Buformin alleviates sepsis-induced acute lung injury via inhibiting NLRP3-mediated pyroptosis through an AMPK-dependent pathway

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Abstract

Background: NOD-like receptor family pyrin domain containing 3 (NLRP3)-mediated macrophage pyroptosis plays an important role in sepsis-induced acute lung injury (ALI). Inhibition of pyroptosis may be a way to alleviate inflammation as well as tissue damage triggered after lipopolysaccharide (LPS) stimulation. The aim of this study was to explore whether buformin (BF), a hypoglycemic agent, could alleviate sepsis-induced ALI by inhibiting pyroptosis.

Methods: Wild-type C57BL/6 mice were randomly divided into control group, BF group, LPS group and LPS+BF group. BF group and LPS+BF group were pretreated with BF at a dose of 25mg/kg, and the changes were observed. In addition, BF was used to interfere with THP-1 cells. The therapeutic effect of BF has been verified by intraperitoneal injection of BF in vivo after LPS stimulation.

Results: Inflammation and injury was significantly reduced in BF pretreated mice, and the indexes related to pyroptosis were suppressed. The phosphorylation of AMPK in lung tissues of mice in the BF and LPS+BF groups was significantly higher. In THP-1 cells, the AMPK inhibitor, Compound C was added to demonstrate that BF worked via AMPK to inhibit NLRP3 inflammasome. It was further demonstrated that BF upregulated autophagy, which in turn promoted NLRP3 inflammasome degradation. On the other hand, BF decreased NLRP3 mRNA level by increasing Nrf2. And BF showed a therapeutic effect after LPS challenge.

Conclusion: Our study confirmed that BF inhibited NLRP3-mediated pyroptosis in sepsis-induced ALI by upregulating autophagy and Nrf2 protein level through an AMPK-dependent pathway. This provides a new strategy for clinical mitigation of sepsis-induced ALI.

Key words: buformin, AMPK, NLRP3, acute lung injury
Introduction

Acute lung injury (ALI) is a serious respiratory disease with high mortality, and there is still no effective treatment at present. (1) Pulmonary edema, increased alveolar permeability, inflammatory cell accumulation and diffuse alveolar injury induced by acute lung injury eventually lead to acute respiratory distress syndrome (ARDS), which is the major cause of intractable hypoxemia. (2) ALI caused by sepsis has the highest level of inflammation among many causes. (3) When lipopolysaccharide (LPS), the most important pathogenic factor of Gram-negative bacteria stimulates, macrophages and neutrophils infiltrating into the lung tissue release large amounts of cytokines and promote pro-inflammatory response, resulting in rapid and severe damage to alveolar epithelium and endothelial cells. (4) Therefore, reducing the degree of inflammatory response and the release of inflammatory cytokines is vital to alleviate LPS-induced acute lung injury.

In recent years, more and more studies have shown that pyroptosis is the direct cause of cytokine storm in acute injury, which leads to the destruction of plasma membrane and the extracellular release of cytokine. (5) NOD-like receptor family pyrin domain containing 3 (NLRP3), one of the intracellular innate immune signaling receptors can function to form the inflammasome, activate pro-IL-1β and IL-18, cleave GSDMD and trigger pyroptosis, once pathogen associated molecular patterns (PAMPs) and damage-related molecular patterns (DAMPs) are recognized. (6) Our team recently found that cytoplasmic DNA promotes lung injury by upregulating NLRP3 expression, and that inhibition of NLRP3 either by drugs or gene knockout can attenuate the destruction of lung tissue in LPS-induced ALI. (7-9) Based on the early results, inhibition of NLRP3 - mediated pyroptosis may be an important measure for alleviating ALI.

AMP-activated protein kinase (AMPK), as an ATP receptor, can directly bind adenine nucleotides and thus sense intracellular energy. (10) When the ratio of ATP to AMP is changed, AMPK is activated to promote the phosphorylation of key proteins of several pathways, thus participating in the regulation of various cellular processes. (11) It has been reported that in ALI, AMPK promotes the expression of SIRT1 and thus regulates the acetylation of pro-inflammatory proteins to alleviate inflammation. (12) In addition, AMPK can play a
mitochondrial protective function by maintaining the expression of mitochondrial antioxidant enzymes against ALI.(13) Of interest is that AMPK activation can affect inflammasome assembly by inhibiting thioredoxin interacting protein (TXNIP) expression and inhibit NLRP3 expression by inhibiting p65 phosphorylation, thereby suppressing pyroptosis.(14, 15) Thus there is sufficient basis to suggest that AMPK is a key protective factor to suppress pyroptosis in ALI.

Buformin (BF) was originally used clinically as a hypoglycemic agent in the treatment of type 2 diabetes as well as metformin (MF) and phenformin (PF), members of the biguanide family.(16) With the exception of MF, which is still clinically used, BF and PF are no longer routinely used in the treatment of diabetes due to the risk of lactic acid poisoning in patients. Recently, there has been renewed interest in the therapeutic effects of BF in other diseases, such as endometrial cancer and breast cancer.(17, 18) Due to the molecular structure, BF is more lipophilic than MF and has a stronger inhibitory effect on mitochondrial complex I, but weaker than PF.(19) Therefore, BF can greatly improve the activation of AMPK without accumulating excessive lactic acid caused by respiratory chain dysfunction. At present, MF has been proved to reduce ALI via inhibiting NLRP3-mediated pyroptosis, but whether BF has protective effect on acute injury is still unknown.(20, 21) Thus, this study aims to explore whether BF pretreatment can reduce ALI induced by sepsis and the related mechanisms.

Materials and Methods

Chemical and reagents

Buformin (BF) was purchased from MedChemExpress (Monmouth Junction, NJ, USA), purity>98.6%. LPS (#L6529, from Escherichia coli 055: B5,) was purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-β-actin (#3700), anti-NLRP3(#15101), anti-p65(#8242), anti-p-p65(#3033), anti-AMPK (#5831), anti-p-AMPK (#2535), anti-mTOR (#2983), anti-p-mTOR (#2971) and anti-LC3 (#12741) were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-GSDMD-N(A20197), anti-caspase p20(A0964),
anti-GSKβ(A6164), anti-p-GSKβ(AP1088), anti-p62(A19700) and anti-Beclin 1(A7353) were provided by Abclonal Technology (Wuhan, Hubei, China). Anti-ASC (67494-1-Ig), anti-Nrf2 (16396-1-AP) and the secondary antibody (SA00001-1, SA00001-2) were purchased from Proteintech (Wuhan, Hubei, China).

Animal model establishment

All experimental protocols received approval from the Animal Care and Use Committee of Renmin Hospital of Wuhan University (no.20210305), Wuhan, China. C57BL/6 male mice (6–8 weeks old, weight: 19.8± 1.5g) were provided by Hubei Province Experimental Animal (Wuhan, Hubei, China). Nrf2-KO (Stock no.017009) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All experimental animals in this study were raised in a specific pathogen free (SPF) environment.

Referring to previous studies,(22) we established the murine ALI model by intraperitoneal injection of LPS at a dose of 10 mg/kg. The control group was injected with the same volume of sterile saline. Supported by reagent instructions and previous studies,(20, 23) Buformin dissolved with 10% DMSO+40% PEG300+5% Tween-80+45% saline was intraperitoneally injected into the mice from BF group and LPS+BF group 30 minutes before modeling at a dose of 25mg/kg. As for the control group and the LPS group, the same volume of solvent was injected intraperitoneally at the same time. After 12 hours of LPS or saline injection, the mice were sacrificed by dislocation of cervical vertebra under deep anesthesia (0.3% pentobarbital sodium injected intraperitoneally, 0.1ml/10g).

In order to verify whether BF can treat ALI, we injected the same dose of BF contralaterally into the peritoneal cavity of mice 6 hours after LPS injection. Similarly, 12h after LPS injection, treated mice were sacrificed under the same anesthetic conditions.

In a blind fashion, left lung tissue from mice was taken for subsequent pathological studies while the right lung tissue was preserved for molecular biology analysis. All mice were injected with drugs in the disposal room, and lung tissues were obtained in the specimen collection room in the Animal Center of Renmin Hospital of Wuhan University.
**Histopathological analysis**

The lung tissues of mice were fixed with 10% neutral formalin and the specimens were embedded in paraffin. The tissue wax blocks were cut into 3-μm sections, which were then stained hematoxylin-eosin (HE). The sections were photographed with an optical microscope, and observers scored semi-quantitatively according to the previously mentioned scoring criteria for the degree of inflammatory cell infiltration in lung tissue, the integrity of the alveolar cavity, septal thickening, hemorrhage, and hyaline membrane formation.(24, 25)

**Assay of MPO activity**

The lung tissue was homogenized under low temperature. MPO activity of the homogenate was measured using an MPO activity assay kit (#ab105136, Abcam) following the instructions and the absorbance at 412nm was measured on a microplate reader.

**Assay of lung wet/dry ratio**

After modeling, the lung tissue was taken and the surface blood was wiped off, and the wet weight of the right lung was measured immediately. The same tissue was placed in the oven at 65°C for 96h, then the weight of the dried tissue was measured again, and the wet/dry weight ratio of the lung was calculated.

**Assay of blood glucose**

Tail vein blood was collected before and after intervention to measure blood glucose levels in mice. Blood glucose content was measured with the glucometer (Roche, Mannheim, Germany) and blood glucose strips according to the instructions.

**Collection and analysis of cells from airway and lung tissues**

After all interventions were completed, the mice were sacrificed by injection of an overdose of pentobarbital and the cervical trachea was exposed. Sterile saline was instilled into the alveoli via the trachea and recovered, and this operation was repeated three times. The obtained bronchoalveolar lavage (BAL) fluid was centrifuged sufficiently at low temperature to collect cells. The cells were resuspended in sterile PBS and counted with a hemocyte
counter, followed by Giemsa staining. According to the staining results, the number of macrophages was calculated and recorded.

**Cell lines and treatment**

The hominine monocytic THP-1 cells were provided by the China Cell Line Bank (Beijing, China). Prior to drug intervention, THP cells cultured in RPIM-1640 medium with 10% FBS (Invitrogen-Gibco, Grand Island, NY) required 100ng/ml PMA (MedChemExpress, Monmouth Junction, NJ, USA) to induce differentiation into adherent macrophages for 12 h. To establish the ALI model in vitro, the differentiated THP-1 cells were stimulated with 1μg/ml LPS for 6h. For purpose of exploring the optimal time and concentration of intervention, cells were cultured at 0-300μM BF for 0h, 6h, 12h, 18h and 24h. The ATP-competitive and selective AMPK inhibitor Compound C (10μM, MedChemExpress, Monmouth Junction, NJ, USA) and the autophagy inhibitor 3-MA (5mM, MedChemExpress, Monmouth Junction, NJ, USA) was added 0.5h before LPS or BF intervention. Nrf2 siRNA (Table 2) was functional to knock down the expression of Nrf2 in THP-1 cells using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA).

**Western Blot and real-time PCR**

Changes in protein levels in mouse lung tissues as well as cells were detected by protein immunoblot (Western Blot). Milled lung tissues or cell precipitates obtained by centrifugation were taken, lysed on ice for 30 min by adding RIPA lysis solution at a certain ratio, quantified and denatured after ultrasonic centrifugation. Protein electrophoresis was performed in SDS-PAGE gels and transferred to membranes. The primary antibody was incubated overnight at 4°C in the refrigerator after 3h of closure, and the secondary antibody was incubated at room temperature the next day. Then the bands were recorded and the results were analyzed by Image J software.

Real-time quantitative PCR to detect the expression of mRNA of related molecules: Trizol method was used to extract total mRNA from tissues or cells, and 2 μg of the obtained mRNA was reverse transcribed to synthesize cDNA. The mRNA expression levels were detected using SYBR®Green Realtime PCR Master Mix (QPK-201, TOYOBO). The relevant primers
are shown in Table 1.

**Immunofluorescence staining**

Tissue section: paraffin sections were placed in the 65°C oven for 2h, dewaxed and hydrated to repair the antigen. THP-1 cells: The treated cells were discarded from the upper layer of medium, washed and fixed with 4% paraformaldehyde for 30 min, and washed again.

Tissue sections or cell specimens were permeabilized with 0.2% Triton X-100 and subsequently blocked, and primary antibodies were diluted 1:100 and incubated overnight at 4°C. The specimens were rewarmed the next day and incubated for 45 min with secondary antibody protected from light. After DAPI re-staining of cell nuclei, the specimens were microscopically photographed.

**PI-Hoechst staining**

The prepared cell specimens were washed with pre-cooled PBS and fixed with 4% paraformaldehyde, then stained with PI (#81845, Sigma-Aldrich, USA) and Hoechst-33342(#14533) for 10 min, respectively. The samples were photographed with fluorescence microscopy after sufficiently washing and protection from light.

**Assay of cell viability**

The differentiated THP-1 cells were plated on 96-well plates for 12h and then cultured with BF at the corresponding dose for 0h, 6h, 12h, 18h and 24h. CCK8 assay kit (#ab228554, Abcam) was used to detect cell viability.

**Assay of IL-1β, IL-18 by ELISA**

Tissue homogenates ground with pre-cooled PBS solution or cell supernatants obtained by centrifugation were operated according to the steps of the kit instructions (#ab197742, #ab216165, Abcam) and tested for absorbance on the microplate reader, and data were collected and analyzed.

**Assay of the activation of p65 by ELISA**
Cell lysates with protein concentrations of 100-500 μg/ml were operated according to the kit instructions (#ab176648, Abcam), and the absorbance of the samples was recorded at 450 nm.

**Statistical Analysis**

Statistical analysis was performed using SPSS 23.0 and statistical graphs were obtained via GraphPad Prism 8. After testing for normality via the Shapiro-Wilk test, the measurement data that conformed to normal distributions were analyzed using the two-tailed Student’s t-test, while data showing skewed distributions were analyzed using the Mann-Whitney U test. When influenced by a single factor, statistical differences between more than two groups were examined using one-way ANOVA, followed by Bonferroni’s analysis or Tamhane’s T2 analysis for multiple comparisons. For data intervened by both factors, the two-factor ANOVA was used. Grouped data were analyzed by the Sidak’s test for within-group differences and the Tukey’s test for multiple comparisons of between-group differences. All data were expressed by Mean ± SD. When p < 0.05, it indicates that the data are statistically different.

**Results**

Protective effect of BF pretreatment on ALI induced by LPS injection in mice lungs

Wild-type mice were given LPS intraperitoneally and pretreated with BF according to the above-mentioned modeling (Figure 1A). Based on the results of pre-experimentation with different doses (Supplement Figure 1A-C), we finally chose a dose of 25 mg/kg for BF injection, while satisfying the requirement of activating AMPK and barely affecting the blood glucose level of mice. H&E staining applied to determine the pathological damage changes in mice lungs (Figure 1B). Lung tissue damage was evident in the LPS group of mice, while the results of the injury score showed significantly less lung damage and a greater reduction in inflammatory cell infiltration in mice injected with BF in advance (Figure 1C). Also, BF reduced the LPS-induced edema in lung tissue of mice (Figure 1D). For the degree of
inflammatory cell infiltration, the activity of MPO, a lysosomal protein highly expressed in neutrophils, was significantly higher in the LPS group than in the control group, however, BF pretreatment greatly mitigated this situation (Figure 1E). BF was first used as a hypoglycemic agent, but the dose used in this study was much lower than the conventional dosage and had a shorter duration of effect and the mode of administration was different. In order to exclude whether BF functioned as a blood glucose regulator in this study, all wild-type mice were measured for their blood glucose before and after injection, and the results showed that this dose of BF did not cause significant changes in blood glucose in mice in a short period of time (Figure 1F). Detection of mRNA levels of proinflammatory cytokines (IL-1β, IL-6, TNF-α) in mouse lung tissues revealed that the expression of proinflammatory cytokines was significantly inhibited in the LPS+BF group compared with the modeling group (Figure 1G-H). In summary, BF inhibits the extent of lung tissue damage as well as the level of inflammation in LPS-induced ALI in vivo.

BF inhibited NLRP3-mediated pyroptosis and activated AMPK in vivo

Key upstream regulators of the NLRP3-mediated pyroptosis pathway include NLRP3, ASC and caspase-1. (26) Upon the inflammasome formation, caspase-1 is hydrolyzed to p20 fragment and the primary target protein, GSDMD, exposes the N fragment to disrupt the cell membrane leading to inflammatory outbreaks. (26) As shown in Figure 2A, the protein levels of NLRP3, Caspase-1 p20, ASC and GSDMD-N in mouse lung tissues were significantly increased after LPS stimulation, which could be suppressed by BF. CD68, a highly glycosylated glycoprotein is often used as a marker of macrophages in immunofluorescence staining. (27) Immunofluorescence staining of mouse lung tissues showed a significant increase in NLRP3 expression and macrophage infiltration in the LPS group, and the two were largely co-localized. The phenomenon could be reversed by BF (Figure 2B). IL-1β and IL-18, which are released when pyroptosis occurs, were found by ELISA to be similarly inhibited by BF pretreatment upon LPS challenge (Figure 2 C and D). From the results from Figure 2 E and F, BF was verified to significantly increase the phosphorylation of AMPK in
mouse lungs.

After LPS stimulation, the number of cells obtained from the BAL fluid of mice was significantly higher, while the number of BAL cells from mice given BF in advance was significantly lower (Supplement Figure 2A and B). At the same time, the degree of macrophage infiltration, an important regulator of the immune environment, was also alleviated (Figure 2G), which echoed the results of immunofluorescence staining.

BF inhibited NLRP3-mediated pyroptosis in an AMPK-dependent manner in macrophages

To further investigate the mechanism of BF in reducing ALI, we used different doses of BF to interfere with THP-1 cells to find out the optimal concentration of MF in macrophages. According to the results (Figure 3A and B), the phosphorylation of AMPK was significantly elevated at a concentration of 30 μM of BF. And the activation of AMPK was strongest after BF was added to the medium for 12h (Figure 3C and D). However, at too high a dose or for too long a duration of action, BF exhibited potent cytotoxicity (Figure 3E). Therefore, in this study, we chose to pretreat THP-1 cells in vitro model with BF at a concentration of 30 μM for 12 h.

After 6 h of LPS stimulation, inflammatory cytokines (IL-6 and TNF-α) were significantly lower in BF pretreated THP-1 cells than in untreated ones (Figure 3F and G). Based on Figure 3H, AMPK activation by BF was accompanied by significant inhibition of NLRP3, caspase-1 p20 and GSDMD-N in LPS-stimulated macrophages. PI-Hoechst staining can be used to determine the extent of plasma membrane damage and cell death. After LPS challenged, the cell membrane was disrupted leading to a large amount of PI entering the cells making the red light significantly enhanced, while the cell membrane of LPS+BF treated cells was more intact and the number of live cells increased (Figure 3I). Similarly, the secretion of mature IL-1β and IL-18 in the cell supernatant, as well as the mRNA level of NLRP3 was inhibited by BF (Figure 3J-L). To verify whether inhibition of NLRP3-mediated pyroptosis is a direct factor in the reduction of inflammation levels by BF, we transfected cells with adenovirus to overexpress NLRP3 and observed the effect of BF (Supplement Figure 3A). It was found that NLRP3 overexpression counteracted the mitigating effect of BF pretreatment on macrophages.
Interestingly, the protein level of NLRP3 did increase somewhat after the addition of Compound C, an AMPK activation inhibitor (Figure 3M). Taken together, we have strong evidence that BF inhibits NLRP3 inflammatory vesicle function and thus alleviates pyroptosis mainly via activating AMPK.

BF enhanced autophagy to inhibit pyroptosis in macrophages via AMPK-mTOR pathway

To explore the mechanism by which BF inhibits pyroptosis by activating AMPK, we next performed experimental assays of AMPK-related pathways. In normal blood glucose, AMPK has been shown to be a crucial regulator that inhibits mTOR activity to promote autophagy.(28) As shown, p-mTOR expression levels were significantly suppressed in the BF group or LPS+BF group. Meanwhile, the expression of autophagy markers LC3 II and Beclin-1 increased and p62 decreased, both indicating that BF can amplify autophagy (Figure 4A and B). To verify whether BF is achieved by AMPK, Compound C was added, and it turned out that the autophagic process can be suppressed by CC (Figure 4C and D). Transfection of GFP-LC3 plasmid in cells allows more visualization of autophagy level. Consistent with the previous results, the expression of LC3 increased after BF treatment and was inhibited by CC (Figure 4E).

To explore the relationship between autophagy and pyroptosis, we added the autophagy inhibitor 3-MA to the original experimental groups. 3-MA inhibits class III PI3K to suppress autophagy, and therefore was added to counteract the level of autophagy promoted by BF (Figure 5 A and B). Moreover, the protein levels of NLRP3, caspase-1 p20 and GSDMD-N in cells rebounded after autophagy was inhibited (Figure 5C), while the cell membrane of THP-1 cells was also more disrupted (Figure 5D). Similarly, IL-1β and IL-18 in the supernatants were significantly higher than those in the BF pretreatment group (Figure 5 E and F). It is noteworthy that although the protein level of NLRP3 was increased, its mRNA level was still relatively low (Figure 5G). This suggests that the autophagy promoted by BF did not inhibit pyroptosis at the transcriptional level of NLRP3, but most likely promoted the degradation of NLRP3 inflammasome.
BF suppressed NLRP3 transcription via up-regulating AMPK-dependent NRF2 expression 

We next continued to explore the ways in which BF regulates the transcriptional level of NLRP3. Nrf2 is a transcription factor that plays a protective role in inflammation. We found that the expression of Nrf2 was significantly inhibited in the in vitro simulated acute lung injury model, while pretreatment with BF was able to promote its expression, while the phosphorylation of glycogen synthase kinase-3β (GSK-3β) showed the same trend (Figure 6 A-C). To determine whether the elevation of Nrf2 was related to AMPK, CC was added and it was found that Nrf2 expression was reduced (Figure 6D). In addition, activation of p65, which is the subunit of NF-κB, another NLRP3 transcription factor, could be inhibited by pretreatment with BF (Figure 6E-G).

In recent years, there is increasing evidence that Nrf2 inhibits inflammatory pathways mainly due to its ability to inhibit p65 activation and nuclear translocation, which in turn inhibits the expression of inflammatory proteins such as NLRP3.(29) After transfection of Nrf2 siRNA in cells, phosphorylation of p65 and consequently NLRP3 expression, as well as protein levels of p20 and GSDMD-N were significantly elevated, even with BF pretreatment (Figure 7A and C). At the same time, the amount of free activated p65 was also increased (Figure 7H). The knockdown of Nrf2 also aggravated cell membrane breakdown and secretion of IL-1β and IL-18, suggesting that the Nrf2 pathway is an important pathway for BF to inhibit pyroptosis (Figure 7B, D and E). The result of immunofluorescence staining showed that NLRP3 expression changed in cells as expected and was elevated after Nrf2 inhibition (Figure 7G). Of interest, Nrf2 knockdown substantially increased the mRNA level of NLRP3 which was reduced by BF (Figure 7F).

Nrf2 deficiency moderately counteracted the preventive effect of BF against ALI in vivo 

To further verify whether BF is partially dependent on Nrf2 to alleviate acute lung injury in mice, wild-type and Nrf2 knockout mice were given BF and solvent pretreatment, respectively, prior to modeling. In the perspective of pathological analysis, Nrf2−/− mice with
or without pretreatment with BF had severe pathological damage and the protective effect of BF was significantly diminished compared to wild-type mice (Figure 8 A and B). According to Figure 8C, the protein expression of p-p65 in the lung tissue of Nrf2-/- group mice was not reduced after BF pretreatment, and the protein levels of NLRP3, p20, and GSDMD-N were much higher than those of WT mice in LPS + BF group. Not surprisingly, IL-1β and IL-18 secretion in the lung tissue of Nrf2-/- mice also remained much higher than in the wild-type group of identically treated mice (Figure 8 D and E). In conclusion, the protective effect of BF was significantly inhibited by Nrf2 deletion in mice, which supports our contention.

BF showed a certain therapeutic effect after LPS challenge

To fully explore the clinical function of BF, we then simulated BF treatment after exposure to infection to observe if ALI could be ameliorated before the peak of injury and inflammation (Figure 9 A). The results of H&E staining of lung tissue showed that injection of BF after LPS stimulation for 6 h could appropriately alleviate damage to lung tissue as well as inflammatory cell recruitment (Figure 9 B and C). MPO activity and inflammatory cytokine secretion in mouse lung tissue were suppressed to a certain extent after BF treatment (Figure 9 D-G). As shown in Figure 9 H and I, AMPK, the main target of BF was efficiently activated while NLRP3 expression was reduced. The results suggested that, although not as significant as pretreatment, BF could still appropriately treat ALI after LPS infection.

Discussion

In acute lung injury induced by lipopolysaccharide, pyroptosis of alveolar macrophages leads to massive release of inflammatory factors in lung tissue and exacerbates tissue damage. In this study, BF was found to inhibit NLRP3-mediated pyroptosis in macrophages via activation of AMPK in sepsis-induced ALI. Pretreatment of BF upregulates AMPK phosphorylation, leading to enhancement of autophagy to degrade NLRP3 inflammasome on the one hand and promotion of Nrf2 expression to repress NLRP3 transcription on the other hand (Figure 10). BF is a member of the widely used biguanide family and has been used as an oral hypoglycemic agent along with metformin and phenformin.(30) However, BF and PF exhibit...
stronger inhibition of mitochondrial respiration than metformin, increasing the accumulation of lactic acid in the body. (19, 31) The toxic effects of PF are the most pronounced, with some studies showing that its risk of causing lactic acidosis is tens of times greater than that of metformin, and even greater than that of BF. (31) Hence, the protective effect of BF is of renewed interest compared to its weaker toxic effects. In cervical cancer, BF has been shown to promote phosphorylation of histones as well as AMPK to promote delayed DNA damage repair and cell cycle arrest, thereby improving the sensitivity of tumor cells to radiotherapy. (32) Similarly, BF plays an anti-proliferation and anti-invasion role in endometrial cancer and breast cancer via activating AMPK. (17, 18) It can be seen that the phosphorylation of AMPK by BF through the inhibition of ATP formation is fundamental to the protective effect. In this study, low-dose BF pretreatment alleviated lung tissue injury, edema and the inflammation level in septicemic mice. We thus explored the mechanisms underlying the protective role of BF in injury.

When LPS stimulates, it is first recognized by Toll-like receptor 4 (TLR4) on the cell membrane, which in turn transmits signals intracellularly to increase NLRP3 expression by activating p65 and promoting its nuclear translocation, leading to pyroptosis. (33) Macrophages, as the key to maintain the stability of the immune environment, will release a large number of inflammatory cytokines and high mobility group box-1 (HMGB1), cell contents, etc. to the extracellular space, triggering an inflammatory cascade response once pyroptosis was activated. (34) It is the direct cause of severe tissue damage and high degree of inflammation in sepsis-induced ALI. In recent years, more and more studies have demonstrated that AMPK acts as not only a receptor for energy metabolism, but also a regulatory factor in many immune pathways. (35) In vitro and in vivo experiments, we preliminarily demonstrated that BF inhibits NLRP3-mediated pyroptosis by activating AMPK.

Autophagy is a highly conserved lysosome-dependent degradation process involved in the regulation of cell development, differentiation, dynamic homeostasis, and plays a protective role in inflammatory invasion. (36) In most physiological or pathological states, AMPK can inactivate mTOR, which in turn promotes the phosphorylation of ULK1 and binds to it, ultimately amplifying autophagy. (37) In the present study, we found that BF did have an
up-regulatory effect on autophagy by activating AMPK. Previous studies have reported that autophagy can scavenge ROS, inhibit the assembly of inflammasomes, and even directly clear inflammatory vesicles to inhibit pyroptosis. In BF pretreated macrophages, the expression of NLRP3 and other proteins was indeed significantly inhibited, and the degree of pyroptosis was significantly restrained, but notably, the mRNA level of NLRP3 was not reduced by the inhibition of autophagy. It strongly suggested that BF-promoted autophagy was not involved in the transcriptional regulation of inflammatory proteins, but rather degraded the NLRP3 inflammasome, which in turn alleviates ALI.

To seek the mechanism by which BF inhibits NLRP3 transcription, we measured changes in another pathway, the GSK3β-Nrf2 pathway. Upon exposure to risk factors, nuclear factor-erythroid 2 related factor 2 (Nrf2) segregates from Keap1 into the nucleus to bind to antioxidant response elements (AREs) and promote expression of various cytoprotective genes (e.g. GCLM and HO-1) to inhibit the activation of pro-inflammatory proteins such as p65. Meanwhile, AMPK promotes phosphorylation (inactivation) of GSK3β and promotes Nrf2 accumulation while inhibiting its degradation out of the nucleus. Encouragingly, our results proved that while enhancing autophagy, BF inhibited p65 activity through the GSK3β-Nrf2 axis and reduced mRNA level of NLRP3.

Although, the palliative effect of BF pretreatment on sepsis-induced ALI and the related mechanisms have been explored as well as validated. The results demonstrate that BF given before the onset of sepsis provides protection against subsequent ALI. And to fully explore the clinical value of BF, we then supplemented the in vivo experiments with the administration of BF after LPS injections. Reassuringly, BF showed some therapeutic effect, but its anti-inflammatory as well as palliative effect was much lower than that of the advance dosing. We speculate that this may be due to the fact that, on the one hand, the systemic inflammation caused by sepsis affects the uptake of BF and the stimulation of downstream targets, and on the other hand, the duration of BF action is insufficient.

In conclusion, our study found that in sepsis-induced acute lung injury, BF could inhibit NLRP3-mediated pyroptosis via an AMPK-dependent pathway that upregulates autophagy and Nrf2 protein level. It provides a new insight and rationale for clinical use of BF, the
AMPK agonist, to alleviate the symptoms of ALI and ARDS.

**Clinical perspectives**

The level of inflammation in sepsis-induced ALI is extremely high, with severe damage and lack of effective treatment modalities.

We demonstrated that BF pretreatment alleviated ALI by inhibiting pyroptosis via AMPK-dependent pathway.

The study provides a new idea for clinical prophylaxis of sepsis-induced ALI and taps into other roles of BF.

**Data Availability Statement**

The data in this study were obtained from experiments, not from the internet, and therefore are not suitable for data sharing.

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Table 1 The Primers Used in Real-Time PCR
Table 2 The siRNA Sequence of the Target Protein used

Figure Legend

Figure 1. Protective effect of BF pretreatment on sepsis-induced ALI in mice lungs

(A) Schematic diagram of animal experiment (B-C) H&E staining of mouse lung tissues at 12h after LPS injection and injury scores based on analysis of pathological section results. (D) Lung wet/dry ratio was calculated after weighing and recording the dry and wet weights of the mouse lung tissues in the indicated groups. (E) MPO activity in the indicated groups. (F) Tail vein blood glucose levels were measured once before and once after the drug intervention in the indicated groups. (G-I) The mRNA levels of IL-1β, IL-6 and TNF-α in the mouse lung tissues of the indicated groups. For (C) and (D), n=3 per group. For (E) and (F), n=6 per group. For (G), (H) and (I), n=5 per group.

(*P<0.05, **P<0.01 or ***P<0.001 compared with indicated group, ns, no significance)

Figure 2. BF inhibited NLRP3-mediated pyroptosis and activates AMPK in vivo

(A) Western blot analysis for NLRP3, caspase-1 p20, ASC and GSDMD-N of mouse lung tissues in the indicated groups. (B) Immunofluorescence staining of mouse lung tissues in the indicated groups. Green: CD68 Red: NLRP3 Blue: DAPI (C-D) Levels of IL-1β and IL-18 in lung tissue homogenates detected by ELISA in the indicated groups. (E-F) Western blot analysis and statistics results for p-AMPK and AMPK of mouse lung tissues in the indicated groups. (G) Macrophage counts collected in the BAL fluid of mice in the indicated groups. For (C), (D) and (F), n=5 per group. For (G), n=3 per group.

(*P<0.05, **P<0.01 or ***P<0.001 compared with indicated group, ns, no significance)

Figure 3. BF inhibited NLRP3-mediated pyroptosis by promoting AMPK activation in macrophages

(A-B) Western blot analysis and statistics results for p-AMPK and AMPK of THP-1 cells respectively at different concentrations of 0μM, 10μM, 30μM, 100μM and 300μM for 12h. (C-D) Western blot analysis and data results for p-AMPK and AMPK of THP-1 cells
respectively for 0h, 6h, 12h, 18h and 24h at a dose of 30μM. (E) Cell viability was determined by CCK8 kit in THP-1 cells treated with BF at different concentrations of 0μM, 10μM, 30μM, 100μM and 300μM for 0h, 6h, 12h, 18h and 24h. (F-G) THP-1 cells were treated with 1 μg/ml of LPS for 6 h or 30 μM of BF for 12 h. The mRNA level of IL-6 and TNF-α in the indicated groups. (H) Western blot analysis and statistics results for p-AMPK and AMPK of THP-1 cells in the indicated groups. (I) PI & Hoechst staining for treated THP-1 cells. (J-K) The levels of IL-1β and IL-18 determined by ELISA in cell supernatant. (L) The mRNA level of NLRP3 in the indicated groups. (M) Western blot analysis for p-AMPK, AMPK and NLRP3 of THP-1 cells in the indicated groups. (Compound C was added 30mins before at a dose of 10μM.) For (B), (D) and (E), n=3 per group. For (F), (G), (J), (K) and (L), n=5 per group. (*P<0.05, **P<0.01 or ***P<0.001 compared with indicated group, ns, no significance)

Figure 4. BF enhanced autophagy via AMPK-mTOR pathway

(A-B) Western blot analysis and statistics results for p-mTOR, mTOR, LC3, p62 and Beclin-1 of THP-1 cells in the indicated groups. (C-D) Western blot analysis and statistics results for LC3, p62 and Beclin-1 of THP-1 cells in the indicated groups. (Compound C was added 30mins before at a dose of 10μM.) (E) Observation of treated THP-1 cells by confocal microscopy after transfection with GFP-LC3 (objective 80×, zoom: 1.5×). For (B) and (D), n=3 per group. (*P<0.05, **P<0.01 or ***P<0.001 compared with indicated group)

Figure 5. BF attenuated pyroptosis by promoting autophagy

(A-B) Western blot analysis and statistics results for LC3, p62 and Beclin-1 of THP-1 cells in the indicated groups. (3-MA was added 30mins before at a dose of 5mM.) (C) Western blot analysis for NLRP3, caspase-1 p20 and GSDMD-N of THP-1 cells in the indicated groups. (3-MA was added 30mins before at a dose of 5mM.) (D) PI & Hoechst staining for treated THP-1 cells. (E-F) The levels of IL-1β and IL-18 determined by ELISA in cell supernatant.
(G) The mRNA level of NLRP3 in the THP-1 cells of the indicated groups. For (B), n=3 per group. For (E), (F) and (G), n=5 per group.

(*P<0.05, **P<0.01 or ***P<0.001 compared with indicated group, ns, no significance)

Figure 6. BF upregulated Nrf2 expression via the AMPK-GSK3β pathway

(A-C) Western blot analysis and statistics results for p-GSK3β, GSK3β and Nrf2 of THP-1 cells in the indicated groups. (D) Western blot analysis for Nrf2 of THP-1 cells in the indicated groups. (Compound C was added 30mins before at a dose of 10μM.) (E-F) Western blot analysis and statistics results for p-p65 and p65 of THP-1 cells in the indicated groups. (G) Absorbance of p65 in cell lysates of each group measured by ELISA. For (B), (C) and (F), n=3 per group. For (G), n=5 per group.

(*P<0.05, **P<0.01 or ***P<0.001 compared with indicated group, ns, no significance)

Figure 7. BF reduced NLRP3 mRNA level by promoting Nrf2 expression

(A and C) Western blot analysis and statistics results for Nrf2, p-p65, p65, NLRP3, caspase-1 p20 and GSDMD-N of THP-1 cells in the indicated groups. (B) PI & Hoechst staining for treated THP-1 cells. (D-E) The levels of IL-1β and IL-18 determined by ELISA in cell supernatant. (F) The mRNA level of NLRP3 in the THP-1 cells of the indicated groups. (G) Immunofluorescence staining of treated THP-1 cells. Red: NLRP3 Blue: DAPI (H) Absorbance of p65 in cell lysates of each group measured by ELISA. For (C), n=3 per group. For (D), (E), (F) and (H), n=5 per group.

(*P<0.05, **P<0.01 or ***P<0.001 compared with indicated group)

Figure 8. Nrf2 deficiency counteracted the preventive effect of BF against ALI in vivo

(A-B) H&E staining of WT and Nrf2 -/- mouse lung tissues at 12h after LPS injection and injury scores based on analysis of pathological section results. (C) Western blot analysis for Nrf2, p-p65, p65, NLRP3, caspase-1 p20 and GSDMD-N of WT and Nrf2-/- mouse lung tissues in the indicated groups. (D-E) Levels of IL-1β and IL-18 in lung tissue homogenates detected by ELISA in the indicated groups. For (B), n=3 per group. For (D) and (E), n=5 per
group.

(*P<0.05, **P<0.01 or ***P<0.001 compared with indicated group)

Figure 9. BF showed a certain therapeutic effect after LPS challenge

(A) Schematic diagram of animal experiment (B-C) H&E staining of mouse lung tissues at 12h after LPS injection and injury scores based on analysis of pathological section results. (D) MPO activity in the indicated groups. (E-G) The mRNA levels of IL-1β, IL-6 and TNF-α in the mouse lung tissues of the indicated groups. (H-I) Western blot analysis and statistics results for p-AMPK and AMPK of mouse lung tissues in the indicated groups. For (C) and (I), n=3 per group. For (D), n=6 per group. For (E), (F) and (G), n=5 per group.

(*P<0.05, **P<0.01 or ***P<0.001 compared with indicated group, ns, no significance)

Figure 10. The possible mechanisms by which BF alleviates sepsis-induced ALI
| Species | Gene | Forward Primer | Reverse Primer |
|---------|------|----------------|----------------|
| Mice    | TNF-α| ACTGAACCTTCGGGGTGATCGGT | TGGTTTGTACGACGTGGCTAT |
| Mice    | IL-6 | CCCAATTTCAGCTCTCC     | CGCAGGTTTTGGCCGAGTA  |
| Mice    | IL-1β| AATGAAGGAGCAGGAGGACCC| CTCCAGCCAAGCTTTTCTTG |
| Mice    | GAPDH| ACTCCACTCACGGCAAATTC | TCTCCTATGGTGGTGCACGACA|
| Human   | NLRP3| ATGTGGGGGAGAATGCTTTG | TGTCTCCGAGAGTGTGTC |
| Human   | TNF-α| TCCAGGCGGTTGCTTTTTC | GTTTGTCACGAGGGGTTTC |
| Human   | IL-6 | AGCCCTGAGAAAGGAGACA  | CCAAAAGACACGTGATGAT |
| Human   | IL-1β| CAGGACTCACAGCAAAAAA  | TTTAACACGCAGGACAGGT |
| Human   | β-actin | ACAGAGCCTCGCCTTTC     | CCACCACACGCCCCCTGG   |
| Species | Gene | sense | antisense |
|---------|------|-------|----------|
| Human   | Nrf2 | AUUGAUGUUUCUGAUCUAUCACUT | AGUGAUAGAUCAGAAACAUCAAU |
|         |      | T     | TT       |
A)

- NLRP3
  - Control: 110 KD
  - BF: 110 KD
  - LPS: 110 KD
  - LPS+BF: 110 KD

- Caspase-1 p20
  - Control: 20 KD
  - BF: 20 KD
  - LPS: 20 KD
  - LPS+BF: 20 KD

- ASC
  - Control: 25 KD
  - BF: 25 KD
  - LPS: 25 KD
  - LPS+BF: 25 KD

- GSDMD-N
  - Control: 29 KD
  - BF: 29 KD
  - LPS: 29 KD
  - LPS+BF: 29 KD

- β-actin
  - Control: 42 KD
  - BF: 42 KD
  - LPS: 42 KD
  - LPS+BF: 42 KD

B)

- CD88
- NLRP3
- DAPI
- merge

C)

IL-1β in lung tissue (pg/mL)

- Control: **
- BF: *
- LPS: ns
- LPS+BF: ns

D)

IL-18 in lung tissue (pg/mL)

- Control: ***
- BF: **
- LPS: ns
- LPS+BF: ns

E)

- p-AMPK
  - Control: 62 KD
  - BF: 62 KD
  - LPS: 62 KD
  - LPS+BF: 62 KD

- AMPK
  - Control: 62 KD
  - BF: 62 KD
  - LPS: 62 KD
  - LPS+BF: 62 KD

- β-actin
  - Control: 42 KD
  - BF: 42 KD
  - LPS: 42 KD
  - LPS+BF: 42 KD

F)

Ratio of p-AMPK/AMPK (Fold Change)

- Control: ns
- BF: ***
- LPS: ns
- LPS+BF: ns

G)

BAL macrophage count (×10^4)

- Control: ns
- BF: **
- LPS: *
