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Original article

Melatonin ameliorates acute necrotizing pancreatitis by the regulation of cytosolic Ca^{2+} homeostasis

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ABSTRACT

Objectives: This study aims to investigate the relationship between the protective effects of melatonin in pancreas and the expression of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and Na^{+}/Ca^{2+} exchanger (NCX) in rats with acute necrotizing pancreatitis (ANP), to verify whether melatonin ameliorates ANP by alleviating calcium overload.

Methods: Ninety-six male Sprague-Dawley rats were randomly divided into four groups (sham operation group, ANP group, melatonin treatment group, melatonin contrast group). ANP was induced by the retrograde injection of 4% taurocholate (1 ml/kg body weight) into the biliopancreatic duct. Melatonin (50 mg/kg body weight) was administered 30 min before the induction of ANP in the melatonin treatment group. Rats in each group were euthanized at 1, 4, and 8 h after ANP induction. Pancreatic tissues were removed to measure SERCA and NCX levels and cytosolic calcium ion (Ca^{2+}) concentration ($[Ca^{2+}]_i$).

Results: At each time point, SERCA and NCX levels in the melatonin treatment group were significantly higher than that in the ANP group, and lower than that in the sham group and the melatonin contrast group. These levels did not differ between the 4- and 8-h time points in the ANP group. $[Ca^{2+}]_i$ in pancreatic acinar cells was higher in the melatonin treatment group than in the sham group and the melatonin contrast group, but lower than in the ANP group, at each time point.

Conclusion: Melatonin can reduce pancreatic damage via the up-regulation of SERCA and NCX expression, which can alleviate calcium overload in pancreatic acinar cells.

1. Introduction

Acute necrotizing pancreatitis (ANP) is a disease associated with high mortality for which no effective treatment is currently available [1]. Supportive and symptomatic treatments are currently used in clinical practice [2]. The major barrier to the efficient treatment of ANP has been our limited understanding of the complicated pathogenesis of this disease, which remains unclear despite the proposal of several explanatory theories. Ward et al. first proposed that calcium overload in pancreatic acinar cells was a “trigger point” of ANP [3], and this process has been generally believed to play a vital role in this disease [4].

Ca^{2+} is a major player in the pathogenesis of ANP. The role of cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ in pancreatic acinar cells is widely recognized as a major mediator of cell injury/death [1]. Calcium oscillations have been found to affect several physiological processes, including exocytosis, mitochondrial adenosine triphosphate (ATP) production, and gene transcription [5]. Increased $[Ca^{2+}]_i$ has been associated with the activation of intracellular digestive enzymes, as well as an inflammatory response signaling cascade [6]. Different patterns of $[Ca^{2+}]_i$ have been shown to influence both apoptotic and necrotic cell death pathways [6]. The role of Ca^{2+} in mediating intracellular trypsinogen activation is accepted as a major step in the pathophysiology of ANP.

Acute pancreatitis, caused by such major accelerators as gallstones and alcohol, has been found to increase calcium entry through the plasma membrane and/or release from intracellular stores [7]. Calcium is the first messenger to induce the release of calcium from intracellular storage through the regenerative process of calcium-induced calcium release. In ANP, the quantity and/or activity of plasma membrane Ca^{2+}-ATPase (PMCA) and/or sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) are reduced, causing the failure of intracellular Ca^{2+} release from cells or return to Ca^{2+} stores in time [8]. Recent findings have shown that bile acids can increase $[Ca^{2+}]_i$ by inhibiting SERCA pumps, which move Ca^{2+} from the cytosol back into internal stores, and consequently can cause

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cell damage through a Ca^{2+}-dependent mechanism [9]. Another report has shown that phosphatidylinositol 3-kinase, which could inhibit SERCA, can increase $[Ca^{2+}]_i$ considerably during agonist stimulation [10].

The Na^+/Ca^{2+} exchanger (NCX) was first described in the late 1960s and has been identified in most types of cells, including pancreatic duct cells [11]. The NCX on the plasma membrane is believed to be the main calcium extrusion system from the cytosol to the extracellular space. However, it remains unclear whether the quantity of NCX in pancreatic acinar cells is affected in ANP. This study sought to identify such an effect.

Melatonin is secreted by the pineal gland although its main source has been found to be the gastrointestinal tract [12,13]. Experimental studies [14–16] have found that melatonin can reduce the occurrence and development of ANP. Melatonin might ameliorate the severity of ANP through its influence on cytokines, such as tumor necrosis factor (TNF)-α [2]. Col et al. [17] found that intraperitoneal melatonin injections reduced the quantity of malonyldialdehyde (MDA) and increased the levels of superoxide dismutase (SOD) and glutathione (GSH), which are associated with oxidative stress, in pancreatic tissue. Recent studies have confirmed that melatonin significantly decreases all investigated inflammatory parameters of ANP, as well as reduces apoptosis and necrosis in pancreatic injury. In the present study, we aim to explore the mechanism of intracellular Ca^{2+} overload by determining the SERCA and NCX changes of pancreatic tissues and further to find out the therapeutic mechanisms of melatonin in rats with ANP.

2. Materials and methods

2.1. Animals

Ninety-six clean-grade male Sprague-Dawley (SD) rats weighing 200–250 g were purchased from the Experimental Animal Center of Wenzhou Medical College. The animals were maintained under standard conditions of 12-h light/dark cycles in a normal temperature-controlled room with ad libitum to standard rat pellets and water. All animals were kept in the laboratory for 1 week and were deprived of rat pellets for 12 h before experimentation, but were allowed free access to water throughout the experimental period. This experiment was approved by and performed in accordance with the guidelines for animal use of the Experimental Animal Center of Wenzhou Medical College.

2.2. Animal groups and procedures

SD rats were randomly assigned to a sham operation group (SO group, $n = 24$), an ANP group ($n = 24$), a melatonin treatment group (MT group, $n = 24$) and a melatonin contrast group (MC group, $n = 24$). In ANP and MT group, ANP was induced through the retrograde infusion of 4% taurocholate (1 ml/kg body weight; Sigma–Aldrich, St. Louis, MO, USA) into the biliopancreatic duct after clamping the hepatic duct. In the SO group, the procedure was terminated after cannulating the biliopancreatic duct by penetrating the duodenum with a 24-gauge catheter. In the MT group, melatonin (50 mg/kg body weight; Sigma–Aldrich) was administered i.p. 30 min before the injection of taurocholate. The MC group was treated in the same way as SO group, but 50 mg/kg of melatonin was given i.p. 30 min before operation.

At 1, 4, and 8 h after ANP induction, rats were anesthetized with 10% chloral hydrate (300 mg/kg body weight; Solarbio-Beijing), the abdomen was opened, and the pancreatic tissues were rapidly collected and divided into four pieces. The first tissue sample from each rat was placed in 4% buffered formalin and prepared for routine paraffin embedding prior to pathological examination, the second sample was used immediately for $[Ca^{2+}]_i$ measurement, and the third and fourth tissue samples were stored at −80 °C for the determination of SERCA and NCX levels, respectively, by real-time reverse-transcription polymerase chain reaction (RT-PCR) and western blotting. Blood samples (2 ml) from each rat in all groups were collected via a postcava puncture and stored in plain biochemistry tubes without anticoagulants (Generay, Shanghai, China). Twenty minutes after withdrawal, the blood samples were centrifuged at 1200 g for 20 min and the serum was collected for the measurement of amylase activity in a biochemical automatic analyzer (Hitachi-Japan). The rats were euthanized by exsanguination after the experiments.

2.3. Pathological examination and scoring of pancreatic tissues

The tissue samples fixed in 4% neutral buffered formalin were embedded with paraffin wax, sectioned (4-μm thickness), stained with hematoxylin & eosin (H&E), and observed under light microscopy. For H&E staining, sections were stained with hematoxylin for 3 min, washed, and then stained with 0.5% eosin for an additional 3 min. After washing under water, the slides were dehydrated in an ethanol series (70%, 96%, and 100%), followed by xylene. Tissue alteration was assessed in 20 fields/section by an experienced histologist who was blinded to the experimental protocol. Ten randomly selected visual fields in each pathological section were observed under a high-powered microscope (CX31, Tokyo, Japan) (H&E, ×400) and scored by three experienced pathologists using Schmidt et al's standards [18]. Briefly, edema was graded as: 0, absent; 1, present in the interlobular space; 2, present in the intralobular space; or 3, isolated-island shape of pancreatic acinus. Inflammatory cell infiltration was scored as: 0, absent; 1, present in ducts (around ductal margins); 2, present in parenchyma (<50% of lobules); or 3, present in parenchyma (>50% of lobules). Acinar cell degeneration was scaled as: 0, absent; 1, focal (<5%); 2, focal and/or sublobular (<20%); or 3, focal and/or lobular (>20%). Parenchymal hemorrhage was scored as: 0, absent; 1, mild; 2, moderate; or 3, severe. The maximum possible score for each visual field was 12, and the pathological score for each section was determined by calculating the mean of the three pathologists' scores for the 10 visual fields.

2.4. Preparation of isolated rat pancreatic acinar cells and $[Ca^{2+}]_i$ measurement

Pancreatic tissue was quickly removed, immediately washed in phosphate-buffered saline (PBS) (Generay, Shanghai, China) to remove blood, blood vessels, and pancreatic duct tissue, and transferred to ice-cold Dulbecco's modified Eagle's medium (DMEM; Gibco BRL-Gaithersburg, MD). The tissue was snipped into small fragments, treated by enzymatic digestion with collagenase (1 mg/ml; F. Hoffmann-La Roche Ltd-Switzerland Basel), incubated at 37 °C under gentle agitation, dispersed with a plastic pipette, filtered through a nylon mesh (200 μm), and suspended in DMEM containing 4% bovine serum albumin (BSA; Gibco BRL). The acinar cells were centrifuged twice for 3 min at 300 g in fresh DMEM containing 2 g/L BSA to remove large tissue. All experimental procedures were carried out at room temperature (23–25 °C).

Acinar cells loaded with 2 μmol/l fluorescent ratiometric Ca^{2+} indicator (Fura-2; Sigma–Aldrich) were suspended by incubating for 30 min at 37 °C. The cells were then washed in fresh physiological solution and used within 1–3 h. $[Ca^{2+}]_i$ was measured at 340- and 380-nm excitation wavelengths and 500-nm emission by a spectrofluorimeter (RF 510; Shimadzu-Japan). The ratio of fluorescence intensities at the two excitation wavelengths ($F_{340/380}$) was used as the $[Ca^{2+}]_i$ value, as described previously [19].