Benzoylaconine Modulates LPS-Induced Responses through Inhibition of Toll-Like Receptor-Mediated NF-κB and MAPK Signaling in RAW264.7 Cells

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Research Article

Keywords: BAC, TLR, TAK1, NF-κB, MAPKs, anti-inflammation.

Posted Date: February 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-190441/v1

Version of Record: A version of this preprint was published at Inflammation on July 16th, 2021. See the published version at https://doi.org/10.1007/s10753-021-01478-z.

Abstract

Previous studies have shown that benzoylaconine (BAC), a representative monoester alkaloid, has a potential anti-inflammatory effect. This study investigated the underlying molecular mechanisms using the mode of LPS-activated RAW264.7 macrophage cells. Our findings showed that BAC significantly suppressed the release of pro-inflammatory cytokines and mediators, including IL-6, TNF-α, IL-1β, ROS, NO, and PGE_2. BAC treatment also effectively downregulated the elevated protein levels of iNOS and COX-2 induced by LPS in a dose-dependent manner. In this study, we found that BAC inhibited LPS-induced NF-κB activation by reducing the phosphorylation and degradation of IκBα by Western blotting and blocking the nuclear translocation of p65 using an immunofluorescence assay. The elevated protein levels of JNK, p38, and ERK phosphorylation after LPS stimulation were restored effectively by BAC treatment. Moreover, LPS-induced phosphorylation of TAK1, which is a crucial upstream regulatory factor of Toll-like receptor-induced MAPK and NF-κB signaling, was inhibited by BAC in activated RAW264.7 macrophages. These findings demonstrated that BAC exhibited an anti-inflammatory effect by inhibition of Toll-like receptor-induced MAPK and NF-κB pathways, indicating that it could potentially be used for treating inflammatory diseases.

Introduction

Inflammation has a protective role when the body is exposed to harmful stimulation, such as tissue injury and infection. Numerous diseases, including cancer, arthritis, atherosclerosis, asthma, and sepsis, are closely associated with an uncontrolled inflammatory response [1, 2, 3]. Thus, exploring new drugs that can target uncontrolled inflammatory processes is considered an effective strategy to treat these diseases [4]. During immune response processes, such as the secretion of cytokines and antigen presentation, macrophages belonging to the group of innate immune cells can be recruited to the site of inflammation [5, 6]. After LPS stimulation, macrophages initiate a series of inflammatory responses by activating specific signaling cascades and releasing both pro-inflammatory cytokines and mediators, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β, reactive oxygen species (ROS), nitric oxide (NO), and prostaglandin E_2 (PGE_2).

Toll-like receptors (TLRs), transmembrane proteins expressed on various immune cells, influence innate immune cell activation [7]. For macrophages to induce inflammatory responses, it is essential that the N-terminal region of a TLR recognizes a pathogen molecule, such as LPS [8]. Then, the LPS-induced TLR activation triggers downstream signaling pathways involving both nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) [9]. The NF-κB pathway has a key role during inflammatory responses by regulating the transcription of certain genes encoding growth factors, pro-inflammatory cytokines, and inducible enzymes, including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [10, 11]. Upstream IκB kinase (IKK) phosphorylation triggered by activated transforming growth factor beta-activated kinase 1 (TAK1) when LPS binds to TLR is a key step for NF-κB activation. IKK activation leads to IκB phosphorylation and ubiquitin-dependent degradation, promoting NF-κB dissociation from the IκB/NF-κB in the cytoplasm and translocation to the nucleus, which subsequently

initiates pro-inflammatory gene expression and the inflammatory response [10, 12]. Furthermore, TAK1 activation also activates the MAPK family, including c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), which then regulates the expression of transcription factor activator protein-1 (AP-1), a protein closely linked to various cellular processes, such as cell survival, differentiation, apoptosis, and pro-inflammatory cytokine production [13]. Therefore, downstream signaling molecules of TLR involving MAPK and NF-κB pathways are effective therapeutic targets of anti-inflammatory drugs for inflammation treatment.

BAC (Fig. 1) is widely found in *Aconitum* plants, which are traditional Chinese medicines that have various pharmacological properties, such as anti-inflammatory, analgesic, and anti-tumor activities. The main pharmacological activity components of *Aconitum* plants are different forms of alkaloids, such as diester-diterpenoid and monoester-diterpenoid alkaloids, which are closely associated with pharmacological effects and toxicity. Compared to aconitine (AC), which belongs to diester-diterpenoid alkaloids possessing highly neurotoxic and cardiotoxic activities with an LD_{50} of approximately 1.8 mg/kg in mice, BAC is a monoester-diterpenoid alkaloid that has a LD_{50} of 1,500 mg/kg, i.e., it possesses a much lower toxicity [14, 15]. The following research focused on BAC due to its considerable pharmacological activity and low toxicity.

Previous studies have reported that BAC possesses potential anti-inflammatory activity, but its molecular mechanisms of anti-inflammatory action have remained unclear until now, preventing BAC's clinical use as a therapeutic drug for inflammation-related diseases. Our current study investigated the molecular mechanisms underlying the anti-inflammatory action of BAC in LPS-activated RAW264.7 macrophage cells. We found that the anti-inflammatory activity of BAC is mediated by the suppression of TLR-induced MAPK and NF-κB activation.

Materials And Methods

Materials

BAC (purity ≥ 98%) and RAW264.7 cells were provided by Yousi Scientific Co., Ltd., and the Chinese Academy of Sciences (Shanghai, China). LPS from *Escherichia coli* and TPCK (NF-κB inhibitor) were obtained from Sigma (St. Louis, MO, USA). SB202190 (p38 MAPK inhibitor), U0126 (ERK inhibitor), SP600125 (JNK inhibitor), and Cell-Counting Kit-8 (CCK-8) were obtained from Medchem Express (St. Louis, MO, USA). Trizol and phenylmethanesulfonyl fluoride (PMSF) were acquired from Ambion (CA, USA) and Aladdin (Shanghai, China). HiScript Reverse Transcriptase and SYBR Green Master Mix were provided by VAZYME (Nanjing, China). ROS Assay Kit, BCA Protein Assay Kit, radioimmunoprecipitation assay (RIPA), and phosphatase inhibitor cocktail were acquired from Beyotime (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Anti-NF-κB, anti-JNK, anti-p38, anti-ERK1/2, anti-TAK1, anti-p-NF-κB, anti-p-JNK, anti-p-p38, anti-p-ERK1/2, and anti-p-TAK1 antibodies were purchased from Abcam (Cambridge, UK). Anti-IκBα and anti-p-IκBα antibodies were obtained from Affinity Bio Reagents (Golden, CO, USA).

RAW264.7 Murine Macrophage Culture

Culture medium was used to dilute the stock solution of LPS to 1 μg/mL before use. Cells cultured in DMEM containing 10% FBS at 37°C in a 5% CO_2 moist atmosphere were pretreated with the indicated concentrations of BAC for 1 h before co-treatment with 1 μg/mL LPS at different time points.

CCK-8 Assay

RAW264.7 cells were plated at 1.5×10^4 cells/well in 96-well plates overnight and treated with BAC (0.1, 1, 10, 100 and 500 μM) for 1 h before 1 μg/mL LPS stimulation in triplicate. Then, 10 mL of CCK-8 buffer was added to each well, and cells were incubated at 37°C for 24 h. FlexStation 3 microplate reader (Molecular Devices, CA, USA) was used to detect the absorbance at the wavelength of 450 nm.

NO Assay

NO levels in activated macrophages were determined using Griess reagent, which could measure the nitrates and nitrites concentrations. U0126, SB202190, TPCK, and SP600125 was administered to cells separately with or without BAC (10 μM) for 1 h before LPS (1 μg/mL) stimulation for an additional 24 h period. Then, 50 μL of Griess reagent was mixed with 50 μL of culture medium in 96-well plates and the reaction was left to proceed for 5 min. After collecting the supernatant, an NO Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China) was used to determine the NO production in activated macrophages, and a microplate reader was used to detect the absorbance at 540 nm.

ROS Assay

Cells were pretreated with BAC (1, 10, and 100 μM) for 1 h before LPS (1 μg/mL) stimulation for 24 h to detect the intracellular ROS levels. The media were then removed before cells were incubated with DCFH-DA (10 μM) for 20 min. Olympus BX53 fluorescence microscope (Olympus, Japan) was used to measure ROS's level as the mean fluorescence intensity after sufficient washing. Excitation and emission were detected at the wavelengths of 488 nm and 525 nm, respectively.

ELISA

Cells were pretreated with BAC (1, 10, and 100 μM) for 1 h before LPS (1 μg/mL) stimulation for 24 h in the medium to detect pro-inflammatory cytokine levels. ELISA kits (Elabscience, Wuhan, China) were used to determine the production of IL-6, IL-1β, TNF-α, and PGE_2 following the manufacturer's instructions.

Quantitative Real-Time PCR Detecting

After treatment, total RNA was extracted with Trizol and converted to cDNA using the HiScript Reverse Transcriptase, according to the manufacturer's recommendations. The primer sequences used in our study were as follows: β-actin (sense: CACGATGGAGGGGCCGGACTCATC, antisense: TAAAGACCTCTATGCCAACACAGT); IL-1β (sense: TCAGGCAGGCAGTATCACTC, antisense:

AGCTCATATGGGTCCGACAG); TNF-α (sense: GCCTATGTCTCAGCCTCTTCT, antisense: TTGTGAGTGTGAGGGTCTGG); and IL-6 (sense: GGAGTTCCGTTTCTACCTGG, antisense: GCCGAGTAGACCTCATAGTG). SYBR Green Master Mix and ABI QuantStudio 6 Real-Time PCR System were used to quantitate the relative mRNA concentrations. β-actin was used as a reference gene, and data from the relative gene expression were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Assay

After BAC or/and LPS treatment, macrophages were washed three times with ice-cold phosphate-buffered saline (PBS), and the whole-cell proteins were extracted using RIPA buffer supplemented with PMSF and phosphatase inhibitor cocktail. Subsequently, the total protein concentration was measured using the BCA protein assay. After separation by electrophoresis, 40 μg protein was transferred onto the PVDF membranes (Millipore, Billerica, MA, USA), which were then blocked for 2 h with 5% nonfat milk Tris-buffered saline Tween 20 (TBST). Subsequently, membranes were incubated with specific primary rabbit antibodies and washed five times with TBST, followed by incubation for 2 h with goat anti-rabbit IgG HRP at room temperature. Finally, each membrane was washed with TBST and visualized using the ECL substrate (Beyotime, Shanghai, China).

Immunofluorescent Assay

After BAC (100 μM) pretreatment for 1 h, LPS stimulation for 20 min, macrophages were stimulated by LPS (1 μg/mL) for 30 min to detect the location of NF-κB p65 in activated macrophages. After washing twice with PBS for 20 min, cells fixed with 4% paraformaldehyde for 15 min were permeabilized with 0.5% Triton X-100 for 30 min. Then cells were blocked with normal goat serum before incubation with rabbit anti-NF-κB p65 antibody (dilution 1:100) at 4°C overnight. After incubation with Cy3-labeled goat anti-rabbit IgG (dilution 1:100) as secondary antibodies in the dark for 30 min and sufficient washing, cells were stained with DAPI (0.5 μg/mL) for 10 min. Stained cells were visualized under a BX53 fluorescence microscope (Olympus, Japan).

Statistical Analysis

All data were presented as mean ± SD. Statistical analyses were performed using SPSS v22 software (IBM, Armonk, NY, USA). P-values of < 0.05 were considered statistically significant.

Results

The effect of BAC on cell viability and production of pro-inflammatory cytokines

The CCK-8 assay was used to detect the viability of activated macrophages. Indicated concentrations of BAC had no obvious cytotoxicity up to 500 μM (Fig. 2a), suggesting that BAC does not affect cells with subsequent experimental concentrations. Next, the effect of BAC on the levels of pro-inflammatory cytokines was investigated by ELISA. LPS treatment significantly increased IL-1β, TNF-α, and IL-6 levels

to 111.30, 1591.12, and 960.90 pg/mL; however, treatment with BAC markedly decreased their levels to 43.34, 75.96, and 83.40 pg/mL (Fig. 2b, c, d), respectively.

Moreover, we used quantitative real-time PCR to determine if the inhibitory effects of BAC on this cytokine release correlated with gene expression regulation. Treatment with BAC dose-dependently downregulated the mRNA transcription of these cytokines (Fig. 2e, f, g). Our results suggested that BAC attenuated the inflammatory response by suppressing these cytokines' synthesis and release in activated macrophages.

The effect of BAC on pro-inflammatory mediators production

ELISA was used to determine the anti-inflammatory effect of BAC on PGE_2, NO, and ROS levels in activated macrophages. The production of PGE_2 was significantly upregulated compared with control cells after LPS stimulation, whereas 1, 10, or 100 μM BAC treatment markedly decreased PGE_2 levels by 8.2%, 46.6%, and 73.0% (Fig. 3a), respectively. LPS treatment induced an observable elevation of NO levels in RAW264.7 macrophages, while BAC treatment significantly suppressed NO production as expected. Meanwhile, treatment with 1, 10, or 100 μM BAC dose-dependently decreased ROS levels (Fig. 3c), indicating that BAC also showed an inhibitory effect on ROS release. Together, these results suggested that the extensive anti-inflammatory effects of BAC were exerted by suppressing these mediators production in LPS-induced macrophages. Furthermore, treatment of BAC combined with SB202190, SP600125, U0126, or TPCK resulted in less LPS-induced NO production in macrophages (Fig. 3b), suggesting that the anti-inflammatory activity of BAC might be associated with effects on the MAPK and NF-κB pathways.

The effect of BAC on the expression of COX-2 and iNOS

COX-2 and iNOS play critical roles in the generation of PGE_2 and NO, respectively [16]. Here, we used Western blot to examine the regulatory effect of BAC on the protein levels of COX-2 and iNOS. LPS-stimulated RAW264.7 cells exhibited remarkably elevated protein levels, whereas treatment with BAC effectively downregulated these expressions, and a dose-dependent response was seen for BAC (Fig. 4). These results provided supportive evidence that suppression of PGE_2 and NO release by BAC was related to the downregulation of COX-2 and iNOS levels.

The effect of BAC on the MAPK Pathway

MAPKs function as critical regulators in the inflammatory process by regulating AP-1 transcription factor activation [17, 18, 19]. Hence, to further explore the possible mechanism of BAC for suppressing pro-inflammatory mediator production, we examined the effect of BAC on the expression of MAPKs in activated macrophages. As expected, LPS induced an increase in the phosphorylation of MAPKs, an alteration that was counteracted effectively by BAC treatment in a dose-dependent manner. For instance, the values of p-p38/p38, p-JNK/JNK, and p-ERK/ERK decreased by 13.6%–58.9%, 18.3%–52.7%, and 39.4%–64.9% with 1, 10, or 100 μM BAC compared with activated macrophages (Fig. 5). Meanwhile, both BAC and LPS did not affect the non-phosphorylated MAPK's levels. Therefore, the above findings suggest

that the BAC's inhibitory effect in activated macrophages might be related to the regulation of the MAPK signaling cascade.

The effect of BAC on activation of NF-κB

Since NF-κB, which can be activated by LPS stimulation in macrophages, is a vital regulatory factor in the expression of inflammation-related mediators and cytokines, we next investigated whether treatment with BAC could affect the NF-κB activation. Moreover, the nuclear fractions of LPS-activated RAW264.7 macrophages were isolated to measure the effect on nuclear translocation. Stimulation by LPS remarkably induced an elevation of p65 phosphorylation levels and nuclear translocation, whereas treatment with BAC could dose-dependently inhibit elevations of the p65 phosphorylation and nuclear translocation (Fig. 6a, b, d). The location of the p65 subunit of NF-κB was detected using immunofluorescence assay, which further supported our above findings (Fig. 6e). Furthermore, BAC significantly dose-dependently attenuated IκBα phosphorylation and degradation in activated macrophages(Fig. 6c). These results suggested that one of the mechanisms by which BAC attenuates inflammatory responses induced by LPS is through inhibition of NF-κB activation.

The effect of BAC on TAK1 phosphorylation

TAK1 is a crucial upstream adaptor protein in TLR-mediated MAPK and NF-κB pathways. Next, the effect of BAC on TAK1 phosphorylation was assessed to investigate whether its inhibition of MAPK and NF-κB was related to TLR signaling regulation. LPS stimulation significantly increased the protein level of phosphorylated TAK1. However, BAC treatment markedly downregulated the elevation of phosphorylated TAK1 in LPS-activated RAW264.7 macrophages (Fig. 7). These findings indicated that BAC exhibited significant anti-inflammatory activity in activated macrophages by inhibiting TLR-mediated MAPK and NF-κB signaling pathways.

Discussion

Classic non-steroidal anti-inflammatory drugs may lead to patient intolerance and cause some side effects when used for inflammatory diseases [20]. Thus, it is necessary to explore and develop new anti-inflammatory drugs. Recent studies have shown that some natural monomer components extracted from herbs exert anti-inflammatory activity, such as BAC, which is widely found in *Aconitum*plants. However, the molecular mechanisms by which BAC regulates the inflammatory process in activated macrophages have not been systemically elucidated. This study confirmed the anti-inflammation property of BAC and investigated its mechanism of action by LPS-treated macrophages. Our study is the first to show that BAC exerts an anti-inflammatory effect in LPS-activated macrophages via inhibition of the TLR-related NF-κB and MAPK pathways. Furthermore, our results revealed that BAC could be used as both a promising anti-inflammatory drug and a new inhibitor of MAPK and NF-κB signaling.

Inflammation is an essential pathological process of living tissues to eliminate causative agents and promote tissue repair [21]. As we know, macrophages can modulate inflammation processes by

regulating the production of various cytokines. Cytokines released by macrophages can cause the neutrophils activation in the early stages of inflammation, leading to inflammatory damage. LPS-activated macrophages are the most common model for studying inflammatory responses. LPS-induced activation of innate immune cells like macrophages could trigger an inflammatory response, leading to excess production of TNF-α, IL-6, ROS, NO, IL-1β, and PGE_2 [22, 23, 24]. An excessive release could cause tissue damage and pathological changes, contributing to the progression and exacerbation of inflammation. Our experiment demonstrated that BAC significantly suppressed the production of these cytokines and mediators in LPS-activated macrophages.

Meanwhile, quantitative real-time PCR results showed that the gene transcription of IL-1β, IL-6, and TNF-α was also significantly and dose-dependently downregulated by BAC. Previous studies have revealed the vital roles of COX-2 and iNOS, which are specific inducible enzymes in cells modulating the production of key inflammatory mediators, such as PGE_2 and NO, during the development of inflammatory diseases [16]. Their expression is upregulated, which serves as a host defense against harmful stimulation. However, their overexpression is associated with various diseases, including cancer, arthritis, and asthma [25, 26, 27, 28]. Thus, the regulation of their expression is a promising anti-inflammatory strategy. We found that BAC effectively inhibited their expression in LPS-activated macrophages, indicating that the suppression of several mediators released by BAC is related to the downregulation of iNOS and COX-2 expression. Above all, our present data showed that BAC has a promising therapeutic potential in clinical inflammatory diseases by regulating iNOS and COX-2 expression.

Our subsequent experiments focused on the molecular mechanisms of the anti-inflammatory properties of BAC. As recognition receptors that initiate defense reactions by sensing various endogenous molecules and invading pathogens, TLRs have a critical effect on the activation of specific signaling cascades, such as NF-κB and MAPKs. LPS is the most important ligand of TLR4, which is one of the TLRs expressed in the surface of macrophages and can cause the oligomerization of TLR4 when binding to it, further leading to the permutations and combinations of Toll-interleukin-1 receptor (TIR) domains to recruit downstream adapter molecules to initiate signal transduction in the cytoplasm. Until now, five adaptor proteins have been identified, namely TIRAP, MyD88, TRAM, TRIF, and SARM [4]. Cell-signaling is initiated by activated TLR4 via MyD88-dependent or independent pathways to synthesize several downstream products [29]. The NF-κB family, which consists of NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel proteins, was previously shown to be closely related to various human diseases and plays a crucial role in inflammatory processes [30]. In non-activated macrophages, the NF-κB dimer is bound to IκBα, which belongs to the IκB family of inhibitory protein, and therefore exists in an inactive form in the cytosol. When LPS binds to TLRs in the macrophage membrane, NF-κB signaling is initiated in response to stimulation by LPS [31]. TLR4 expression in macrophages induced by LPS leads to the IκB kinase activation during the process of NF-κB activation, and then triggers the IκBα phosphorylation and degradation. The nuclear localization signals on the NF-κB subunits are unmasked by the IκBα degradation, permitting the p65 subunit, which is separated from NF-κB/Rel complexes, to translocate from the cytoplasm to the nucleus and bind to a specific DNA site to initiate the expression of genes

associated to the inflammatory process [10]. The most critical step is the NF-κB p50/p65 dimer translocation to the nucleus for the NF-κB pathway activation [32]. In this study, an immunofluorescence assay was used to observe the p65 subunit location, and we concluded that BAC significantly inhibited p65 translocation in activated macrophages by inhibiting IκBα phosphorylation and degradation. Moreover, the above results demonstrated that BAC could decrease pro-inflammatory cytokines and enzyme expression by suppressing the activation of NF-κB p65.

The MAPK signaling pathway is linked to some biological processes, such as gene transcription, apoptosis, proliferation, and differentiation. Besides the NF-κB pathways, MAPKs have a major role in numerous pro-inflammatory gene expression. For example, iNOS and COX-2 expression induced by some inflammatory products like LPS is regulated by MAPKs. Furthermore, multiple lines of evidence have demonstrated that MAPKs are associated with regulating the transcriptional activity of NF-κB and are considered a promising target for reducing inflammation. The phosphorylation of JNK, p38, and ERK triggered by TLR4 that transduces LPS signaling, is linked with the activation of the MAPK pathways, which can activate transcription factors and induce the expression of inflammatory molecules in the nucleus [33, 34]. Our present study revealed that treatment with BAC has a significant inhibitory effect on the expression of p-JNK, p-p38, and p-ERK protein in LPS-activated macrophages. Moreover, LPS-activated macrophages treated with BAC combined with p38, NF-κB, JNK, or ERK inhibitor produced less NO than when treated with BAC alone. Hence, our data indicated that BAC mitigated the LPS-induced inflammatory response through the inhibition of the MAPK and NF-κB signaling pathways.

TAK1, a vital signaling molecule belonging to the MAP kinase kinase kinase (MAP3K) family, is activated by a TLR-TIRAP-MyD88 dependent pathway, triggering downstream pathways, including JNK, p38, ERK, and NF-κB [34, 35, 36]. LPS stimulation recruits TAK1 to TLR4 and leads to NF-κB activation via IκB proteins phosphorylation and degradation. Meanwhile, MEK1/2MKK3/6 and MKK4/7 are directly phosphorylated by p-TAK1, which could activate the MAPK signaling pathway [35, 36]. Thus, TAK1 is believed to be a crucial adaptor protein connecting TLR4 with two vital downstream cascade branches, MAPK and NF-κB, in the macrophage model stimulated by LPS. In our present study, whether BAC affected the activation of TAK1 was investigated in LPS-activated macrophages. The results demonstrated that BAC could significantly downregulate the elevation of p-TAK1, which subsequently suppressed downstream MAPK and NF-κB pathways. As mentioned above, we found that blocking the activation of a key upstream factor, TAK1, was a reason for inhibiting TLR-mediated NF-κB and MAPK pathways by BAC. Signaling pathways related to the anti-inflammatory activity of BAC are shown in Fig. 8.

To sum up, our results first demonstrate that the mechanism underlying the attenuation of inflammatory responses by BAC in activated macrophages is through the inhibition of the TLR/NF-κB and MAPK pathways, contributing to the decrease in the levels of pro-inflammatory mediators and cytokine production. Our novel findings provide valuable information for the potential of BAC as a therapeutic agent able to target specific signal transduction for inflammation-related diseases.

Declarations

Funding

This work was supported by the Major Science and Technology Innovation Project of Shandong Province (2018CXGC1304), Science and Technology Development Project of Traditional Chinese Medicine of Shandong Province (2019-0400), Technology Development Project of Traditional Chinese Medicine of Qingdao (2020-zyy031).

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval and Consent to participate

Not applicable

Consent for publication

All the authors agree to submit the final version of manuscript for publication.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Code availability

Not applicable

Authors' contributions

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Figures

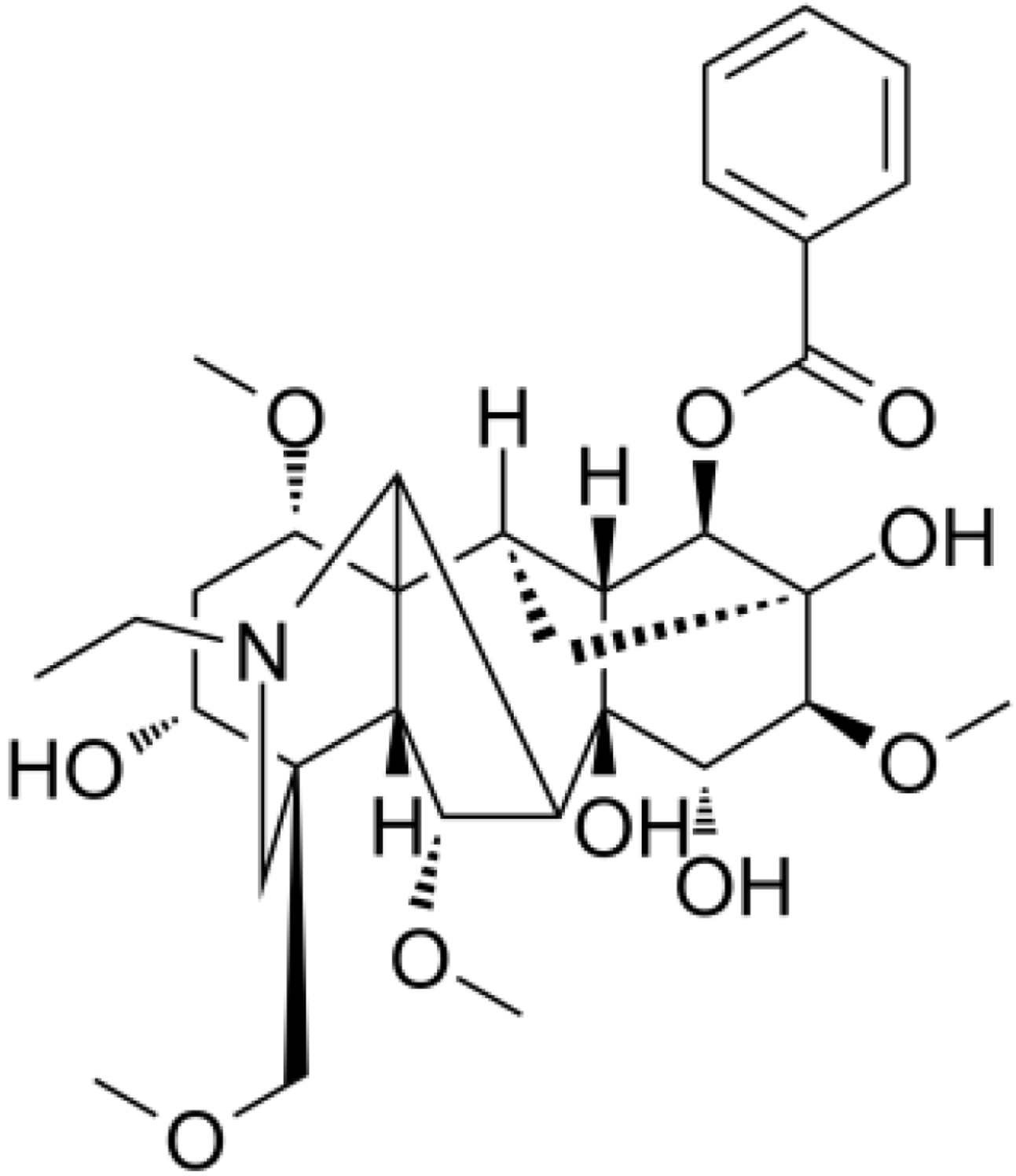

Figure 1

Chemical structure of BAC

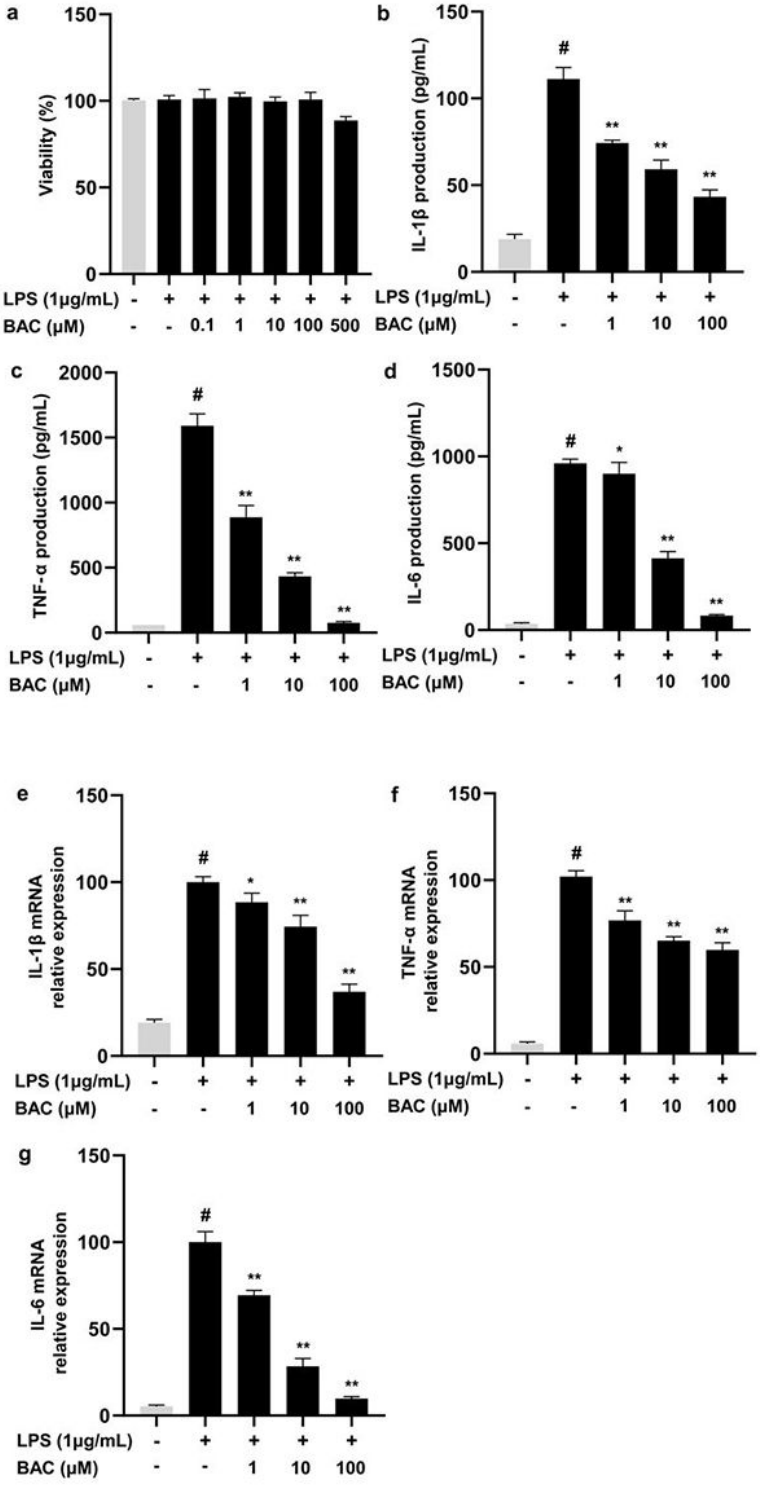

Figure 2

Effect of BAC on cell viability and LPS-induced pro-inflammatory cytokines production in macrophages. a Macrophages were treated with BAC (0.1, 1, 10, 100, 500 μM) for 1 h and followed by LPS (1 μg/mL) stimulation for 24 h. Cell viability was measured by CCK-8 assay. b–d Macrophages were incubated with BAC (1, 10, 100 μM) for 1 h before LPS (1 μg/mL) stimulation for 24 h. ELISA was used to determine IL-1β, TNF-α, and IL-6 production. e–g Macrophages were incubated with BAC (1, 10, 100 μM) for 1 h before

LPS (1 μg/mL) stimulation for 24 h. The mRNA expression of these pro-inflammatory cytokines was determined by real-time PCR. Data are mean ± SD of three independent experiments (#P < 0.05 vs. the control group, *P < 0.05, **P < 0.01 vs. LPS-treated group).

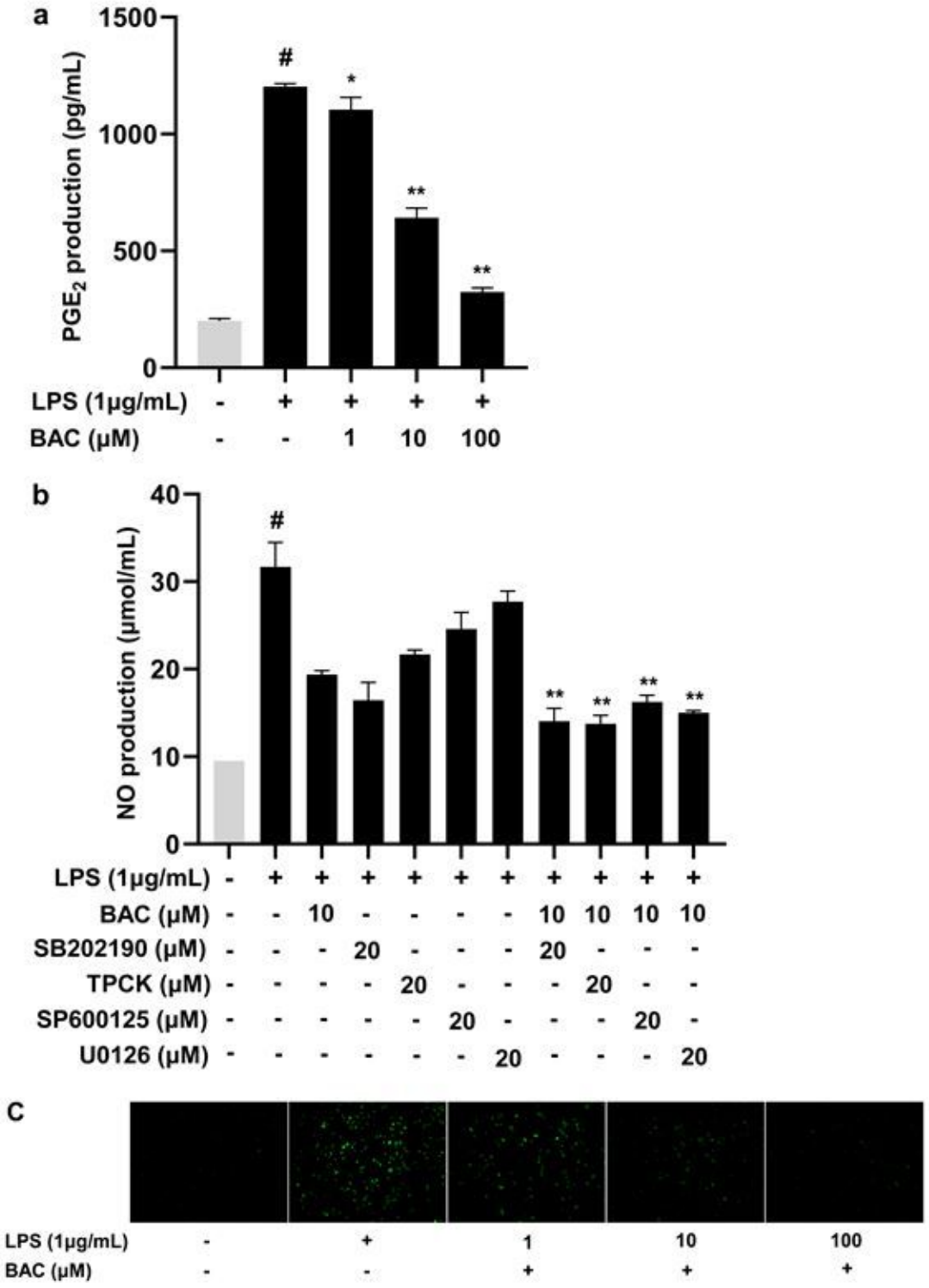

Figure 3

Effect of BAC on LPS-induced pro-inflammatory mediators production in macrophages. Macrophages were pretreated with BAC (1, 10, 100 μM) 1 h and followed by LPS (1 μg/mL) stimulation for 24 h. ELISA

and fluorescence microscopy were used to detect a PGE2 and c ROS production, respectively. b U0126, SB202190, TPCK, and SP600125 were administered to cells separately with or without BAC (10 μM) for 1 h before LPS (1 μg/mL) stimulation for 24 h. A microplate reader was used to detect the absorbance at 540 nm.

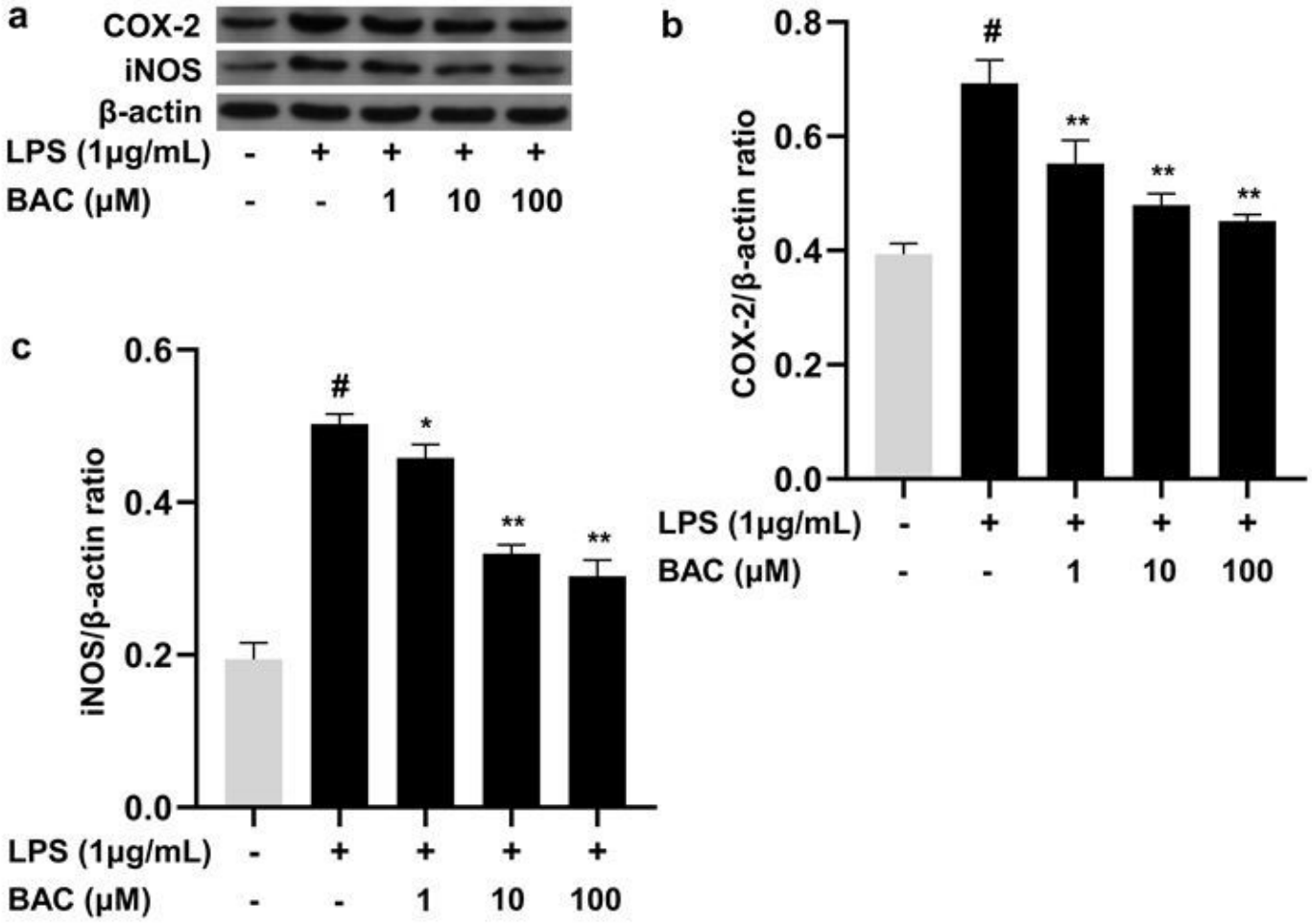

Figure 4

Effect of BAC on LPS-induced COX-2 and iNOS protein expression in macrophages. Macrophages were pretreated with BAC (1, 10, 100 μM) for 1 h and followed by LPS (1 μg/mL) stimulation for 24 h. a Western blotting was used to determine the protein levels. b The relative protein level of COX-2. c The relative protein level of iNOS. Data are mean ± SD of three independent experiments (#P < 0.05 vs. the control group, *P < 0.05, **P < 0.01 vs. LPS-treated group).

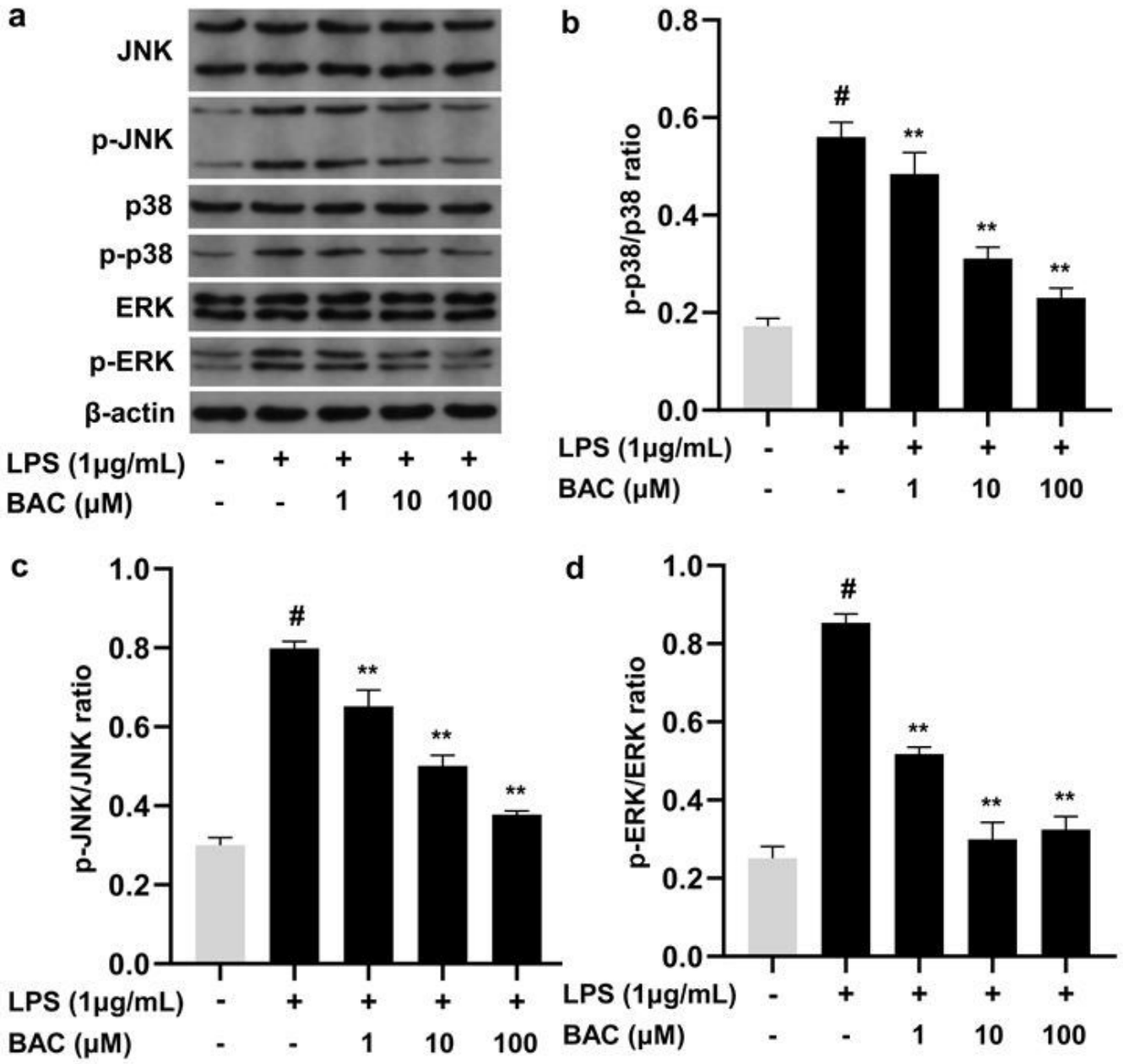

Figure 5

Effect of BAC on LPS-induced p38, JNK, and ERK protein expression in macrophages. Macrophages were pretreated with BAC (1, 10, 100 μM) for 1 h and followed by LPS (1 μg/mL) stimulation for 24 h. a Western blotting was used to determine protein levels. b The phosphorylation of p38. c The phosphorylation of JNK. d The phosphorylation of ERK. Data are mean ± SD of three independent experiments (#P < 0.05 vs. the control group, *P < 0.05, **P < 0.01 vs. LPS-treated group).

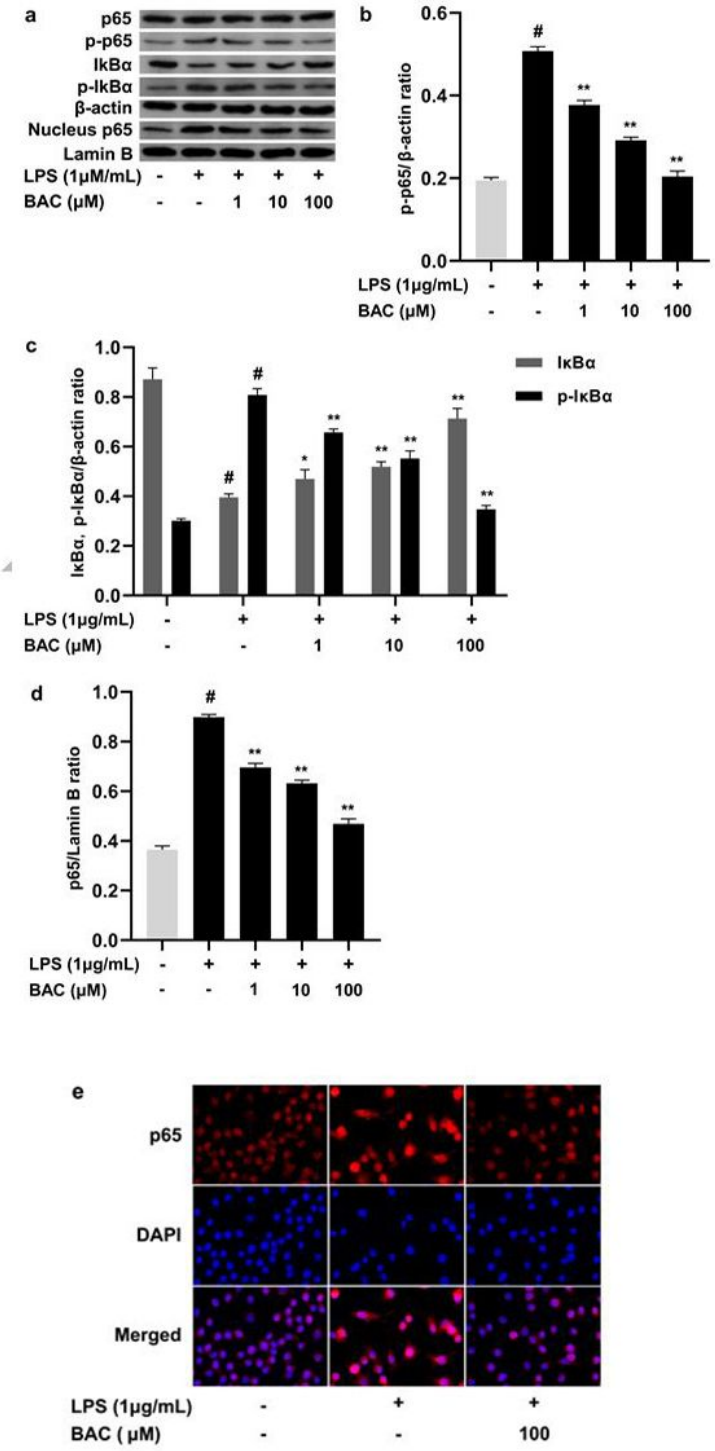

Figure 6

Effect of BAC on LPS-induced NF-κB activation in macrophages. Macrophages were pretreated with BAC (1, 10, 100 μM) for 1 h and then stimulated by LPS (1 μg/mL) for 30 min (d) and 24 h (b, c). Western blotting was used to determine protein levels. b LPS-induced p65 phosphorylation in macrophages. c LPS-induced IκBα phosphorylation and degradation in macrophages. d The LPS-induced translocation of p65 in the nucleus. e Images of NF-κB p65 (red) and nucleus (blue) were obtained using a fluorescence

microscope (400×). Data are mean ± SD of three independent experiments (#P < 0.05 vs. the control group, *P < 0.05, **P < 0.01 vs. LPS-treated group).

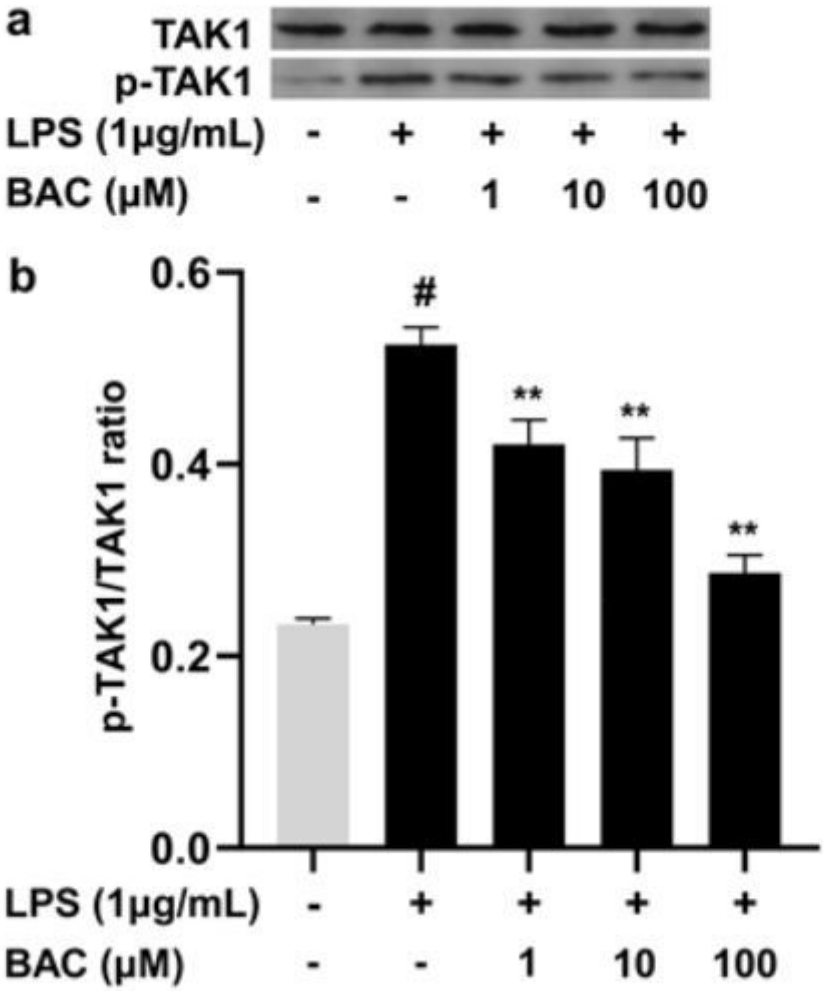

Figure 7

Effect of BAC on LPS-induced TAK1 phosphorylation in macrophages. Macrophages were pretreated with BAC (1, 10, 100 μM) for 1 h and followed by LPS (1 μg/mL) stimulation for 6 h. a Western blotting was used to determine protein levels. b The phosphorylation of TAK1. Data are mean ± SD of three independent experiments (#P < 0.05 vs. the control group, *P < 0.05, **P < 0.01 vs. LPS-treated group).

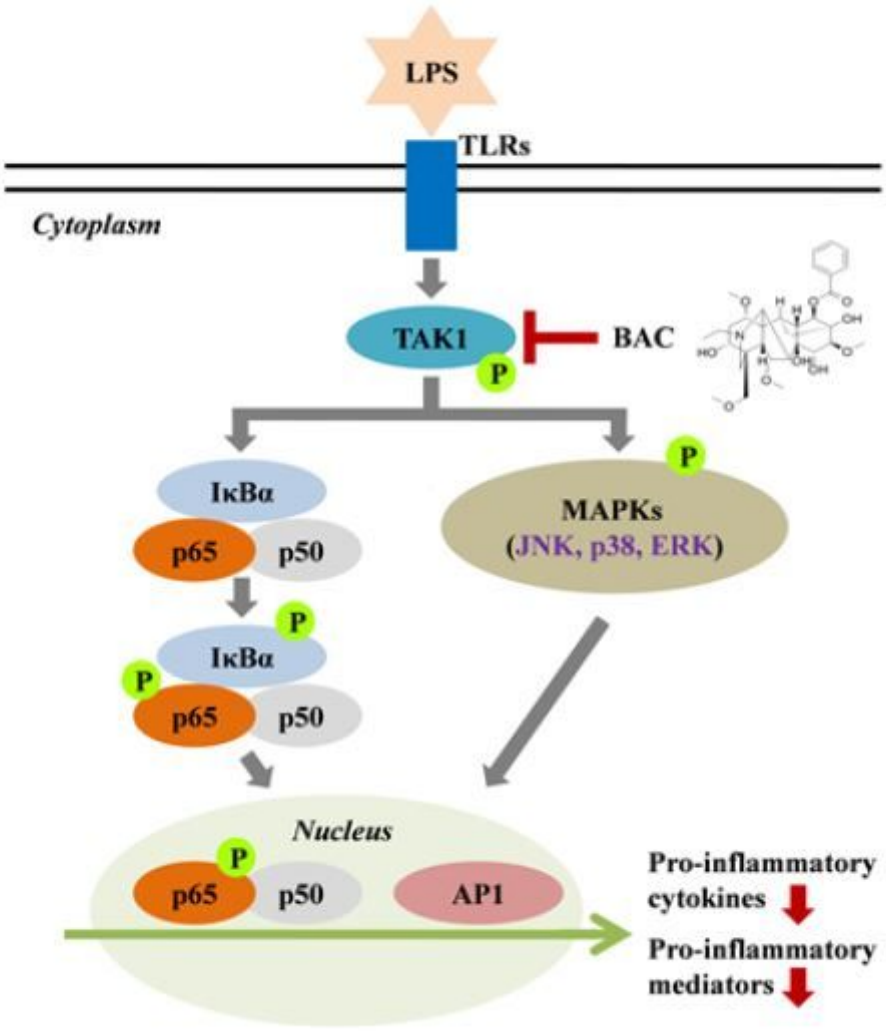

Figure 8

The potential molecular mechanism of the anti-inflammatory effect by BAC.