF-α and IFN-γ were not detectable in the control and berberine control group. LPS induced a significant increase in plasma IL-12 level, but there was no significant difference in plasma IL-12 level between berberine+LPS group and LPS group (Figure 4). Plasma levels of NO metabolites at 8 h after LPS injection increased dramatically, pretreatment with 50 mg/kg neutral sulfate berberine once a day for 5 d significantly reduced plasma NO

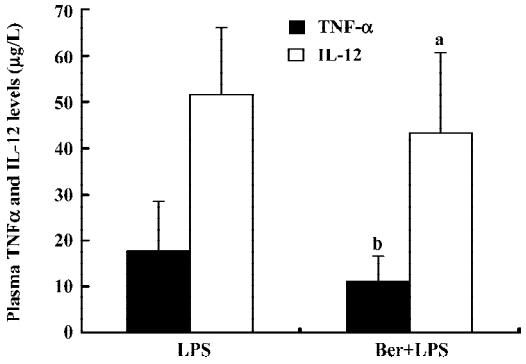

Figure 4. The levels of plasma TNF-α (2 h after LPS injection) and IL-12 (4 h after LPS injection) in mice in LPS and berberine+LPS groups. Plasma TNFα and IL-12 was not detectable in control and berberine alone group. n=14 for TNFα; n=15 for IL-12. Mean±SD. ^aP>0.05, ^bP<0.05 *vs* LPS group. Ber, neutral sulfate berberine.

levels 8 h after LPS challenge (Figure 5). As noted in Figure 6, mice in the control (n=10) and berberine alone group (n=10) had no detectable plasma IL-10 levels. Plasma IL-10 levels were markedly higher at 2 h after LPS challenge in berberine+LPS group (n=14) than LPS group (n=15). However, there was no difference in plasma IL-10 levels at 8 h after LPS challenge between the LPS (n=14) and berberine +LPS group (n=13).

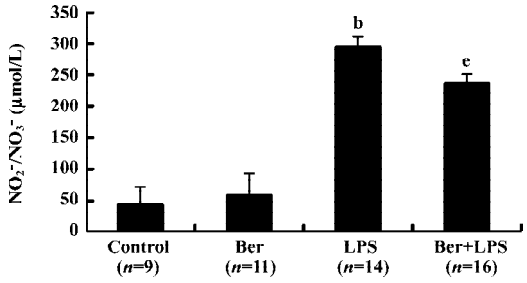

Figure 5. The effects of neutral sulfate berberine pretreatment on plasma NO_2^-/NO_3^- levels in mice 8 h after LPS (28 mg/kg, ip) challenge. Mean±SD. ^bP<0.05 *vs* control group. ^eP<0.05 *vs* LPS group, Ber, neutral sulfate berberine.

Discussion

In the present study, we demonstrated that pretreatment with neutral sulfate berberine protected mice from LPS-induced lethality and inhibited LPS-induced acute lung and intestine injury. LPS has proinflammatory properties and plays a crucial role in the pathogenesis of Gram-negative bacterial sepsis[7]. As an anti-microbial agent[9], berberine

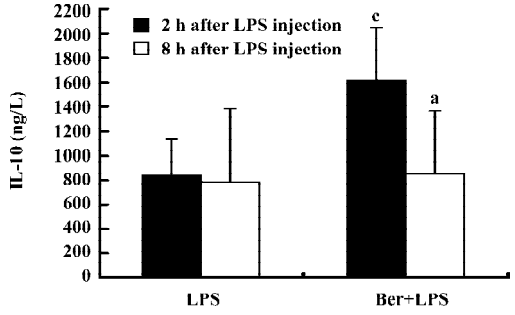

Figure 6. The effect of neutral sulfate berberine (Ber) pretreatment on plasma IL-10 levels in mice 2 and 8 h after LPS (28 mg/kg, ip) challenge. n=14 in LPS group. n=16 in Ber+LPS group. Mean±SD. ^aP>0.05, ^eP<0.01 *vs* LPS group.

can inhibit the biological toxicity of LPS, this suggests the application of berberine may provide a new therapeutic strategy for the treatment of sepsis.

LPS induces progressive production of pro-inflammatory mediators including TNF-α, IL-1β, IL-6, IL-12, and NO; at the same time it also stimulates the release of anti-inflammatory mediators such as IL-10. Inflammatory response disequilibrium causes leukocytic activation, microthrombus formation, refractory hypotension, circulatory failure and even wide tissue injury[7]. In order to explore the mechanisms underlying the protective action of berberine against endotoxemia, we investigated the regulatory effects of berberine on production of pro-inflammatory and anti-inflammatory mediators induced by LPS. In the survival study, we demonstrated that pretreatment with berberine at a dose of 50 mg/kg once a day for 5 d produced the best inhibitory effects on the mortality rate of mice challenged with LPS. Thus, we observed the effects of berberine at this dosage on plasma TNF-α, IL-12 (p70), IFN-γ, NO, and IL-10 levels in mice exposed to LPS. The results demonstrated that pretreatment with berberine remarkably inhibited TNF-α, IFN-γ and NO release and upregulated IL-10 in mice challenged with LPS, but pretreatment with berberine *in vivo* did not reduce plasma IL-12 (p70) levels in endotoxemic mice.

Kang *et al*[11] reported that treatment with berberine did not suppress TNF-α, IFN-γ, and IL-6 mRNA expression induced by LPS in mouse macrophages *in vitro*. In addition to the difference in the treatment protocol, differences in conditions between *in vivo* and *in vitro* experiments may be responsible for this discrepancy. In the present study, we found that pretreatment with berberine augmented IL-10 secretion stimulated by LPS. It is well known that IL-10 downregulates the production of TNF-α, IL-1β, IL-6, IL-12, IFN-γ, and NO[8,17,18]. As a powerful anti-inflammatory

cytokine, IL-10 plays an important role in improving survival in animals challenged with LPS. Administration of IL-10 protects animals against LPS-induced lethality. In contrast, treatment with monoclonal antibody to IL-10 increases lethality in endotoxemic animals[8,19]. Recently, some researchers observed the effects of immunomodulator with simultaneously regulating pro-inflammatory and anti-inflammatory mediators on endotoxemia. The results indicated that glycine, ester prodrug of mycophenolic acid, phenypiperazine derivatives and pirfenidone not only suppress TNF-α release but also increase IL-10 production stimulated by LPS. These agents all increase the survival rate of endotoxemic mice[20–23]. Therefore, IL-10 augmenting activity of berberine may be responsible for the inhibitory effect of pretreatment with berberine on TNF-α, IFN-γ, and NO production in endotoxemic mice. This might be one of reasons why pretreatment with berberine improved the survival of mice exposed to LPS.

However, in this study, we showed that pretreatment with berberine did not inhibit LPS-induce IL-12 (p70) production in mice. Kang *et al* demonstrated that berberine was able to induce IL-12 p40 production through activating α_2-adrenergic receptor and p38 mitogen-activated protein kinase not modulating transcription factor NF-κB activity, and significantly enhanced IL-12 p40 production in a dose-dependent manner in mouse macrophages when combined with LPS[11]. IL-12 is a heterodimeric cytokine consisting of 35 (p35) and 40 (p40) kDa subunits. The highly coordinated expression of p40 and p35 forms p70. Because the IL-12 p35 gene is constitutively expressed and p40 or p70 production is limited in activated macrophages and monocytes, p40 expression controls bioactive p70 secretion[11,24]. Accordingly, it seemed reasonable that pretreatment with berberine could not reduce plasma IL-12 p70 levels in endotoxemic mice, because direct upregulation of IL-12 p40 production by berberine itself might diminish the action of IL-10. However, some studies have shown that α_2-adrenergic agonist increases LPS-induced TNF-α mRNA expression and lethality[25,26], while specific α_2 adrenoceptor antagonists protect against lethality and organ injury induced by LPS[26]. Therefore, activation of α_2-adrenergic receptor by berberine may be harmful in endotoxemia. Our recent observation demonstrated that the α_2 adrenoceptor antagonist, yohimbine, enhanced protective effects of berberine against endotoxemia in mice (data not shown), large dose berberine might activate α_2 adrenoceptor *in vivo*, which might explain why the mortality rate of endotoxemic mice in the 100 mg/kg berberine treatment group was higher than the 50 mg/kg berberine treatment group in this experiment. This remains to be further investigated.

We have shown that pretreatment with neutral sulfate berberine decreases mortality and attenuates lung and intestine injury in mice exposed to LPS, probably through a regulating balance of pro-inflammatory and anti-inflammatory cytokines. These findings may provide an useful therapeutic strategy for endotoxemia.

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