NOD receptor and TLR9 modulation in severe acute pancreatitis-induced intestinal injury

YUPENG YAN1, BIN LU2, PENGYANG LI3 and JI WANG1

1Intensive Care Unit, China Meitan General Hospital, Beijing 100028; 2Department of Anesthesiology, Beijing Hospital of Traditional Chinese Medicine; 3Department of Orthopedics, Dongzhimen Hospital, Beijing 100010, P.R. China

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Abstract. Severe acute pancreatitis (SAP) has a rapid onset and may cause multiple organ dysfunction syndrome (MODS), which has high mortality. Nucleotide binding oligomerization domain (NOD) receptor and Toll-like receptor 9 (TLR9), a pattern recognition receptor in innate immunity, are involved in inflammation, immunity and pathogen recognition. The role and mechanism of the NOD receptor and TLR9 in early MODS of SAP-induced intestinal injury, however, remain unclear. Wistar rats were divided into control, SAP, TLR9 inhibitor and NOD receptor activation groups. Reverse transcription-quantitative polymerase chain reaction was used to analyze the expression of TLR9, NOD1 and NOD2 in the experimental treatment groups. Serum amylase, creatinine and alanine aminotransferase indices were measured, ELISA was used to determine the expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and western blot analysis was used to assess nuclear factor (NF)-κB levels compared with the control group. Furthermore, ROS production was increased, SOD activity was decreased and higher serum indices were exhibited, compared with the control group. The NOD receptor group presented more significant differences compared with the SAP group. The TLR9 inhibitor group exhibited opposite effects, with markedly decreased TLR9, NOD1, NOD2, TNF-α, IL-1β and NF-κB levels. The TLR9 inhibitor group also presented reduced ROS production, increased SOD activity and lower serum indices compared to the SAP group. The present study therefore indicated that NOD receptor and TLR9 may modulate the inflammatory response and further impact upon intestinal injury of SAP, via the regulation of NF-κB expression and the oxidation/antioxidation balance, suggesting therapeutically targeting NOD receptor and TLR9 might be a useful approach for the treatment of severe acute pancreatitis.

Introduction

Acute pancreatitis (AP) is commonly observed in clinics (1). Severe acute pancreatitis (SAP) can cause necrosis of peri-pancreatic tissues, injury and dysfunction of several organs, thus rapidly aggravating the patient's condition. Due to the lack of effective and specific treatment, SAP has an unfavorable prognosis and a high mortality rate (2). SAP is frequently caused by a secondary bacterial infection, and can cause injury to the intestinal mucosal structure or functioning, thus disrupting the intestinal barrier and resulting in systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (3,4). During SAP, the mucosal barrier becomes damaged, which increases the permeability of the intestinal tract and allows the release of gut bacteria, thus resulting in intestinal infection. Bacterial contamination can cause the body to release large amounts of inflammatory mediators and cytokines, causing endotoxemia and further early injuries related to MODS (5). During the occurrence and progression of AP, intestinal mucosal barrier injury is a critical factor following intestinal injury; the inhibition of SAP-related mucosal barrier injuries is a primary target for the prevention and treatment of early MODS.

The innate immune system is the initial line of defense against microbial invasions that cause intestinal mucosal injury (6). During this process, pattern recognition receptors (PRR) serve an important role in defending against infection (7). The extracellular PRR, Toll-like receptor (TLR) and the intracellular PRR, nucleotide oligomerization domain (NOD) receptor, are two important members of the innate immune system for recognizing and fighting against microbial pathogens (8,9). NOD-like receptors (NLRs) display highly conserved structures (10) and participate in recognition and defense against microbial pathogens. Furthermore, they modulate the homeostasis of intestinal symbiotic microbes (11), thus exhibiting a bifunctional role. As one of the transmembrane PRRs involved in pathogen recognition, TLRs serve important roles in signal transduction, phagocytosis and cell apoptosis.
during acute inflammation (12,13). A previous study correlated TLR4 expression with the pathogenesis of AP (14), however, TLR9 is indispensable for inflammation, immunity and pathogen recognition (15,16). The role of TLR9 in AP and its interaction with NOD, or other related mechanisms, has not currently been elucidated. Therefore, the present study aimed to investigate the role of the NOD receptor and TLR9 in MODS-induced intestinal injury during early SAP. As NOD receptor and TLR9 are both play an important role in microbial pathogens recognition and inflammation, and the close association of TLR9 with AP, the present study investigated the role of TLR9 and NOD receptor in rats with SAP through blocking TLR9 or activating the NOD receptor.

Materials and methods

Experimental animals. A total of 40 healthy male Wistar rats (age, 2 months; body weight, 250±20 g) were purchased from Laboratory Animal Unit of Chinese Medical Sciences University (Shenyang, China) and were kept in a specific-pathogen-free grade facility. The room temperature was maintained at 21±1°C and the relative humidity was maintained at 50-70%. Animals were kept on a 12-h light/dark cycle with free access to food and water. All procedures were approved by the Animal Ethics Committee of China Meitan General Hospital (Beijing, China).

Reagents and instruments. Sodium taurocholate, glutamate-meso-diaminopimelic acid (DAP), muramyl acid dipeptide (MDP) and chloroquine were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Polyvinylidene difluoride membrane was purchased from Pall Life Sciences (Port Washington, NY, USA). Western blotting lysis buffer was purchased from Beyotime Institute of Biotechnology (Haimen, China). Enhanced Chemiluminescence (ECL) reagent was purchased from Amersham (GE Healthcare Life Sciences, Little Chalfont, UK). Rabbit anti-rat nuclear factor (NF)-κB monoclonal antibody (cat. no. 4764) and horseradish peroxidase-labelled IgG secondary antibody (cat. no. 7074) were purchased from Cell signaling Technology, Inc. (Danvers, MA, USA). Tumor necrosis factor (TNF)-α (cat. no. RTA00) and interleukin (IL)-1β (cat. no. RLB00) ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The TRIzol reagent for RNA extraction, reverse transcription (RT) cDNA first chain synthesis kit and superoxide dismutase (SOD) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The surgical microscope was purchased from Suzhou Sunan Zimmered Medical Instrument Co., Ltd. (Suzhou, China). The microplate reader was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The Gene Amp PCR System 2400 DNA cycler was purchased from PerkinElmer, Inc. (Waltham, MA, USA). Other common reagents were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

Animal grouping and treatment. Wistar rats were randomly divided into four groups (n=20/group). The four groups were: Control group, which received an equal volume of saline inside the bile duct; SAP model group; TLR9 inhibitor group, which received an intraperitoneal injection of the TLR9 inhibitor chloroquine, following SAP model induction; and NOD receptor activation group, which received an intraperitoneal injection of the NOD receptor agonists MDP and DAP, following SAP model induction.

Sample collection. Rats were anaesthetized with ketamine-zylazine and blood samples were collected from the abdominal aorta using vacuum tubes at 12 h after SAP. Blood was incubated at room temperature for 30 min, followed by 4°C centrifugation at 1,200 x g for 10 min to collect the super- nant. Serum was frozen at -20°C for further use. Injured intestinal tissues were collected from all groups and stored at -80°C.

Serology indexes assay. An automatic biochemical analyzer (AU680, Beckman Coulter) was used to test the serum amylase (AMY), creatinine (Cr) and alanine aminotransferase (ALT) levels according to manufacturer's protocol.

ELISA test for serum levels of inflammatory factors TNF-α and IL-1β. Serum samples were tested for inflammatory factors including TNF-α and IL-1β levels using ELISA kits, following the manufacturer's protocol. In brief, 50 μl serially diluted standards were added into a 96-well plate, and test samples (50 μl) were applied in triplicates and incubated for 2 h. Following gentle washing (5 times) with washing buffer and a 30 sec vortex, 50 μl enzyme labeling reagent was added into each well and incubated at 37°C for 30 min. Following a further 5 washes, chromogenic substrates A and B (50 μl each) were added and developed in the dark at 37°C for 10 min. The reaction was quenched with 50 μl stopping buffer. A microplate reader was used to measure absorbance values at 450 nm. A standard curve was plotted based on the standard concentrations and respective optical density (OD) values, followed by calculation of the sample concentrations using the sample OD values.
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for TLR9, NOD1 and NOD2 mRNA expression in SAP intestines. Intestinal tissues were collected and rinsed in PBS. Tissues were homogenized in liquid nitrogen and total RNA was extracted using TRIzol reagent. cDNA was synthesized using cDNA first chain synthesis kit. A fluorescent qPCR kit (Verso 1-Step RT-qPCR SYBR Green kit; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to collect data and determine the Cq value, with reference to GAPDH. PCR amplification was performed in a total volume of 20 µl, including 10 µl SYBR Green qPCR Super Mix, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 5 µl cDNA and 4 µl sterile water under the following conditions: 52˚C for 1 min, followed by 35 cycles of 90˚C denaturation for 30 sec, 58˚C annealing for 50 sec and 72˚C elongation for 35 sec. Primers are presented in Table I. The relative expression level was determined using the 2^(-DDCq) method (17).

Western blotting for NF-κB protein expression. Total proteins were extracted from intestinal tissues after homogenization on liquid nitrogen, mixed with lysis buffer (Beyotime Institute of Biotechnology) for 15-30 min and incubated on ice. Using ultrasonic rupture (5 sec, 4 times) and centrifugation (10,000 x g for 15 min) at 4˚C, proteins were collected and stored at -20˚C for subsequent western blotting. Proteins (20 mg/lane) were separated by 10% SDS-PAGE, and were transferred to PVDF membranes using the semi-dry method. Non-specific binding sites were blocked by 5% non-fat milk powder for 2 h. The membrane was incubated with anti-NK-κB monoclonal antibody (1:1,000) at 4˚C overnight. Goat anti-rabbit IgG (1:2,000) was subsequently added for 30 min at room temperature. Following 0.1% (v/v) PBS-Tween washing and ECL development for 1 min, the membrane was exposed to X-ray film. An image analyzing system (ImageQuant LAS 500, GE Healthcare Life Sciences) and Quantity One software version 4.3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to scan the X-ray films and to detect the density of bands, from repeated experiments (n=4).

Reactive oxygen species (ROS) content assay. Intestinal tissue homogenates were denatured at 95˚C for 40 min, cooled in tap water, and centrifuged at 1,500 x g for 10 min at 4˚C. Homogenates were incubated at 37˚C in 2',7'-dichlorofluorescein diacetate for 15 min. Following centrifugation at 4,000 x g for 15 min at room temperature, the precipitates were re-suspended in sterilized PBS buffer, and incubated at 37˚C for 60 min. Spectrometry was used to detect the ROS levels at a wavelength of 520 nm, and data were expressed as a ROS production percentage.

Statistical analysis. SPSS v16.0 software (SPSS, Inc., Chicago, IL, USA) was used to analyze all data. Measurements were expressed as the mean ± standard deviation. One-way analysis of variance with Newman-Keuls multiple comparison post-hoc analysis was used to compare the means across groups. P<0.05 was considered to indicate a statistically significant difference.

Results

TLR9 expression in rat intestinal tissues. RT-qPCR was used to measure TLR9 mRNA expression levels in rat intestinal tissues from all treatment groups (Fig. 1). The results indicated significantly elevated levels of TLR9 mRNA in the SAP group and NOD receptor activation groups (P<0.05 compared with the control group); the NOD receptor activation group exhibited the greatest increase of TLR9 mRNA (P<0.05 compared with the SAP group). The TLR9 inhibitor group significantly

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
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<tbody>
<tr>
<td>GADPH</td>
<td>AGTGCCAGGCTTCGTCTCATA</td>
<td>ACTTGCAACTTGGCGTGGGTAG</td>
</tr>
<tr>
<td>TLR9</td>
<td>CTCATCTAAAGGGCAACCAATGG</td>
<td>GCACATTCTCTCGTGATACCG</td>
</tr>
<tr>
<td>NOD1</td>
<td>TAACGTCAAGGGAACGGAATG</td>
<td>ACATTCTCTCATCTA</td>
</tr>
<tr>
<td>NOD2</td>
<td>TCATAGGCCCTCATCT</td>
<td>ACCTTGCACTTGCGGG</td>
</tr>
</tbody>
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TLR9, Toll-like receptor 9; NOD, nucleotide oligomerization domain receptor.
inhibited TLR9 mRNA expression (P<0.05, compared with the SAP group).

NOD1 and NOD2 expression change in rat intestinal tissues. RT-qPCR was performed to measure the mRNA levels of NOD1 and NOD2 in rat intestinal tissues from all treatment groups (Fig. 2). The results demonstrated significantly elevated NOD1 and NOD2 mRNA expression levels in the SAP model and NOD receptor activation groups (P<0.05, compared with the control group); the NOD receptor activation group exhibited a stronger increase of NOD1 and NOD2 mRNA (P<0.05, compared with the SAP group). The TLR9 inhibitor group significantly inhibited NOD1 and NOD2 mRNA expression (P<0.05, compared with the SAP group). These results indicated an inter-regulation between TLR9 and NOD in SAP-induced intestinal injury.

Serology index analysis. Serology indices were measured 12 h following SAP in all treatment groups (Table II). The results indicated significantly elevated AMY, Cr and ALT in the SAP and NOD receptor activation groups (P<0.05, compared with the control group); the NOD receptor activation group exhibited the greatest increase of the measured indices (P<0.05, compared with the SAP group). Treatment with the TLR9 inhibitor significantly inhibited the elevation of these serology indices (P<0.05, compared with the SAP group), however, the measured levels were higher than the control group. These results demonstrated that modulation of the NOD receptor and TLR9 may improve the serology indices in the early phase of MODS related with SAP.

Effects of TLR9 inhibition and NOD receptor activation on the expression of serum inflammatory factors TNF-α and IL-1β. ELISA tests were used to investigate the effects of TLR9 inhibition and NOD receptor activation on the serum levels of the inflammatory factors TNF-α and IL-1β (Fig. 3). The results indicated significantly elevated levels of TNF-α and IL-1β in the SAP and NOD receptor activation groups (P<0.05, compared with the control group), and the NOD receptor activation group exhibited the greatest increase of these factors (P<0.05, compared with the SAP group). The TLR9 inhibitor group significantly inhibited secretion of these inflammatory factors (P<0.05, compared with the SAP group). These results demonstrated that modulation of the NOD receptor and TLR9 may ameliorate SAP-induced intestinal injury by altering the secretion of serum inflammatory factors.
Table III. Effects of TLR9 and NOD receptor on oxidative stress indices of pancreatic tissues.

<table>
<thead>
<tr>
<th>Parameter (relative value)</th>
<th>Control</th>
<th>SAP</th>
<th>NOD receptor</th>
<th>TLR9 inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROS</td>
<td>56±14</td>
<td>259±31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>289±67&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>162±42&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD</td>
<td>137±23</td>
<td>85±12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58±6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>117±21&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.05 vs. control group; <sup>b</sup>P<0.05 vs. SAP group; <sup>c</sup>P<0.05 vs. NOD receptor group. SAP, severe acute pancreatitis; TLR9, Toll-like receptor 9; NOD, nucleotide oligomerization domain receptor; ROS, reactive oxygen species; SOD, superoxide dismutase.

Figure 4. Effects of TLR9 and NOD receptor modulation on intestinal expression of NF-κB in SAP. (A) Western blot analysis. A, control group; B, SAP group; C, NOD receptor group; D, SAP group. (B) Semi-quantitation of western blot analysis. *P<0.05 vs. control group; #P<0.05 vs. SAP group. SAP, severe acute pancreatitis; TLR9, Toll-like receptor 9; NOD, nucleotide oligomerization domain receptor; NF-κB, nuclear factor-κB.

Discussion

TLRs can recognize molecular markers from a wide range of pathogens. There are currently 11 known members of the TLR family with unique ligands. The endotoxin lipopolysaccharide from gram-negative bacteria can be recognized by TLR3, however, the major ligand of TLR9 is CpG-DNA (18,19). Upon activation, TLR9 transduces signals via Toll-interleukin receptor structural domains to activate NF-κB, thereby regulating gene transcription, inducing the release of inflammatory factors, such as TNF-α and IL-1β, and leading to an increased inflammatory response (20). The NOD receptor family serves a similar function to the TLR family. Amongst these, NOD1 and NOD2 are associated with the induction of inflammation, with DAP and MDP as their ligands, respectively (21,22). A recent study has revealed a correlation between NOD receptors and TLR or pancreatitis (23). The role and mechanism of the NOD receptor and TLR9 in SAP-induced intestinal injury, however, has not been elucidated. The present study established a SAP rat model, following by treatments with either NOD ligand agonists or a TLR9 inhibitor in order to investigate the impact of their activity modulation on serological and inflammatory factors. TLR9, NOD1 and NOD2 expression in the SAP and NOD receptor activation groups were significantly elevated, with the greatest effect observed in the NOD receptor activation group. The TLR9 inhibitor group exhibited decreased TLR9, NOD1 and NOD2 expression. These results suggested that TLR9 and NOD may have inter-regulatory effects on intestinal injury during SAP.

SAP commonly occurs in early MODS and late infectious necrosis (24), and the pathogenesis can facilitate the abundant release of inflammatory factors by lymphocytes, neutrophils and macrophages. The upregulation of anti-inflammatory factors further interferes with the pro-inflammatory/anti-inflammatory balance, eventually causing mortality as a result of SIRS and multiple organ failure (25). Therefore, a core explanation for SAP-related intestinal injury is induction of the inflammatory response. Furthermore, SAP can damage the liver, resulting in the release of enzymes synthesized by liver cells into the hepatic portal vein, from which they are distributed to the tissues and organs via the circulation, causing elevated serum AMY, Cr and ALT, and aggravating intestinal injury (26). The present study demonstrated that TLR9 and NOD receptor modulation may modify the inflammatory response via alterations to the serum inflammatory factor release in SAP. Regulation of these receptors may improve the serology index in early MODS of SAP, and potentially limit SAP pathogenesis and SAP-related intestinal injury.

The present study also investigated related inflammatory mechanisms, and demonstrated elevated ROS and decreased SOD levels during SAP pathogenesis. Under normal functioning of the cellular antioxidant system, ROS is continuously cleared, thus preventing and alleviating tissue injury. SOD is an important antioxidant enzyme involved in the clearance of free oxygen radicals, and serves an important role in maintaining the oxidation and antioxidation balance (27). Modulation of TLR9 and NOD receptor activity may impact upon the oxidation/antioxidation balance, as SOD upregulation will accelerate ROS clearance; therefore, regulation of TLR9 and NOD may potentially decrease SAP-related intestinal tissue injury. TLR and NOD receptor function as important innate immune receptors, and can recruit innate immune cells under pathogenic invasion, thus participating in the immune response. NF-κB, as a target gene for facilitating expression and transcription, is a critical mediator (28). The present study...
demonstrated significantly elevated NF-κB expression in rat intestinal tissues in SAP and NOD receptor groups, whilst TLR9 inhibition significantly depressed the intestinal expression of NF-κB, suggesting that TLR9 and NOD may modulate SAP-related intestinal injury by regulating NF-κB expression in SAP.

In conclusion, the NOD1 and NOD2 receptors and TLR9 demonstrated an ability to regulate NF-κB expression and the oxidation/antioxidation balance to modulate the inflammatory response, which may affect SAP-related intestinal injury. The NOD receptors and TLR9 may function synergistically to accomplish this effect. The present study investigated the SAP-related intestinal injury at a molecular level, thus providing a molecular mechanism for the investigation of novel clinical treatments for SAP-related intestinal injury.

References