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ORIGINAL ARTICLE

Emodin Attenuates Severe Acute Pancreatitis *via* Antioxidant and Anti-inflammatory Activity

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Abstract— There is no specific drug to treat severe acute pancreatitis (SAP), which induces substantial medical and social burden. Many studies have reported the beneficial effects of emodin against SAP *in vivo* and *in vitro*. However, the underlying mechanism has been unclear. This paper described the design and implementation of anti-inflammatory and antioxidant activity of emodin. Emodin restored the pathological damage of SAP and simultaneously decreased the high levels of serum amylase, lipase, TNF-α, and IL-18 in the peripheral blood of SAP rat. Emodin reversed reactive oxygen species (ROS) in neutrophils derived from SAP rat. The levels of voltage-dependent anion channel 1 (VDAC1), NOD-like receptor protein 3 (NLRP3), caspase-1, and IL-18 were examined to analyze the change of inflammasome-related mediators between SAP and emodin treatment. These findings suggest that emodin plays its protective role on SAP against oxidative stress and inflammasome signals.

KEY WORDS: emodin; reactive oxygen species; severe acute pancreatitis; inflammasome signals.

INTRODUCTION

Severe acute pancreatitis (SAP) is a lethal disease with a severe inflammatory condition. SAP patients pose a significant clinical challenge with increased mortality [1]. During the process of SAP, there are accumulation and infiltration of multiple inflammatory cells, such as neutrophils. The neutrophils infiltrate into the inflammation site of the pancreas and are stimulated by trypsin, releasing a large amount of potent substances, such as inflammatory mediators and reactive oxygen species (ROS) [2, 3]. A great deal of previous research into ROS has focused on its vital role in the process of inflammation [4, 5]. There is a consensus among scientists that ROS produce can be initiated by the inflammatory process and can also promote proinflammatory signaling and produce oxidative stress. Therefore, it would be of significant clinical value in approaching a medicine involved in the regulation of inflammation and ROS generation.

Historically, rhubarb as an active ingredient in traditional decoctions was used to treat acute pancreatitis in China. Emodin, one main active component of rhubarb, appears to be positively related to inflammatory regulation [6–8]. Several lines of clinical and experimental evidence suggest that emodin can ameliorate the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) that are usually caused by SAP [9]. Much of the research up to now has been described in the molecular mechanism underlying inflammatory response to emodin [10, 11]. However, the exact mechanism that underpins the effect of emodin is not yet clear. This paper attempts to show

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that emodin can be exercised to inhibit ROS with further regulation of downstream inflammatory signaling.

Mitochondria is the source of ROS, which can initiate the oxidation of almost all kinds of substance in organisms, including lipids, proteins, and nucleic acids [12]. The inflammation would lead to ROS overproduction in neutrophil cells and gradual accumulation in the body. ROS further result in the membrane peroxidation and further damage the organelles, such as mitochondria. Voltage-dependent anion channel (VDAC), posed as mitochondrial porin, is found to manifest mitochondrial permeability [13, 14]. ROS derived from mitochondria can regulate the oxidation of VDAC and promote the inflammasome activation [15, 16]. NOD-like receptor protein 3 (NLRP3) is packed into portfolios of different biological processions, including aging, inflammation, physical inactivity, and overnutrition [17, 18]. When activated under inflammatory stress, NLRP3 can stimulate the downstream production of caspase-1. Furthermore, NLRP3 boosts numerous inflammatory cytokines, such as IL-18 [19]. The serial reaction contributes to the aggravation of systemic inflammatory response.

The *in vivo* experimental assay was organized in order to study the molecular mechanisms of anti-inflammatory and antioxidative effects of emodin. The aim of this study is also to explore the relationship between emodin and inflammasome-related mediators under severe inflammatory stress. The rats were grouped into sham, SAP, and emodin groups. The next section presents the analysis of morphological and pathological change, ROS concentration, and expression of signal members. This research provides an important opportunity to advance the understanding of inflammatory response to emodin treatment under SAP.

MATERIALS AND METHODS

Animal Model and Groups

Many researchers have utilized Sprague Dawley rats to measure SAP animal model. Healthy adult male Sprague Dawley rats (300–350 g) were obtained from the Laboratory Animal Center of Dalian Medical University (Dalian, China). Before the experiment, all rats were housed under controlled day-night cycles and adapted to the experimental environment for 1 week. The temperature was maintained at 22–24 °C, and humidity was maintained at 60–70%.

The rats were randomly divided into three groups, including sham operation group (sham group), SAP model group (SAP group), and emodin-treated group (emodin group). As the traditional method described in previous studies [9, 20], pancreatic and biliary duct retrograde infusions of 5.0% sodium taurocholate (0.1 ml/100 g) were set out to make a SAP rat model with preoperative fasting for 12 h. The sham rats were applied with the same surgical method without sodium taurocholate infusion. The rats in the emodin group were performed with three-time intragastric administration of 6 mg/ml emodin (1 ml/100 g) after SAP. The sham and SAP rats were intragastrically administered with 0.4% CMC-Na (1 ml/100 g). Sodium taurocholate was purchased from Sigma Chemical (St. Louis, USA). Emodin was purchased from Solarbio (Peking, China).

ELISA Assay for Detecting Serum Amylase, Lipase, TNF-α, and IL-18

Blood samples were collected and clotted at room temperature. The levels of serum amylase, lipase, TNF-α, and IL-18 were measured using ELISA kit following the manufacturer's protocol. ELISA kits were obtained from Langdun Biotech (Shanghai, China). The optical density (OD) reading at 450 nm was determined as activation of serum inflammatory factors. Erythrocyte was administrated with 30–45 min natural sedimentation.

Isolation and Purification of Neutrophils

Neutrophils were isolated by negative magnetic bead sorting kit (Miltenyi Biotec, Germany). Blood samples were collected within heparin sodium then added to an equal volume of Dextran T500. The supernatant was gently transferred to a new tube with 10-fold volume erythrocyte lysate reagent. The supernatant was centrifuged at 200*g* for 5 min. After being washed off erythrocyte debris, the resulting cells were counted and then administrated with pre-cooling buffer in order to be mixed well with anti-granulocyte-APC reagent (Miltenyi Biotec, Germany). After the 10-min incubation and 300*g* centrifugation for 10 min, pre-cooling buffer was added again. Prior to next buffer, the cells were mixed with anti-APC MultiSort microbeads (Miltenyi Biotec, Germany). After the 300*g* centrifugation for 10 min, the cells were applied by a 200-mesh filter and then added to the sorting column which was connected to a VarioMACS magnet (Miltenyi Biotec, Germany). Neutrophils was collected and obtained. The whole process was operated in 4 °C.

ROS Detection

The neutrophils were divided into 5 tubes, including negative tube, positive tube, sham tube, SAP tube, and emodin tube. Each tube was centrifuged at 300*g* for 5 min, and then the supernatant was added up with RPMI 1640 medium with 10% FBS. The administration was as follows: a negative tube without a ROS label was used, and a positive tube was added with a ROS inducer and placed at room temperature for 30 min. The cells were mixed with ROS assay buffer and gradually centrifuged at 300*g* for 5 min. The cells were performed with a 200-mesh filter, and ROS was detected with analysis of flow cytometry (BD Fortessa, USA).

Western Blot for Detecting VDAC1 and NLRP3

The neutrophils were collected with magnetic bead sorting. The supernatant was obtained with centrifugation of neutrophil cell suspension. After being washed with PBS twice, cell lysing buffer was used to extract protein sample. The total protein was mixed with loading buffer in a 5-min boil. The lysates were loaded in equal protein amounts determined by BCA (Beyotime Biotechnology, China). Proteins were carried out using SDS-PAGE system followed by transfer onto a nitrocellulose membrane using a dry blotting system. Prior to block with blocker solution, the membrane was carefully washed in T-TBS for 3 min. The membrane was incubated with the primary antibody in 2% BSA for 8–10 h at 4 °C.

T-TBS was used to wash off the unbound primary antibody. The membrane was incubated with HRP-labeled species-specific secondary antibodies for 1 h at room temperature. After PBS was washed for 3 times, positive signals were detected by ECL substrate and recorded in gel imaging system (Bio-Rad, USA).

PCR Assay for Detecting Caspase-1 and IL-18

The neutrophil cell suspension was added with lysate, then the supernatant was obtained with centrifugation. The mRNA-extracted cell form was transcoded into cDNA, which was the sequence template in the PCR system. PCR was performed using a 20-μl reaction (Takara, Japan) containing 10 μl SYBR Premix Ex Taq II, 6 μl template, 0.4 μl ROX Reference Dye II, and 0.8 μl primers. The PCR instrumentation was the 7500 Fast Dx Real-time PCR machine (Applied Biosystems, USA). Initial denaturation was at 95 °C for 30 s followed by 40 cycles of 95 °C denaturation for 5 s, and 60 °C annealing and extension for 34 s. Data was collected during the annealing and extension step. The data was determined with cycle threshold (Ct).

Statistics Analysis

All statistical analyses were carried out using GraphPad Prism 6.0 with statistical significance set at $P < 0.05$ or $P < 0.01$. The pathological score was shown as the mean ± 95% Cl and were assessed using nonparametric tests. Unpaired Student's *t* tests were used for two-group comparisons, and one-way ANOVAs were used for multi-group comparisons.

RESULTS

Emodin Attenuates Pathological Profile of SAP Animal Model

The first set of study aimed to determine the phenotypic response of SAP rat to emodin treatment. Figure 1 provides an overview of the pathological change between different groups. In the sham group as shown in Fig. 1a, there was no abnormality in the rat pancreatic tissue with light red color. The rat intraperitoneal exhibition showed a small amount of ascites and mild flatulence, and there was no saponification spot. Figure 1b provides that there were massive ascites and intestinal dilatation with intestinal adhesion, and the pale-yellow saponification spot scattered in intestine tube and mesentery. Compared with the SAP group, the emodin group shown in Fig. 1c had fewer amount of abdominal ascites and less saponification spot without intestinal adhesion.

From hematoxylin and eosin (HE) staining in Fig. 1d–f, we could see that there was a significant inflammatory evidence in SAP rat compared with sham rat. Figure 1g presents a remarkable increase in the pathological score of SAP rat. After emodin treatment, HE staining showed an edema reduction in interstitial tissue. In the emodin group exhibited in Fig. 1e and f, the necrotic area and inflammatory area were reversed to be shrunk, and the pathological score of the pancreas descended significantly. The next section was continually concerned with molecular evidence under emodin treatment.

Emodin Reduced the Molecular Indicators in SAP

Based on the change in pathological profile, molecular indicators were performed in order to determine the biological effect of emodin. Figure 2 compares the levels of molecular indicators (serum amylase, lipase,

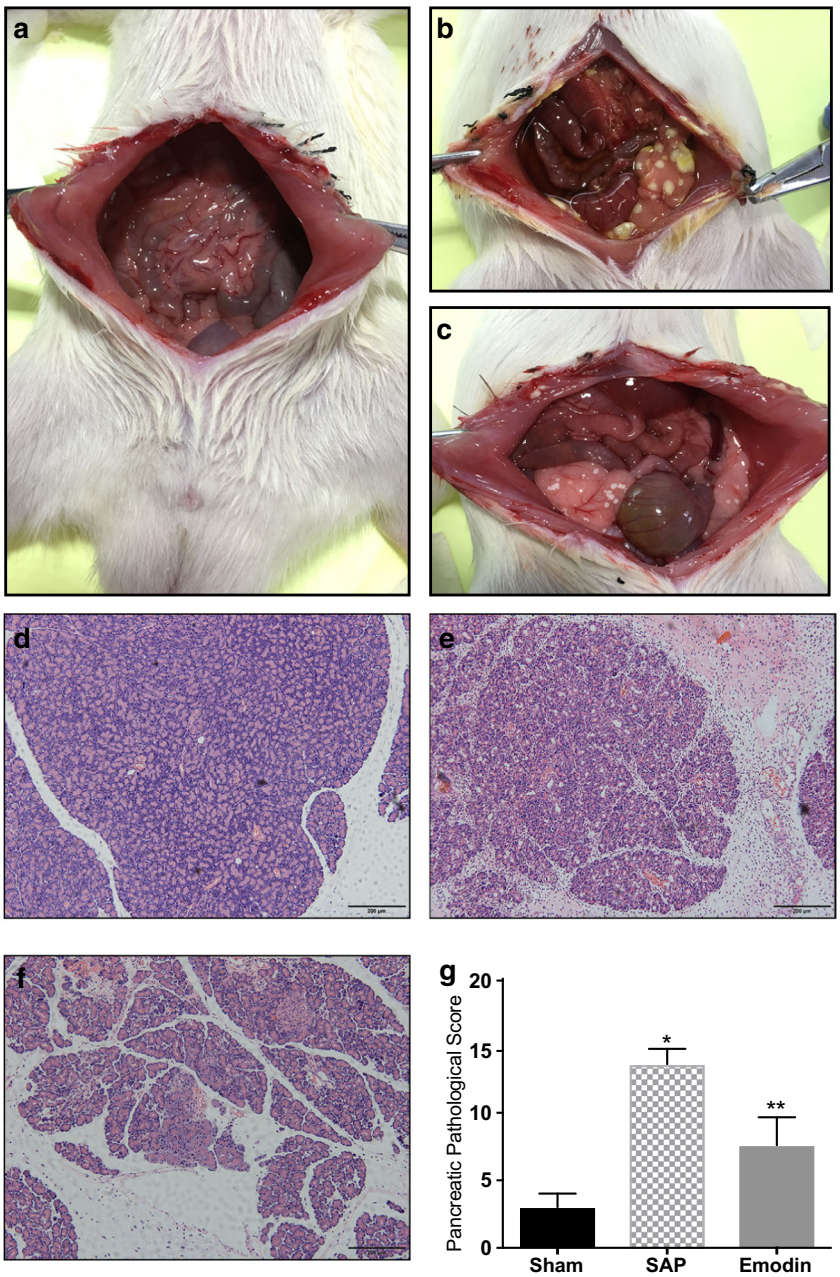

Fig. 1. Pathological phenotype of SAP rat with and without emodin treatment. **a**–**c** Anatomical observation of the rat abdominal cavity in different groups: **a** sham group, **b** SAP group, and **c** emodin group. **d**–**g** HE staining of the pancreatic tissue in rat. **d** Sham group. **e** SAP group. **f** Emodin group. **g** Pathological score of the pancreas. $*P < 0.01$ *vs.* the sham group; $**P < 0.01$ *vs.* the SAP group.

TNF-α, IL-18) among the three groups with utilization of ELISA. In the sham group, there was 14.42 ± 0.50 ng/μl in serum amylase, 56.25 ± 1.88 ng/ml in lipase, 287.1 ± 25.59 ng/l in TNF-α, and 3.95 ± 1.20 pg/ml in IL-18. In the SAP group, there was 24.48 ± 1.03 ng/μl in serum amylase, 194.8 ± 1.01 ng/ml in lipase, 808.8 ± 65.24 ng/l in TNF-α, and 14.45 ± 3.43 pg/ml in IL-18. In the emodin group, there was 24.48 ± 1.03 ng/μl in serum amylase, 126.9 ± 7.84 ng/ml in lipase, 421.7 ± 82.69 ng/l in TNF-α, and 5.35 ±

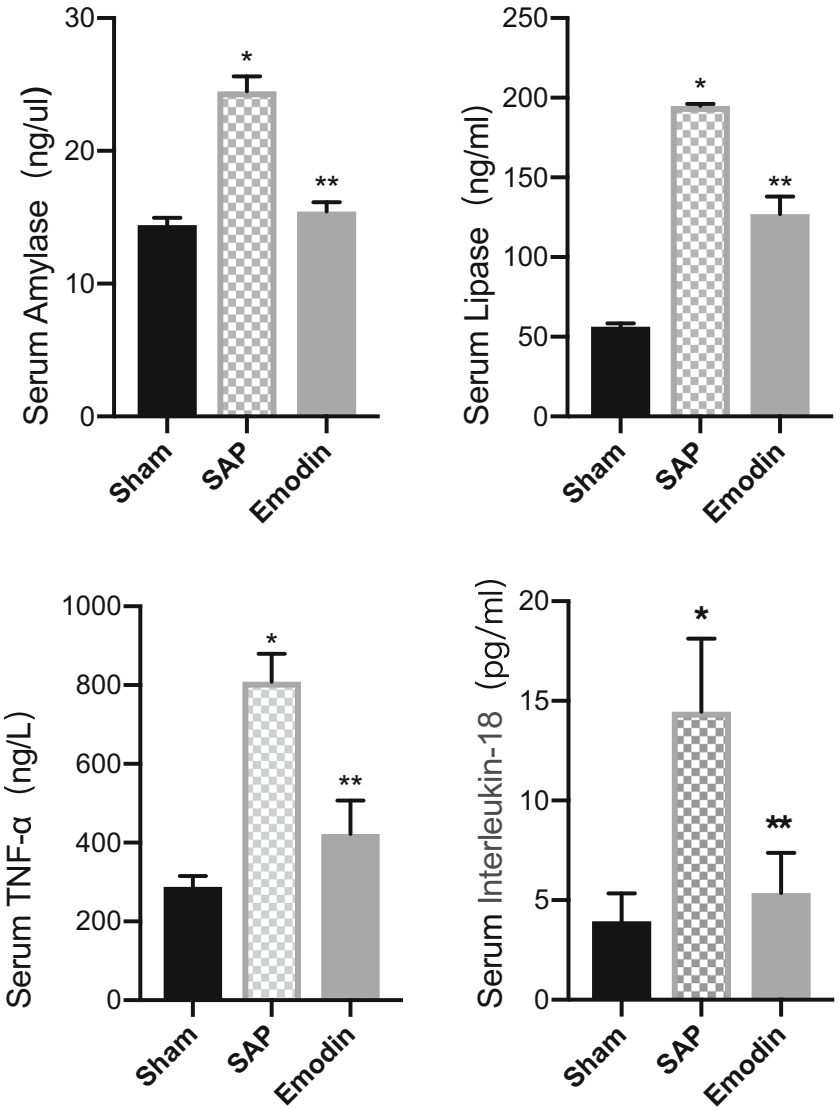

Fig. 2. Four indicators of pancreatitis in the rat peripheral blood. **a** Serum amylase. **b** Lipase. **c** TNF-α. **d** IL-18. *$P < 0.01$ *vs.* the sham group; **$P < 0.01$ *vs.* the SAP group.

1.91 pg/ml in IL-18. All four indicators increased strongly in SAP rat compared to sham ones and were successfully reduced by emodin, suggesting a positive correlation between emodin and anti-inflammatory.

The Elevated ROS Level in SAP Was Reversed with the Treatment of Emodin

To better understand the function of emodin, this set of study was applied to analyze its underlying causes of anti-inflammatory effects. At first, the purity of neutrophil isolated by the magnetic bead approach was identified using flow cytometry assay. Figure 3a shows the negative control result. Figure 3b–d show that the cell purity was 97.05% in the sham group, 98.78% in the SAP group, and 98.48% in the emodin group. The purity in each group was up more than 95%.

From Fig. 3e and f, the significant evidence was quite revealed in the ROS of neutrophils extracted after different treatments. Compared to the level in the sham group, ROS increased substantially in the SAP group and decreased after emodin treatment.

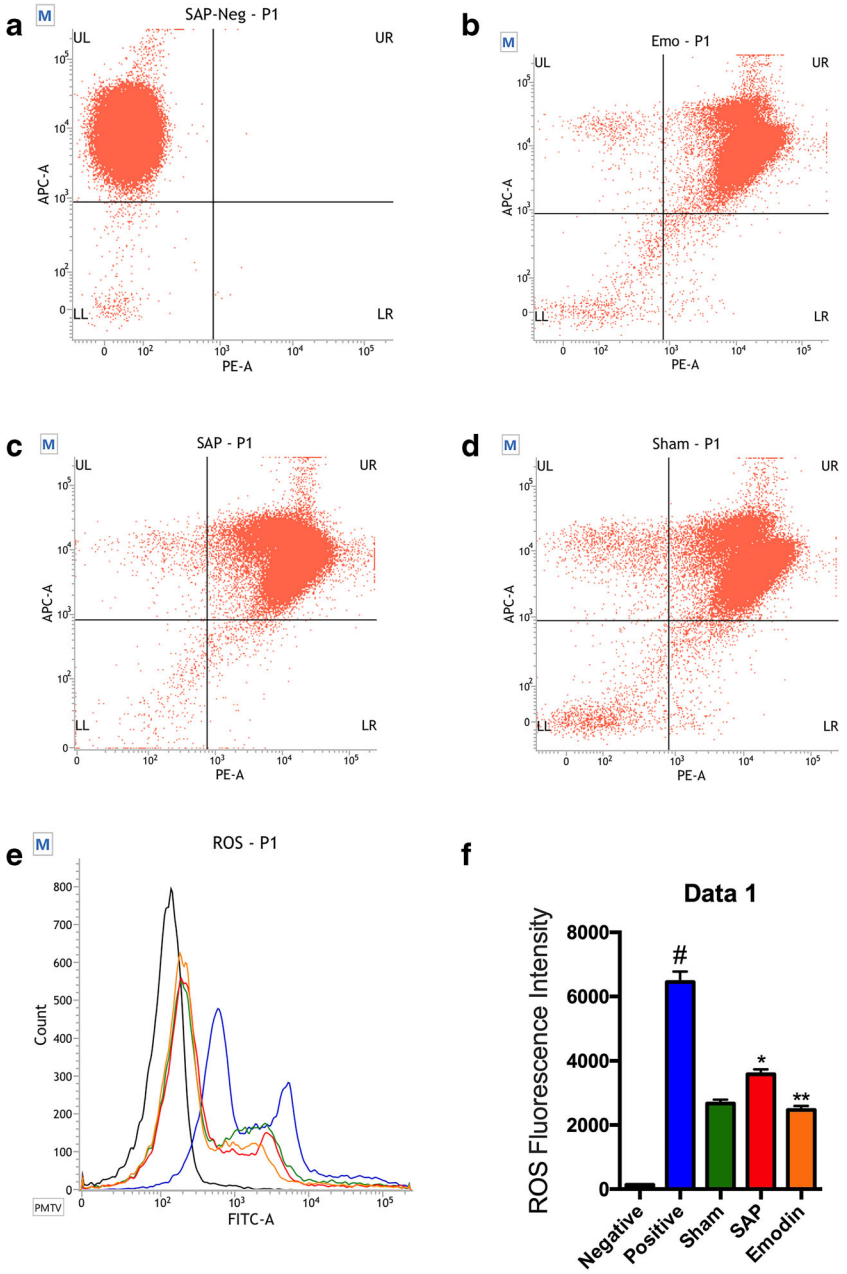

Fig. 3. ROS level in neutrophil. **a**–**d** The purity of neutrophil cell. **a** Negative control. **b** Sham group. **c** SAP group. **d** Emodin group. **e**, **f** The fluorescence intensity of ROS level in neutrophil. $^{\#}P < 0.01$ *vs.* the negative group; $^{*}P < 0.01$ *vs.* the sham group; $^{**}P < 0.01$ *vs.* the SAP group.

Emodin Was Involved in Regulating Inflammasome-Related Mediators in SAP

Through the investigation of anti-inflammatory and ROS above, VDAC1, NLRP3, caspase-1, and IL-18 were detected. It is exhibited in Fig. 4a and b that VDAC1 was downregulated in the SAP group and the level was reversed in the emodin group. On the contrary, the expression of NLRP3 was elevated under SAP while depressed by emodin treatment.

Figure 4c presents the other two inflammatory mediators: caspase-1 and IL-18, detected by qPCR. Both of them were upregulated under SAP. However, these two mediators were lowered under emodin treatment.

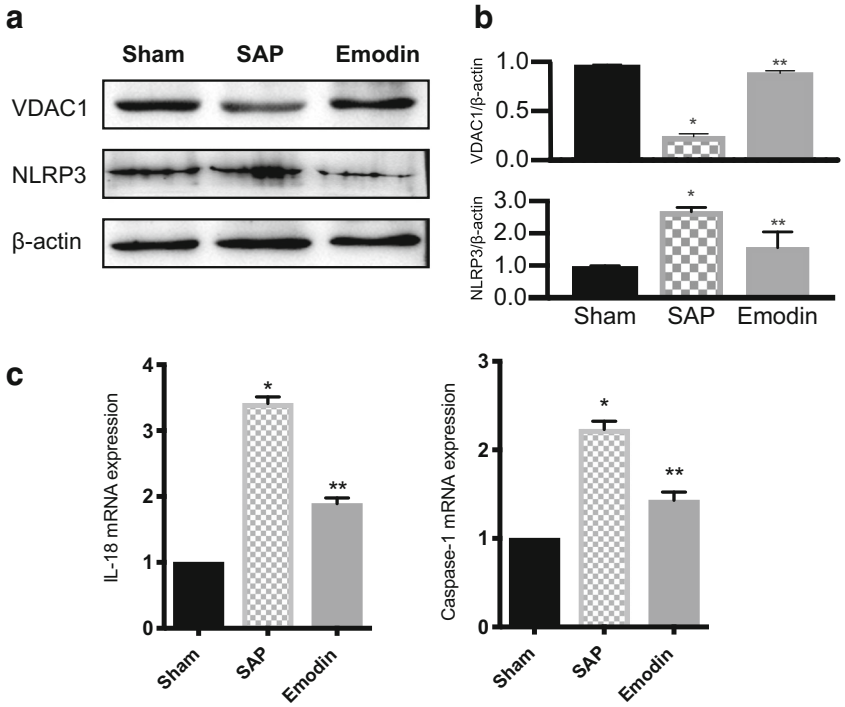

Fig. 4. The levels of inflammasome-related mediators. The expression profile of VDAC1, NLRP3, caspase-1, and IL-18. **a** Western blot imaging data of VDAC1 and NLRP3. **b** The relative expression of VDAC1 and NLRP3 proteins. **c** The mRNA detection of caspase-1 and IL-18. *$P < 0.01$ *vs.* the sham group; **$P < 0.01$ *vs.* the SAP group.

DISCUSSION

SAP is associated with a mortality rate of 30% due to rapid inflammatory development and complex pathophysiological condition [21, 22]. At present, there is a less effective way to ameliorate the outcome of SAP patients. It is generally known that inflammation, ROS stress, and microvascular dysfunction are integrated characteristics of SAP. Among these characteristics, ROS and its related mediators play a major role in the aggravation of SAP during the severe inflammation which persists in any period of this disease [23]. The presence of ROS has been thought to be involved in the pathogenesis of SAP. The present study was thus designed to determine the effect of emodin on ROS change in order to improve our understanding on the underlying emodin-induced reduction of SAP mortality *in vivo*.

As mentioned in the literature report, the effect of emodin on inflammation has been investigated in clinical and laboratory research [24, 25]. Among various types of inflammation, emodin was determined to combine with an autophagy inhibitor in order to relieve the symptoms of ankylosing spondylitis [10]. Emodin was also reported to weaken hepatitis with its anti-inflammatory influence [26]. It was identified from an assay of acute kidney injury (AKI) rats that emodin could regulate the imbalance of gut microbiota in AKI rats [27]. According to Chinese traditional medical records, rhubarb, as a component of classical decoctions, was used to treat the abdominal symptoms [28]. Emodin, its main ingredient of rhubarb, was widely investigated in the field of abdominal inflammation [11, 29]. On the question of anti-inflammatory effect of emodin on SAP, our results showed that pathological phenotype was attenuated by emodin. All elevated serum amylase, lipase, TNF-α, and IL-18 levels in SAP rat were decreased obviously after the 24-h emodin treatment, which was consistent with pathological score change. Generally, TNF-α was known as one of the cytokines that stimulated neutrophil activation in the process of SAP [30]. IL-18, one of the IL-1 family members, could be generated by neutrophils during acute inflammation. The academic literature on TNF-α and IL-18 revealed that both of them were positively related to the severity of SAP [30]. These current findings corroborated the results of a great deal of the previous work in the anti-inflammatory effect of emodin.

Convincing evidence has documented that neutrophils play a key role in the induction of SAP. Once inflammatory stimulation occurs, neutrophils release potent substances, including IL-18 and ROS [31, 32]. Subsequently, ROS begin to attack and damage the organelles, such as lysosome and mitochondria [33, 34]. In the outer membrane of mitochondria, VDAC is oxidized by ROS and then regulates the ROS production of mitochondria, further activating NLRP3 inflammasome [16, 18, 35]. NLRP3 is the typical member of NOD-like receptor family, which recognizes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) with the C-terminal domain of NLRP3 [36–38]. Activated NLRP3 and ATP are polymerized to assemble the complex with pro-caspase-1 by its CARD domain, which can induce the activation of caspase-1 and IL-18 [39–41]. In this study, ROS and NLRP3 inflammasome–related pathway were performed using flow cytometry, western blot, and PCR assay to compare the different influences of accumulated ROS on NLRP3 inflammasome–related pathway before and after emodin administration. The expression of VDAC1 protein was decreased in neutrophil extracted from SAP rat but increased by emodin treatment. On the contrary, the low level of NLRP3 in SAP rat was reversed by emodin. It was determined from PCR data that caspase-1 and IL-18 were upregulated in the SAP group compared to the sham group and then significantly downregulated in the emodin group. These changes of ROS and inflammasome pathway attributed to the response of neutrophils in SAP to emodin.

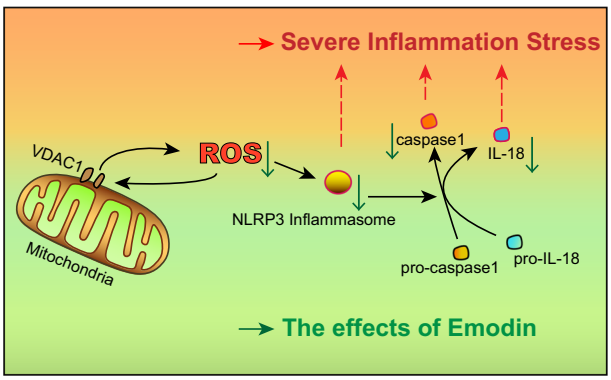

Fig. 5. Schematic representation of antioxidant and anti-inflammatory effects induced by emodin. Under severe inflammation stress, the pore of mitochondria opened and released ROS to the cytoplasm, where the development of NLRP3 inflammasome was promoted by ROS. Then, caspase-1 and IL-18 became mature and aggravated inflammation. On the other hand, emodin can decrease the ROS activity and involved in the downregulation of NLRP3, caspase-1, and IL-18. The response of inflammatory cell to emodin can reverse the severe inflammatory condition.

Taken together, the current study has revealed that ROS in SAP rat was inhibited by emodin with the regulation of NLRP3, VDAC1, caspase-1, and IL-18. Therefore, it seemed that emodin is used to relieve the symptoms of SAP rat through its function on ROS and inflammasome pathway. These findings may help us to understand the underlying mechanism of emodin treatment in severe inflammatory response.

CONCLUSION

As shown in Fig. 5, these results provided a further insight into the role of emodin on SAP rat with the inhibitory effect of ROS and inflammatory response. These *in vivo* experiments confirmed that SAP was obviously attenuated by emodin with pathological evidence and the change of inflammatory mediators. An implication of this is the possibility that emodin inhibited ROS and NLRP3 inflammasome–related pathway simultaneously.

The study was limited by the lack of information on the expression of additional mediators, which would be helpful to illustrate the inflammasome-related response to emodin. An additional uncontrolled factor was the possibility that these data were supposed to be identified with more assays and approaches, such as *in vitro* experiment. Notwithstanding these limitations, the study suggested that emodin reduced ROS level and then downregulated NLRP3 inflammasome–elated mediators.

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AUTHOR CONTRIBUTIONS

Shilin Xia and Dong Shang participated in the design of this study. Shilin Xia performed the manuscript review. Yujia Ni exerted the main experiment and performed the statistical analysis. Shilin Xia and Han Liu drafted the manuscript. Qi Zhou, Hong Xiang, and Hua Sui provided assistance for the data acquisition and data analysis. All authors read and approved the final manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflicts of interest.

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