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The Three Main SCFAs Inhibit the Inflammatory Response of A549 Cells Induced by *Acinetobacter baumannii*

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Abstract

Objective: The objective is to explore the mechanism of inhibitory effect of three main SCFAs (acetate, propionate and butyrate) on inflammatory response of A549 cells. **Methods:** Human lung adenocarcinoma cells (A549 cells) were cultured, and were divided into normal control group (NC group), *A. baumannii* infection group (*A. baumannii* group), NF-κB inhibitor group (JSH group), *A. baumannii* infection + sodium acetate group (NaAc group), *A. baumannii* infection + sodium propionate group (NaPc group) and *A. baumannii* infection + sodium butyrate group (NaB group). Real-time quantitative PCR was used to detect the mRNA expression of *NLRP3*, *Caspase*-1, *IL*-1*β*, *IL*-6, and *TGF-β* in A549 cells. Western blotting assay was used to determine the expression of autophagy and "pyroptosis" related proteins of NRLP3, cleaved-Caspase-1 (P20), GSDMD (P30), LC-3 and Beclin-1. At the same time, the expression of NF-κB p65 protein in nucleus and cytoplasm of A549 cells was detected. The level of reactive oxygen species in A549 cells was detected by flow cytometry. **Results:** Compared with *A. baumannii* group, the mRNA expression of *NLRP3*, *IL*-1*β* and *IL*-6 in NaAc group, NaPc group and NaB group decreased significantly, the mRNA expression of *Caspase*-1 in NaPc group and NaB group decreased significantly, only the mRNA expression of *TGF-β* in NaB group increased significantly; LC3-II expression increased significantly in NaPc group and NaB group, only Beclin-1 expression increased and GSDMD (p30) expression decreased significantly in NaB group. All three kinds of SCFAs could significantly inhibit the expression of cleaved-Caspase-1 (p20) after *A. baumannii* infection, but there was no significant change in the protein expression of NLRP3. Compared with NC group, the production of reactive oxygen species in *A. baumannii* group increased significantly at 3 h after *A. baumannii* infection. Compared with *A. baumannii* group, NaB could significantly suppress the production of reactive

oxygen species induced by *A. baumannii*. Compared with *A. baumannii* group, the expression of NF-κB p65 in nucleus was significantly decreased and the expression of NF-κB p65 in cytoplasm was significantly increased after 24 h pre-incubation with NaB, NaPc and NaAc, respectively. **Conclusion:** *A. baumannii* can induce inflammatory injury of pulmonary epithelial cells, and the three major SCFAs can inhibit the activation of NLRP3 inflammasome and the release of pro-inflammatory factors through NF-κB/ROS/NLRP3 pathway, which provides a new way for clinical prevention of severe inflammatory injury caused by *A. baumannii* infection.

Keywords

Acinetobacter baumannii, SCFAs, A549 Cells, Inflammatory Injury

1. Introduction

As an important hospital-acquired pathogen, *Acinetobacter baumannii* is the main pathogen inducing ventilator-associated pneumonia in patients in the department of respiratory medicine and intensive care unit (ICU), which can cause acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) [1]. Clinical syndrome of acute respiratory failure, with a high fatality rate, has not yet been effectively prevented. The main cause of ALI/ARDS is the uncontrolled expression of a large number of pro-inflammatory cytokines, which leads to lung tissue damage. Our previous results showed that three major short chain fatty acids (SCFAs) including acetate, propionate and butyrate inhibit macrophage inflammation through the NF-κB/ROS/NLRP3 pathway, inhibit the release of various pro-inflammatory cytokines, and reduce the degree of inflammatory damage in normal tissues and cells [2]. As an important anti-inflammatory metabolite of intestinal flora, SCFAs play an important role in maintaining systemic immune balance and inhibiting excessive inflammation. However, there is still a lack of research on the abundant lung epithelial cells that can secrete cytokines and chemokines [3]. In this study, *A. baumannii* infected A549 cells to induce the secretion of various cytokines, and to investigate the inhibitory effect of three main SCFAs on the inflammatory response of A549 cells, providing a new idea for the prevention and treatment of ALI/ARDS based on the strategy of "lung disease and intestinal therapy".

2. Materials and Methods

2.1. Bacterial and Cell Cultures

The *A. baumannii* strain (ATCC 19606) was donated by Dr. Zhou Wanqing (Department of clinical laboratory, Nanjing Gulou Hospital). The A549 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, and cultured stably in 37˚C and 5% CO_2 until the 6th generation for this study. The A549 cells in a good growth state were digested by trypsin and inoculated into

6-well cell plates with 1 × 10^6 cells in each well. Normal control group (NC group), *A. baumannii* infection group (*A. baumannii* group), NF-*κ*B inhibitor (JSH-23, AbMole USA) group (JSH group), *A. baumannii* infection + sodium acetate group (NaAc group), *A. baumannii* infection + sodium propionate group (NaPc group) and *A. baumannii* infection + sodium butyrate group (NaB group). *A. baumannii* group cells were infected with *A. baumannii* (bacteria: cells = 10:1) for 1 h, 3 h, and 6 h, JSH group cells were treated with JSH-23 with final concentration of 30 μM for 24 h, NC group cells were not treated with any treatment. Cells in the three SCFAs treatment groups were treated with NaAc, NaPc and NaB at the final concentration of 100 μmol/L for 24 h, respectively, and then infected with *A. baumannii* (bacteria: cells = 10:1). The experiment was terminated after 1 h, 3 h and 6 h. Cells were collected for subsequent research.

2.2. Western Blotting

The cell culture medium was discarded and 100 μL RIPA lysis solution was added to each well for ice lysis for 15 min. The lysis products were centrifuged at 12,000 r/min for 10 min. The supernatant was collected for Western blotting. LC-3 antibody, Beclin-1 antibody, NLRP3 antibody, pro-caspase-1 antibody, Cleaved caspase-1 (P20) antibody, actin antibody, Gasdermin D (GSDMD) (P30) antibody was diluted at 1:1000 and incubated at 4˚C overnight. The 1:5000 diluted anti-rabbit/mouse IgG-HRP was incubated at room temperature for 1 h. The ECL chemiluminescence kit was used, and the expression levels of LC-3, Beclin-1 and GSDMD (P30) were analyzed by Image J software.

2.3. qRT-PCR

Total RNA was extracted by Trizol method, and cDNA chains were synthesized by oligo$(dT)_n$ primers and PrimeScriptTM RT Master Mix, and corresponding mRNA expression levels were detected by qRT-PCR. The PCR primers sequence was listed in Table 1. The total reaction system was 20 μL, including SYBR Green Master Mix 10 μL, upstream and downstream primers 0.4 μL (10 μmol/L), cDNA 2 μL. Pre-denaturation at 95˚C for 1 min, denaturation at 95˚C for 5 s, and annealing at 60˚C for 30 s were performed for 40 cycles. The *Ct* values of sample genes in the experiment were homogenized by the *Ct* values of GAPDH.

Table 1. The sequence of qRT-PCR.

Genes	Forward (5’ → 3’)	Reverse (5’ → 3’)
GAPDH	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG
NLRP3	AACAGCCACCTCACTTCCAG	CCAACCACAATCTCCGAATG
Caspase-1	GCACAAGACCTCTGACAGCA	TTGGGCAGTTCTTGGTATTC
IL-1*β*	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
IL-6	CCTTCGGTCCAGTTGCCTTCT	GAGGTGAGTGGCTGTCTGTGT
TGF-β	TACCTGAACCCGTGTTGCTCTC	GTTGCTGAGGTATCGCCAGGAA

2.4. NF-κB p65 Detection

The nuclei and cytoplasmic proteins of A549 cells were isolated strictly according to the instructions of the nuclear/plasma protein separation kit (Beyotime Biotechnology, China) for Western blotting analysis. The anti-NF-κB P65 antibody was diluted at 1:1000 and incubated overnight at 4˚C. The rabbit IgG-HRP (1:5000) was incubated at room temperature for 1 h. After washing with TBST for 3 times, the expression of NF-κB p65 in the nucleus and cytoplasm was analyzed by grayscale scanning analysis software.

2.5. ROS Detection

The levels of reactive oxygen species (ROS) in A549 cells were detected by flow cytometry combined with 2',7'-dichlorofluorescein yellow diacetate (DCFH-DA) probe. Serum-free medium diluted DCFH-DA at 1:1000 ratio until the final concentration was 10 μmol/L. The cells were suspended in the diluted DCFH-DA probe solution at a concentration of 1×10^7/mL. The cells were incubated in a cell culture box at 37˚C, sheltered from light, for 20 min. The cells were mixed upside down at an interval of 5 min to make full contact between the probe and the cells. Serum-free cell culture medium was washed for 3 times to remove the DCFH-DA probes that did not enter the cells, and the cells were re-suspended with sterile PBS buffer for detection.

2.6. Statistical Analysis

SPSS 22.0 statistical software was used for data analysis, measurement data were expressed as mean±standard deviation ($\bar{x} \pm s$). The ANOVA was used for inter-group comparison, and the LSD-T test was used for inter-group multiple comparison, and the statistical graph was generated by GraphPad Prism 7 software with $P < 0.05$ was considered statistically significant.

3. Results

3.1. Three Major SCFAs Inhibited the Activation of NLRP3 Inflammasome in A549 Cells

The NLRP3 inflammasome composed of the NLRP3 protein, Caspase-1, and ASC. The mRNA expression of NLRP3 and Caspase-1 in A549 cells treated with NF-κB inhibitor (JSH) was significantly lower than that in *A. baumannii* group ($P < 0.05$). Compared with *A. baumannii* group, NLRP3 mRNA expression in NaAc group, NaPc group and NaB group was significantly decreased ($t = 11.37$, $P = 0.008$; $t = 6.92$, $P = 0.020$; $t = 11.90$, $P = 0.007$). Compared with *A. baumannii* group, NaPc and NaB, except NaAc, significantly inhibited caspase-1 mRNA expression ($t = 7.67$, $P = 0.017$; $t = 14.78$, $P = 0.005$). NF-κB inhibitor (JSH) can inhibit NLRP3 and Caspase-1 mRNA ($P < 0.05$) (**Figure 1**).

3.2. Three Major SCFAs Regulate the Expression of A549 Cytokines

Compared with *A. baumannii* group, the mRNA expression of IL-1β was

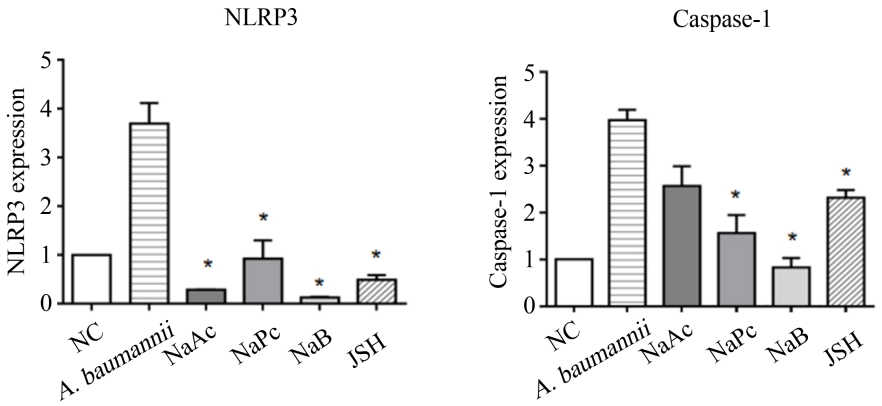


Figure 1. The mRNA expression of NLRP3 inflammasome in A549 cell. Compared with *A. baumannii* group, *P < 0.05.

significantly inhibited after 24 h pre-incubation with NaAc, NaPc and NaB (NaAc: t = 22.59, P = 0.002; NaPc: t = 6.55, P = 0.023; NaB: t = 52.82, P = 0.000); Il-6 mRNA expression was also inhibited (NaAc: t = 6.05, P = 0.026; NaPc: t = 5.29, P = 0.034; NaB: t = 8.79, P = 0.013); NaB alone significantly increased TGF-β mRNA expression in A549 cells (t = 5.25, P = 0.034) (**Figure 2**).

3.3. Three Major SCFAs Regulate the Expression of NLRP3 Inflammasome and Autophagy Related Proteins

Compared with *A. baumannii* group, LC3-II expression of A549 cells pre-incubated with NaPc and NaB was significantly increased (t = 4.23, P = 0.048; t = 4.52, P = 0.046). Both NaB and NF-κB inhibitor (JSH) enhanced Beclin-1 expression (t = 4.80, P = 0.041; t = 6.44, P = 0.023), and significantly inhibited the expression of activated GSDMD (P30) fragment (t = 5.16, P = 0.036; t = 6.72, P = 0.021). All three SCFAs significantly reduced the expression of Caspase-1 (P20) after *A. baumannii* infection (NaAc: t = 5.13, P = 0.036; NaPc: t = 4.49, P = 0.046; NaB: t = 4.62, P = 0.044), but the three SCFAs had no significant effect on NLRP3 expression (**Figure 3**).

3.4. Three Major SCFAs Inhibited ROS Production in A549 Cells

When infected with *A. baumannii* for 3 h, the production of ROS in A549 cells in *A. baumannii* group was significantly increased compared with NC group. Compared with *A. baumannii* group, both NaB and NF-κB inhibitor (JSH) significantly reduced ROS induced by *A. baumannii* (t = 4.37, P = 0.048; t = 4.65, P = 0.043) (**Figure 4**).

3.5. Three Major SCFAs Inhibited the Activation of NF-κB Pathway in A549 Cells

Compared with *A. baumannii* group, NaB, NaPc and NaAc were pre-incubated for 24 h, respectively, and the expression of NF-κB P65 was significantly decreased (P < 0.05). NaPc, NaB and NF-κB inhibitor (JSH) had similar effects,

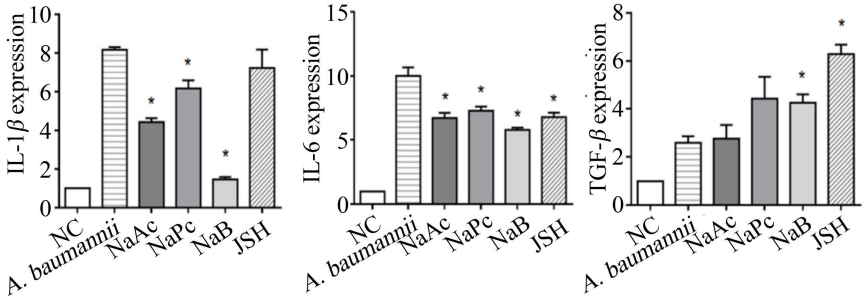
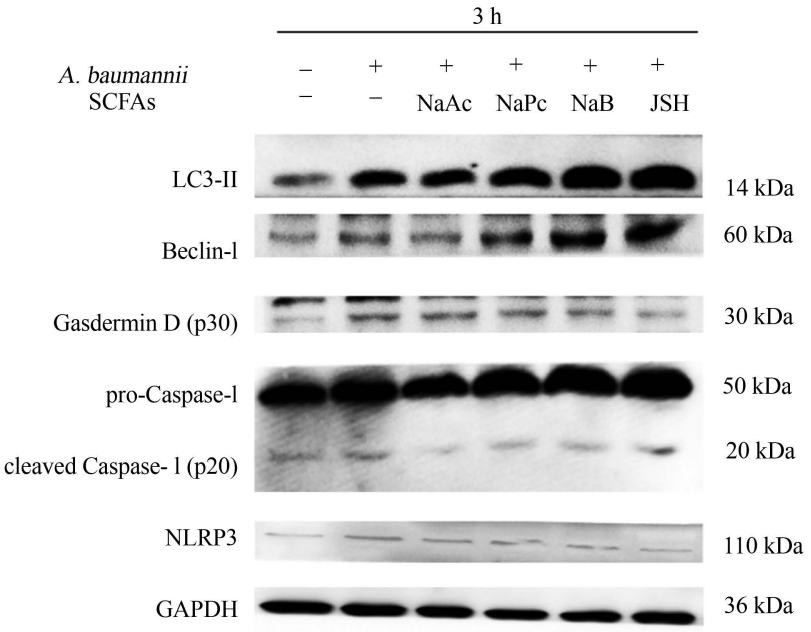


Figure 2. The mRNA expression of IL-1β, IL-6 and TGF-β in A549 cell. Compared with *A. baumannii* group, *P < 0.05.



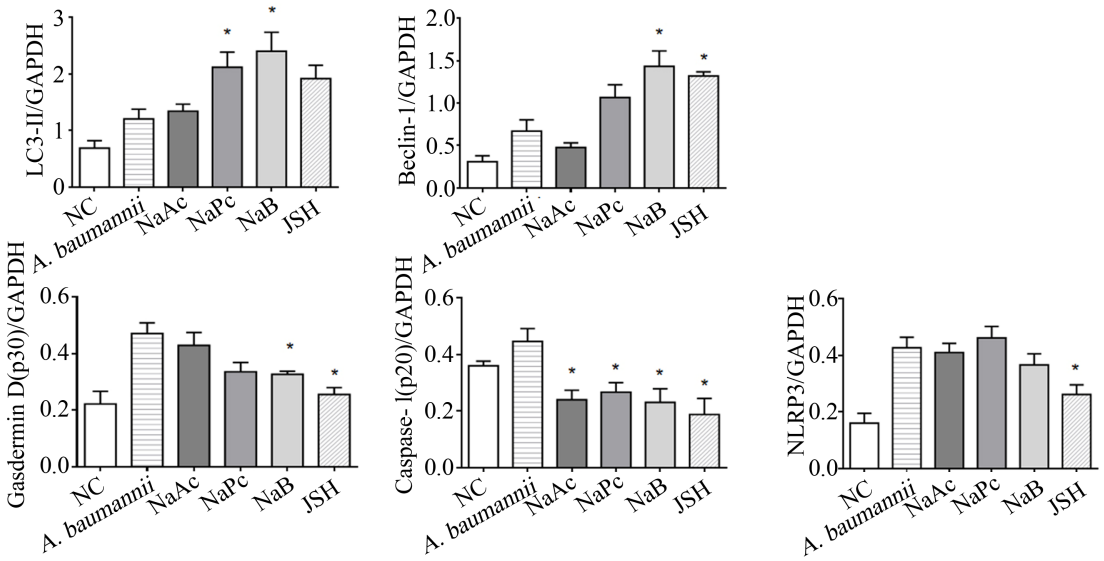


Figure 3. The mRNA expression of inflammasome and autophagy related proteins in A549 cell. Compared with *A. baumannii* group, *P < 0.05.

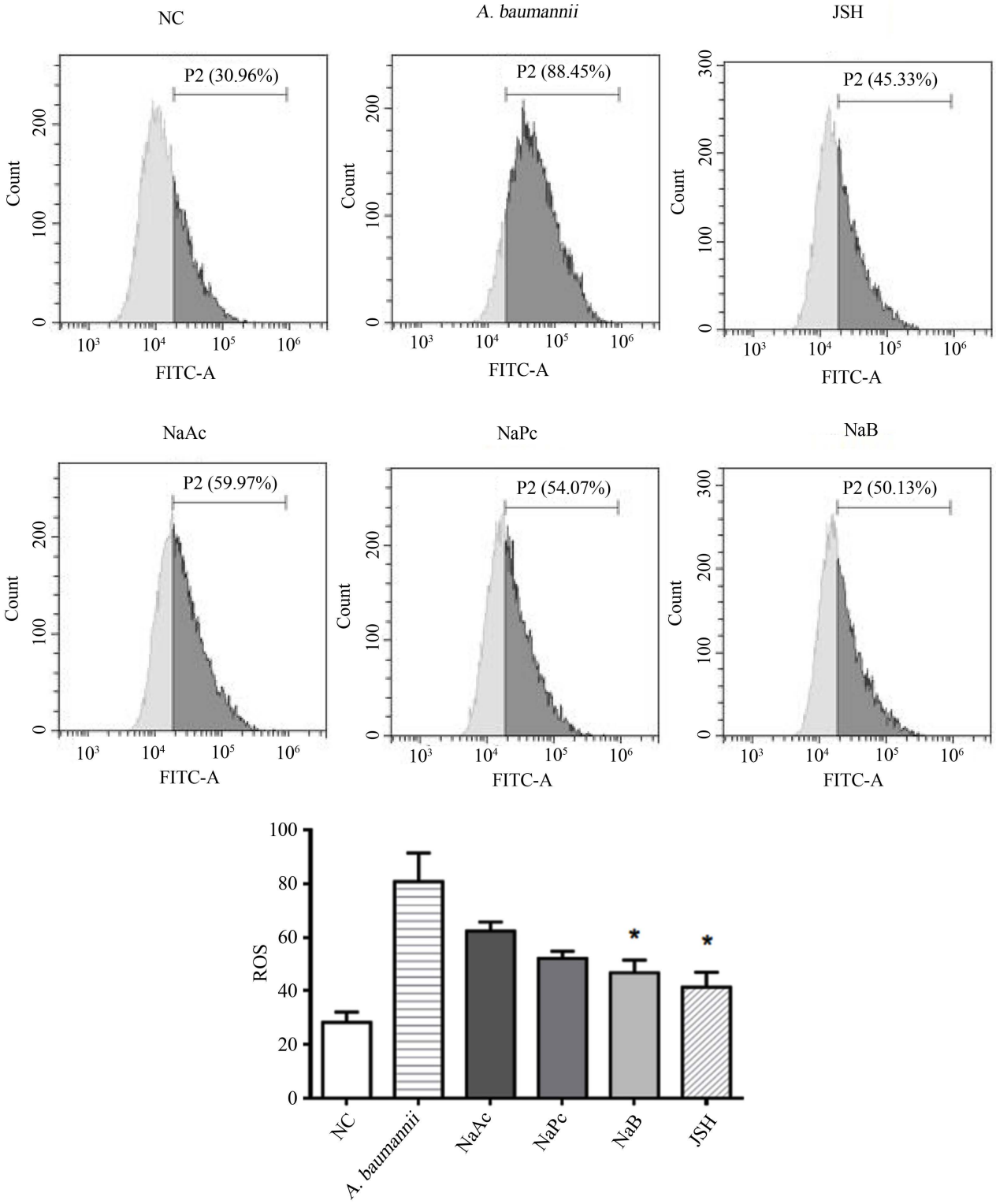


Figure 4. The ROS expression in A549 cell. Compared with *A. baumannii* group, *P < 0.05.

which inhibited NF-κB P65 entry into the nucleus and increased the expression of NF-κB P65 in the cytoplasm (t = 6.05, P = 0.026; t = 4.59, P = 0.044; t = 4.74, P = 0.042) (**Figure 5**).

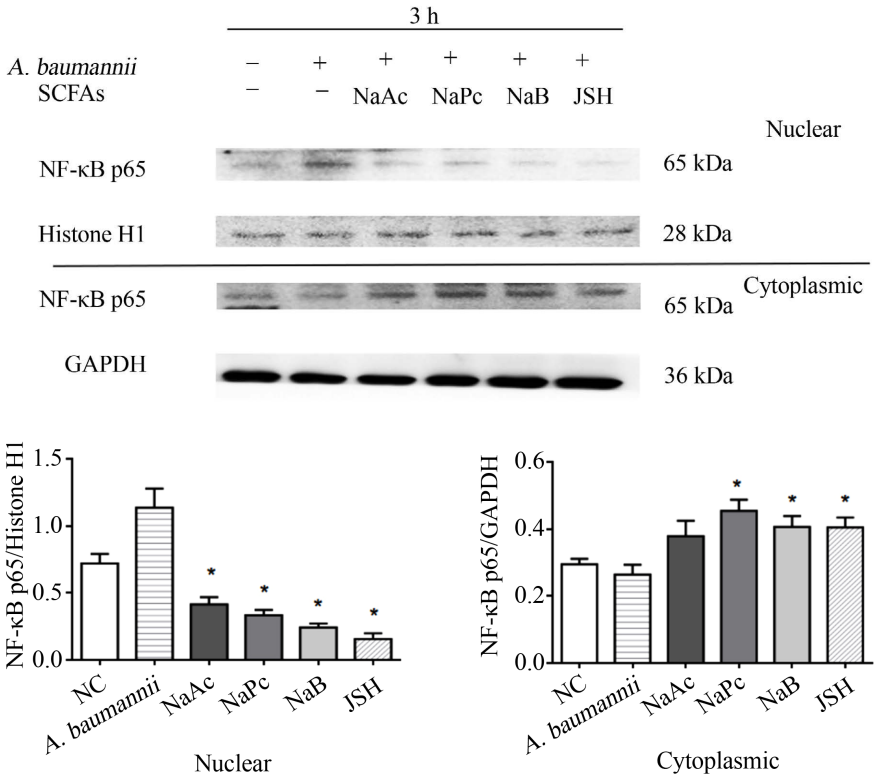


Figure 5. The expression of NF-κB p65 in nucleus and cytoplasm. Compared with *A. baumannii* group, *P < 0.05.

4. Discussion

This study showed that *A. baumannii* infection significantly increased the expression of autophagy-related protein, NLRP3 inflammasome and NF-κB pathway activation, and released a large amount of ROS to induce "pyroptosis" of A549 cells. All three SCFAs inhibited the NLRP3 inflammasome and NF-κB pathway activation of A549 cells, and NaB and NaPc had more inhibitory effects, while NaAc had weaker inhibitory effects. NaB treated A549 cells also significantly decreased ROS, but NaAc and NaPc did not significantly inhibit the generation of ROS. It was found that the production of ROS and the activation of NF-κB pathway are the key to inducing "pyroptosis" and inflammation [4]. We believe that the inhibition of inflammation of epithelial cells by three major SCFAs is closely related to the inhibition of intracellular ROS production and the activation of NF-κB pathway.

Moderate inflammation to protect the body from the inside and outside environment of pathological damage plays an important role, but excessive inflammation is a multiple system disease occurrence and development of pathology foundation [5]. In addition to classical immune cells, such as macrophages, dendritic cells, T cells, B cells, NK cells, mast cells, eosinophils and basophils, various non-immune cells, such as glial cells, endothelial cells, epithelial cells and fibroblasts, are involved in the above reactions [6]. Epithelial cells are cells located on the surface of skin or lumen, which play an important role in maintaining

organ homeostasis and defending against pathogen invasion [7]. However, the mechanism by which pathogens induce secretion of pro-inflammatory cytokines from respiratory epithelial cells and SCFAs from intestinal to inhibit inflammation and maintain mucosal immune microenvironment is still unclear. The mechanism of maintaining the balance between excessive inflammation and immune defense and maintaining the homeostasis of the body's internal environment is extremely complex [8]. Our respiratory mucosa is exposed to hundreds of millions of antigen particles every day, and the epithelial cells are under great pressure. Studies have shown that epithelial-derived TGF-β plays a central role in the pulmonary immune response, and Th2 cells in mice lacking epithelial-derived TGF-β are reduced, leading to reduced IL-13 secretion, presenting airway hypersensitivity, bronchial asthma, pulmonary interstitial fibrosis and acute lung injury, etc. [9]. In this study, it was found that butyrate (NaB) could significantly up-regulate TGF-β expression in A549 cells and effectively prevent airway hypersensitivity, bronchial asthma, pulmonary interstitial fibrosis and acute lung injury.

NLRP3 inflammasome recognizes pathogen-related molecular patterns or ROS and undergoes auto-oligomerization and recruitment of adaptor protein ASC to recruit and activate Caspase-1 precursors to generate mature Caspase-1, and ultimately shear IL-1β precursors and release mature one [10]. In this study, it was found that when A549 cells were infected with *A. baumannii*, NLRP3 inflammasome was greatly expressed, and various pro-inflammatory cytokines and ROS were released, while autophagy was enhanced. The three SCFAs could significantly inhibit the activation of Caspase-1, but had no significant effect on the expression of NLRP3 inflammasome in A549 cells. The results of qPCR showed that the NLRP3 mRNA expression of A549 cells was significantly decreased after the pre-treatment of the three SCFAs. NLRP3 inflammasome can activate Caspase-1, which further activates GSDMD protein, and ultimately induce cell "pyroptosis" [11]. The results showed that NaB significantly inhibited the activation of GSDMD compared with NaAc and NaPc. The "pyroptosis" is a new programmed cell death mode discovered and confirmed in recent years, which is characterized by dependence on inflammatory Caspase-1 accompanied by the release of a large number of pro-inflammatory factors [12]. The morphological characteristics, occurrence and regulation mechanism of "pyroptosis" are different from other cell death modes such as apoptosis and necrosis [13]. The "pyroptosis" mainly relies on NLRP3 inflammasome to activate part of the Caspase family proteins, causing them to cut GSDMD protein and activate GSDMD protein. The activated GSDMD protein is translocated to the cell membrane and forms holes, and cell swelling leads to cytoplasmic outflow, ultimately leading to "pyroptosis". In the process of "pyroptosis", pores are formed on the cell membrane, resulting in a large outflow of various inflammatory factors along with the contents and inducing severe inflammation [14]. We believe that the three SCFAs can inhibit the occurrence of "pyroptosis" and reduce inflammatory injury by inhibiting the activation of NLRP3 inflammasome.

Autophagy is a cellular macromolecular clearance mode that occurs when eukaryotic cells are faced with certain stressors, enabling cells to maintain dynamic balance under various stress conditions [15]. In recent years, it has been found that autophagy can inhibit the activation of NLRP3 inflammasome to reduce inflammation. Our study found that both NaPc and NaB can up-regulate the expression of autophagy-related proteins in A549 cells, and NaB has the most significant up-regulation effect on autophagy-related proteins, but NaAc has no significant effect on the expression of autophagy-related proteins in A549 cells. We believe that NaPc and NaB can also inhibit inflammation by up-regulating the autophagy of A549 cells [16]. In addition, the release of mitochondria-derived ROS can be inhibited by autophagy, and the activation of NF-κB pathway can also up-regulate autophagy, accelerate the clearance of damaged mitochondria, and inhibit the production of NLRP3 inflammasome dependent IL-1β. Our results suggest that NaB inhibits the activation of NF-κB pathway and NLRP3 inflammasome, reduces the release of ROS and the occurrence of "pyroptosis", and ultimately alleviates the inflammatory injury of lung mucosal epithelial cells induced by *A. baumannii* infection.

The imbalance of pro-inflammatory and anti-inflammatory factors is the main manifestation of uncontrolled inflammation, and more and more evidence shows that the amount of SCFAs production in the intestinal tract is closely related to pulmonary inflammation [17]. Our study showed that all three SCFAs inhibited the expression of IL-1β and IL-6 in A549 cells, and NaB significantly up-regulated the expression of TGF-β. Other studies have also shown that NaB can significantly inhibit the Th1 cellular immune response, while the effects of NaAc and NaPc are not obvious, which is consistent with the best clinical effect of NaB in the treatment of inflammatory bowel disease.

In our mind, the concentration of each component in intestinal SCFAs production is greatly affected by intestinal flora and diet, and no one has a fixed proportion. This study avoids this problem and adopts the method of studying each SCFA separately, which can provide a theoretical reference for the functional study of these mixtures. In the next step, we will use *Lactobacillus* to regulate the intestinal flora of experimental mice to increase the production of SCFAs in the intestinal and the SCFAs absorbed into the blood, and study the anti-inflammatory effect of the mixed 3 kinds of SCFAs through animal models to provide direct experimental evidence for the "intestinal-lung axis".

In conclusion, three major SCFAs, especially NaB, can enhance the level of autophagy in lung epithelial cells, inhibit the activation of NLRP3 inflammasome and the occurrence of "pyroptosis" through the NF-κB/ ROS/NLRP3 pathway, and inhibit the release of pro-inflammatory cytokines in *A. baumannii* induced inflammatory injury of lung epithelial cells. Reducing lung mucosal inflammation provides a new idea for the clinical treatment of ALI/ARDS, while the exact mechanism of SCFAs in the treatment of inflammatory diseases remains to be further studied.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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