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Methane-rich saline alleviates cerulein-induced acute pancreatitis by inhibiting inflammatory response, oxidative stress and pancreatic apoptosis in mice

Qun Xie[a,1], Miaomiao Fei[a,1], Zhenzong Fa[b,1], Liping Wang[c], Jun Wang[a], Yan Zhang[a], Jiafeng Wang[a,*], Xiaoming Deng[a,*]

[a] Department of Anesthesiology and Intensive Care, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
[b] Shanghai Key Laboratory of Molecular Mycology, Department of Dermatology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China
[c] Department of Anesthesiology, Fuzhou General Hospital of Nanjing Military Region, Fuzhou 350025, Fujian Province, China

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ABSTRACT

Background: Acute pancreatitis (AP) is a potentially life-threatening gastrointestinal disease involving intracellular activation of digestive enzymes and pancreatic acinar cell injury. The present study was performed to investigate whether methane-rich saline (MS) was involved in the regulation of AP.
Methods: MS (16 ml/kg) was administered at different dosing frequencies on mice with cerulein-induced AP. Serum amylase, lipase and histopathological changes in the pancreas tissue were measured. Serum cytokine TNFα, IL-6, IFNγ and IL-10 were detected by ELISA. The mRNA levels of these inflammatory cytokines in the pancreas were detected by real time-PCR. Myeloperoxidase (MPO) and superoxide dismutase (SOD) were determined using commercial kits. Apoptosis was assessed by immunohistochemistry and Western blot.
Results: MS treatment reversed the increased serum level of amylase and lipase, alleviated the pathological damage in the pancreas, and decreased the expression of TNFα, IL-6, IFNγ and IL-10 in cerulean-induced AP mice. In addition, MPO was down-regulated and SOD was up-regulated in the MS treated pancreas, indicating that MS had an anti-oxidant effect against AP. Furthermore, MS protected pancreatic cells against cerulean-induced apoptosis and abolished cleaved caspase-3.
Conclusion: MS exerted anti-inflammatory, anti-oxidant and anti-apoptotic effects on cerulein-induced AP in mice and may proved to be a promising therapeutic agent for the clinical treatment of pancreatitis.

1. Introduction

Acute pancreatitis (AP) is a potentially life-threatening gastrointestinal disease clinically characterized by abdominal pain and elevation of serum pancreatic enzymes [1,2]. Intracellular activation of digestive enzymes and pancreatic acinar cell injury are believed to be the initial pathophysiologic course, followed by pancreatic inflammation as represented by local infiltration and activation of immune cells, including neutrophils, lymphocytes, monocytes and macrophages, which produce overwhelming loads of inflammatory mediators [3,4]. Excessive inflammatory cytokines result in systemic inflammatory response syndrome (SIRS) and/or organ failure resulting in death [5]. Although AP has been recognized for more than one hundred years and great efforts have been made to elucidate its pathogenesis, treatment of this devastating disease remains a clinical challenge [6].

Different animal models have been generated to investigate AP pathogenesis [7], among which consecutive intraperitoneal injection of cerulein has been extensively adopted to establish experimental AP models since it was reported by Lampel et al. in 1977 [8]. Cerulein, a diethylamine salt of the decapeptide originally isolated from the skin of the Australian frog, shares seven of its eight C-terminal amino acids with the C-terminal octapeptide of cholecystokinin. Administration of cerulein was reported to cause pancreatic zymogen activation, secretion of lipase and amylase, acinar cells death and infiltration of inflammatory cells [9,10].

Methane is the simplest alkane commonly used as a type of attractive fuel. It facilitates the control of the hydroxyl amount in the troposphere and also plays a role in global warming. Although the flammability and explosiveness of methane gas limit its clinical use, methane can dissolve in normal saline (NS) at the proper concentration

* Corresponding authors.
E-mail addresses: jfwang@smmu.edu.cn (J. Wang), deng_x@yahoo.com (X. Deng).
[1] These authors contributed equally to this work.

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without significantly altering its chemical qualities [11]. In recent years, many researchers have focused on the biological characteristics of methane and found that methane can slow down intestinal transit, augment small intestinal contractile activity and exert a protective effect by reducing ROS production and inhibiting inflammatory response in ischemia/reperfusion (IR)-induced intestinal injury [12,13]. Most recently, methane has been reported to play a positive role in hepatitis, myocardial ischemia injury, sepsis and even diabetic retinopathy, exhibiting many pharmacological effects such as anti-inflammatory, antioxidant and anti-apoptotic properties [14–19]. Although methane has been studied in many medicinal fields, no study has examined the protective effect of methane against pancreatic injury.

The aim of the present study was to investigate whether methane could alleviate pancreatic injury in mice with cerulein-induced AP when they were treated at different dose frequencies and further explore the underlying mechanisms including anti-inflammatory, anti-apoptotic and anti-oxidative effects of methane in AP.

2. Materials and methods

2.1. Mice and reagents

Male wide-type C57BL/6 mice aged 8–10 weeks and weighing 20–26 g were obtained from the Animal Experimentation Center of the Second Military Medical University (Shanghai, China). All experiments were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with the approval of the Scientific Investigation Board of Second Military Medical University. Cerulein was purchased from Sigma Chemical (Sigma-Aldrich, St. Louis, MO). All the other chemicals and reagents were of standard commercially available biochemical quality.

2.2. Preparation of methane-rich saline

Methane-rich saline (MS) was provided by the Department of Diving Medicine of the Second Military Medical University of Navy Medicine (Shanghai, China) as previously described [14]. To obtain a supersaturated level of methane, methane was dissolved in NS for 6 h under high pressure (0.4 MPa). The concentration of methane in NS was measured by gas chromatography (Gas chromatography-9860, Qiyang Co., Shanghai, China). MS was freshly prepared one day before the animal experiments to ensure a steady concentration in the injection and stored under atmospheric pressure at 4 °C for 24 h.

2.3. Animal model and experimental design

AP was induced by intraperitoneal (i.p.) injection of cerulein (50 μg/kg, Sigma) every hour for eight consecutive hours. MS was administered i.p. at a dose of 16 ml/kg. Mice were randomly allocated into five groups: NS group receiving equal volume of NS instead of cerulein; AP group receiving cerulein to induce AP; MS1 group receiving cerulein to induce AP and single-dose of MS (given 30 min before the first cerulein injection); MS2 group receiving twice MS (given 30 min before the first injection and 30 min after the eighth injection); MS3 group receiving three times MS (given 30 min before the first injection, 30 min after the fourth injection and 30 min after the eighth injection). NS group and AP group also received three times NS instead of MS at the subsequent time points. Mice were sacrificed 16 h after the last cerulein injection (Fig. 1A).

2.4. Measurement of serum enzymes and cytokines

Mice were anesthetized by sevoflurane (2.0 MAC) 16 h after the last cerulein injection and blood was collected by heart puncture and anticoagulated by heparin. Plasma was separated after centrifugation at 12,000 rpm for 10 min. To assess the level of pancreas injury following cerulein challenge, serum levels of amylase and lipase were measured by Amylase Activity Assay Kit (Sigma, St. Louis, MO) and Lipase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum concentrations of TNFα, IL-6, IFNγ and IL-4 were detected by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA) according to the manufacturer's instructions.

2.5. Histological examination

Pancreas tissues were harvested 16 h after the last cerulein administration, fixed in 4% formalin for at least 48 h, paraffin embedded sliced into 5 μm sections, and stained with hematoxylin and eosin (H & E). All the assessments were performed by an experienced pathologist blinded to the experiment design according to the criteria described previously [10]. Specimens were scored as 0–3 from normal to severe in terms of the extent and severity of pancreatitis, including the degree of edema, inflammatory cell infiltration, necrosis, and hemorrhage (Table 1). All images were captured and processed using Olympus light microscopy (IX71) at the Second Military Medical University.

2.6. RNA isolation and real-time PCR analysis

Total RNA was extracted from the pancreatic tissue using TRIzol Reagent (ambion by *Life* technologies) according to the manufacturer's instructions. To determine the expression of target genes in the pancreas, real-time PCR reactions were performed using Reverse Transcriptase (Takara) and SYBR green master mix (Takara) in a 7900 Real-Time PCR system (Applied Biosystems). Primer sequences used to amplify mRNAs are shown in Table 2. All samples were analyzed in triplicate. GADPH was used as an endogenous reference “housekeeping” gene, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Measurement of tissue myeloperoxidase (MPO) and superoxide dismutase (SOD) levels

Pancreas tissues (100 mg) were homogenized in NS. The homogenate was then centrifuged at 12,000 rpm for 10 min at 4 °C to obtain the supernatant. The MPO and SOD levels in the pancreas tissue were determined by MPO detection kit and SOD detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. MPO activity, an indicator of PMN (polymorphonuclear neutrophils) accumulation in the pancreas, was expressed as U/g tissue; SOD activity was expressed as U/mg tissue.

2.8. TUNEL assay and immunohistochemistry

The terminal desoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) assays were performed on paraffin-embedded sections for cell apoptosis using an in situ cell death detection kit POD (Roche, Switzerland). Negative (by omitting TdT) and positive (by pretreating the sections with DNase I) controls were used for contrast. Immunohistochemical staining was performed using mouse anti-Caspase3 (Cell Signaling Technology) antibody with a dilution ratio of 1:200.

2.9. Western blot analysis

The pancreas was harvested, homogenized into lysis buffer (Thermo, USA), and centrifuged at 12,000 rpm for 10 min at 4 °C. The protein concentration was determined by Bradford protein assay kit (Thermo, USA) with bovine serum albumin as standard. Equal amounts of protein extracts were separated discontinuously onto 10% polyarylamide gels (Life Technologies, Carlsbad, CA) and transferred to nitrocellulose membranes (Life Technologies, Carlsbad, CA). After

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