Resolvin D1 protects against inflammation in experimental acute pancreatitis and associated lung injury

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Acute pancreatitis is an inflammatory condition that may lead to multisystemic organ failure with considerable mortality. Recently, resolvin D1 (RvD1) as an endogenous anti-inflammatory lipid mediator has been confirmed to protect against many inflammatory diseases. This study was designed to investigate the effects of RvD1 in acute pancreatitis and associated lung injury. Acute pancreatitis varying from mild to severe was induced by cerulein or cerulein combined with LPS, respectively. Mice were pretreated with RvD1 at a dose of 300 ng/mouse 30 min before the first injection of cerulein. Severity of AP was assessed by biochemical markers and histology. Serum cytokines and myeloperoxidase (MPO) levels in pancreas and lung were determined for assessing the extent of inflammatory response. NF-κB activation was determined by Western blotting. The injection of cerulein or cerulein combined with LPS resulted in local injury in the pancreas and corresponding systemic inflammatory changes with pronounced severity in the cerulein and LPS group. Pretreated RvD1 significantly reduced the degree of amylase, lipase, TNF-α, and IL-6 serum levels; the MPO activities in the pancreas and the lungs; the pancreatic NF-κB activation; and the severity of pancreatic injury and associated lung injury, especially in the severe acute pancreatitis model. These results suggest that RvD1 is capable of improving injury of pancreas and lung and exerting anti-inflammatory effects through the inhibition of NF-κB activation in experimental acute pancreatitis, with more notable protective effect in severe acute pancreatitis. These findings indicate that RvD1 may constitute a novel therapeutic strategy in the management of severe acute pancreatitis.

acute pancreatitis; resolvin D1; inflammation; nuclear factor-κB; lung injury

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Omega-3 polyunsaturated fatty acids (n-3 PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are enriched in some fish oils, are believed to exert beneficial effects on a wide range of inflammatory disorders (30, 40), including acute pancreatitis (1, 24). EPA and DHA originate the lipid mediators known as resolvins, which regulate critical cellular events in the resolution of inflammation (32, 33). Resolvin D1 [7S, 8R, 17S-trihydroxy DHA (RvD1)] is one of the resolvins and is derived from DHA (34). RvD1 can inhibit neutrophil activation (16, 38), regulate cytokines (18, 39), and inhibit the activation of NF-κB pathway in endotoxin (lipopolysaccharide, LPS)-induced inflammatory response (4, 6, 18, 21, 42). It has also been identified to reverse chronic pancreatitis-induced chronic pain (27).

Overall, these observations prompted us to hypothesize that RvD1 may have protective effects on acute pancreatitis by suppressing inflammatory response. To test this hypothesis, we induced pancreatitis in mice, producing different degrees of severity by repeated injections of cerulein with or without LPS. Local injuries of pancreas and lung were assessed by established parameters, and systemic inflammation was determined through assaying the serum TNF-α and IL-6 levels and my-
eloperoxidase (MPO) activity. The effects of RvD1 on inflammatory response in acute pancreatitis were studied in detail. Our findings provide a novel therapeutic approach for anti-inflammatory treatments in acute pancreatitis.

MATERIALS AND METHODS

Animals and reagents. Adult male C57BL/6 mice (20–25 g) were obtained from the Animal Centre of Sichuan University (Chengdu, China), maintained on a 12-h light-12-h dark cycle at 22°C, given water ad libitum, fed standard laboratory chow, and allowed to acclimatize for a minimum of 1 wk. Mice were randomly assigned to control or experimental groups. All experiments were conducted with the approval of the Animal Research Committee at Sichuan University. Cerulein and LPS were purchased from Sigma Chemical (Sigma-Aldrich, St. Louis, MO). RvD1 was purchased from Cayman Chemical (Ann Arbor, MI). Antibodies against NF-κB p65 subunit and histone H3.1 were purchased from Cell Signaling Technology (Danvers, MA). Other items were purchased from standard suppliers or as indicated in text.

Induction of experimental pancreatitis. For cerulein pancreatitis, C57BL/6 mice were treated by seven hourly intraperitoneal injections of cerulein (50 μg·kg⁻¹·h⁻¹). A more severe acute pancreatitis model was induced by administration of cerulein in combination of LPS (20): mice were injected intraperitoneally with cerulein in the same way as those in the cerulein acute pancreatitis model except that LPS was added (10 mg/kg) with the last injection of cerulein. Controls received comparable injections of normal saline. RvD1 (dose of 300 ng/mouse ip based on preliminary data) was administered to the mice 30 min before the first injection of cerulein. Mice were killed 8 and 24 h after the first injection of cerulein. RvD1 therapeutic treatment group, RvD1 (dose of 300 ng/mouse ip) was administered to the mice 4 h after the last injection of cerulein. Mice were killed 24 h after the first injection of cerulein.

Histological examination. Fresh specimens of murine pancreas and lung were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Tissues were embedded in paraffin, and 5-mm sections were processed for hematoxylin-eosin staining by standard procedures. Then multiple randomly chosen microscopic fields from at least three mice in each group were examined by two pathologists in a blinded
manner. For pancreatic injury, the scoring was on a scale of 0–3 (0 being normal and 3 being severe) according to four items: presence of vacuolization, interstitial edema, interstitial inflammation, and number of acinar cell necroses, as previously described (17). For lung injury, vacuolization, interstitial edema, interstitial inflammation, and number being normal and 3 being severe) according to four items: presence of alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in air space or the vessel wall, and thickness of the alveolar wall/hyaline membrane formation, as previously described (14).

Measurement of amylase and lipase. Serum amylase and lipase were determined by means of a commercially available kit (R&D Systems, Minneapolis, MN), and expressed as units per liter.

Measurement of cytokines. The proinflammatory cytokines TNF-α and IL-6 in serum were measured by using a Luminex assay kit according to the manufacturer’s instructions (R&D Systems). Assays were performed in duplicate using the Luminex 100 System (Austin, TX) Digital images of the bead array were captured following laser excitation and were processed on a computer workstation. Standard curves and reports of unknown samples were prepared with Master QT software (MiraBIO, Alameda, CA).

Measurement of MPO activity. The extent of neutrophil infiltration was measured in both pancreatic and lung tissue by quantifying MPO activity as previously described (35). The enzyme activity was determined by using an MPO detection kit according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activity was expressed as units per milligram of wet tissue and calculated as percent of control as previously described (20).

Western blot analysis. Pancreatic tissue samples collected at 8 h after first injection were homogenized, and nuclear protein was extracted separately by using the Nuclear Protein Extraction Kit (Viagene Biotech, Ningbo, China) according to the manufacturer’s instructions. The concentrations of protein were determined by the BCA method (Pierce, Rockford, IL). Each 20 μg aliquot of protein was loaded in a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After complete protein transfer, the membranes were blocked with 5% milk powder solution for 1 h at room temperature and incubated at 4°C overnight with rabbit monoclonal anti-NF-κB p65 subunit diluted at a 1:1,000 dilution in 5% milk powder solution. For internal reference, a rabbit monoclonal anti-histone H3.1 antibody (1:1,000 dilution) was used. After washing the membranes, we applied goat polyclonal anti-rabbit immunoglobulin G secondary antibody (Cell Signaling Technology) conjugated to horseradish peroxidase in a 1:5,000 dilution and incubated for 1 h at room temperature. Finally, antibody binding was visualized by using the enhanced chemiluminescence system (Pierce).

Statistical analysis. Data are expressed as means ± SE. All data were analyzed by one-way ANOVA with a posttest analysis (Newman-Keuls). In all cases, a P value of < 0.05 was selected as criterion for statistical significance.

RESULTS

Effects of RvD1 on the severity of experimental acute pancreatitis. We evaluated the severity of experiment pancreatitis through histological score based on the extent of tissue edema, vacuolization, inflammation, and necrosis. The pancreas histological picture was normal in both control and RvD1 alone-treated mice. Cerulein-treated mice displayed histological signs of acute pancreatitis characterized by interstitial edema, vacuolization, and infiltration of neutrophil and mononuclear cells with little parenchymal necrosis and hemorrhage. The treatment of cerulein in combination with LPS caused more severe pathological changes in the pancreatic tissue, with an obvious edema, inflammation, vacuolization, and, in many local acinar cells, necrosis (Fig. 1). In contrast, treatment with RvD1 significantly reduced the morphological changes seen in both models of acute pancreatitis and decreased the severity of experimental acute pancreatitis (Fig. 2D). Some parameters used to quantify the severity of acute pancreatitis were also measured, such as amylase and lipase. Low levels of serum amylase and lipase activity were evidenced in control and RvD1 alone-treated mice. Injection of cerulein with or without LPS enhanced serum amylase and lipase activity compared

![Fig. 3. Effects of RvD1 on inflammatory cytokines in experimental acute pancreatitis. The inflammatory cytokines TNF-α (A) and IL-6 (B) in the serum were determined at 8 and 24 h after the first injection of cerulein by Luminex assay. Results are expressed as means with the SE of at least 3 separate experiments with statistical significance at *P < 0.05.](http://ajpgi.physiology.org/)

![Fig. 4. Effects of RvD1 on NF-κB activation in experimental acute pancreatitis. The activation of NF-κB was determined by detecting the levels of NF-κB p-65 subunit expression (B) in pancreatic tissue at 8 and 24 h after the first injection of cerulein was shown. Data are expressed as percentage of the mean control value. Results are expressed as means with the SE of at least 3 separate experiments with statistical significance at *P < 0.05.](http://ajpgi.physiology.org/)
with control mice. Treatment with RvD1 showed a marked reduction in the activity of these pancreatitis markers (Fig. 2, A and B).

**Effects of RvD1 on inflammatory cytokines in experimental acute pancreatitis.** To assess pancreatic inflammatory response, we investigated the proinflammatory cytokines TNF-α and IL-6, two of the main mediators of the acute-phase response whose levels are useful for predicting the severity of acute pancreatitis (19, 22). Low TNF-α and IL-6 levels were evidenced in the serum of control and RvD1 alone-treated mice. The TNF-α and IL-6 serum levels revealed a moderate increase only at early stages after cerulein treatment compared with control animals, whereas the serum obtained from cerulein plus LPS-injected mice showed a marked increase in both cytokines levels. In contrast, treatment with RvD1 significantly reduced the cerulein- or cerulein plus LPS-induced increase of TNF-α and IL-6 levels, especially in cerulein plus LPS-injected mice (Fig. 3).

**Effects of RvD1 on pancreatic MPO activity in experimental acute pancreatitis.** MPO was assessed as a quantitative marker of neutrophil infiltration in pancreatic inflammatory disease. Low levels of MPO were detected in the pancreas of control and RvD1 alone-treated mice. In contrast, the pancreas obtained from mice injected with cerulein with or without LPS showed a marked increase in MPO activity. Treatment with RvD1 inhibited cerulein with or without LPS-induced increase in MPO pancreatic levels, and the inhibition role of RvD1 on MPO activity seems more effective in cerulein plus LPS-induced mice (Fig. 2C).

**Effects of RvD1 on pancreatic NF-κB activation in experimental acute pancreatitis.** Pancreatic tissue samples were collected at 8 h after first cerulein administration, and the levels of NF-κB p65 subunit in the nucleus were measured by Western blot (Fig. 4A). Cerulein and cerulein plus LPS both induced increased levels of NF-κB p65 subunit in the nucleus, with a more pronounced increase of NF-κB p65 subunit in cerulein plus LPS-induced mice. In contrast, treatment with RvD1 inhibited cerulein with or without LPS induced NF-κB p65 subunit increase in the nucleus (Fig. 4B).

**Effects of RvD1 on severity of acute pancreatitis-associated lung injury.** We also evaluated the severity of acute pancreatitis-associated lung injury based on histological alternation including alveolar congestion, hemorrhage, neutrophil infiltration in air space, and thickness of the alveolar wall. The lung histological picture was normal in control and RvD1 alone-treated mice. In mice treated by cerulein with or without LPS, lung damage was characterized by alveolar congestion, obvious hemorrhage, infiltration of neutrophil in air space, and increased thickness of the alveolar wall (Fig. 5). Treatment with RvD1 significantly reduced the histological alterations of lung injury (Fig. 6B). MPO was also assessed as a quantitative marker of neutrophil infiltration in lung inflammatory response. Low levels of MPO were detected in the lung tissues of control and RvD1 alone-treated mice. In contrast, the lung tissues obtained from cerulein with or without LPS-injected mice showed an increase in MPO activity, with a more pronounced increase of MPO activity in cerulein plus LPS-induced mice. In lung tissues of additional RvD1-treated mice,
there was significantly less MPO activity compared with cerulein or cerulein plus LPS-induced mice, respectively (Fig. 6A). Overall, these finding indicated that pretreatment of RvD1 reduced the severity of acute pancreatitis-associated lung injury.

Therapeutic treatment of RvD1 reduced the severity of experimental acute pancreatitis. We further evaluated the severity of experimental pancreatitis with RvD1 therapeutic treatment. Application of RvD1 4 h after induction of acute pancreatitis, either by cerulein or cerulein plus LPS, could significantly alleviate pancreatic inflammation, which was evaluated by pancreatic morphological changes (Fig. 7), pancreatic injury scoring, pancreatic MPO activity, and serum amylase and lipase activity (Fig. 8).

DISCUSSION

Acute pancreatitis is a potentially fatal disease characterized by wide clinical variation, ranging from a mild self-limiting to severe disease complicated by sepsis and multiorgan failure, leading to high morbidity and mortality rates (8, 41). Currently, despite the development of new diagnostic tools and treatment options, there are several problems in the therapy of severe acute pancreatitis (26, 44). In this study, we induced acute pancreatitis in two different mouse models characterized by different degrees of severity. In mice, acute pancreatitis induced by cerulein alone represents relatively a mild type, with focal necrosis that possibly could be detected but customarily little parenchyma necrosis occurs. Acute pancreatitis is a kind of special inflammatory disease that in could arouse systemic inflammatory responses (SIRS), which cause “injury in distant organ more severe than that in pancreas”; this is typically observed clinically. In pancreatitis-induced SIRS, lung is the most susceptible organ and significant changes are primarily detected in lungs. Our results demonstrate that cerulein alone induced a relatively mild model of acute pancreatitis with moderate increased leukocyte infiltration in lung tissues. In contrast, combination of cerulein and LPS injection induced a more severe model with deteriorated pancreatic inflammation and evident local acinar necrosis, as well as drastic systemic inflammatory responses, accompanied by more severe lung injury. Despite the difference in the two models...

Fig. 7. Therapeutic effects of RvD1 on histological changes in experimental acute pancreatitis. The histological examination was done at 24 h after the first injection of cerulein. Representative micrographs of H&E-stained pancreatic sections are shown. Bar indicates 50 μm. Acute pancreatitis was induced by cerulein with the presence or absence of LPS. Controls were injected with normal saline. RvD1 (300 ng/mouse) was administered 4 h after the last injection of cerulein.

Fig. 8. Therapeutic effects of RvD1 on the severity of experimental acute pancreatitis. Acute pancreatitis was induced by cerulein with the presence or absence of LPS. Controls were injected with normal saline. RvD1 (300 ng/mouse) was administered 4 h after the last injection of cerulein. Serum amylase (A) and lipase (B) activity were measured at 24 h after the first injection of cerulein. Serum amylase (A) and lipase (B) activity were measured at 24 h after the first injection of cerulein. MPO activity (C) in pancreatic tissue was measured at 24 h after the first injection of cerulein. Data are expressed as % of control in each group. Histological changes of pancreatic injury 24 h were scored as shown (D). Results are expressed as means with the SE of at least 3 separate experiments with statistical significance at *P < 0.05.
of acute pancreatitis, we discovered the significant therapeutic role of RvD1 in both models no matter the presence or absence of LPS, although it is a little more protective in the severe form of the disease. Thus RvD1 could be a representative agent of a novel class of drugs to be proposed for an innovative treatment of severe acute pancreatitis.

As the results showed, RvD1 administration in mice subjected to cerulein and LPS-induced severe acute pancreatitis caused a marked reduction in the level/activity of the markers of pancreatitis severity. In this study, RvD1 treatment decreased serum lipase and amylase activity in experiment pancreatitis. The results of the present study also show that cerulein and LPS caused a significant enhancement in the serum levels of TNF-α and IL-6. These inflammatory cytokines are two of the principal mediators of the acute-phase response, and they have been suggested as markers for predicting the severity of acute pancreatitis (19, 22). In view of the well-established anti-inflammatory properties of RvD1 (2, 34), in this study the administration of RvD1 significantly inhibited the production of TNF-α and IL-6. Evidence also demonstrated that blockades of these inflammatory cytokines attenuate the disease process in experimental pancreatitis (3, 25). Furthermore, TNF-α and IL-6 are basic regulators of all neutrophil functions and MPO is a well-known marker of neutrophil infiltration in inflammatory disease (10, 37). In the present study, cerulein and LPS stimulation caused enhanced MPO levels in both pancreatic and lung tissue, whereas RvD1-treated mice showed a significant decrease of the enzymatic activity, thus suggesting a reduced recruitment of neutrophils inside the pancreatic and lung tissue. Both biochemical and molecular data correlated very well with the histological results. Indeed, in pancreas samples obtained from cerulein and LPS-injected mice, we observed a marked edema, an increased neutrophil infiltration, local necrosis, and a high degree of vacuolization that were abated by treatment with RvD1. Moreover, cerulein and LPS-induced lung injury characterized by alveolar congestion, obvious hemorrhage, infiltration of neutrophil in air space, and increased thickness of the alveolar wall, which were also attenuated by treatment with RvD1. The effects of RvD1 were confirmed in cerulein alone-induced mild acute pancreatitis. Therefore, our findings indicate that pre-treatment of RvD1 produces beneficial effects on the disease process.

The signaling pathway responsible for the role of RvD1 in regulating inflammatory response during the course of acute pancreatitis has been of interest. One important signaling molecule, NF-κB, was identified as an important regulator of the expression of many inflammatory mediators in the pancreas (5). There is an emerging body of evidence that suggests that NF-κB plays an important role in the early stage of acute pancreatitis and that inhibiting this transcription factor reduces the disease severity (7, 29, 31). Most researchers agree that blocking NF-κB activation is beneficial in acute experimental pancreatitis (13, 28). Here, we show that NF-κB activation gradually increased after induction of pancreatitis, especially in cerulein and LPS-induced severe acute pancreatitis, and positively correlated with an increase in serum proinflammatory cytokines, serum amylase, and lipase, as well as the influx of inflammatory cells into the pancreas. It is remarkable that recent researches have shown RvD1 is involved in the regulation of NF-κB activation in the context of inflammation. Wang et al. (42) demonstrated that RvD1 markedly inhibited the activation of NF-κB and mitogen-activated protein kinases (MAPKs) in a mouse model of LPS-induced acute lung injury. Consistent with their findings, Liao et al. (21) reported that RvD1 attenuate lung inflammation of LPS-induced acute lung injury by suppressing NF-κB activation through a mechanism partly dependent on peroxisome proliferator-activated receptor gamma (PPARγ) activation. Chen et al. (4) reported that RvD1 inhibited endotoxin-induced NF-κB activation and suppressed inflammation in LPS-induced kidney injury.

In line with these observations, our data showed that RvD1 significantly inhibited both the cerulein- and cerulein in combination with LPS-induced NF-κB activation in experimental pancreatitis in mice; as a result, proinflammatory cytokines, amylase and lipase in plasma, and neutrophil infiltration in both pancreas and lung were reduced, ameliorating the acute pancreatitis and associated lung injury.

Furthermore, Wang et al. (43) also showed that RvD1 has a therapeutic effect 8 h after LPS administration. Indeed, our further data confirmed that therapeutic treatment of RvD1 4 h after induction of acute pancreatitis also significantly reduced the severity of experimental acute pancreatitis. Mice treated with therapeutic RvD1 showed significant decrease of digestive enzyme activity and pancreatic MPO activity, as well as the histological results. In pancreas samples obtained from cerulein and LPS-injected mice, we observed a marked edema, an increased neutrophil infiltration, local necrosis, and a high degree of vacuolization that were abated by therapeutic treatment with RvD1 4 h after induction of acute pancreatitis. These data suggested that RvD1 has a therapeutic effect in cerulein-induced experimental acute pancreatitis even though the pancreatic inflammation has been originated.

In conclusion, our data demonstrate that RvD1 is capable of improving injury of pancreas and lung and exerting anti-inflammatory effects through the inhibition of NF-κB activation in experimental acute pancreatitis in mice, with an even more notable protective effect in severe acute pancreatitis. Therefore, our present findings provide the potential for the development of an innovative therapeutic approach for the management of severe pancreatitis in humans.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


