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Bidirectional Effects of Pyrrolidine Dithiocarbamate on Severe Acute Pancreatitis in a Rat Model

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Abstract

Introduction: The mechanism by which intestinal mucosal barrier is damaged in severe acute pancreatitis (SAP)-associated impairment is not fully understood.

Methods: We established an L-arginine-induced SAP rat model, pretreated with or without pyrrolidine dithiocarbamate (PDTC). Hematoxylin and eosin staining was performed to evaluate the pathological alterations. Western blotting was conducted to detect the expression of autophagy-related proteins. Oxidative stress was assessed by the levels of malondialdehyde and superoxide dismutase.

Results: We found significant injury of the intestinal mucosal barrier in SAP rats, with overexpression of Beclin-1, LC3, and p65. Pyrrolidine dithiocarbamate showed a bidirectional effect in protecting SAP rats. A high dose of PDTC aggravated disease in rats, while a low or medium dose of PDTC pretreatment, was able to alleviate tissue damage. Pyrrolidine dithiocarbamate changed the expression of Beclin-1, LC3, and p65 in the intestines. The fatty acid-binding protein level was increased in SAP rats with high-dose PDTC or without PDTC pretreatment and was reduced in SAP rats with low- or medium-dose PDTC exposure.

Conclusions: Autophagy is involved in the impairment of intestinal mucosal barrier during SAP. A suitable dose of PDTC (1 or 10 mg/kg) may decrease the severity of SAP by inhibiting autophagy in intestinal mucosal cells.

Keywords

autophagy, intestinal mucosal barrier, pancreatitis, inflammation, oxidative stress

Introduction

Pancreatitis is initiated by activation of trypsin within the pancreas, resulting in further activation of various proteases that can damage cells. Acute pancreatitis is commonly induced by gallstones and alcohol, although drugs and certain genetic mutations can also cause this intractable disease.[1] Acute pancreatitis is characterized by edema, hemorrhage, and necrotic inflammation of different severities. Patients suffering from this disease can present with abdominal pain, nausea and vomiting, and fever. An elevated serum level of amylase is the most common sign of acute pancreatitis and is very informative for diagnosis. The prognosis of acute pancreatitis is highly dependent on the severity. Mild acute pancreatitis only displays pancreatic edema and patients had a good prognosis. Severe acute pancreatitis (SAP), however, may have hemorrhage and necrosis in pancreas, leading to secondary abdominal infection, peritonitis, and even shock. The mortality of SAP patients can

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be high. In clinic, anti-inflammation and supportive care are main strategies for the treatment of SAP. Thus, more detailed mechanisms of SAP are urgently needed to develop a novel treatment.

It has been reported that intestinal mucosa was damaged in case of SAP. The epithelial cells of intestinal mucosa become denatured and necrotic, leading to ischemia and reperfusion of the intestine, dysregulation of intestinal microbiota, and increased permeability of mucosal barrier. These pathological alterations can further induce bacteria and toxin translocation and inflammatory cytokine release, which change SAP from a local disease to a systemic disease. However, the mechanisms of disturbance of intestinal mucosa barrier are far from understood. Previous studies have shown enhanced apoptosis of intestinal epithelial cells in SAP. Autophagy, also known as type II programmed death, is different with but closely connected to apoptosis.[2] In this study, we aimed to investigate whether autophagy plays a role in SAP-related death of intestinal epithelial cells.

Autophagy is a cellular process exerted by lysosomes and is essential for cell survival, differentiation, and homeostasis.[3] The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling is deeply involved in the regulation of autophagy, and a complicated crosstalk between NF-κB signaling and autophagy has been revealed.[4] Although in many cases, inhibition of NF-κB signaling showed induction of autophagy,[5,6] pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor, was reported to be able to suppress autophagy.[7] In addition, using a taurocholate-induced acute necrotizing pancreatitis rat model, Yang and colleagues proved that NF-κB signaling stimulated autophagy in the pancreas.[8] However, in the background of SAP, the interplay between NF-κB signaling and autophagy was less studied, especially in other organs injured by SAP.

Here, we used a rat model to study the autophagy of epithelial cells in terminal ileum. The associations among autophagy, intestinal mucosa permeability, and oxidative stress under PDTC treatment were demonstrated in this study.

Materials and Methods

Animal Model

Sprague-Dawley rats ($\sim$2 months old) weighing 250 $\pm$ 30 g were obtained from Animal Center of Hebei Medical University. Rats were cultured for at least 1 week before subjected to experiments. Rats were randomly assigned to 5 groups with 24 rats per group. The normal control group was cultured normally with only necessary vehicle (normal saline) treatment. The SAP group was peritoneally injected with 20% L-arginine (2.5 g/kg) for twice, with an interval of 1 hour between 2 injections. The PDTC groups were peritoneally pretreated with PDTC (Sigma-Aldrich, St. Louis, Missouri, USA), followed by L-arginine injection for twice 1 hour later. Pyrrolidine dithiocarbamate groups contained high-dose group (P100, with 100 mg/kg PDTC), medium-dose group (P10, with 10 mg/kg PDTC), and low-dose group (P1, with 1 mg/kg PDTC). Six mice in each group were scarified and analyzed after 12 hours of the second L-arginine injection, and the left 6 mice in each group were analyzed after another 12 hours. The protocol for animal experiments was approved by the ethical committee of Hebei Medical University.

Hematoxylin and Eosin Staining

The histological analysis was performed as previously described.[8] In brief, formalin-fixed paraffin-embedded tissue was cut into 4-μm thick sections. The sections were then stained with hematoxylin and eosin (H&E) using a standard method. The slides were blindly reviewed by an experienced pathologist.

Western Blotting

Intestine tissues (100 mg) were collected after 12 or 24 hours of L-arginine exposure. Then, they were cut into small pieces on ice and washed twice with cold phosphate buffered saline (PBS). Tissue was homogenated in 1 mL RIPA buffer (Thermo Fisher Scientific, Waltham, Massachusetts) for 15 minutes on ice, and the supernatant was collected by centrifugation. Total proteins were quantified using the bicinchoninic acid assay (Thermo Fisher Scientific). A total of 50 μg proteins per sample were subjected to electrophoresis using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to a polyvinylidene fluorid membrane. The membrane was blocked with nonfat milk at room temperature for 1 hour and was incubated with indicated primary antibodies (1:1000 dilution) at 4°C overnight. After washing with PBS with Tween 20 3 times, the membrane was further incubated with appropriate secondary antibodies (1:2000 dilution) at room temperature for 2 hours. Following another 5 washes, enhanced chemiluminescent western blotting substrate (Thermo Fisher Scientific) was added to the membrane. β-actin was used as a control. Beclin-1, LC3, p65, and β-actin primary antibodies were purchased from Cell Signaling Technology (Danvers, Massachusetts). Photodensity of bands was evaluated using Image J (NIH, Bethesda, Maryland) and was normalized to that of β-actin.

Fatty Acid-Binding Protein Assay

Peripheral blood was taken from rats, and serum was isolated. The fatty acid-binding protein (FABP) level was detected using Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions.

Superoxide Dismutase and Malondialdehyde Detection

Intestine tissue homogenate was acquired after 12 or 24 hours of L-arginine exposure as mentioned earlier. The superoxide dismutase (SOD) and malondialdehyde (MDA) levels were detected using the SOD Assay Kit and the Lipid Peroxidation Assay Kit (both from Sigma-Aldrich), respectively, according to the manufacturers' instructions.

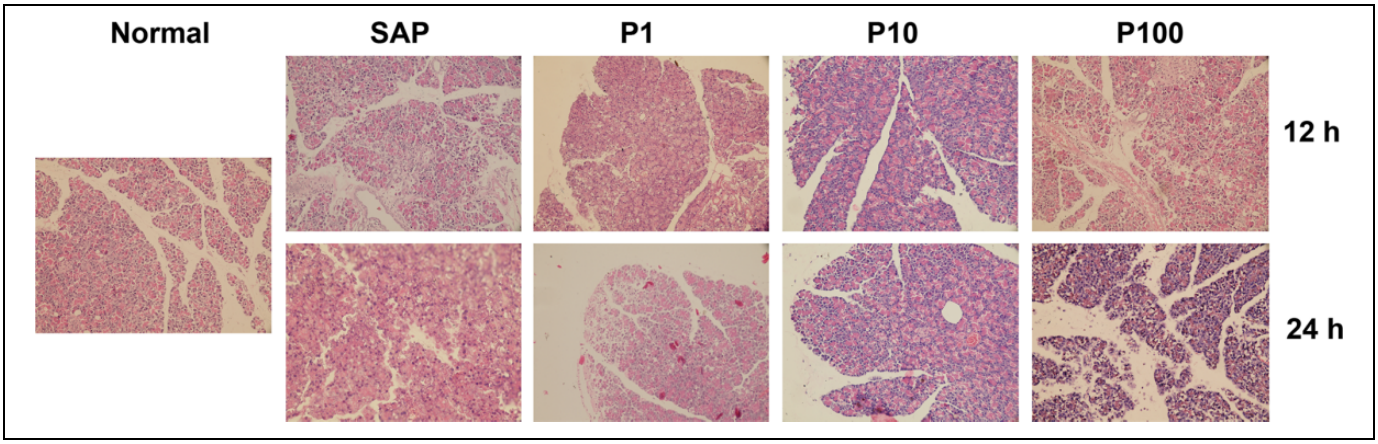

Figure 1. Histology of pancreas. Rats were treated as indicated. After 12 or 24 hours of L-arginine exposure, the pancreas was collected and the pathological changes were evaluated.

Statistical Analysis

All data are presented as the mean (standard deviation). Multiple group comparison was performed using one-way analysis of variance. Comparison of different time points in mice with the same treatment was conducted using unpaired Student's *t*-test. Statistical analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, Illinois). $P < .05$ was considered as statistically significant.

Results

Pyrrolidine dithiocarbamate Ameliorates L-Arginine-Induced Pancreatic Damages in Rats

Intraperitoneal administration of L-arginine is a well-established methodology for induction of experimental SAP in animals.[9] After 12 hours of L-arginine injection, we observed pancreatic edema with bleeding spots on the surface of pancreas in rats. Ascites was found in some rats merely 12 hours of L-arginine exposure. At 24 hours post injection of L-arginine, these observations became more significant, and bloody ascites were noticed. These findings were typical characteristics after administration of L-arginine.[10] However, in SAP rats with low- and medium-dose of PDTC pretreatment, pancreatic edema and bleeding were ameliorated, and these signs were similar at 12 and 24 hours of L-arginine treatment. Unexpectedly, the rats that received high-dose PDTC injection also had severe pancreatic injury, showing edematous, hemorrhagic, and jelly pancreas. Such injury even aggravated in rats exposed to L-arginine for 24 hours than that for 12 hours. Consistent with the gross observations, rats without PDTC pretreatment and with high-dose PDTC pretreatment showed edema and necrosis of acinars with infiltration of inflammatory cells under microscopic examination (Figure 1). In addition, lack of pancreatic lobules and occasional massive necrosis were observed. Severe pathological alterations were found in rats with long-term treatment of PDTC (24 hours vs 12 hours), showing fat necrosis and isolated acinars. While in rats that received low- and medium-dose PDTC pretreatment, these pathological changes were largely alleviated, especially after 24 hours of L-arginine exposure. Altogether, these results suggested that a suitable dose of PDTC was able to improve pancreatic injury induced by L-arginine in rats. An improperly high dose of PDTC was toxic to animals.

Pyrrolidine dithiocarbamate Improves Intestinal Damage in SAP Rats

To investigate whether PDTC can influence intestines in case of SAP, we first examined the pathological alterations of intestines in the rats. In contrast to the healthy manifestations of intestines in control rats, SAP rats displayed intestinal pneumatosis. Hematoxylin and eosin staining of rat intestines showed damage of intestinal villi and loss of epithelial cells (Figure 2). Furthermore, congestion, edema, and inflammation were observed in the intestinal lamina propria. All these pathological changes were severe in rats with long-term L-arginine exposure than those in rats with short-term exposure. Intriguingly, rats in the P100 group showed similar intestinal alterations with SAP rats. However, rats in the P1 and P10 groups had significantly less damage compared to rats in the SAP and P100 groups, and no significant difference was found between rats with long- and short-term of L-arginine exposure.

Fatty acid-binding protein was reported to be correlated with gut dysfunction and could be used for evaluating the severity of SAP in patients.[11,12] We then tested serum level of FABP in rats. As expected, FABP level of the SAP group was much higher than that of the control group (Figure 3). Consistently, the P100 group also had an elevated FABP level, which was comparable to that of the SAP group. Of note, the FABP level in both P1 and P10 groups was reduced compared to that of the SAP group, although it was still significantly higher than that of the control group. At 12 hours after L-arginine exposure, FABP level was similar between the P1 and P10 groups; however, at 24 hours after L-arginine exposure, the P10 group showed a lower FABP level than the P1 group. In addition, long-term exposure of L-arginine induced a higher

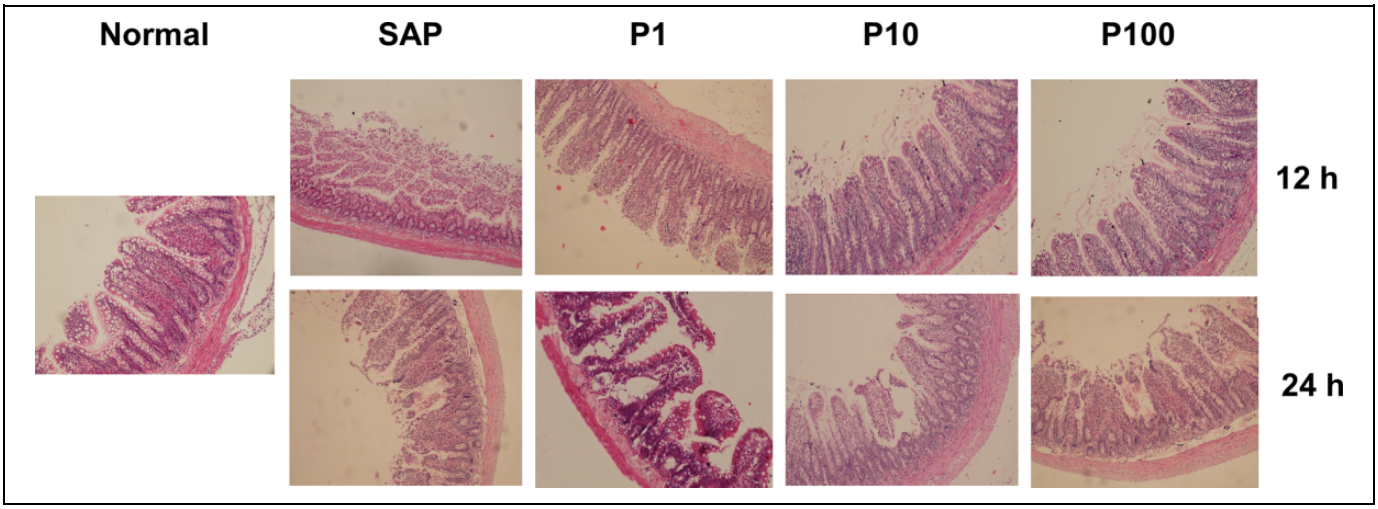

Figure 2. Histology of intestine. Rats were treated as indicated. After 12 or 24 hours of L-arginine exposure, the intestine was collected and the pathological changes were evaluated.

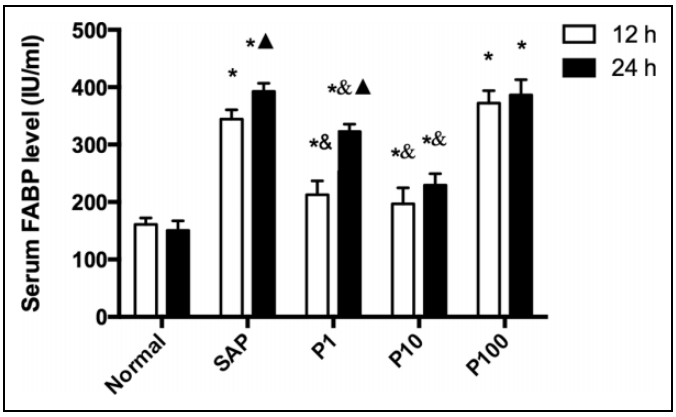

Figure 3. The FABP level in different groups. Rats were treated as indicated. After 12 or 24 hours of L-arginine exposure, serum FABP level in rats was determined. *, $P < .05$ as compared with the normal control group at the same time point; &, $P < .05$ as compared with the SAP group at the same time point; ▲, $P < .05$ as compared to 12 hours in the same group. N = 12. FABP indicates fatty acid-binding protein.

FABP level in both SAP and P1 groups compared to short-term exposure. These findings suggested that though low- and medium-dose PDTC pretreatment partially reserved gut function, the medium dose of PDTC showed better effects in preventing disease aggravation.

Pyrrolidine dithiocarbamate Reduces p65 Expression in the Intestine of SAP Rats

Since PDTC is a known NF-κB inhibitor[13] and we showed that PDTC reduced intestinal damage in SAP rats, we thus hypothesized that NF-κB might play a role in SAP-associated intestinal damage. We detected overexpression of p65 in the intestine tissue from SAP and P100 rats compared to that from the control rats (Figure 4A and B). Pyrrolidine dithiocarbamate significantly decreased p65 expression in P1 and P10 groups, and p65 expression in P10 group was even lower than that in P1 group. Interestingly, while p65 expression was higher in long-term L-arginine exposure compared to short-term exposure in SAP, P1, and P100 groups, no significant difference of p65 expression was detected in P10 rats between 12 and 24 hours after L-arginine treatment. Thus, PDTC was likely to function by inhibiting p65 overexpression induced by L-arginine, although a high dose of PDTC could cause more p65 expression.

Pyrrolidine dithiocarbamate Inhibits SAP-Associated Autophagy in Rat Intestines

To study the roles of autophagy in SAP-associated intestinal injury, we investigated the expression of 2 autophagic markers Beclin-1 and LC3.[14] In general, both proteins were induced by L-arginine and a high dose of PDTC and were partially decreased by low- and medium-dose PDTC pretreatment (Figure 4A, C and D). A slight difference was that LC3 expression was further upregulated in long-term exposure of L-arginine compared to that in short-term L-arginine exposure. No time-dependent Beclin-1 expression was observed, except for the P100 group. Thus, PDTC could limit autophagic activity in the intestines of SAP rats.

Pyrrolidine dithiocarbamate Enhances Anti-Oxidative Activity in the Intestines of SAP Rats

Oxidative stress has crosstalk with both autophagy and the NF-κB signaling.[15,16] We found that L-arginine exposure with or without a high dose of PDTC pretreatment dramatically increased MDA in intestinal homogenate (Figure 5A). In parallel, the level of SOD, a critical anti-oxidant enzyme, greatly reduced in SAP and P100 rats (Figure 5B). In the presence of low- or medium-dose PDTC, the changes of MDA and SOD levels were partially reversed. Noticeably, the medium-dose PDTC showed the best performance in control of oxidative stress because no further elevation of MDA level was detected

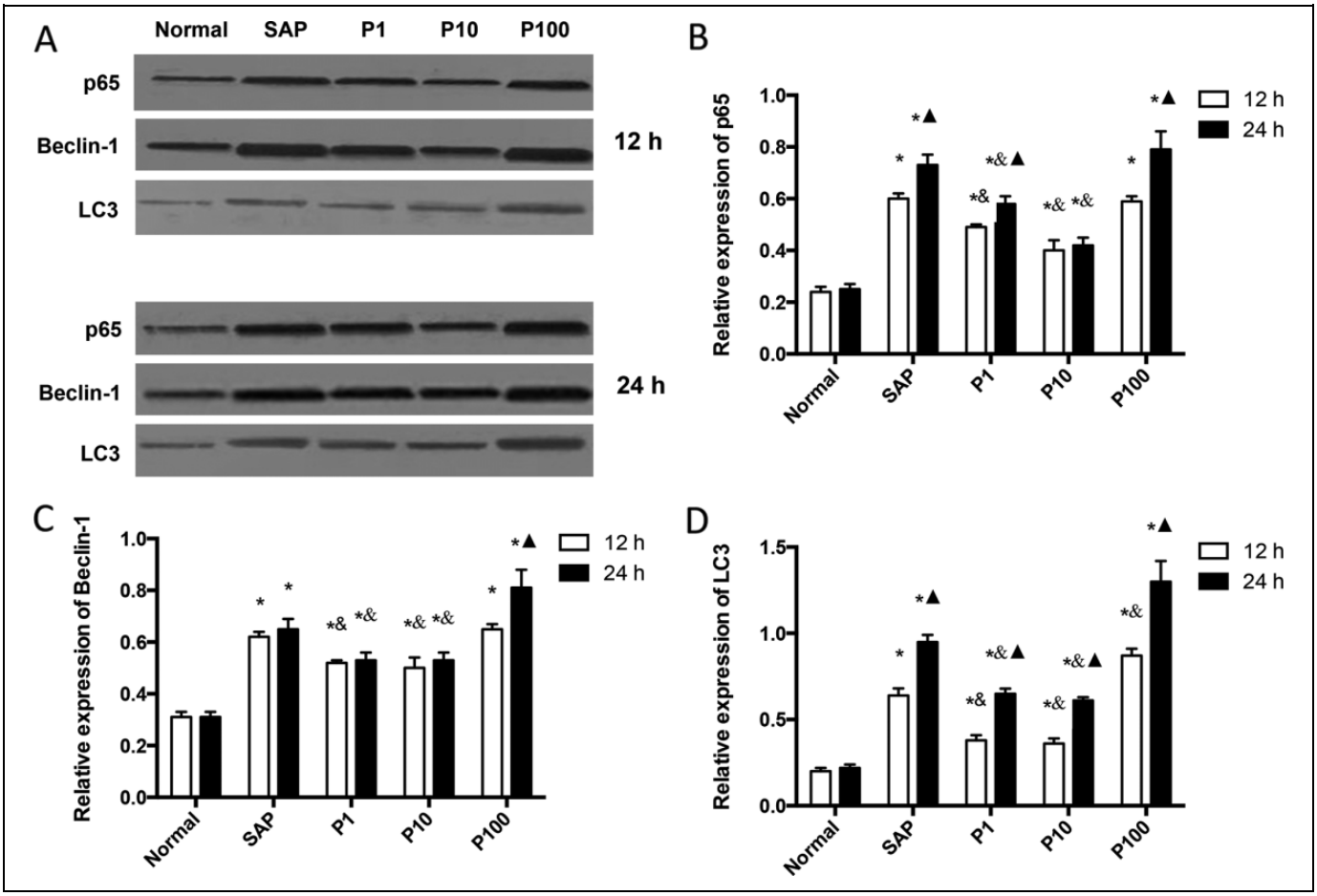

Figure 4. Changes of p65 and autophagy-associated proteins in different groups. A, Representative bands of western blotting. B-D, Statistical analysis of western blotting bands for each protein. *, $P < .05$ as compared to the normal control group at the same time point; &, $P < .05$ as compared with the SAP group at the same time point; ▲, $P < .05$ as compared with 12 hours in the same group. N = 12. SAP indicates severe acute pancreatitis

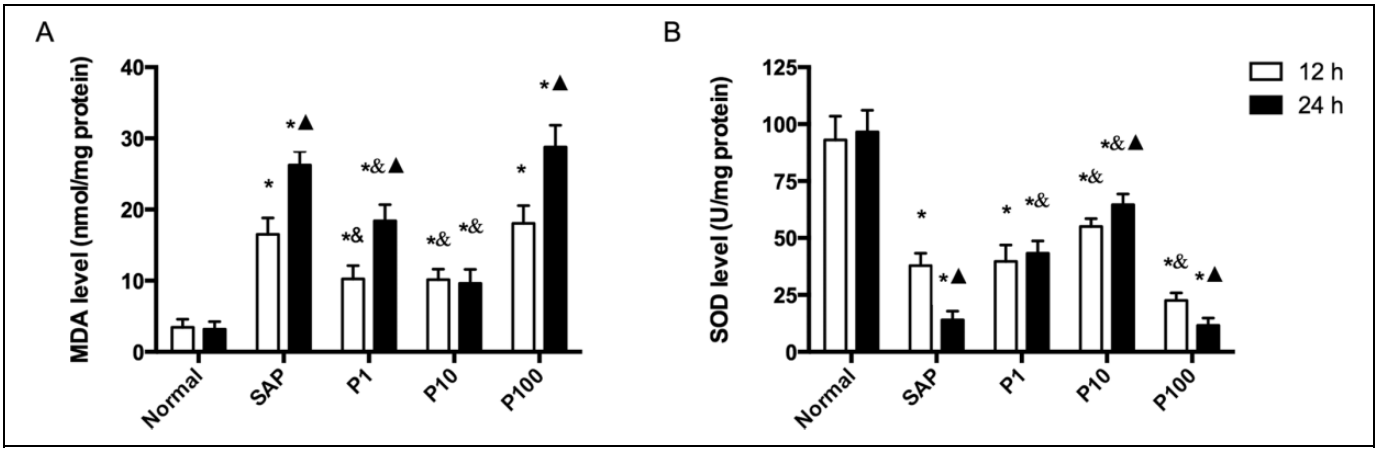

Figure 5. Oxidative stress was evaluated in the guts of rats. The levels of (A) MDA and (B) SOD were determined in the indicated groups. *, $P < .05$ as compared to the normal control group at the same time point; &, $P < .05$ as compared with the SAP group at the same time point; ▲, $P < .05$ as compared to 12 hours in the same group. N = 12. MDA indicates malondialdehyde; SOD, superoxide dismutase; SAP, severe acute pancreatitis.

from 12 to 24 hours after L-arginine treatment. These results implicated that PDTC might alleviate intestinal damage in SAP rats by inhibiting oxidative stress.

Discussion

The close relationship between intestine and pancreas in pancreatitis can be explained by both anatomic and functional reasons. Translocation of bacteria within the intestine is considered a second hit following SAP, leading to multiorgan dysfunction. However, how pancreatitis induces bacterial translocation has not been fully understood. It was well-known that increased permeability of intestinal mucosal barrier can greatly increase the possibility of bacterial translocation. Thus, understanding the mechanism by which SAP causes impairment of intestinal mucosal barrier is important to develop novel strategy to treat SAP.

Many efforts have been put to answer this question and tried to design therapeutic strategy. For example, high-mobility group box 1 (HMGB1)-induced toll-like receptor (TLR) 4 and TLR9 in ileum contributed to the dysfunction of intestinal mucosal barrier, and anti-HMGB1 displayed a significant preventive role in an SAP mouse model.[17] Increased NF-κB and decreased occludin, an intestinal tight junction protein, also contributed to impairment of intestinal barrier function during SAP, and inhibition of NF-κB mitigated intestinal damage and inflammation.[18] High-mobility group box 1 is a classic damage-associated molecular pattern (DAMP) and causes sterile inflammation.[19] Therefore, local inflammation caused by DAMPs and bacteria during SAP is important for the impairment of intestinal mucosal barrier. To this end, PDTC can be reasonably applied to protect SAP rats.

Most existing literatures indicated apoptosis as the main type of death for intestinal mucosal cells during SAP.[20,21] However, few studies have been focused on the autophagy of intestinal mucosal cells in case of SAP. A recent report showed that autophagy was activated in the intestinal epithelial cells.[22] The authors demonstrated that a high level of LC3 decreased the risk of bacterial translocation with a healthier intestinal barrier. Although we found a similar overexpression of LC3 in our rat model, we, on the contrary, showed a harmful rather than protective role of autophagy in intestinal mucosal barrier. Our conclusion was supported by the fact that inactivation of autophagy using PDTC alleviated injury of intestinal mucosal barrier. It is well known that autophagy has physiological roles in the maintenance of intestinal homeostasis by promoting cellular survival.[23] Intriguingly, the effects of autophagy inactivation were highly dependent on the dosage of PDTC. A high dose of PDTC (100 mg/kg) did not alleviate and even worse the impairment of intestinal mucosal barrier. This bidirectional effect of PDTC coincided with the distinctive roles of physiological and pathological autophagy. We thus speculate that a suitable level of autophagy is critical for the homeostasis of intestinal mucosal cells, and an increased level of autophagy in these cells is induced by SAP. When a low or medium dose of PDTC is used, overactivated autophagy can be properly weakened to the physiological level; however, in the presence of high-dose PDTC administration, autophagy may be completely inhibited, resulting in decreased survival of intestinal mucosal cells. Our present study showed that 10 mg/kg PDTC displayed the best performance in inhibiting autophagy and protecting intestinal mucosal integrity. The bidirectional efficacy of PDTC also implicates that the therapeutic window of PDTC, or other autophagy inhibitors, needs to be carefully explored in humans. In addition, the underlying effects and mechanisms of enhanced autophagy in intestinal mucosal cells during SAP are still elusive and need further investigation.

In agreement with previous studies, we demonstrated increased oxidative stress in the guts of SAP rats. For example, Deng et al reported that the NADPH oxidase inhibitor apocynin attenuated intestinal barrier dysfunction using a sodium taurocholate-induced SAP rat model.[24] Oxidative stress including reactive oxygen species and reactive nitrogen species is considered the converging point of autophagy-inducing stimuli.[15] However, an improperly high level of oxidative stress harms intestinal mucosal barrier since antioxidative materials can alleviate the impairment of gut barrier.[24,25] Since NF-κB induces oxidative stress generation,[16] the NF-κB inhibitor PDTC is able to function as an antioxidative stress agent.[26,27] In fact, PDTC was reported to improve the survival of taurocholate-induced SAP rats.[28] Additionally, recently some investigators showed preliminary results of improved intestinal mucosal barrier with treatment of 40 mg/kg PDTC.[18] We used pretreatment of PDTC ranging from 1 to 100 mg/kg and found a bidirectional effect of PDTC. More importantly, we noticed simultaneous alterations of NF-κB expression and MDA and SOD levels. Our results suggested that inhibition of NF-κB by a suitable dose of PDTC was probable to protect SAP-associated intestinal injury by suppressing autophagy and oxidative stress. Nevertheless, gut is not the only organ that impaired during SAP. Interestingly, PDTC also reduced SAP-associated lung injury by inhibition of NF-κB.[29] However, whether autophagy plays a role in such process is unclear.

Conclusions

We used an L-arginine-induced SAP rat model to demonstrate that autophagy is upregulated in the intestines of rats. A suitable dose of PDTC (1 or 10 mg/kg) could alleviate intestinal impairment by inhibiting autophagy. Regulating autophagy may decrease the risk of secondary multiple organ dysfunction syndrome in patients with SAP.

Author Contribution

Huan Yang and ShuCan Ma contributed equally.

Declaration of Conflicting Interests

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