Biliverdin administration protects against endotoxin-induced acute lung injury in rats

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Biliverdin administration protects against endotoxin-induced acute lung injury in rats. Am J Physiol Lung Cell Mol Physiol 289: L1131–L1137, 2005. First published September 9, 2005; doi:10.1152/ajplung.00458.2004.—Given the high morbidity and mortality rates associated with pulmonary inflammation in sepsis, there is a pressing need for new therapeutic modalities to prevent acute respiratory distress. The enzyme heme oxygenase-1 (HO-1) provides potent cytoprotection against lung injury; however, the mechanism by which it does so is unclear. HO-1 catabolizes heme into biliverdin (BV), which is rapidly converted to bilirubin by BV reductase. We tested the hypothesis that BV administration could substitute for the effects observed with HO-1. Using the well-described rat model of LPS-induced shock, we demonstrated that exposure to BV imparts a potent defense against lethal endotoxia systemically, as well as in the lungs, and effectively abrogates the inflammatory response. BV administration before a lethal dose of LPS leads to a significant improvement in long-term survival: 87% vs. 20% in sham-treated controls. BV treatment suppressed LPS-induced increases in lung permeability and lung alveolitis and significantly reduced serum levels of the LPS-induced proinflammatory cytokine IL-6. Moreover, bilirubin administered just after LPS also abrogated lung inflammation. BV treatment also augmented expression of the anti-inflammatory cytokine IL-10. Similar effects on production were observed with BV treatment in vitro in mouse lung endothelial cells and RAW 264.7 macrophages treated with LPS. In conclusion, these results in mouse lung endothelial cells suggest that BV imparts a potent defense against lethal endotoxia systemically, as well as in the lungs, and effectively abrogates the inflammatory response. BV administration before a lethal dose of LPS leads to a significant improvement in long-term survival: 87% vs. 20% in sham-treated controls. BV treatment suppressed LPS-induced increases in lung permeability and lung alveolitis and significantly reduced serum levels of the LPS-induced proinflammatory cytokine IL-6. Moreover, bilirubin administered just after LPS also abrogated lung inflammation. BV treatment also augmented expression of the anti-inflammatory cytokine IL-10. Similar effects on production were observed with BV treatment in vitro in mouse lung endothelial cells and RAW 264.7 macrophages treated with LPS. In conclusion, these results in mouse lung endothelial cells suggest that BV imparts a potent defense against lethal endotoxia systemically, as well as in the lungs, and effectively abrogates the inflammatory response. BV administration before a lethal dose of LPS leads to a significant improvement in long-term survival: 87% vs. 20% in sham-treated controls. BV treatment suppressed LPS-induced increases in lung permeability and lung alveolitis and significantly reduced serum levels of the LPS-induced proinflammatory cytokine IL-6. Moreover, bilirubin administered just after LPS also abrogated lung inflammation.

MATERIALS AND METHODS

Animals. Pathogen-free male Sprague-Dawley rats (225–250 g; Harlan Sprague-Dawley, Indianapolis, IN) were allowed to acclimate for 1 wk before experimentation. The animals were fed rodent chow and water ad libitum. They were briefly anesthetized with isoflurane (2% vol/vol) before intravenous LPS (Escherichia coli serotype 0127: B8, Sigma Chemical, St. Louis, MO) administration: 50 mg/kg iv for survival studies and 3 mg/kg iv for sublethal studies.

BV hydrochloride (Frontier Scientific, Logan, UT; 35 mg/kg ip) was administered 16 h and again 1 h before LPS (3 mg/kg iv). Bilirubin (Frontier Scientific) was administered once (35 mg/kg iv).

Experiments were carried out according to protocols approved by the Animal Care and Use Committee at the University of Pittsburgh School of Medicine. A commercially available assay kit (Sigma Chemical) was used to determine serum bilirubin.

Cecal ligation-and-puncture model. BV was dissolved in 0.1 N NaOH and adjusted to final pH 7.4 with HCl. After dilution in PBS, BV (35 mg/kg ip) was injected before, during, and after the operation: 8, 6, and 3 h before the operation, at the time of the operation, and 15 h after the operation. The appropriate saline volume was administered intraperitonealately at the same time points to the control animals. The rats were anesthetized with continuous isoflurane inhalation. The operative procedures were performed under sterile conditions. Animals underwent a small midline laparotomy; the cecum was everted and partially ligated using a 4-0 silk tie and punctured once with a 20-gauge needle.

THE ENZYME HEME OXYGENASE (HO) is the rate-limiting step in the degradation of heme to biliverdin (BV), which is rapidly converted to bilirubin by BV reductase (BVR). During this process, CO and free iron are also generated in equimolar quantities, the latter being promptly sequestered into ferritin [the iron storage protein (9)] or removed via an induced iron channel (3).

It has become apparent that one or more of its three products mediates the effects of HO-1 expression. Although extensive data suggest that CO can often substitute for HO-1 (5, 6, 13, 17, 18, 20, 24, 31), CO does not do so in all cases. In addition, in those situations in which CO and BV have been tested, it appears that they affect different components of the pathophysiological processes underlying disorders such as ischemia-reperfusion injury of the small intestine and that they do not function entirely by the same signaling pathways, as found with inhibition of smooth muscle cell proliferation (16). We thus tested the hypothesis that administration of BV could also substitute for the effects otherwise observed with HO-1 and CO in a model of endotoxin-induced acute lung injury, as well as in cecal ligation and puncture, a clinically relevant model of sepsis and acute lung injury (10). We show that BV does ameliorate the injury in these situations and that, at least in part, it does so by blocking NF-κB activation.

On the basis of work done with HO-1 in vivo and in vitro in macrophages (22), a cell that is intimately involved in contributing to induction and effecting the inflammatory response, we hypothesized that BV would suppress the LPS-induced inflammatory response. BV is rapidly converted to bilirubin when administered in vivo (30), and with the potential for a BV-bilirubin redox cycle (2), the generation of each product should be ample. An advantage to BV administration is endogenous generation of bilirubin by BVR, which is present in most cells.
a 21-gauge needle. Peritonitis was induced by cecal ligation and puncture as described elsewhere (10, 29). After 24 h, bronchoalveolar lavage (BAL) was performed (see below).

**BAL analyses.** The following procedure was performed for in vivo studies of LPS- and BV-treated rats and rats subjected to cecal ligation and puncture. Animals were anesthetized with isoflurane, killed by transection of the heart, and tracheotomized. BAL (8 ml/lavage) was performed three times via the tracheal catheter with ice-cold PBS (pH 7.4). From the first BAL, total protein was measured via Bradford assay (Bio-Rad, Hercules, CA). Cell pellets were pooled from the first and subsequent two BALs and centrifuged at 800 g for 10 min. The supernatant was discarded, and the cells were resuspended in PBS. Cell counts were performed in triplicate using a Neubauer hemocytometer. For differential analyses, 20 μl of the cell resuspension were cytocentrifuged and stained with Diff Quick (IMEB, San Marcos, CA). For each sample, 200 cells (~16 different fields) were identified by standard cellular morphological characteristics and counted.

**Cell culture and reagents.** RAW 264.7 mouse peritoneal macrophages (American Type Culture Collection, Rockville, MD) were maintained and grown in DMEM with 10% fetal bovine serum and 0.5% gentamicin and stimulated with 1 μg/ml LPS (E. coli serotype 0127:B8). Mouse lung endothelial cells (generous gift of Dr. B. Pitt, School of Public Health, University of Pittsburgh) were grown in DMEM-HEPES with endothelial cell growth supplement and supplemented with 1 μg/ml LPS.

BV hydrochloride was dissolved in 0.2 N NaOH and adjusted to pH 7.4 with 1 N HCl. It was diluted in PBS and used at 1–100 μM before administration of LPS. Because BV is light sensitive, the solution was prepared and the experiments were carried out in dim light.

**Cytokine measurement.** The concentration of cytokines released by LPS-stimulated macrophages into the culture supernatant and from the serum of the rats was measured by enzyme-linked immunosorbent assay according to the manufacturer’s instructions (R & D Systems, Minneapolis, MN).

**Electrophoretic mobility gel shift assay.** Nuclear protein extracts from the lung of rats were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) according to the manufacturer’s instructions. Nuclear proteins (10 μg) were incubated in binding buffer (Promega, Madison, WI) with 3.5 pmol of double-strand DNA oligonucleotide containing an NF-κB consensus binding sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′), which was labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega). Binding reactions were completed by incubation of the reaction solution for 30 min at room temperature. Protein-DNA complexes were separated from the unbound DNA probe by electrophoresis through 5% native polyacrylamide gels containing 0.5× Tris-borate-EDTA. The gel was dried and exposed to Biomax MR film (Kodak, Rochester, NY).

**RNA extraction and SYBR green real-time RT-PCR analysis.** Total RNA was isolated using the Trizol method, with homogenization of the lung tissue in Trizol lysis buffer followed by chloroform extraction (Life Technologies, St. Paul, MN). Total RNA (10 μg) was separated by 1% agarose gel electrophoresis and then transferred to a GeneScreen Plus membrane (NEN, Boston, MA) by capillary action. Ethidium bromide staining of the gel was used to confirm the integrity of the RNA.

The mRNA for IL-6 and IL-10 were then quantified in duplicate by SYBR green two-step real-time RT-PCR. After removal of potentially contaminating DNA with DNase I and Superscript II (Life Technologies), first-strand cDNA was generated. SYBR green PCR Master Mix (PE Applied Biosystems, Foster City, CA) was used to prepare the PCR mixture using the primer sequences designated below. Thermal cycling conditions were 10 min at 95°C to activate the AmpliTaq Gold DNA polymerase followed by 40 cycles at 95°C for 15 s and 60°C for 1 min on a sequence detection system (ABI Prism 7000, PE Applied Biosystems). Expression of each gene was normalized with GAPDH mRNA content.

**RESULTS**

**BV protects against lethal endotoxic shock.** We tested whether pretreatment with BV (35 mg/kg ip) before injection of LPS would confer protection against endotoxic shock. We chose the 35 mg/kg dose of BV for two reasons: 1) the levels of BR generated after this dose of BV would be elevated but not outside the high normal range of <1 mg/dl (Fig. 1B), and 2) in models of organ transplantation, BV has been shown to exert protective effects at this dose (30). LPS administered at 50 mg/kg iv to rats resulted in 80% mortality within 20 h. Conversely, 87.5% of rats survived as evaluated at 72 h when pretreated with BV 16 h and again 1 h before LPS injection (Fig. 1A; *P < 0.03 vs. vehicle). Historically, rats that survive >72 h survive long term. We measured serum bilirubin in rats treated with BV or vehicle: at 1 h, serum bilirubin increased threefold in animals treated with BV but remained at normal levels in control (vehicle-treated) rats (Fig. 1B). This confirmed the conversion of BV to bilirubin as expected and suggests that bilirubin may act in part to explain the protection conferred by BV administration.

**BV modulates LPS-induced cytokine production in vivo.** We used a sublethal dose of LPS (3 mg/kg iv) to study the effects
of BV on the acute-phase inflammatory response (production of cytokines) that accompanies lung injury. Serum levels of the LPS-induced proinflammatory cytokine IL-6 were significantly suppressed (>40%) by BV (Fig. 2A), whereas serum levels of the anti-inflammatory cytokine IL-10 were elevated (250%; Fig. 2B) compared with the sham controls. IL-6 is accepted as the serum marker most predictive of survival (15, 21). Measurements of LPS-induced TNF-α production were not significantly modulated by BV (data not shown) as otherwise observed with HO-1 and CO. These data perhaps show the different roles of each product in preventing or limiting the inflammatory response.

**BV modulates LPS-induced cytokine mRNA production in vivo.** RT-PCR analysis of lung tissue RNA revealed a significant increase in IL-6 and IL-10 mRNA expression in whole tissue homogenates 1 h after LPS injection compared with control animals. In animals pretreated with BV, the mean expression of IL-6 in response to LPS was significantly reduced, whereas IL-10 expression was significantly elevated (Fig. 3).

**BV inhibits LPS-induced lung inflammation and leukocyte accumulation in the BAL.** LPS injection results in acute alveolitis characterized by increased neutrophil (PMN) influx into the lung and increased pulmonary edema. BV pretreatment significantly reduced the number of total leukocytes and PMNs recovered in the BAL and as measured by total tissue myeloperoxidase accumulation (data not shown) by >60% and decreased protein accumulation assessed 24 h after LPS compared with controls (Fig. 4). On the basis of the rapid conversion of BV to bilirubin (Fig. 1B), we next tested whether bilirubin administered just after LPS to simulate more closely the clinical setting would result in similar anti-inflammatory effects in the lung. We observed a marked diminution of LPS-induced airway inflammation by bilirubin (Fig. 4C). BAL PMN counts were reduced >50% compared with sham-treated controls. Similar results were observed with BV administered at the same time as LPS (data not shown). We conclude from these experiments that bilirubin generated from BV imparts protection otherwise observed with BV and, in fact, may be the mechanism by which BV abrogates the inflammatory response. These data show a protective effect of BV that has functional importance in maintaining lung barrier function, which ordinarily is compromised after administration of endotoxin.

As an alternative and more clinically relevant method by which to induce inflammation and acute lung injury, we used the well-characterized model of cecal ligation and puncture in a separate group of rats. The symptoms and signs in this model are very similar to those observed with LPS administration. In this model as well, similar to the results found with LPS, the total cell count in the BAL 24 h after septic insult was significantly lower (75.8%, P < 0.02) in rats treated with BV than in the controls (Fig. 4D).

**BV reduces NF-κB binding in the lung.** To test potential signaling pathways and mechanisms by which BV might act, we hypothesized that the transcription factor NF-κB would be involved. LPS administration results in very strong activation of NF-κB as measured by electrophoretic mobility gel shift assay in the lungs of rats. Administration of BV before LPS injection resulted in a marked decrease in NF-κB binding compared with the LPS-treated animals (Fig. 5).

**BV modulates LPS-induced IL-6 production in vitro.** RAW 264.7 mouse peritoneal macrophages and mouse lung endothelial cells were pretreated with BV (10 μM) for the last 16 h before LPS. BV significantly decreased LPS-induced IL-6 production in both cell types (P < 0.01 vs. vehicle; Fig. 6, A and B). We tested the effects of BV in both cell types, because from the in vivo data it was not clear which cell type might be targeted by BV.

**BV requires HO-1 activity to prevent endotoxin-induced alveolitis.** BVR has been shown to function as a transcription factor on activation, and HO-1 is one of the genes it has been shown to regulate (1). We tested the hypothesis that BV administration would function via BVR and, in part, mediate its protective effects in the lung by increasing HO-1 expres-
In an earlier report from our laboratories, we showed that LPS potently upregulates HO-1 expression in the lungs of rats (19). We therefore administered the selective chemical inhibitor tin protoporphyrin (Sn-PP), which we have also previously shown to block HO-1 activity (18), in the presence and absence of BV administered via the regimen described above. We then evaluated LPS-induced PMN influx into the air space in each treatment group (Fig. 7). Blockade of HO-1 resulted in loss of the BV inhibitory effects, supporting the concept that HO-1 activity is necessary in mediating the function of BV. We cannot rule out the possibility that Sn-PP might interfere with other BVR functions. Future studies are needed to investigate the role of BVR in more detail.

DISCUSSION

We have examined the exogenous administration of BV as a potential therapy for endotoxic shock. Bile pigments have long been regarded as toxic products that are rapidly conjugated to glucuronic acid for urinary excretion (26). In recent years, however, it has become clear that bilirubin can also serve as a potent antioxidant in the brain, acting to scavenge peroxyl radicals with at least as much efficiency as \( \alpha \)-tocopherol or vitamin E (12). It is also well accepted that bilirubin is the most powerful antioxidant in serum (4). It is also been suggested that jaundice, whereby plasma bilirubin levels become significantly elevated, may prove beneficial to neonates and is a normal reaction whereby the newborn eliminates free radicals produced by the sudden influx of oxygen in the first breaths. That bilirubin has beneficial protective properties is becoming obvious from several studies. Individuals with Gilbert’s syndrome, a genetic disorder resulting in a mild hyperbilirubinemia, have a fivefold lower risk for developing vascular disease than the general population (27). Individuals with high to just-above-normal levels of bilirubin also have less atherosclerosis-type disorders than individuals with just-below-normal bilirubin levels. In bleomycin-induced pulmonary fibrosis, continuous administration of bilirubin, resulting in hyperbilirubinemia, attenuated lung inflammation and transforming growth factor-\( \beta \) production (28). Our finding that bilirubin administered along with LPS is protective supports a protective role in abrogating endotoxic shock-induced lung inflammation. Upregulation of HO-1 activity in an ischemia-reperfusion model significantly restored myocardial function and mini-
and lethal endotoxic shock in rats (22). We have shown that a low concentration of CO protects against LPS-induced lung injury (16a).

Bovine serum (BV) has been shown to inhibit the proliferation, which is a major contributor to chronic rejection (31). BV has also been shown to block smooth muscle cell proliferation, which is a major contributor to chronic rejection (16). BV acts similarly: pretreatment with BV saved 87% of the rats from lethal endotoxic shock (Fig. 1A). To examine the anti-inflammatory effects of BV, we injected rats with a sublethal dose (3 mg/kg) of LPS. BV pretreatment effectively decreased the serum levels of the proinflammatory cytokine IL-6 while increasing the production of the anti-inflammatory cytokine IL-10 ($P < 0.05$ vs. control; Fig. 2, A and B). This correlated with the effects of BV treatment on expression of these cytokines in the lung tissue (Fig. 3). In contrast, in a model in which a syngeneic small intestine was transplanted (16), BV administration did not alter production of the anti-inflammatory cytokine IL-10 or HO-1 expression, suggesting that these effects of BV might be tissue, cell, and/or model specific.

The morbidity and mortality associated with sepsis and septic shock have not changed over the past 50 years. Use of the cecal ligation model allowed us to evaluate the effects of BV in a clinically relevant model of sepsis (10). BV prevented the lung injury that is a prominent feature of sepsis and, thus, might be considered a potential therapeutic modality against sepsis-induced acute respiratory distress. Additionally, bilirubin administration after LPS injection was equally effective in preventing endotoxin-induced airway inflammation, which clinically is very relevant, inasmuch as many septic patients present after inoculation with the pathogen.

Fig. 7. Administration of tin protoporphyrin (Sn-PP) to block heme oxidase 1 (HO-1) prevents protection of the lung by BV. Sn-PP (50 μmol/kg sc) was administered 5 h before the first administration of BV (35 mg/kg iv); 2 h after the second injection of BV, animals were treated with LPS (3 mg/kg iv). PMN influx was evaluated 24 h later by BAL. BV prevents PMN influx, which is lost in animals pretreated with Sn-PP to block HO-1. Values are means ± SD of 4 replicate treatment groups. *$P < 0.05$ vs. LPS and LPS/BV/Sn-PP in PMNs.

Studying the mechanisms that could explain the protective anti-inflammatory effect of BV, we examined NF-κB, a transcription factor by which LPS modulates cytokine production. In our previous studies (22, 23), we showed that CO, another product of HO-1 action, inhibits LPS-induced NF-κB activation in macrophages, which contributes to the cytoprotection. Here we showed that BV also inhibits NF-κB activation in the lung (Fig. 5), as we also found in a model involving transplantation of the small intestine in a syngeneic combination (16). These findings are consistent with the concept that activation of NF-κB may contribute to injury and that its suppression by BV may be part of the mechanism whereby BV elicits its salutary effects. Further studies are needed to test this concept.

Our in vitro studies are consistent with the in vivo findings and provide additional insights into the mechanisms by which BV affords protection. BV has anti-inflammatory effects inmediated infarct size and mitochondrial damage on reperfusion. This same effect was observed when bilirubin was administered exogenously at concentrations as low as 100 nM (7).

A recent report of a model of kidney and liver ischemia-reperfusion injury further supports a protective response of BV against tissue injury (11).

It has recently been suggested that bilirubin can be reconverted to BV when bilirubin is oxidized by peroxyl radicals and then recycled by BVR to bilirubin (2). This redox cycle might explain the antioxidant cytoprotection assigned to bilirubin and BV. The antioxidant activity of BV is predominantly due to its ability to scavenge free radicals (8). BVR has pleiotropic effects, in that it has been shown to modulate cellular functions by acting not only as an enzyme that converts BV to bilirubin but also as a transcription factor as well as a kinase (1). BV administration has a salutary effect in other systems as well. This same effect was observed when bilirubin was administered 5 h before the first administration of BV (35 mg/kg ip); 2 h after the second injection of BV, animals were treated with LPS (3 mg/kg iv). PMN influx was evaluated 24 h later by BAL. BV prevents PMN influx, which is lost in animals pretreated with Sn-PP to block HO-1. Values are means ± SD of 4 replicate treatment groups. *$P < 0.05$ vs. LPS and LPS/BV/Sn-PP in PMNs.

Previous work from our laboratories showed that induction of HO-1 by administration of heme or exposure to a low concentration of CO protects against LPS-induced lung injury and lethal endotoxic shock in rats (22). We have shown that BV affords protection. BV has anti-inflammatory effects in a clinically relevant model of sepsis (10). BV prevented the lung injury that is a prominent feature of sepsis and, thus, might be considered a potential therapeutic modality against sepsis-induced acute respiratory distress. Additionally, bilirubin administration after LPS injection was equally effective in preventing endotoxin-induced airway inflammation, which clinically is very relevant, inasmuch as many septic patients present after inoculation with the pathogen.

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cultured macrophages and endothelial cells; BV led to a significant decrease in LPS-induced IL-6 production in these cells (Fig. 6, A and B). The anti-inflammatory effect was seen at only 1 μM. Our studies of the administration of BV, expression of HO-1, and protection gave what might, at first analysis, appear to be conflicting data. We suggest that HO-1 is normally induced by LPS and that the products of HO-1 action, including BV and CO, elicit protection from endotoxic shock and acute lung inflammation. BV may act directly, perhaps through its antioxidant properties, to protect from endotoxic shock. The Sn-PP data suggest that BV requires HO-1 and, perhaps, BVR to exert its effects. Perhaps administration of BV triggers a feedforward cycle, whereby upregulation of HO-1 and/or increase in BVR activity leads to the generation of endogenous products such as CO, ferritin, or more BV, which can then participate in the redox cycling proposed by Sedlak and Snyder (25). Why does the physiological response of HO-1 to the stress of LPS administration or cecal ligation and puncture not prevent death of the animals? We have argued before, and do so again here, that the physiological response might be too late and/or too weak.

Kutty and Maines (14) suggested that BV in the intestine increases HO-1 expression. Unpublished results from our laboratory indicate that BV administration to LPS-stimulated macrophages leads to inhibition of the induction of inducible nitric oxide (NO) synthase and, thus, NO production. NO is a powerful stimulus for the upregulation of HO-1. Therefore inductive NO synthase blockade suggests an NO-independent mechanism by which HO-1 is upregulated, perhaps, in this case, by BVR.

The following question remains unanswerable: By which mechanism(s) does BV exert its effects? Our data with bilirubin would argue against a direct role for BVR; however, we do not see a complete abrogation of the inflammation, suggesting that BVR has a potential role in gene regulation or that bilirubin may enter the redox cycle as mentioned above and generate BV. The redox cycling concept of Sedlak and Snyder (25) supports a powerful antioxidant mechanism. A decrease in oxidative stress compared with the situation normally present with LPS could be one explanation. Alternatively, BVR, which has been elegantly described to have multiple functions, including gene regulation, could be regulating a battery of protective genes after administration of its substrate. Our results found no change in BVR expression, but we did not investigate whether BVR was translocated to the nucleus, for instance, for gene regulation or as an activated kinase. It is possible that, on addition of BV, BVR is redirected to different compartments of the cell. Unpublished observations suggest that this may indeed occur in vitro in macrophages exposed to BV, which supports the findings by Ahmad et al. (1). It is provocative to hypothesize that BVR might be the central mediator of the protection observed with HO-1. With each product of HO-1 activity imparting different protective mechanisms and cellular targets, our understanding of the potent cytoprotective properties of HO-1 induction is beginning to emerge as each of these products begins to be dissected separately. The correlation between HO-1 induction and endogenous generation of the products and exogenous administration of each product needs to be better resolved and remains a shortcoming of these results. Further characterization of dosing regimens and kinetic assessment of true HO-1 activity (separate from the artificial in vitro assay), particularly in the in vivo setting, will begin to shed light on these important questions.

In summary, our study shows that administration of BV provides effective protection in endotoxic shock and a clinically relevant cecal ligation-and-puncture sepsis model in rats. BV improves survival rates and suppresses the inflammation in vivo and in vitro. We speculate that blockade of NF-κB activation by BV is an important factor in these effects.

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REFERENCES


