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Apocynin alleviates lung injury by suppressing NLRP3 inflammasome activation and NF-κB signaling in acute pancreatitis

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ABSTRACT

Mounting evidence has demonstrated that acute pancreatitis (AP) is one of the causes of multiple organ damage. NADPH (nicotinamide adenine dinucleotide phosphate) act as a substrate of NADPH oxidase (NOX) to generate reactive oxygen species (ROS), but the role NADPH oxidase signaling pathway plays in AP-induced acute lung injury remains unclear. Apocynin, an inhibitor of NOX, is highly effective in suppressing the production of ROS. Here, we used rat model of severe acute pancreatitis (SAP) to explore whether the NOX inhibitor apocynin produced protective effects in against SAP-induced lung injury via inhibition of inflammation and oxidation. We observed that apocynin significantly attenuated severe acute pancreatitis-induced increase of NOX2, NOX4 and ROS expressions in lung tissues. In addition, the phosphorylation and degradation of IκBα, and the nuclear localization of NF-κB p65 in SAP-induced lung injury were also inhibited after using apocynin. Simultaneously, down-regulation of NOX suppressed the levels of inflammasome proteins including NLRP3, ASC, pro-Caspase-1 and cleaved-Caspase-1 in the lung. Serum levels of TNF-α, interleukin (IL)-1β and IL-6 were also reduced. Our findings suggest that beyond anti-oxidative effects, apocynin may also have anti-inflammatory effects by suppressing NLRP3 inflammasome activation and NF-κB signaling in acute pancreatitis. Therefore, apocynin may have therapeutic potential in the treatment of SAP and SAP-induced lung injury.

1. Introduction

Acute pancreatitis (AP), a multifactorial disease, causing complex inflammatory reaction, is followed by multiple organ damage [1]. Acute respiratory distress syndrome (ARDS), which is induced by acute lung injury (ALI), appears to be a predominant cause of death in AP. Besides, ALI is common in critically ill patients due to various conditions including SAP [2]. The production of inflammatory mediators, including complement activation products, cytokines and chemokines, as well as ROS, are fundamental causes of SAP-induced ALI. These mediators contribute to macrophage and neutrophil accumulation in the lung, and subsequently trigger a cascade of pathological events. During these pathophysiological events, vascular endothelial and alveolar epithelial cells are compromised by oxidative stress, leading to disruption of the blood-alveolar barrier, resulting in severely impaired gas exchange, pulmonary edema, and even intrapulmonary hemorrhage [3]. Presently, the detrimental effects of oxidative stress and excessive inflammatory cascade reaction in the pathogenesis of ALI associated with SAP have been extensively studied [4,5]. However, the pathogenesis of SAP-induced ALI is complicated, and its complex pathological mechanism has not been fully elucidated.

Numerous studies have revealed that the increase in reactive oxygen species (ROS) production contributes to the development of acute respiratory distress syndrome [6,7]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is considered the main source of reactive oxygen species (ROS) in ALI [8]. The Nox family is a multicomponent enzyme, comprised of seven members including Nox1-5 and Duox 1 and 2. Among them, Nox2 and Nox4 are highly expressed in professional phagocytes to produce a large amount of ROS in lung tissue [9]. Apocynin, the most widely NOX inhibitor in experiment, is well-known for its anti-oxidant and anti-inflammatory capability. Our previous studies found that apocynin could reduce intracellular oxidative stress and protect SAP-associated intestinal mucosal barrier injury [10]. However, whether Nox is involved in SAP-associated acute lung injury remains to be investigated.

Recent studies demonstrated that the inflammatory cascade induced

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by sterile inflammasome activation is a major component in a wide range of disease, including AP and ALI [11–13]. NLRP3 inflammasome, one of the most well-characterized inflammasomes, is an oligomeric molecular complex which can be activated by various "danger signals" (e.g. ATP and ROS) [14]. Several studies have revealed that inflammation is mediated by the intracellular NLRP3 (one member of NOD-like receptor family) which form an intracellular multiprotein complex with apoptosis-associated speck-like protein containing a CARD (ASC) and active caspase-1, to provide a platform for regulating secretion of mature IL-1β and IL-18 [15,16]. IL-1β is closely related to SAP, and its increase is positively correlated with the severity of the disease [17]. The pathological damage of the pancreas, the degree of inflammation, and the severity of SAP-ALI have been shown to be significantly reduced after blocking IL-1β expression in SAP [18]. In addition, NLRP3 inflammasome can be activated by many factors, including environmental irritants, endogenous danger signals, pathogens, and various pathogen-associated molecular patterns (PAMPs) [19]. ROS have been identified as an important NLRP3 inflammasome activator in settings of various diseases, such as hepatic ischemia/reperfusion injury [20]. Thus, it is of great interest to investigate if the potential detrimental effects of NLRP3 inflammasome in SAP-induced ALI can be prevented by the NOXs inhibitor apocynin.

ALI/ARDS, a clinical syndrome with few therapies and high mortality, has already been proven to be closely connected to oxidative stress and inflammation [21]. Similar results have been demonstrated that the oxidative stress participated in inflammatory reaction of ALI/ARDS, and then strengthened inflammation by the activation of pro-inflammatory pathways, such as the NLRP3 inflammasome and nuclear factor-kappa B (NF-κB) signaling [22]. NF-κB, a ubiquitous inducible transcription factor, is a robust regulator of various pro-inflammatory cytokine gene expressions in response to external stimuli. Several important pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α were also activated by the NF-κB signaling pathway [23,24]. Intriguingly, as a classical pro-inflammatory pathway, NF-κB signaling pathway also provides original insights due to its key role in initiating NLRP3 activation [25]. Studies have demonstrated that pharmacologic or genetic inhibition of NF-κB alleviated NLRP3-dependent inflammation in preclinical animal models and humans [26,27].

Previous studies have demonstrated that ROS may act as a "kindling factor" to activate NLRP3 inflammasomes and act as "bonfire" or "effector" molecules, resulting in pathological processes [28]. Among the cellular response mechanisms, ROS is considered a damage factor that promotes oxidative injury, and both the NLRP3 and NF-κB pathways are pro-inflammatory pathways that cause cellular damage [22]. These pathways are both involved in the process of ALI. Our previous studies demonstrated that apocynin is involved in the regulation of NF-κB signaling pathway in sepsis-induced acute pancreatic and intestinal injury [10]. However, there is no evidence elucidating how apocynin interacts with the NF-κB/NLRP3 pathways and how apocynin exerts protective effect to against ALI associated with SAP. Therefore, we hypothesized that apocynin can attenuate lung injury and inflammation by inhibiting NLRP3 inflammasome activation and regulating NF-κB signaling pathway in SAP model induced by sodium taurocholate (STC) treatment.

2. Materials and methods

2.1. Rat model of severe acute pancreatitis

Animal protocols were approved by the Committee on Ethics of Animal Experiments of Wuhan University (No. WDRM-20171019), and in accordance with the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male SPF Wistar rats (7–8 weeks) were subjected to SAP using retrograde infusion of 5% sodium taurocholate (STC; Sigma-Aldrich, St. Louis, USA) into the biliary pancreatic duct as described previously [29]. Given libitum access to fresh water, the rats were fasted overnight. Anesthesia was induced with 4% isoflurane in 2 L/min oxygen in a sealed container, and maintained with 2% isoflurane in 2 L/min oxygen during surgery. Freshly prepared 5% STC solution (50 mg/kg) was retrogradely infused into biliary-pancreatic duct through the duodenum, and then isotonic saline solution (20 mL/kg) was injected into the back subcutaneously to compensate for fluid loss. All rats were obtained from the Hunan SJA Laboratory Animal Co. (Changsha, China), and maintained at room temperature under a 12 h light-dark cycle, with free access to standard laboratory rodent chow and sterile water.

2.2. Drug administration and experimental groups

Apocynin (Santa Cruz Biotechnology, Cat: sc-203321A) was dissolved in 10% dimethyl sulfoxide (DMSO) and then diluted in sterile saline. 30 min before the model of SAP, apocynin was injected (50 mg/kg, i.v.) and the optimal dose was selected based on previous studies [10]. A total of 24 male rats were randomly assigned into three groups (n = 8 per group): The sham operation (SO) group, the SAP group, and the SAP plus apocynin treatment (APO) group. In the SO group, rats received a sham surgery during which the pancreas and duodenum were flipped a number of times instead of infused STC. In the APO group, rats receiving 10% dimethyl sulfoxide (DMSO) containing apocynin were injected very slowly through the femoral vein 30 mins prior to SAP induction. In the SO and SAP group, rats received a volume-matched 10% DMSO substituted for apocynin.

2.3. Serum assay

Rats from each group were euthanized 12 h after treatment. All blood samples were collected through the posterior vena cava. After centrifuging at 1500*g* × 10 min, the supernatants were collected and stored at −80 °C. Serum amylase (AMY) and lipase (LIPA) levels were measured using an automatic biochemistry analyzer with standard techniques (ADVIA 2400 Clinical Chemistry System; Siemens Healthcare Diagnostics Inc. New York, USA). Serum levels of tumor necrosis factor (TNF)-α (Ebioscience Inc. cat: #BMS622), interleukin (IL)-1β (Ebioscience Inc. cat: #BMS630) and IL-6 (Ebioscience Inc. cat: #BMS625) were quantified using specific ELISA kits, according to the manufacturer's protocols. All samples were run in duplicate three times.

2.4. Lung and pancreas wet/dry (W/D) weight ratio

After undergoing severe acute pancreatitis, lungs were obtained from rats under anesthesia. The blood stains on the surface of the lungs were cleaned, then the wet weight of lung was measured. After heated at 70 °C for 48 h, the dry weight of lung was obtained [30]. A portion of pancreatic tissue was analyzed for pancreatic water content by measuring the ratio of initial wet weight to final dry weight (70 °C for 48 h) of the pancreas. The W/D weight ratio = wet weight/dry weight.

2.5. Evaluation intracellular reactive oxygen species (ROS) formation

ROS was assessed by dihydroethidium (DHE) staining. The fresh lung tissue frozen sections were rewarmed, and the liquid was flown away around the tissue. The DHE (Sigma-Aldrich Inc. cat: D7008) diluted with PBS was incubated at 37 °C for 30 min in the dark. The slides were placed in PBS (pH 7.4) and washed 3 times in the dark on a decolorizing shaker for 5 min each time. The sections were dried slightly and then sealed with an anti-fluorescence quenching capsule. Sections were observed under a fluorescence microscope (Olympus Optical Ltd., Tokyo, Japan) and images were acquired. The generation of ROS in each group were normalized to the sham operation group, in which the level of ROS is represented as 100%.

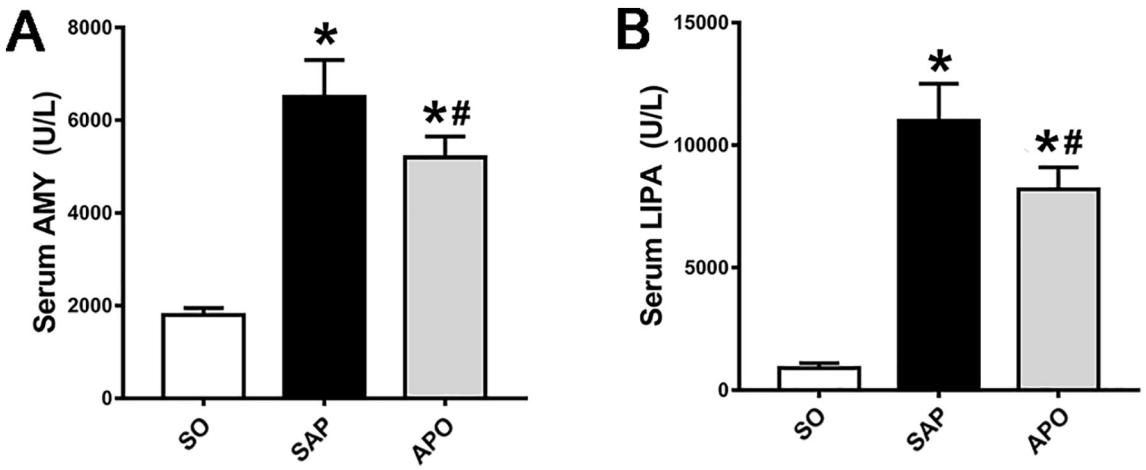

Fig. 1. Apocynin treatment attenuates serum AMY and LIPA. Serum AMY (A) and LIPA (B) levels of each group. The values were presented as mean ± SEM of three independent experiments. $^{*}P < 0.05$ vs. sham operation, $^{\#}P < 0.05$ vs. severe acute pancreatitis group.

2.6. Histopathological examination

Paraffin-embedded samples from the pancreas and lung following SAP, APO and sham treatment were fixed in 4% phosphate-buffered formaldehyde, dehydrated in the concentration series of alcohol at room temperature, and infiltrated in melted paraffin. After stained with hematoxylin and eosin (H&E) for 10 min at room temperature, the samples were made. All tissue sections were examined under optical microscope (Olympus Optical Ltd., Tokyo, Japan). Histopathological alterations of the pancreas were evaluated according to the criteria proposed by Schmidt et al [31]. Evaluation of pulmonary injury was assessed for interstitial and intra-alveolar edema, leukocyte infiltration, and hemorrhage, as described by Osman et al [32].

2.7. Immunofluorescent staining

Paraffin-embedded sections were incubated at 60 °C for 1 h, deparaffinized with xylene twice, then subjected to decreased concentration of ethanol for rehydration. After boiled 10 min in sodium citrate buffer (pH 6.0), the samples experienced antigen retrieval. Paraffin-embedded sections were incubated for 1 h with blocking solution (0.1 M PBS, 0.2% Triton X-100 and 5% fetal bovine serum), then overnight at 4 °C with anti-MPO antibody(1:500, Cat: GB11224; Servicebio), anti-IL-1β antibody (#50350T, 1:200, Biolegend), anti-NLRP3 antibody (#ab4207, 1:200, Abcam), anti-Caspase-1 antibody (#67314, 1:100, CST), and anti-p-NF-κB p65 antibody (#3033S, 1:200, CST) in a humidity box. After rinsed with PBS 3 times, incubated 1 h at room temperature with Alexa Fluor 647–conjugated Goat Anti-Rabbit (#ab150083, 1:300, Abcam), then counterstained the Nuclei with 4,6-diamidino-2-phenylindole (DAPI, #ab104139, Abcam). To evaluate the level of immunologic infiltration, the numbers of MPO-positive cells were counted and the fluorescent intensities of NLRP3 inflammasome, Caspase-1, IL-1β and p-p65 were measured using ImageJ (National Institutes of Health, Bethesda, MD, USA). The numbers of positive cells and the fluorescent intensities were quantified under high-power field, six different fields for each rat and eight rats for each group.

2.8. Western blotting

Total protein extraction kit (Beyotime Bio-technology) was used to extract total proteins in different group, and protein concentrations were determined using BCA kit. 20-μg protein samples were electrophoresed on 10% SDS polyacrylamide gels and then polyvinylidene fluoride (PVDF) membrane. After incubated with blocking solution (0.1 M TBS,0.1% Tween-2, 0.5% skim milk) at room temperature for 1 h, the anti-NOX2 (#ab80508, 1:700, Abcam), anti-NOX4 (#ab133303, 1:500, Abcam), anti-NLRP3 (#ab4207, 1:500, Abcam), anti-ASC (#8242S, 1:500, CST), anti-cleaved-Caspase-1 (#67314, 1:200, CST), anti-pro-Caspase-1 (#ab207802, 1:1000, Abcam), anti-p-IκB-α (#ab133462, 1:500, Abcam), anti-PCNA (#1130S, 1:800, CST), anti-p-NFκB-p65 (#3033S, 1:200, CST) and anti-β-actin (#ab52614, 1:1000, Abcam) were added, and incubated for 12 h at 4 °C. The membranes were rinsed with TBST for 3 times, followed by 1.5 h incubation with corresponding secondary antibody (#926-32211, 1:5000, LI-COR) at room temperature. The membranes were scanned using an LI-COR-Odyssey infrared scanner and Odyssey 3.0 analytical software (LiCor).

2.9. Statistical analysis

All statistical tests were performed using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). Data is presented as the mean ± standard deviation for continuous variables. Differences between groups were compared by one-way-analysis of variance (ANOVA). $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Apocynin reduced the severity of SAP-induced pancreas and lung injury

Compared with SO group, the levels of serum AMY and LIPA were significantly increased at 12 h in SAP group (Fig. 1), indicating that the SAP model was successfully generated. To study the protective mechanism of apocynin, the rats were treated with apocynin 30 min before SAP modeling. The levels of serum AMY and LIPA were markedly attenuated as compared with those in the SAP group, the difference was significant ($P < 0.05$). Then, we analyzed histopathological changes in pancreatic and pulmonary tissues. The results showed that SAP group rats were more vulnerable to inflammatory injury in the pancreas and lung than SO group rats. As shown in (Fig. 2), the severity of pancreas and lung injury decreased in APO rats compared with SAP rats ($P < 0.05$). The scores of pancreas and lung were lower in APO rats than SAP rats ($P < 0.05$).

3.2. Apocynin attenuated lung and pancreas edema in SAP rats

The wet/dry weight ratio was widely used for detecting and measuring the degree of lung and pancreas edema. To determine whether SAP led to lung injury, we compared the wet/dry weight ratio of lungs

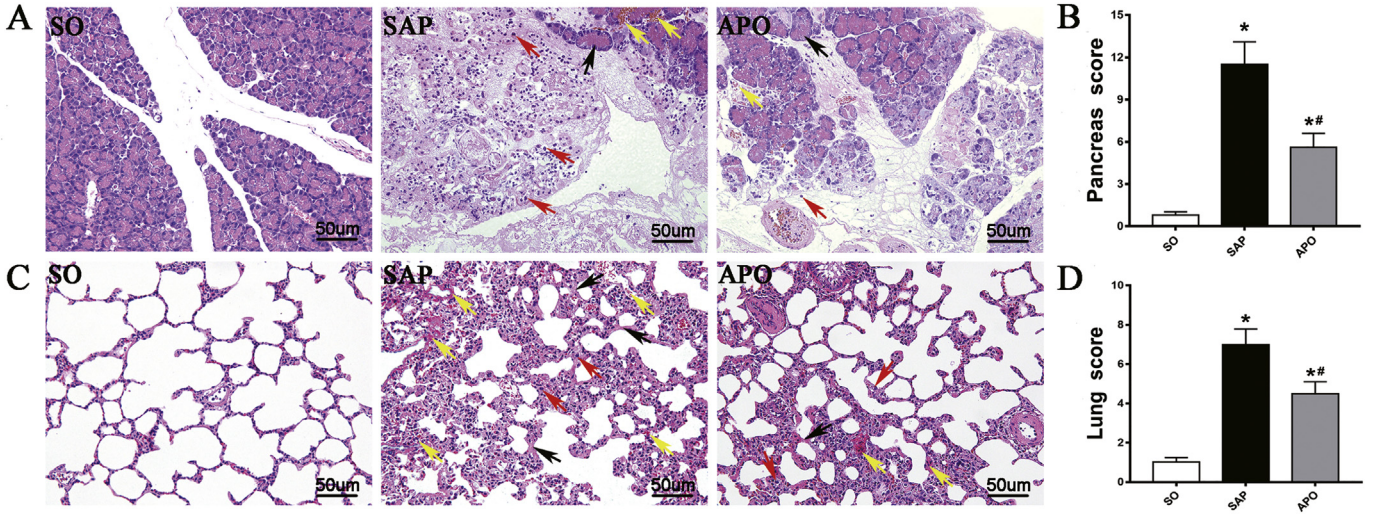

Fig. 2. Effects of Apocynin on pancreas and lung histopathological changes. Representative histopathological changes of pancreas and lung tissues were obtained from rat of different groups. A: Pancreas, edema (black arrow), massive areas of acinar necrosis, inflammatory cell infiltration (red arrow), and intrapancreatic hemorrhage (yellow arrow) in pancreas were observed in SAP and APO group. B: Pancreas score, C: Lung, interstitial and intra-alveolar edema (black arrow), inflammatory cell infiltration (red arrow), and hemorrhage (yellow arrow) were detected. D: Lung score (Hematoxylin and eosin staining, magnification 200×). $^{*}P < 0.05$ vs. sham operation, $^{\#}P < 0.05$ vs. severe acute pancreatitis group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

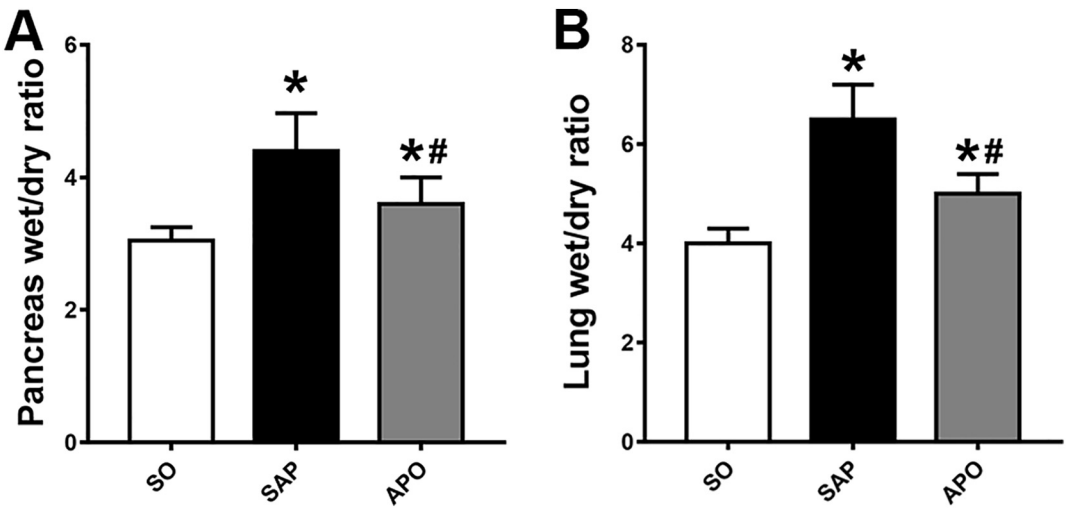

Fig. 3. Effects of Apocynin on the lung and pancreas W/D ratio. A: Pancreas W/D ratio, B: Lung W/D ratio. The values were presented as mean ± SEM of three independent experiments. $^{*}P < 0.05$ vs. sham operation, $^{\#}P < 0.05$ vs. severe acute pancreatitis group.

between SO group and SAP group (Fig. 3). The results indicated that SAP group showed higher wet/dry weight ratio of lung than SO group ($P < 0.05$). However, the wet/dry weight ratio of lung was significantly reduced by treatment with apocynin ($P < 0.05$). In addition, the pancreas edema showed the same trend as lung edema.

3.3. Apocynin decreased the production of proinflammatory cytokines

Serum concentrations of proinflammatory cytokines were analyzed in order to evaluate the effects of NOX inhibition on inflammatory process following SAP. As presented in Fig. 1, concomitant with the pathological damage incited by SAP, serum levels of TNF-α, IL-1β and IL-6 were significantly increased compared with those in the SO group ($P < 0.05$; Fig. 4). However, the increased levels of these cytokines following SAP induction were markedly reversed by the pharmacological blockade of NOX, using the NOX inhibitor apocynin ($P < 0.05$; Fig. 4).

3.4. Apocynin suppressed the expressions of NOX2, NOX4 and production of ROS in lung of SAP rats

Exact evidence has suggested that intracellular NADPH oxidase is the main source of ROS. Western blotting of NOX2 and NOX4 were used to evaluate whether apocynin can successfully attenuate SAP-induced NOX2 and NOX4 expressions in lung (Fig. 5, A–C). We respectively examined the effect of NADPH oxidases by detecting NOX2 and NOX4 protein levels in SO, SAP and APO rats. In SO rats' lung tissue, the expressions of NOX2 and NOX4 were exhibited little by Western blotting (Fig. 5, D and E). However, NOX2 and NOX4 protein levels were increased in SAP rats and decreased in the lung of rats that had undergone apocynin pretreatment. We also detected the production of reactive oxygen species (ROS), similarly, apocynin can decrease the ROS level in rats suffering from severe acute pancreatitis ($P < 0.05$).

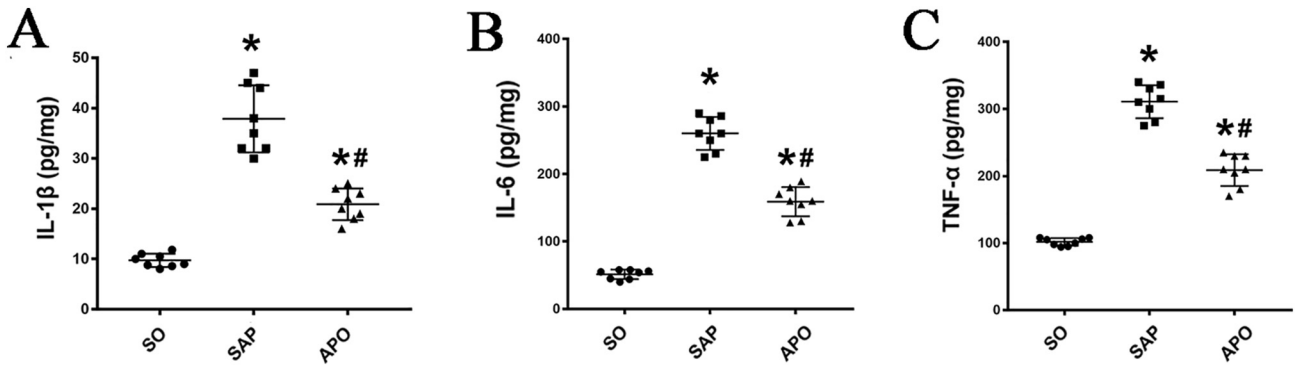

Fig. 4. Apocynin treatment attenuates serum IL-1β, IL-6 and TNF-α. The values were presented as mean ± SEM of three independent experiments. A: IL-1β, B: IL-6, C: TNF-α. $^{*}P < 0.05$ vs. sham operation, $^{\#}P < 0.05$ vs. severe acute pancreatitis group.

3.5. Apocynin decreased lung neutrophil infiltration and IL-1β expression in SAP model

We examined lung neutrophil infiltration and IL-1β expression in all rats (Fig. 6). Pulmonary neutrophils were detected using immunofluorescence staining with anti-MPO antibodies. Compared with SO rats, the lung neutrophil infiltrations were increased in SAP rats ($P < 0.05$), while apocynin can decrease the lung neutrophil infiltrations following SAP ($P < 0.05$). Similarly, the results showed that SAP can enhance the expression of IL-1β ($P < 0.05$). APO rats showed less IL-1β fluorescent intensity in the lung compared to SAP rats ($P < 0.05$).

3.6. Apocynin decreased the NLRP3 inflammasome and NF-kB signaling activation in SAP model

Accumulated evidence has revealed that the NLRP3 inflammasome and NF-κB pathway are essential for the development of ALI (Fig. 7). Compared with SO rats, the levels of NLRP3 inflammasome activation and Caspase-1 expression in cytoplasm of lung were increased in SAP rats ($P < 0.05$). Meanwhile, the group treated with apocynin had lower levels of these proteins ($P < 0.05$). Similarly, APO rats showed decreased fluorescent intensity of p-p65 compared to SAP rats ($P < 0.05$).

To further study the correlation between NLRP3 inflammasome and NF-κB signaling activation in SAP rats, we examined the NLRP3 inflammasome and NF-κB signaling activation in the lung tissue by Western blotting analysis (Fig. 8). The results showed that the association of the NLRP3 complex, pro-Caspase-1 and cleaved-Caspase-1 in the lung substantially increased after the administration of STC ($P < 0.05$). Increased activation of the NLRP3 inflammasome was observed in SAP and APO rats, suggesting that the NLRP3 inflammasome was activated in the SAP model ($P < 0.05$). Phosphorylation of IκB-α is required for the initiation of NF-κB signaling activation. Hence, the levels of phosphorylated IκB-α (p-IκB-α) and p-p65 in the lung were examined. The levels of p-IκB-α and p-p65 in SAP rats significantly increased 12 h after STC treatment. Importantly, the levels of p-IκB-α and p-p65 in the lung from APO rats were lower compared to SAP rats. These results indicated that apocynin can inhibit the NLRP3 inflammasome and NF-κB signaling activation and plays a vital role in

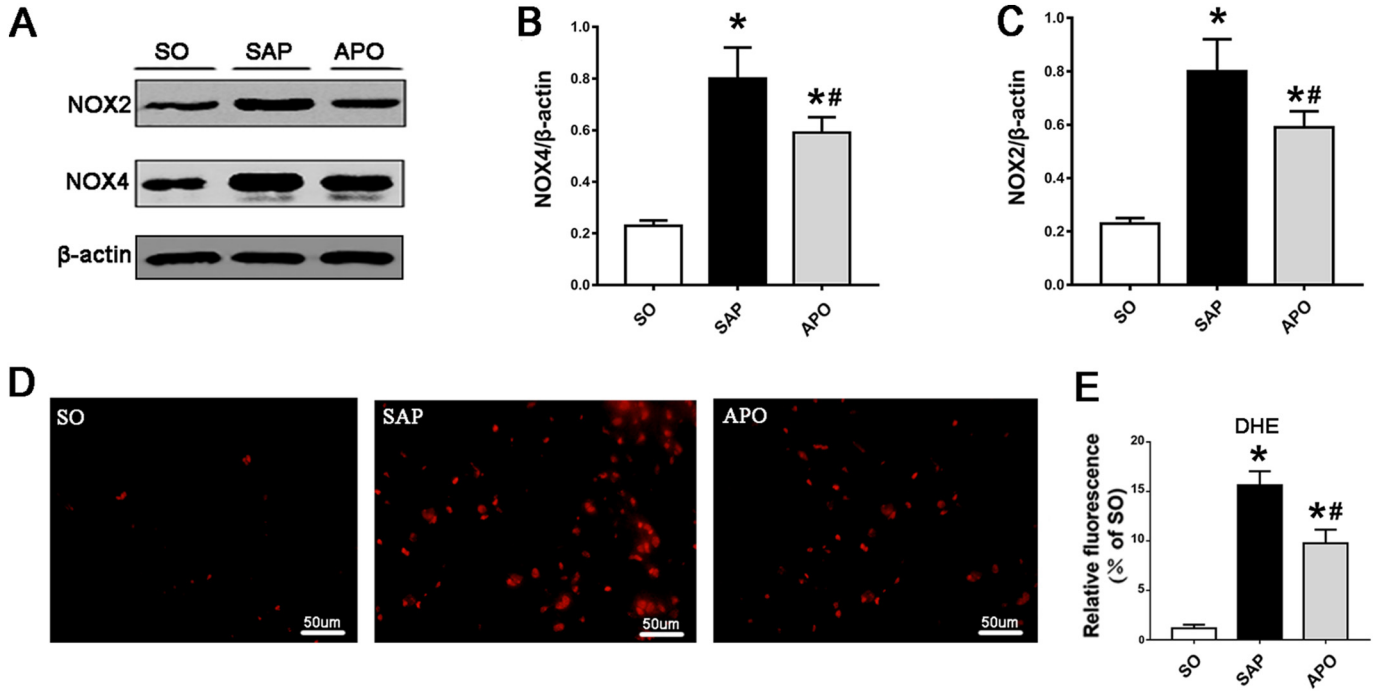

Fig. 5. Apocynin treatment reduces NADPH oxidases and reactive oxygen species (ROS) levels in the lung of rat. A: NADPH oxidases were detected by western blotting with NOX2 and NOX4 antibody in the lung, B: Quantitative analysis of NOX4, C: Quantitative analysis of NOX2, D: Reactive oxygen species staining, E: Quantitative analysis of ROS. (Immunofluorescence staining, magnification 200×). $^{*}P < 0.05$ vs. sham operation, $^{\#}P < 0.05$ vs. severe acute pancreatitis group.

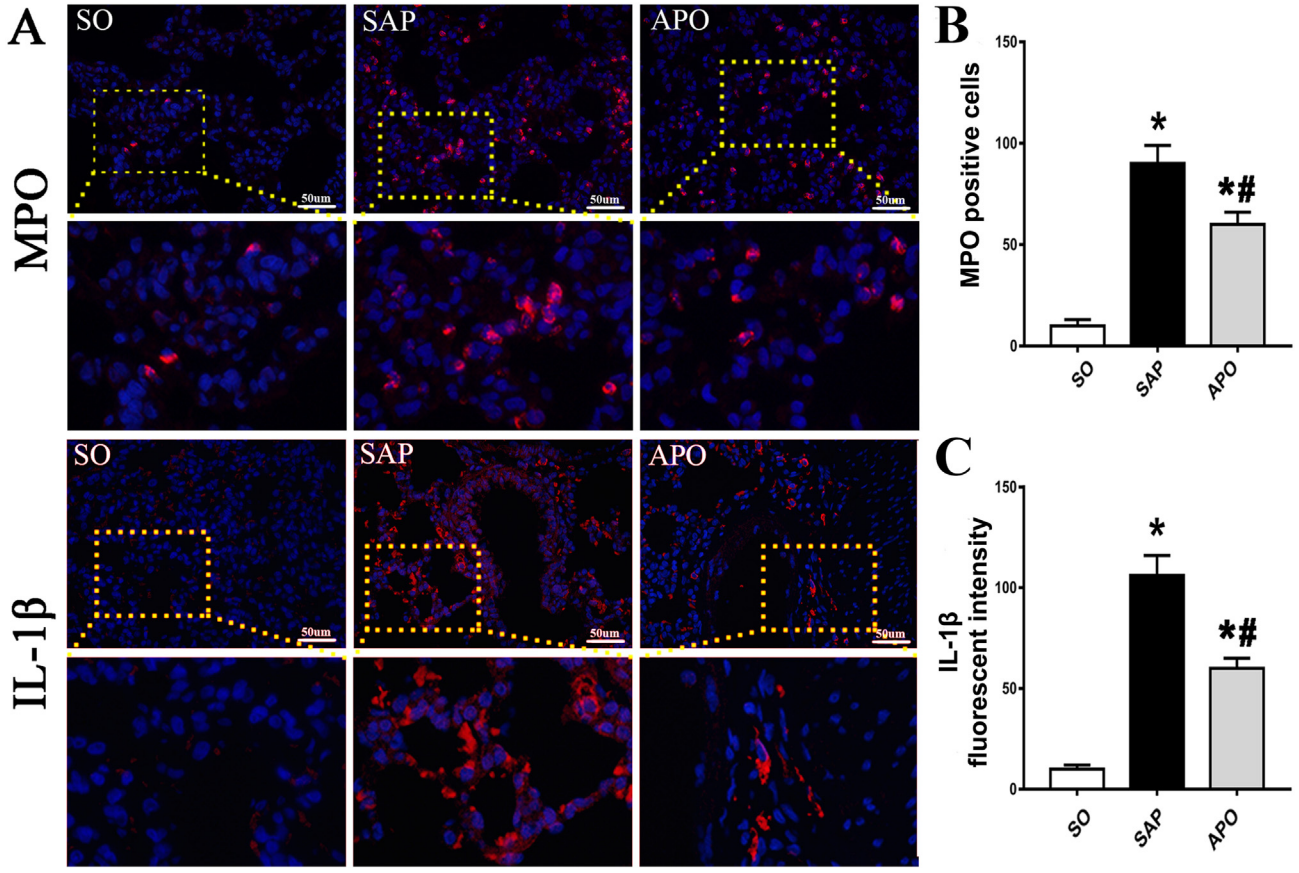

Fig. 6. Apocynin treatment prevents inflammatory cells infiltration and IL-1β in lung tissues. A: Representative immunofluorescence staining for MPO and IL-1β in the lung tissues. B: Quantitative analysis of MPO^+ cells, C: Quantitative analysis of IL-1β fluorescent intensity. (Immunofluorescence staining, magnification 200×). $^*P < 0.05$ vs. sham operation, $^{\#}P < 0.05$ vs. severe acute pancreatitis group.

SAP-induced ALI.

4. Discussion

Oxidative stress, interconnected with a myriad of pathogenesis, can elevate intracellular levels of reactive oxygen species (ROS) and represents a perturbed redox equilibrium [33,34]. SAP, accompanied by a subsequent infectious attack, often leads to systemic inflammation and multiple organ failure. Acute lung injury (ALI) is the commonest distant organ complication with high rates of morbidity and mortality in SAP. The molecular mechanisms of SAP-induced ALI are rather complicated and remain poorly understood, therapeutic remedies targeting the inflammatory modulators and anti-oxidative pathways appear to be the most appropriate approaches for treating AP and AP-induced ALI [35]. In this study, we established a rat model of SAP using STC, and we assessed the extent of SAP-induced ALI. As expected, our results showed that the expression of NOX2, NOX4 and production of ROS were significantly increased, and inflammatory reaction was exacerbated in lung tissues. Our data from the current study showed that the inhibitor of NADPH oxidase, apocynin, exerted anti-inflammatory and anti-oxidative effect by reducing ROS production and inhibiting NLRP3 inflammasome activation, then reduced the severity of SAP-induced pancreas and lung injury. Therefore, these findings provided new insights into the pathogenic mechanism of SAP-induced ALI, which may offer a potential therapeutic target and a preventive strategy for this complication.

NOX enzymes contribute to a wide range of pathological processes, such as post translational processing of proteins, regulating of gene expression and cell differentiation in SAP [36]. Additionally, NOX enzymes are major sources of reactive oxygen species (ROS) in SAP. It is closely related to various diseases, including ALI/ARDS, a kind of syndrome renowned for diffuse inflammation as well as respiratory failure [21]. Apocynin, also called 4-hydroxyl-3-methoxyacetophenone or acetovanillone, was originally extracted from the roots of *Apocynum cannabinum* L. (Apocynacaeae) and was also discovered in Picrorhiza kurroa Royle ex Benth. (Scrophulariaceae). Unlike other traditional anti-inflammatory drugs, such as dexamethasone, one of the glucocorticoids, which is immunosuppressive, anti-inflammatory and anti-allergic by reducing the expression of cytokines, chemokines, adhesion molecules and other inflammatory proteins. Apocynin is a specific inhibitor of NADPH oxidase whose function mechanism lies in the specific inhibition of NOX. Our previous study, apocynin exerts protective effects on AP-induced intestinal injury [10]. Therefore, it is of great interest to explore whether the NOX inhibitor apocynin offers the same protective effects on SAP-induced lung injury. Here, we presented that the levels of serum AMY and LIPA, the histopathological changes of pancreatic and pulmonary tissues and the degree of lung edema were alleviated by apocynin treatment during SAP. In addition, the expressions of NOX2, NOX4 and production of ROS in lung of SAP rats were also suppressed by apocynin treatment.

Inflammation is a main contributor to the pathogenesis of AP-induced lung injury. Neutrophil activation is detected as a result of cytokine production, complement activation, adhesion molecule expression, as well as alveolar macrophage activation [37]. Activated neutrophils in the lung produce proteolytic enzymes and reactive oxygen species (ROS) that result in the development of ALI [38]. In the present study, we examined the infiltration of neutrophil, IL-1β expression in lung by immunofluorescence staining and measured the serum levels of TNF-α, IL-1β and IL-6 to assess inflammasome activity. We found that SAP initiated lung inflammatory response, which was

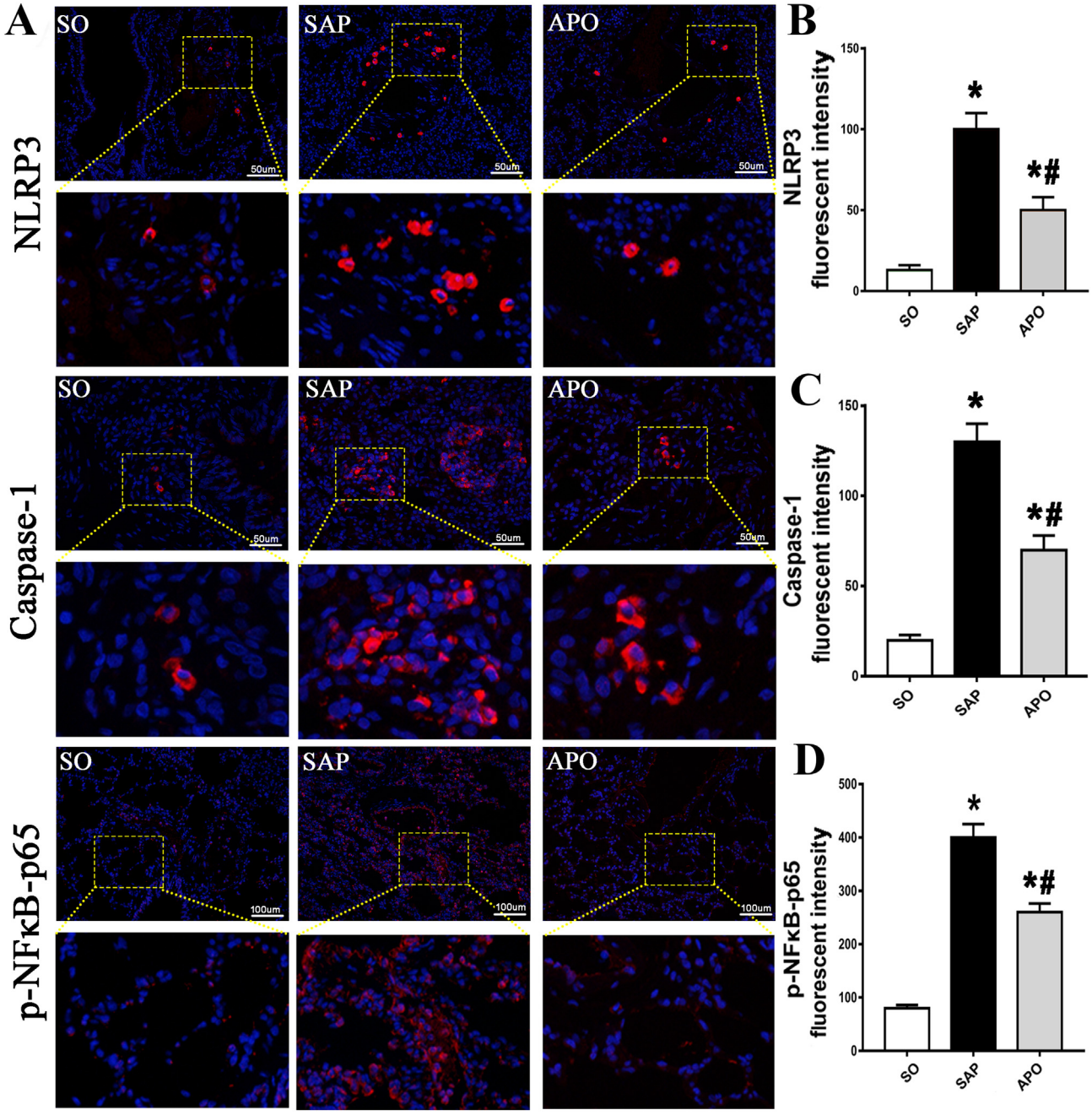

Fig. 7. Effects of Apocynin on NLRP3 inflammasome, Caspase-1 and p-NF-κB-p65 expression in the lung. A: Representative immunofluorescence staining for NLRP3 inflammasomes, Caspase-1 and p-NF-κB-p65 in the lung tissues. B: Quantitative analysis of NLRP3 fluorescent intensity, C: Quantitative analysis of Caspase-1 fluorescent intensity, D: Quantitative analysis of p-NF-κB-p65 fluorescent intensity. (Immunofluorescence staining, magnification 200×). $^{*}P < 0.05$ vs. sham operation, $^{\#}P < 0.05$ vs. severe acute pancreatitis group.

inhibited by apocynin treatment. Additionally, recent studies pointed out that a macromolecular complex, named NLRP3 inflammasome, could exert regulatory role on the secretion of pro-inflammatory cytokines IL-1β and IL-18, thus playing a vital role in acute pancreatitis [39,40]. NLRP3 forms an inflammasome complex and responds to a wide range of infections and stress stimuli [41]. Once NLRP3 becomes activated, there will be the activation of caspase-1, recruitment of ASC, and processing of pro-IL-1β into mature forms [14].. Besides, accumulating evidence has showed that as a key inducer of inflammation through NF-κB, NLRP3 inflammasome complex was mainly activated by ROS overproduction [28,42]. In this present study, immunofluorescence and immunoblotting exhibited that the expression levels of NLRP3 inflammasome proteins and IL-1β showed increment 12 h after acute pancreatitis. Apocynin remarkably inhibited acute pancreatitis-induced expression of NLRP3 inflammasome-associated proteins, including NLRP3, pro-Caspase-1 and IL-1β leading to a corresponding decrease of the pro-inflammatory cytokines in lung.

NF-κB is a vital transcription factor acting as a trans-activator of genes related to inflammation, playing a pivotal role in controlling the inflammatory cascade [43]. Our previous research demonstrated that acute pancreatitis can induce the degradation of IκBα protein by the phosphorylation of IκBα. Acute pancreatitis also lead to the phosphorylation of NF-κB p65 and the subsequent upregulation of NF-κB p65. Therefore, NF-κB translocated into the nucleus, where it plays its regulatory role in gene expression related to inflammation. Our previous studies also demonstrated that blocking NF-κB signaling pathway

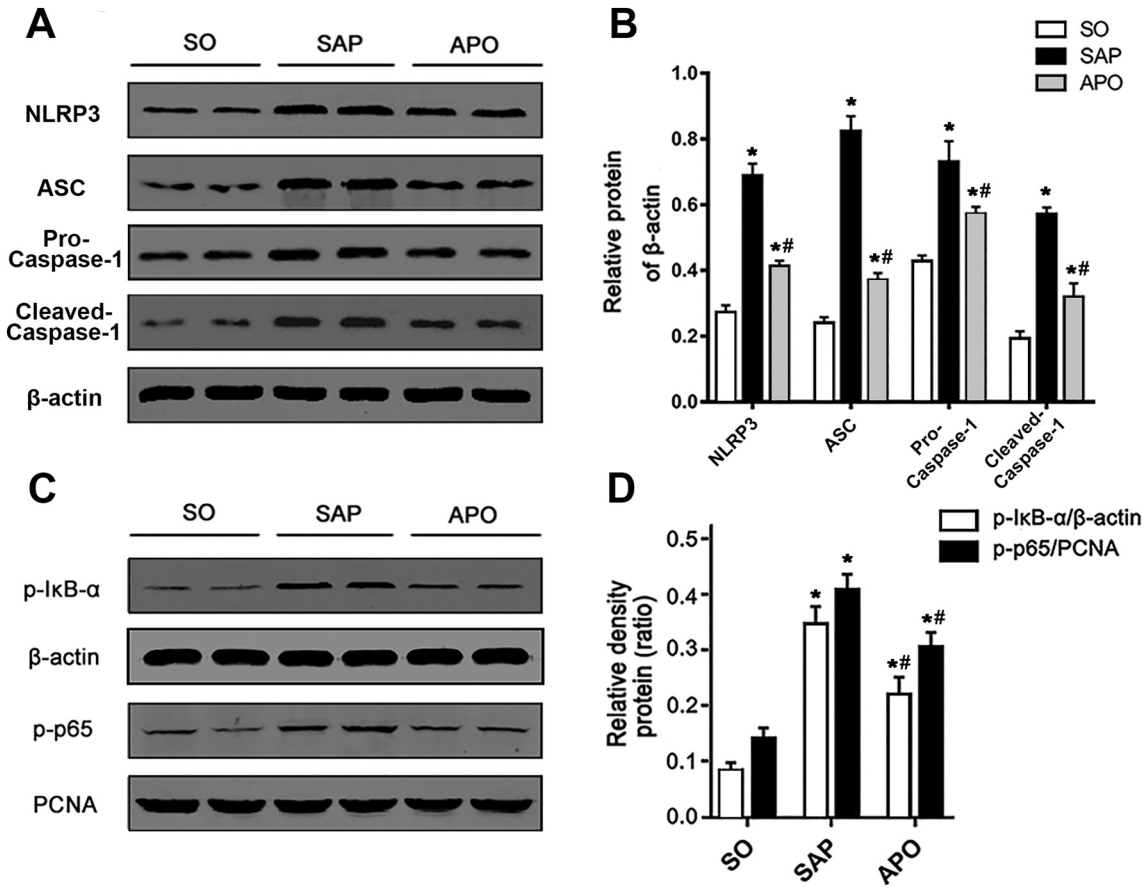

Fig. 8. Effects of Apocynin administration on lung activation of NLRP3 inflammasome complex and NF-κB activation in SAP rat. A: Representative western blots of NLRP3, ASC, pro-Caspase-1 and cleaved-Caspase-1, B: Quantitative analysis of NLRP3, ASC, pro-Caspase-1 and cleaved-Caspase-1, C: Representative western blots of p-IκB-α, and p-p65, D: Quantitative analysis of p-IκBα and p-p65. The values were presented as mean ± SEM of three independent experiments. $^{*}P < 0.05$ vs. sham operation, $^{\#}P < 0.05$ vs. severe acute pancreatitis group.

resulted in anti-inflammatory effect of apocynin in rodent models of acute pancreatitis-induced intestinal injury [10]. Furthermore, antioxidant enzymes and antioxidants suppressed the activation of NF-κB through decreasing the intracellular accumulation of ROS [44]. As an intracellular target of ROS, NF-κB plays a pivotal role in SAP, and inhibition of NF-κB can protect lung tissue following SAP [45]. Chemokines are produced in response to signals including proinflammatory cytokines, and they play a leading role in recruiting neutrophils, monocytes, and lymphocytes selectively. In particular, this study also showed that apocynin can significantly alleviate pancreas damage and reduced serum levels of TNF-α, IL-1β and IL-6 caused by SAP. That is possibly because of the widely distribution of the NOX family members throughout the organism [9,46], including the pancreas. After apocynin treatment, the degree of pancreas damage was significantly alleviated, neutrophil and mononuclear cell infiltration were reduced and inflammatory exudates were improved. To data, the main mechanism in SAP-associated ALI was the inflammatory cytokines released into blood circulation reaching the lung tissue [47]. Combined with our findings, we confirmed the protective role of apocynin in alleviating lung injury, which not only directly reduced the levels of ROS in lung but also indirectly reduced the inflammatory cytokines released into the blood during SAP.

Although NF-κB signaling is vital to NLRP3 activation, the process of NLRP3 inflammasome formation and activation is extremely complicated according to various studies. Diverse extracellular stimuli, such as mitochondrial dysfunction, K^+ efflux, Ca^{2+} overload, and lysosomal leakage [19], which are often referred to as NLRP3 agonists, trigger the assembly of the inflammasome complex and its eventual activation. However, it is unclear whether apocynin regulates the activation of NLRP3 inflammasome by direct interaction with the NLRP3 inflammasome or through indirect effects via other priming signals. This remains to be further studied in the future. In the present study, apocynin may exert protective in SAP-induced lung injury by inhibiting the activation of NLRP3 inflammasome, which induced by down-regulated NF-κB signaling. In summary, we demonstrated that NADPH oxidase inhibitor apocynin dampened SAP-induced ALI as well as pancreatic injury in an experimental SAP model. The mechanism underlying these protective effects might include the inhibition of the activation of NLRP3 inflammasome and NF-κB signaling. These findings support the notion that NADPH oxidases may act as a potential target for preventing SAP and SAP-induced ALI.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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