Bullatine A Exerted Anti-Inflammatory Effects by Inhibiting JNK/ROS/NF- κ B Pathway and Attenuates Systemic Inflammatory Response in LPS-Challenged Mice

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Research

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Abstract

Background: The genus *Aconitum* has rich pharmacological characteristics. *Aconiti brachypodi* Radix (Xue-shang-yi-zhi-hao) is a dried root of aconitum, which is considered to be analgesic and anti-inflammatory in modern medical and pharmaceutical clinical studies. Bullatine A (BA), a major active ingredient of this plant, has been reported for its significant anti-analgesic effect in previous studies. However, the role of BA in inflammation is unknown. In the current study, we aimed to explore the effect of BA on lipopolysaccharide (LPS)-induced inflammatory response both *in vitro* and *in vivo* and its potential anti-inflammatory mechanism.

Materials and Methods: The anti-inflammatory effect of BA was evaluated in two different types of LPS-induced macrophages, including BV-2 microglial cells and immortalized murine bone marrow-derived macrophages (iBMDMs), and in acute inflammation mouse models induced by LPS. Immunofluorescence, flow cytometry, quantitative RT-PCR, western blot and Hematoxylin-Eosin staining were used to determine the anti-inflammatory properties of BA.

Results: The results showed that BA significantly reduced the mRNA levels of several pro-inflammatory cytokines induced by LPS both in BV-2 cells and iBMDMs. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in response to LPS were also decreased by BA. Further investigations indicated BA significantly blocked the phosphorylation of I κ B kinase, degradation of the inhibitor I κ B α and the nuclear translocation of nuclear factor- κ B (NF- κ B) p65. BA also reduced c-Jun N-terminal kinases (JNK) phosphorylation and ROS generation in iBMDMs activated with LPS, but had no effect on other mitogen-activated protein kinases (MAPKs) family proteins such as extracellular signal-regulated kinase (ERK) or p38. Furthermore, BA treatment alleviate liver and lung tissue damage, reduce inflammatory cell infiltration, and inhibit the expression of inflammatory cytokines in LPS-challenged mice.

Conclusions: This study illustrated that BA has obvious anti-inflammatory effects both *in vitro* and *in vivo*, and its underlying anti-inflammatory mechanism may be via inactivating JNK/ROS/NF- κ B pathway. Therefore, BA may have a certain therapeutic potential for inflammatory-related diseases.

Introduction

Phytopharmaceuticals has gained increased attention in worldwide due to its beneficial role in treating a variety of human diseases and also because of the presence of a huge number of natural compounds with various chemical properties¹. The preparations of the *Aconitum* genus have been extensively used for the treatment and prevention of various diseases, including pain, inflammation and neurological and cardiovascular diseases in China and other Asian countries². In China, approximately 76 *Aconitum* species are utilized to be the medicinal plants based on their toxic and side effects, medicinal effects and phytochemical properties^{3,4}. Among them, *Aconiti brachypodi* Radix (Xue-shang-yi-zhi-hao)-the dried roots of *Aconitum brachypodum* Diels, as a traditional Chinese herbal medicine has been listed in the Chinese Pharmacopoeia in 1977 for its treatment of rheumatism and pain⁵.

Bullatine A (BA, C₂₂H₃₃N₂O₂, shown in Figure. 1A), an alkaloid, is one of the major bioactive compounds isolated from *Aconiti brachypodi* Radix. In recent years, the alkaloids of aconitum has been proved to have therapeutic analgesic effects⁶. Evidence from Ren *et al.*³ systematically administered the ethanol extract of *Aconiti brachypodi* Radix (including BA) and found that it effectively attenuated pain response in mice tests with hot-plate, acetic acid, and formalin. Further study reveals that BA is the active compound, which attenuates the pain hypersensitivity in a variety of rat pain models⁷. Recently, BA is found to inhibit ATP-induced BV-2 microglial cell death and P2X receptor-mediated inflammatory responses². These studies suggest BA might have the anti-inflammatory activity. Not only that, BA has great potential to be a candidate drug for the prevention or treatment of inflammation-mediated diseases, since studies has shown that it is less toxic than most other alkaloids isolated from aconitum^{7,8}. However, up to now, few studies have investigated whether BA has a potential effect in lipopolysaccharide (LPS) -induced inflammatory responses *in vivo*, and its underling mechanism remains unclear.

Accordingly, the aimed of present study was to explore the effect of BA on LPS-induced inflammation in macrophages and microglia, with a protective effect of BA pretreatment on LPS inducing systemic inflammation in model mice. Furthermore, the study also sought to elucidated the underlying molecular mechanism during these processes.

Materials And Methods

Reagents and antibodies

BA (purity≥98%) was purchased from Psaitong (Beijing, China). Dimethyl sulfoxide (DMSO) and LPS was obtained from Sigma (St. Louis, MO, USA). Antibodies used for western blotting in the present study were as follows: the primary antibodies against inducible Nitric Oxide Synthase (iNOS) (#13120), anti-cyclooxygenase-2 (COX-2) (#12282), anti-phospho-IKKα/β (S176/180) (16A6) (#2697P), anti-phospho-IκBα (Ser32) (#2859), anti-IκBα (44D4) (#4814), anti-phospho-ERK1/2 (Thr202/Tyr204) (#9101), anti-ERK1/2 (#9102), anti-phospho-p38 MAPK (Thr180/Tyr182) (#4511), anti-p38 MAPK (#9212), anti-phospho-SAPK/JNK (Thr183/Tyr185) (#9251), anti-JNK2 (56G8) (#9258), anti-NF-κB p65 (D14E12) (#8242) were both purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-IKKα (CHUK) (#A2062) was from ABclonal Technology (Wuhan, HB, China), β-tubulin (#CW0098A) and GAPDH (#CW0266A) were from CWBiotech (Beijing, China). Anti-Histone H2B were obtained from Santa Cruz Biotechnology Co., Ltd. (Shanghai, China).

Mice

Male C57BL/6 mice (8-10 weeks old, weight 20-25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Before experiments, three to four mice were housed per cage under a 12 h/12 h light/dark cycle at 22-24 °C with free access to water and food. All the animal experimental procedures

were approved by the Institutional Animal Care and Use Committee of the Beijing Institute of Basic Medical Sciences.

Cell culture and treatment

Immortalized murine bone marrow-derived macrophages (iBMDMs) and BV-2 microglial cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (#11965-092, Life Technologies, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, #04-001-1A, Biological Industries, Beit Haemek, Israel) and 1% penicillin-streptomycin solution (#03-031-1B, Biological Industries) at 37 °C in a humidified atmosphere with 5% CO₂. Cells were treated with various concentrations of BA (10, 20, 40, 80 μM) or 0.1% DMSO as control for 1 h, followed by 1 ug/ml LPS stimulation for 6 h.

Cell viability assay

Cell Counting Kit-8 (CCK-8) assay (ab228554, Abcam, USA) evaluates the cell viability of BV-2 according to the manufacturer's instructions. In brief, cells were plated at a density of 1×10^5 cells/mL in 96-well plates, and then were exposed to different concentrations of BA for 24 h. Subsequently, 10 μl of CCK-8 reagent was supplemented to each well and incubated at 37 °C for an additional 2 h. Finally, a Spectra Max i3x (Molecular Devices, Sunnyvale, CA, USA) was measured to the optical density at 450 nm.

Measurement of intracellular ROS

The intracellular ROS was measured by staining cells with 2',7'-Dichlorofluorescein diacetate (DCFH-DA, Beyotime). Briefly, cells were treated as indicated above, and incubated with 10 μM DCFH-DA at 37 °C for 20 min. Cells were then washed twice with serum-free medium and analyzed by FACS Vantage (BD Biosciences) flow cytometer.

Immunofluorescence Assay

Cells grown on coverslips were treated with BA for 1h and were given LPS stimulation for the next 2h. After 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100, and blocked with 1% bovine serum albumin (BSA). For staining of NF-κB p65, the cells were further incubated with anti-NF-κB p65 (1:400) antibodies at 4°C overnight, followed by incubation with a secondary TRITC-conjugated antibody at room temperature for 1 h. Cell nuclei were counterstained with Hoechst (1 ng/ml) for 5 min. Images were obtained under a confocal microscope (Leica, Germany).

In vivo LPS challenge

LPS (5 mg/kg) was injected into the peritoneal cavity of mice after pretreatment with 10 mg/kg BA (diluted with saline) i.p. injection for two times. Four hours later, all mice were killed, liver and lung tissue were collected and stored -80 °C.

Histological assessment

The liver and lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin. After that, the sections of various organs were stained with hematoxylin & eosin (H&E). The pathological scores for the liver and lung were determined according to previous studies with minor modifications⁹. The score was mainly determined as the degree of immune cell infiltration and structure disruption as follows: 0 = none, 1 = mild, 2 = moderate, and 3 = severe.

Quantitative real-time PCR

Total RNA was collected from tissues and cultured cells by extraction with TRIzol™ Reagent (#15596026, Life Technologies) according to the manufacturer's instructions. Tissues were finely chopped in TRIzol and immediately homogenized using a homogenizer. Then, mRNA from each sample was converted to cDNA according to cDNA synthesis kit (AE311-03, TransGen Biotech, Beijing, China). Quantitative PCR was performed using SYBR Green master mix (#A304-10, GenStar, Beijing, China) with ABI studio Q3 Real Time PCR system (Life technologies, USA). The primer sequences used were listed in Table 1.

Western blotting

Western blot analyses were conducted as previously described¹⁰. In briefly, tissues or cells were lysed with Radioimmunoprecipitation assay (PIRA) buffer, which contains a cocktail of protease and phosphatase inhibitors. The protein was isolated by SDS-PAGE and then transferred to polyvinylidene fluoride membrane (PVDF) membrane (#ISEQ00010, Millipore, Darmstadt, Hessen, Germany). The membrane was blocked with 5% nonfat milk in Tris-buffered saline and incubated overnight with primary antibodies at 4 °C. Finally, the protein was detected by horseradish peroxidase-conjugated secondary antibodies.

Statistical Analysis

We used GraphPad Prism software (version 8) for statistical analyses. All data were presented as means ± SD unless otherwise noted. The significance of differences was assessed by unpaired Student's *t*-test or one-way ANOVA analysis of variance. $p < 0.05$ was considered statistically significant.

Results

Bullatine A inhibits transcription levels of pro-inflammatory cytokines in LPS-activated microglia and macrophages

To evaluate the potential cytotoxicity of BA (Figure 1A), BV-2 microglial cell lines were incubated with or without BA for 24 h. Cell viability assay confirmed that incubation with BA for 24 hours had no significant cytotoxicity on BV-2 cells at concentrations below 80 μM (Figure 1B). Therefore, this concentration range was used to investigate the following anti-inflammation effect of BA *in vitro*. To further investigate confirm the anti-inflammation effect of BA, BV-2 cells were pretreated with BA (40 and 80 μM) for 1 h and then incubated with 1 ug/mL LPS for 6 h. Results showed mRNA levels of several pro-

inflammatory cytokines, including IL-6, iNOS and TNF- α , were significantly upregulated after LPS stimulation. However, this effect was significantly weakened when BA concentration reached 80 μ M (Figure 1C to E). Furthermore, we also examined the effect of BA on iBMDMs, macrophages that play critical roles in host innate immunity and are therefore commonly used for *in vitro* cell-based assays examining the mechanisms of innate immune activation¹¹. Consistent with the results in BV-2 cells, BA inhibited IL-1 β , IL-6 and iNOS mRNA expression occurred in a dose-dependent manner in LPS-stimulated iBMDMs (Figure 1F, G, and H). Together, these results indicate that BA inhibited LPS-induced inflammation effectively in microglia and macrophages.

Bullatine A reduced the high expression of iNOS and COX-2 proteins induced by LPS

We further determined the effects of BA on the protein levels of iNOS and COX2 induced by LPS. The experimental results were shown in Figure 2A, the protein levels of iNOS and COX-2 proteins were significantly increased after LPS stimulation. However, 80 μ M of BA significantly inhibited the high expression of iNOS and COX-2 protein and the density ratio of iNOS and COX-2 proteins to the corresponding GAPDH bands was shown in the Figure 2B and C. These results further confirmed the anti-inflammation activity of BA.

Bullatine A inhibits the activation of NF- κ B pathway

It is well known that NF- κ B signaling pathway plays a critical role in cellular inflammatory response, which is mainly manifested by the NF- κ B transcription factor regulating the expression of pro-inflammatory cytokines, iNOS and COX-2 proteins and involved in oxidative stress and inflammation¹². To further explore the anti-inflammatory mechanisms of BA, we evaluated changes in several key components of NF- κ B signaling. As displayed in the Figure 2D, LPS stimulation markedly increased the protein levels of p-IKK α / β and p-I κ B α . These effects, however, were significantly inhibited after BA pretreatment, especially after 1h of LPS stimulation, and the band density corresponding to the phosphorylation levels of IKK α / β and I κ B α proteins were normalized on the basis of their total proteins (Figure 2E and F).

In response to LPS stimulation, the translocation of NF- κ B p65 subunit to the nucleus was dramatically increased, which is considered to be an important feature in the detection of NF- κ B signaling pathway. Our immunofluorescence assay results showed that BA preconditioning inhibited the NF- κ B p65 subunit transport to the nucleus after 2 hours of LPS stimulation (Figure 2G and H). Western blot also supported this phenomenon, as shown in the Figure 2I, LPS exposure resulted to the translocation of the NF- κ B p65 from the cytosol to the nucleus, whereas a significant reduction of nuclear p65 protein and a significant increment of cytoplasmic p65 protein was observed after pretreatment with BA (Figure 2J and K). Taken together, these results indicate that BA inhibits the release of pro-inflammatory factors induced by LPS probably through the inhibiting activation of NF- κ B signaling pathway.

The effect of Bullatine A on the activation of MAPKs signaling pathway

Mitogen-activated protein kinases (MAPKs) also play an important role in extracellular signal transduction into cellular responses¹². Therefore, we next to explored the effect of BA on the MAPKs signaling pathway by Western blot. As shown in Figure 3A, all phosphorylation pathways of MAPKs, such as p38, JNK and ERK1/2, were significantly increased after 0.5 h or 1.0 h stimulated by LPS. The results showed that pretreatment BA significantly inhibited the phosphorylation of JNK, while did not show remarkable inhibitory effect on p38 and ERK1/2 (Figure 3B to D). These results indicated that BA inhibits the activation of NF- κ B pathway by impairing the phosphorylation of JNK.

Bullatine A reduced LPS-induced stimulation of reactive oxygen species (ROS)

It's reported that JNK could be activated by ROS, and ROS is a pro-inflammatory factor in LPS-induced inflammation. To investigate whether BA could inhibit the ROS production, we measured intracellular ROS levels was assayed using DCFH-DA. As shown in Figure 3E, LPS induced ROS rise was significantly as compared with control group. However, pretreatment with BA (80 μ M) markedly attenuated LPS induced ROS rise (Figure 3F). These results confirmed that BA could obviously restrain intracellular ROS production, which is consistent with the effect on the activation of JNK pathway.

Bullatine A protected liver and lung injuries in LPS-challenged mice

The above studies proved that BA had an anti-inflammatory activity *in vitro*, we then intend to study its activity *in vivo*. To investigated whether BA pretreatment could suppress inflammatory responses *in vivo*, we set out induced production of systemic inflammatory disturbance in mice via intraperitoneal injection LPS. The effect of BA on LPS-induced tissue damage was demonstrated by H&E staining. As shown in Figure 4A, after LPS administration, neutrophils, characterized by dark nuclei (indicated by arrows) were infiltrate the liver wall. However, pretreatment with BA inhibited neutrophil infiltration. Similarly, LPS administration caused disruption of alveolar space compared with lung of saline-treated mice. However, pretreatment with BA improved LPS-induced disruption of alveolar space structure (Figure 4A). All the above pathological changes were statistically significant by histological assessment (Figure 4B).

Bullatine A reduced inflammation gene expression in LPS-challenged mice

Next, we investigated whether BA pretreatment inhibited the LPS-induced gene expression of the pro-inflammatory cytokines in liver and lung. Our results demonstrated that intraperitoneal injection of LPS induced the increased the mRNA levels of inflammation-related genes (IL-1 β , IL-6 and iNOS) in the liver of model mice, consistent with the induction of acute inflammation. However, pretreatment with BA significantly reduced the expression of the above indicators (Figure 4C). A similar effect was observed in the lungs, but the effect was not significant (Figure 4D). Taken together, these results indicating that BA pretreatment also exerted favorable anti-inflammatory effects *in vivo*.

Discussion

In recent decades, a large number of studies have sought natural compounds isolated from plants, which often have advantages in treating diseases due to their relatively low toxicity and side effects. BA, one of the major components extracted from *Aconiti brachypodi* Radix, has various pharmacological activities. It has been reported that BA can selectively inhibit P2X7 receptor mediated inflammatory response, suggesting its anti-inflammatory potential². However, until now, its effect on LPS-induced inflammatory response whatever *in vitro* or *in vivo* and the potential mechanism of its anti-inflammatory activities have not been investigated in detail. Herein, we confirmed that BA exerts anti-inflammatory effects by inactivating the transcriptional activity of NF- κ B, which provides a theoretical basis for the development of new drugs that targeting NF- κ B signaling pathway in the clinical application of inflammatory diseases.

Macrophages are not only an important innate immune cell in the body's defense system, but also a major cell involved in the inflammatory response¹³. The reason why macrophages play an important role in the process of inflammatory response is that it involved in the acute phase of inflammatory diseases by releasing pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6, etc)¹⁴. If the activated macrophages continue to release superabundant proinflammatory factors and inflammatory mediators, an excessive or uncontrolled inflammatory response can result, leading to severe tissue damage and septic shock¹⁵. Therefore, therapies aimed at inhibiting proinflammatory cytokines is of great significance in inflammatory-related diseases. As the main component of endotoxins and the main molecular basis of pathogenesis, LPS is often used in experiments to activated macrophages to evaluate the anti-inflammatory activity of some plant extracts¹⁶. To investigate the anti-inflammatory effects of BA *in vitro*, the effects of BA on the production of LPS-induced pro-inflammatory cytokines were examined in two different types of macrophages. The results of our study showed BA reduced the RNA and protein levels of inflammatory factors in LPS activated macrophages. Especially, the inhibitory effect of BA on the excessive production of pro-inflammatory factors in iBMDMs is much more pronounced, suggesting that it has a better medicinal effect on peripheral inflammation.

Next, we explored the mechanisms underlying the anti-inflammatory effects of BA. It is well known that NF- κ B is a dimer formed by the p50 and p65 subunits, which plays an important role in the regulation of pro-inflammatory mediators. Its dysfunction has been associated with many choric diseases, including asthma, cancer, diabetes, rheumatoid arthritis, inflammation, and neurological disorders, and is therefore considered as a potential drug intervention target for the treatment of inflammation^{17–19}. The nuclear translocation of p65 is thought to key signal for NF- κ B activation²⁰. While in the resting condition, NF- κ B combined with its inhibitor protein I κ B α and co-locates in the cytoplasm²¹. Once stimulated, I κ B kinase engages in phosphorylation of NF- κ B P65 and ubiquitin-mediated degradation of this product via the proteasome pathway, then translocated p65 into the nucleus where it triggers the transcription of specific target genes such as TNF- α , IL-1 β , and IL-6^{22,23}. The protective effects of BA on LPS-induced inflammatory response may be attributed to the inhibition of NF- κ B pathway. Results in the current study showed that BA inhibited the degradation of I κ B- α and also inhibited the translocation of NF- κ B p65 to the nucleus. In addition, iNOS and COX-2, the downstream targets of NF- κ B²⁴, which are believed to be closely related to the occurrence and development of a variety of inflammatory diseases²⁵, and also

suppressed by BA. These findings suggest that BA may inhibit transcriptional activity of NF- κ B, thereby could normalize the expression of pro-inflammatory cytokine and inflammatory mediator.

MAPKs are also involved in LPS-induced inflammation. Activated MAP kinases trigger the expression of target genes in controlling the synthesis and release of cytokines during the inflammatory response, leading to biological responses, including the expression of pro-inflammatory mediators²⁶. Here, we found that BA significantly inhibited the phosphorylation of JNK and the production of ROS. ROS levels that exceed the antioxidant capacity of cells are thought to be involved in the pathogenesis and development of various pathological conditions, including cardiovascular disease, cancer, and aging²⁷. Moreover, it has been reported that ROS serves as an upstream molecule that directly activates the NF- κ B pathway^{28,29}. Previous studies suggested some natural components of plants can exert their pharmacological effects by inhibiting ROS/NF- κ B pathway. For example, Yao *et al.*³⁰ reported that Marein protected human nucleus pulposus cells against high glucose-induced injury and extracellular matrix degradation by inhibiting the ROS/NF- κ B pathway. Barbaloin was also reported possesses a protective effect on LPS-induced acute lung injury by inhibiting the ROS-mediated PI3K/AKT/NF- κ B pathway³¹. In the present study, we found that LPS exposure led to ROS levels rise in iBMDMs, which was attenuated by BA, suggesting that BA exert anti-inflammatory activity may be through regulating the JNK/ROS/NF- κ B pathway (Fig. 5).

In conclusion, we demonstrated that BA effectively inhibits the expression pro-inflammatory cytokines by inactivating JNK/ROS/NF- κ B pathway *in vitro*. More importantly, BA preconditioning attenuates the inflammation and tissue injury of liver and lung in LPS-induced acute inflammation model mice. These findings provide a theoretical basis for the clinical application of BA in the treatment of inflammatory diseases, reveal the pharmacological activities of different active components of *Aconiti brachypodi* Radix.

Abbreviations

BA, Bullatine A, iBMDMs, immortalized murine bone marrow-derived macrophages, IL-1 β , interleukin-1 β , TNF- α , tumor necrosis factor- α , iNOS, inducible nitric oxide synthase, COX-2, cyclooxygenase-2, LPS, lipopolysaccharide, NF- κ B, nuclear factor kappa B, MAPKs, mitogen-activated protein kinases.

Declarations

Ethics approval and consent to participate

Animal procedures were approved by the Animal Care and Use Committee of the Minzu University of China (ECMUC2019001AO).

Consent for publication

Not applicable.

Availability of data and material

Please contact author for data requests.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

SHL performed the experiments and wrote the manuscript. YJL and YC conceived and designed the experiments and reviewed the manuscript.

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Tables

Table 1. Primer pairs used for qPCR analysis

Target	Sequence (5'-3')
<i>β-actin</i>	Forward: GGCTGTATTCCCCTCCATCG Reverse: CCAGTTGGTAACAATGCCATGT
<i>IL-1β</i>	Forward: GTCGCTCAGGGTCACAAGAA Reverse: CTGCTGCCTAATGTCCCCTT
<i>IL-6</i>	Forward: GCTACCAAACCTGGATATAATCAGGA Reverse: CCAGGTAGCTATGGTACTCCAGAA
<i>iNOS</i>	Forward: GTTCTCAGCCCAACAATAACAAGA Reverse: GTGGACGGGTGCATGTCAC
<i>TNF-α</i>	Forward: CAGGCGGTGCCTATGTCTC Reverse: CGATCACCCCGAAGTTCAGTAG

Figures

Figure 1

Effect of Bullatine A on the expression of inflammatory genes in LPS-activated microglia and macrophages. (A) The chemical structure of BA. (B) The cell viability of BV-2 cells incubated with BA in different concentrations. The mRNA levels of IL-6 (C), TNF- α (D) and iNOS (E) in the BV-2 cells treated

with LPS (1 ug/ml) and BA (40 or 80 mM) for 6 h, and mRNA levels of IL-1 β (F), IL-6 (G) and iNOS (H) in iBMDMs cells treated with LPS (1 ug/ml) and BA (10-80 mM) for 6 h were detected by quantitative RT-PCR. *P < 0.05, **P < 0.01, ***P < 0.001 vs. LPS treatment group.

Figure 2

Effects of Bullatine A on the NF- κ B pathway activation in LPS-stimulated iBMDMs cells. (A) Effects in BA on the high expression of iNOS and COX-2 proteins in LPS-activated iBMDMs cells. (B, C) Data were

standardized on the basis of GAPDH levels. (D) Effect of BA on the phosphorylation of IKK α / β and I κ B α proteins detected by western blotting. (E, F) Data were standardized on the basis of percentages of levels of unphosphorylated forms (total-IKK α / β and total-I κ B α). (G) Effect of BA on the translocation of NF- κ B p65 into nucleus was detected by immunofluorescence assay. After BA (80 μ M) treatment for 1h, iBMDMs cells treated with LPS (1 ug/ml) for 2h. (H) Relative NF- κ B p65 luciferase activity was analyzed by Image J software. (I) Expressions of NF- κ B p65 in cytosol and nuclear were measured by western blotting, GAPDH and H2B was taken as control. (J, K) Data were standardized on the basis of percentages of GAPDH and H2B levels. *P < 0.05, **P < 0.01 vs. LPS treatment group.

Figure 3

Effects of Bullatine A on the MAPKs pathway activation in LPS-stimulated iBMDMs cells. (A) Effect of BA on the phosphorylation of p38, JNK and ERK proteins detected by western blotting. (B-D) Densitometric analysis data were standardized on the basis of percentages of levels of unphosphorylated forms (total-p38, total-JNK and total-ERK). (E) Effects of BA on the production of ROS in LPS-stimulated iBMDMs cells. Cells were pretreated with BA at 80 mM for 1 h and then treated with LPS at 1 ug/ml for 24 h. (F) Data were standardized on the basis of ROS levels in the control group. $**P < 0.01$ vs. LPS treatment group.

Figure 4

Bullatine A attenuated liver and lung inflammation and damage in LPS-challenged mice. BA (i.p., 10 mg/kg) was administrated to mice two times (once every 2h) prior to treatment with LPS (i.p., 5 mg/kg). (A) Representative pictures of H&E staining of the sections of liver and lung after administration of saline, LPS, and/or BA (scale bars 100 μ m). (B) The pathological score of liver and lung (n = 7). The mRNA level of IL-1 β , IL-6 and iNOS of liver (C) and lung (D) was measured by quantitative RT-PCR (n = 7). Data are presented as mean $\pm$ S.E.M. *P < 0.05 vs. LPS treatment group.

Figure 5

Schematic diagram of our study.