Research Article

Protective Effects of Emodin-Induced Neutrophil Apoptosis via the Ca\(^{2+}\)-Caspase 12 Pathway against SIRS in Rats with Severe Acute Pancreatitis

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Severe acute pancreatitis (SAP) results in high mortality. This is partly because of early multiple organ dysfunction syndromes that are usually caused by systemic inflammatory response syndrome (SIRS). Many studies have reported the beneficial effects of emodin against SAP with SIRS. However, the exact mechanism underlying the effect of emodin remains unclear. This study was designed to explore the protective effects and underlying mechanisms of emodin against SIRS in rats with SAP. In the present study, cytosolic Ca\(^{2+}\) levels, calpain 1 activity, and the expression levels of the active fragments of caspases 12 and 3 decreased in neutrophils from rats with SAP and increased after treatment with emodin. Delayed neutrophil apoptosis occurred in rats with SAP and emodin was able to reverse this delayed apoptosis and inhibit SIRS. The effect of emodin on calpain 1 activity, the expression levels of the active fragments of caspases 12 and 3, neutrophil apoptosis, and SIRS scores were attenuated by PD150606 (an inhibitor of calpain). These results suggest that emodin inhibits SIRS in rats with SAP by inducing circulating neutrophil apoptosis via the Ca\(^{2+}\)-calpain 1-caspase 12-caspase 3 signaling pathway.

1. Introduction

Acute pancreatitis (AP) is a common acute abdominal disease. Clinically, AP can be classified as mild acute pancreatitis (MAP), moderately severe acute pancreatitis (MSAP), or severe acute pancreatitis (SAP). SAP is extremely dangerous, and its mortality rate is approximately 38.4% [1]. The reason that SAP is difficult to treat is that it has two peak time points at which the risk of mortality is high; the first one occurs owing to early multiple organ dysfunction syndromes (MODS) that are usually caused by the systemic inflammatory response syndrome (SIRS), and the second one occurs owing to late MODS, which are usually caused by necrosis in the pancreas and sepsis [2–4]. Therefore, the prevention of SIRS is undoubtedly important in the early treatment of SAP.

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) is the main active component of rhubarb. In China, rhubarb has been used for many years to treat AP. Many findings support the idea that emodin has benefits as a pharmaceutical treatment for SAP [5–9] and that it can ameliorate SIRS and MODS [10]. However, the exact mechanism underlying the effect of emodin is not yet clear. The aim of this study was to explore the mechanism by which emodin ameliorates SIRS in SAP. Delayed neutrophil apoptosis is proinflammatory and has been demonstrated to be associated with SIRS [11]. Several studies have shown that emodin promotes apoptosis in cancer cells [12–15]. Therefore, we hypothesized that the promotion of neutrophil apoptosis could serve as a potential mechanism for the protective effect of emodin against SIRS in SAP.
Apoptosis has two main pathways: one is the death receptor pathway, and the other is the mitochondrial pathway. Recent studies suggest a third pathway that is triggered in response to endoplasmic reticulum (ER) stress.

Caspase 12, C/EBP homologous protein (CHOP), and c-Jun N-terminal kinase (JNK) are three primary molecules that play a critical role in ER stress-induced apoptosis [16]. ER stress-induced apoptosis via the CHOP or JNK signaling pathways is mitochondria-dependent [17, 18]. Caspase 12, a member of the caspase family of proteases, is located on the ER. ER stress-induced apoptosis via the caspase 12 signaling pathway is independent of mitochondria. The activation of ER-resident caspase 12 directly results in the activation of cytoplasmic caspase 3 during ER stress-induced apoptosis [19, 20].

Caspase 12 can be activated by calpain. Calpain cleaves caspase 12 and promotes its activation during ER stress-induced apoptosis [21, 22]. It belongs to a family of thiol proteases and has more than ten isoforms. The two major isoforms are calpain 1 and calpain 2. Calpain is regulated by many mechanisms, one of which is changes in Ca$^{2+}$ levels [23]. Calpain 1 belongs to the $\mu$-calpain group and requires micromolar changes in Ca$^{2+}$ levels to induce its activity. Calpain 2, however, belongs to the m-calpain group and requires millimolar changes in Ca$^{2+}$ levels to induce its activity. The binding of Ca$^{2+}$ to calpains 1 and 2 induces a regulatory subunit to be released, thereby activating a catalytic subunit [24]. Increases in Ca$^{2+}$ correlate with an increase in calpain activation [25]. Based on the above knowledge, we hypothesized that emodin would induce neutrophil apoptosis via a Ca$^{2+}$-calpain 1-caspase 12-caspase 3 signaling pathway. This study was performed to explore whether emodin could inhibit SIRS by inducing apoptosis in circulating neutrophils via the Ca$^{2+}$-calpain 1-caspase 12-caspase 3 pathway in SAP.

2. Materials and Methods

2.1. Reagents. Sodium taurocholate was obtained from Sigma (St. Louis, USA). Emodin was purchased from Dalian Food and Drug Administration (Dalian, China). The calpain inhibitor (PD150606) was obtained from Alexis Biochemicals (San Diego, USA). The amylase, TNF-α, and IL-6 assay kits were obtained from Lengton Bioscience Co. (Shanghai, China). 4% paraformaldehyde was purchased from Solarbio (Beijing, China). FITC/PI was purchased from Solarbio (Beijing, China). 3-AM assay kit was obtained from Dojindo Laboratories (Minoh, Japan). The apoptosis detection kit (Annexin V-FITC/PI) was purchased from Solarbio (Beijing, China).

2.2. Experimental Model and Groups. 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 the rats were housed under controlled day-night cycles and adapted to the experimental environment for 1 week.

Intraperitoneal injections of 1% pentobarbital sodium (4 mg/100 g) were used for surgical anesthesia. Pancreatic and biliary duct retrograde infusions of 3.0% sodium taurocholate (0.1 mL/100 g), as described by Paranjpe et al. [26], were used to induce a model of SAP with SIRS. Rats in the sham-operation group only had their abdomen opened and closed.

Sham-operation rats were assigned to an SO group that received intraperitoneal injections of equivalent amounts of solvent for emodin and PD150606, and SAP rats were assigned to an SAP group that received intraperitoneal injections of equivalent amounts of solvent for emodin and PD150606, a PD group that received intraperitoneal injections of PD150606 (3 mg/kg) and equivalent amounts of solvent for emodin, an EM group that received intraperitoneal injections of emodin (10 mg/kg) and equivalent amounts of solvent for PD150606, or a PE group that received intraperitoneal injections of PD150606 (3 mg/kg) and emodin (10 mg/kg). Twelve hours after establishing the SAP model and the administration of the respective drugs, intraperitoneal injections of 1% pentobarbital sodium (4 mg/100 g) were used for surgical anesthesia. Heart rate was measured with a tail cuff (BIOPAC MP150, USA), body temperature was measured via the anus (BIOPAC MP150, USA), and blood samples were taken from the abdominal aorta. PaCO$_2$ was measured in the samples using a blood gas analyzer (Abbott, i-STAT300, USA) and white cells were counted using a hematology analyzer (Mindray, BC-3000 Plus, China). Neutrophils were isolated.

SIRS criteria and score calculations followed the description of Lu and Giannoudis [27, 28]. A point was scored for a heart rate increase of 50% above the normal value, a 2-fold increase or 50% decrease in the white cell count compared with normal values, a core temperature increase or decrease of 1°C relative to the normal value, and a PaCO$_2$ decrease of 25% below the normal value, resulting in scores ranging from 0 to 4. When the score was 2 or more, the rat was defined to be in a state of SIRS.

This study was conducted in strict accordance with the criteria in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Ethics Committee of Dalian Medical University.

2.3. Isolation and Purification of Neutrophils. The neutrophils were isolated and purified using negative immunomagnetic separation as described by Hu [29]. Blood samples (5 mL) were added to an equal volume of Dextran T500 (30 g/L, w/v in saline) and bathed in 37°C water for 30 min to allow erythrocytes sedimentation. The leukocyte-rich supernatant was taken and centrifuged at 800 rpm (Eppendorf, Centrifuge 5804 R, Germany) for 5 min. 0.2% NaCl (7 mL) solution was added to remove the residual erythrocytes. 1.6% NaCl (7 mL) supplemented with 0.1% glucose was added for hypertonic
rescue of leukocyte. The leukocyte was collected, washed off erythrocyte debris, and suspended in PBS (1 mL). Anti-CD 2 (15 μg), anti-CD 5 (20 μg), anti-CD 45R (100 μg), anti-F4/80 (2 μg), and anti-ICAM-1 (6 μg) were added to the cells, and the cells were incubated at 4°C for 30 min. Excess antibodies were removed, and cells were suspended in PBS (80 μL) and incubated with goat anti-rabbit IgG microbeads (20 μL) at 4°C for 15 min. The leukocyte-microbead mixture was then added to the column which was connected to a VarioMACS Magnet (Miltenyi Biotec, Germany). Neutrophils were collected.

### 2.4. Serum Amylase, TNF-α, and IL-6 Assays
Blood samples were collected and clotted at room temperature, and serum samples were obtained following centrifugation. Serum amylase activity, TNF-α levels, and IL-6 levels were assayed using ELISA kits. The intensity of the color was measured using the Multiskan Spectrum system.

### 2.5. Evaluation of Pancreatic Pathologies
Pancreases were removed immediately after bleeding the animals and fixed in 4% paraformaldehyde. The fixed pancreases were embedded in paraffin and sectioned at 4 μm with a microtome. The sections were stained with hematoxylin and eosin (H-E). The scoring system applied to evaluate the histological examinations followed previous description [30, 31] as shown in Table 1. Five fields in each sample were observed and the total scores of the five fields were averaged.

### 2.6. Cytosolic Ca²⁺ Determination
The collected cells were washed with HBSS and loaded with 3 μmol/L of Fluo-3-AM staining solution at 37°C for 30 min. Then, the mix was centrifuged and cells were collected. The collected cells were suspended in HBSS and assayed via flow cytometry (BD Accuri C6, USA).

### 2.7. Western Blot Analyses
Cells were collected, added to an ice cold cell lysis buffer with a fresh protease inhibitor cocktail, and incubated for 30 min on ice. The lysate was clarified via centrifugation at 12,000 rpm for 10 min at 4°C (Eppendorf, Centrifuge 5430R, Germany). The supernatant was collected, and the protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA).

Protein samples (50 μg) were loaded in each lane for western blotting. Calpain 1, caspase 12, and caspase 3 proteins were separated via 10% SDS-PAGE. The protein was run for approximately 1 hour, with a low voltage (75 V) for the stacking gel and a higher voltage (100 V) for the separating gel, until the dye front ran off the bottom of the gel. The protein was then electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with a 5% solution of nonfat milk powder and then incubated overnight with primary antibodies at 4°C. The primary antibodies were diluted as follows: anti-β-tubulin, 1:2,000; anti-calpain 1, 1:4,000; anti-caspase 12, 1:2,000; anti-caspase 3, 1:1,000. The next day, the membranes were washed with TBST (15 min, ×3) and incubated with an anti-rabbit IgG (H+L) secondary antibody (1:5,000) at room temperature for 2 h. The membranes were then washed with TBST (15 min, ×3). An ECL mix (the proportion of solutions A and B was 1:1) was prepared, and the membrane was incubated for 1-2 min to ensure that the ECL mix covered the membrane completely. The results were visualized using the gel imager system (Bio-Rad ChemiDoc MP, USA).

### 2.8. Calpain 1 Activity Measurement
Protein samples were obtained and the protein concentrations were measured as described for the western blot analyses. Calpain 1 activity was evaluated by measuring the hydrolysis of its substrate SLLVY-AMC, which has been described by previous studies [32, 33]. The steps followed the manufacturer’s instructions. The samples were detected at an Ex = 380 nm and Em = 460 nm (SpectraMax Paradigm, Multi-Mode Detection Platform, USA). Calpain 1 activity was expressed as nmol AMC released per hour of incubation time per milligram of protein.

### 2.9. Flow Cytometric Analysis of Apoptosis
Neutrophils were washed with PBS, suspended in 200 μL of binding buffer, and stained with 10 μL of Annexin V-FITC and 10 μL of propidium iodide (PI). After staining for 15 min in the dark, 500 μL of binding buffer was added. The samples were then assayed via flow cytometry (BD Accuri C6, USA). The excitation wavelength of the argon laser was set at 488 nm.

### 2.10. Statistical Analysis
SPSS 17.0 was used for statistical analyses. The SIRS scores are expressed as the mean ± 95% CI and were assessed using nonparametric tests. The Mann–Whitney U test was used for the analysis of two independent samples, and Kruskal–Wallis H tests were used for the analysis of multiple independent samples. Other dates are expressed as the mean ± standard. Unpaired Student’s t-tests were used for two-group comparisons, and one-way ANOVAs were used for multigroup comparisons.

### Table 1: Histological scoring for SAP

| Condition | Score | Description |
|-----------|-------|-------------|
| 0         | Absent|             |
| 1         |       | Diffuse expansion of interlobar septa |
| 2         |       | ++ diffuse expansion of interlobular septa |
| 3         |       | +++ diffuse expansion of interlobular septa |
| 4         |       | ++++ diffuse expansion of interlobular septa |
| 0         | Absent|             |
| 1         |       | Around ductal margins |
| 2         |       | In parenchyma (≤50% of lobules) |
| 3         |       | In parenchyma (51–75% of lobules) |
| 4         |       | In parenchyma (>75% of lobules) |
| 0         | Absent|             |
| 1         |       | Blood in parenchyma (≤25%) |
| 2         |       | Blood in parenchyma (26–50%) |
| 3         |       | Blood in parenchyma (51–75%) |
| 4         |       | Blood in parenchyma (>75%) |
| 0         | Absent|             |
| 1         |       | Periductal parenchymal destruction |
| 2         |       | Focal parenchymal necrosis (≤20%) |
| 3         |       | Diffuse loss of lobules (21–50%) |
| 4         |       | Severe loss of lobules (>50%) |
3. Results

3.1. SAP Model Assay. Pancreases in the SAP group presented with different degrees of damaged lobules, hemorrhaging, necrosis, and infiltration by neutrophils and monocytes (Figure 1(a)). The pathological scores in the SAP group were significantly higher than those in the SO group (Figure 1(b)). The levels of serum amylase, TNF-α, and IL-6 in the SAP group were 14.38 ± 1.93 (ng/mL), 493.43 ± 57.16 (ng/L), and 59.90 ± 8.45 (ng/L), respectively, which were significantly
higher than those in the SO group (Figures 1(c), 1(d), and 1(e)). The SIRS score for the SAP group was also significantly higher than that of the SO group (Figure 1(f)).

3.2. Effect of Emodin on Cytosolic Ca$^{2+}$. As shown in Figure 2, cytosolic Ca$^{2+}$ levels decreased in the SAP group compared with that in the SO group. It increased in the EM group and PE group compared with that in the SAP group.

3.3. Effect of Emodin on Calpain, Caspase, and Apoptosis. As shown in Figures 3(a)–3(e), each group showed a comparable level of calpain 1 protein expression, but calpain 1 activity varied. Calpain 1 activity decreased in rats with SAP and increased in emodin-treated rats. PDI50606 attenuated the effects of emodin. The same trend was observed for the changes in the expression levels of cleaved fragments of caspases 12 and 3.

As expected, rates of apoptosis in neutrophils were also different among the groups: they decreased in rats with SAP and increased in emodin-treated rats. PDI50606 attenuated the effects of emodin (Figure 4).

3.4. Effect of Emodin on SIRS Scores. The SIRS scores from each group mentioned above were calculated and are shown in Figure 5. The SIRS scores were obviously higher in the SAP group than in the SO group. Compared with those in the SAP group, the SIRS scores decreased in the EM group. The rats with SAP cotreated with emodin and PDI50606 exhibited a slight decrease in their SIRS scores.

4. Discussion

Although there are several ways to establish models of AP, retrograde infusions of taurocholate into the pancreatic duct is the primary method used to establish a rat model of necrotizing AP. Paszt et al’s study [34] suggests that infusing 3% taurocholate is sufficient to establish a rat model of severe hemorrhagic pancreatitis with SIRS. TNF-α is produced by activated macrophages. Its concentration in both tissues and serum correlates positively with the severity of pancreatic damage and inflammation [35]. IL-6 is derived from a wide range of cells, such as monocytes/macrophages, fibroblasts, and endothelial cells. The IL-6 level rises in patients with AP, and it correlates with the severity of AP [36]. IL-6 is able to predict the SIRS state, and both IL-6 and SIRS scores correlate with systemic complications [28]. In the present study, pancreases in the SAP group presented with different degrees of damaged lobules, hemorrhaging, necrosis, and infiltration by neutrophils and monocytes. The pathological scores in the SAP group were significantly higher than those in the SO group. The levels of serum amylase, TNF-α, and IL-6 in the SAP group were significantly higher than those in the SO group. The SIRS score for the SAP group was also significantly higher than that of the SO group. These results confirm that we successfully established a rat model of SAP with SIRS using 3% taurocholate. This method was repeatable and produced effects similar to those observed in clinical pancreatitis.

Our results showed that cytosolic Ca$^{2+}$ levels decreased in the SAP group compared with that in the SO group and it increased in the EM group and PE group compared with that in the SAP group. Cytosolic Ca$^{2+}$ is an important second messenger and is involved in many cell events, including apoptosis. Cytosolic Ca$^{2+}$ maintenance depends on extracellular calcium influx via voltage-gated Ca$^{2+}$ channels, or calcium release from organelles via the inositol 1,4,5-trisphosphate receptor (IP3R). Previous studies have reported that emodin could elevate intracellular Ca$^{2+}$ concentration through Ca$^{2+}$ influx from extracellular medium and Ca$^{2+}$ release from intracellular stores in smooth muscle cells [37, 38]. Similar to those studies, our results indicated that emodin raises cytosolic Ca$^{2+}$ levels in the neutrophils of rats with SAP. The different cytosolic Ca$^{2+}$ levels in neutrophils between the SO
and SAP groups suggested that the capability of neutrophils to regulate cytosolic Ca$^{2+}$ was suppressed when SAP with SIRS occurred. Emodin elevating cytosolic Ca$^{2+}$ levels suggested that emodin partly remedies the suppressed regulatory capability of neutrophils. According to previous literature [39], we inferred that emodin regulates cytosolic Ca$^{2+}$ in neutrophils mainly via IP3R in ER membranes.

The protein expression levels of calpain 1 in each group were not significantly different. However, protein activity was suppressed in the SAP group and upregulated in the EM group. The effect of emodin on calpain 1 activity was attenuated by PD150606 [3-(4-iodophenyl)-2-mercapto-(Z)-2-propenoic acid], which is a selective calpain inhibitor and binds to the Ca$^{2+}$-binding domain of calpain 1 or calpain 2 [40–42]. These results indicate that emodin does not regulate the protein expression of calpain 1 but has the ability to alter calpain 1 activity. Since PD150606 binds to the Ca$^{2+}$-binding domain of calpain 1 and attenuates the effect of emodin, the results suggest that emodin increases calpain 1 activity by elevating intracellular Ca$^{2+}$ levels.

The levels of cleaved fragments of caspases 12 and 3 were different in each group. They were decreased in the SAP group compared with those in the SO group and increased in the EM group compared with those in the SAP group. The increases of cleaved fragments of caspases 12 and 3 were attenuated in the PE group compared with those in the EM group.

Figure 3: Emodin did not alter protein expression levels of calpain 1 but did increase calpain 1 activity and alter the expression levels of cleaved fragments of caspases 12 and 3. (a) Calpain 1 activity was different in each group. It was decreased in rats with SAP and was increased in emodin-treated rats. PD150606 attenuated this effect of emodin. (b), (c), (d), and (e) Each group showed a comparable level of calpain 1 protein expression. However, the levels of cleaved fragments of caspases 12 and 3 were different in each group. They were decreased in the SAP group and increased in the EM group. PD150606 attenuated this effect of emodin. **P < 0.01 versus SO. ##P < 0.01 versus SAP. B P < 0.05 and BB P < 0.01 versus EM.
Figure 4: Emodin promoted neutrophil apoptosis, which was attenuated by PD150606. Rates of neutrophil apoptosis decreased in rats with SAP and increased in emodin-treated rats. PD150606 attenuated this effect of emodin. **P < 0.01 versus SO. ##P < 0.01 versus SAP. ###P < 0.01 versus EM.

Figure 5: Emodin reduced SIRS scores in rats with SAP. The SIRS scores were obviously high in the SAP group, but they decreased significantly after treatment with emodin. The rats in the PE group exhibited a slight decrease in SIRS scores compared with those in the EM group. **P < 0.01 versus SO. ##P < 0.01 versus SAP. ###P < 0.01 versus EM.

Such results indicated that the active fragments of caspases 12 and 3 change according to calpain 1 activity. As calpain 1 activity decreased in the SAP group, the levels of the active fragments of caspases 12 and 3 decreased. In addition, since calpain 1 activity was increased by emodin, the levels of the active fragments of caspases 12 and 3 increased. Furthermore, as calpain 1 activity was attenuated by PD150606 in the PE group, the levels of the active fragments of caspases 12 and 3 were attenuated. These results confirm that calpain cleaves caspase 12 and promotes caspase 12 activation and that the activation of caspase 12 results in the activation of caspase 3.

The degree of apoptosis in the SAP group was lower than that in the SO group. In addition, the SIRS scores were markedly higher in the SAP group than those in the SO group. This phenomenon suggests that neutrophil apoptosis is delayed in SAP with SIRS. The pathophysiological mechanisms responsible for SIRS are not fully understood. Cytokines are considered to be involved in the occurrence and progression of SIRS. Activated neutrophils induce macrophages to release inflammatory cytokines, amplifying the inflammatory response and increasing the risk of developing MODS [43]. Therefore, it can be concluded that delayed neutrophil apoptosis results in SIRS. A study by Jimenez et al. [11] supports this, reporting that large numbers of activated neutrophils or delayed neutrophil apoptosis are associated with the pathogenesis of SIRS.

As mentioned above, many studies have shown that emodin promotes apoptosis in cancer cells. However,
whether emodin promotes apoptosis in neutrophils remains unknown. The results of the present study showed that rates of neutrophil apoptosis decreased in rats with SAP and increased in emodin-treated rats. Such results support that emodin promotes neutrophil apoptosis. In line with previous results, our results support the hypothesis that emodin increases cytosolic Ca\(^{2+}\) levels, leading to the induction of neutrophil apoptosis via the Ca\(^{2+}\)-calpain 1-caspase 12-caspase 3 signaling pathway.

Compared with those in the SAP group, the SIRS scores decreased in the EM group. The rats with SAP cotreated with emodin and PD150606 exhibited a slight decrease in their SIRS sores. Therefore, we conclude that emodin has protective effects against SIRS in rats with SAP and that these effects depend on emodin-induced neutrophil apoptosis via the Ca\(^{2+}\)-calpain 1-caspase 12-caspase 3 signaling pathway. Protective effects of emodin on SAP were also confirmed by Wu et al. [44] and Yao et al. [45]. According to their studies, emodin promotes neutrophil apoptosis. In line with previous results, our results support the hypothesis that emodin promotes apoptosis in neutrophils. Increased cytosolic Ca\(^{2+}\) levels, calpain 1 is activated by increased cytosolic Ca\(^{2+}\) levels, activated calpain 1 cleaves caspase 12 localized to the ER, and the activation of ER-resident caspase 12 causes the activation of cytoplasmic caspase 3.

5. Conclusions

Delayed neutrophil apoptosis causes SIRS in rats with SAP and emodin-induced neutrophil apoptosis protects rats with SAP against SIRS. The mechanism underlying this effect includes the following steps: emodin increases cytosolic Ca\(^{2+}\) levels, calpain 1 is activated by increased cytosolic Ca\(^{2+}\) levels, activated calpain 1 cleaves caspase 12 localized to the ER, and the activation of ER-resident caspase 12 causes the activation of cytoplasmic caspase 3.

Competing Interests

The authors declare no competing interests.

Acknowledgments

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