Inflammatory stimuli promote oxidative stress in pancreatic acinar cells via Toll-like receptor 4/nuclear factor-κB pathway

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Abstract. The Toll-like receptor 4/nuclear factor-κB (TLR4/NF-κB) pathway is vital to the pathogenesis of acute pancreatitis (AP). The aim of the present study was to identify the mechanism of the activation of the TLR4/NF-κB signaling pathway in the viability of primary pancreatic cells. The cells were stimulated with lipopolysaccharide (LPS) for the activation of NF-κB signaling. Next, the reactive oxygen species (ROS) level was evaluated by detecting the concentration of malondialdehyde and glutathione peroxidase. Cell viability was measured by Cell Counting Kit-8 and MTT assays, while the percentage of apoptosis was detected by flow cytometry. Quantitative polymerase chain reaction was used to detect TLR4, B-cell lymphoma 2 (Bcl2), Bcl2-associated X protein (Bax) and phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) expression levels. Western blot assay was also conducted to detect TLR4 protein expression, while the activity of NF-κB signaling was measured by determining the p65 and phosphorylated p65 protein levels. In addition, the effect of TLR4 overexpression or treatment with TLR4 antagonists in the presence of LPS stimulation was investigated. The results revealed that ROS levels were increased and cell viability was decreased in LPS-stimulated pancreatic acinar cells. TLR4, Bax and PMAIP1 levels were increased, Bcl2 expression was decreased and NF-κB signaling was activated in LPS-stimulated pancreatic acinar cells. Furthermore, pancreatic cells with TLR4 overexpression exhibited increased ROS level and decreased viability. Finally, the effect caused by LPS stimulation was partially reversed by treatment of pancreatic acinar cells with TLR4 antagonists. In conclusion, the current study investigated a novel regulatory mechanism of the TLR4/NF-κB pathway in LPS-stimulated pancreatic cells, which may contribute to pancreatitis. The damage of these cells due to increased ROS levels was observed to occur through activation of the TLR4/NF-κB pathway.

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Introduction

Severe acute pancreatitis (SAP) is one of the most common acute abdominal diseases. It is caused by pancreatic local inflammation, necrosis and infection, and is accompanied by a systemic inflammatory response and multiple organ dysfunction. Although important progress has been made in the comprehensive treatment of SAP, the mortality rate remains as high as 17% (1,2). Thus, the study of the pathogenesis of acute pancreatitis (AP) is helpful in its clinical treatment.

Oxidative stress is the cause and consequence of numerous diseases. Disequilibrium between the formation of reactive oxygen species (ROS) and antioxidant defense systems can cause disease directly or indirectly through the signal transduction pathway activated by excessive ROS in organs (3). An increasing number of studies have identified that ROS-induced oxidative stress serves an unfavorable role not only in the pathogenesis of SAP, but also in the damage of other organs, such as the heart, liver, lung, kidney and digestive tract (4,5). ROS and active nitrogen produced by oxidative stress can cause inflammation and microcirculation disorders, and activate the pathways of cell necrosis or apoptosis, resulting in pancreatic and other organ dysfunction (6). Pro-inflammatory factors and oxidative stress serve a synergistic role in triggering signal transduction in the inflammatory response of AP. Activated mitogen-activated protein kinase and nuclear factor (NF)-κB signaling can then lead to a cascade of amplification of inflammation (7,8). It has been reported that ROS produced by pancreatic follicles increased significantly in an early SAP rat model, while the pancreatic glutathione levels were simultaneously decreased (9). After 6 h of pancreatic obstruction, lipid peroxidation was significantly enhanced (10); however, monocytes produced TNF-α until 12 h after AP, which promoted the accumulation and activation of white blood cells in the inflammatory site (10,11). Thus, the interaction of inflammatory factors and oxidative stress in SAP results in detrimental effects. Studying the mechanism of oxidative

stress in pancreatitis is, therefore, important for eliminating ROS and treating pancreatitis.

It has been reported that the levels of interleukin (IL)-1β, IL-6 and TNF-α in the serum of SAP patients were significantly increased, indicating that a systemic inflammatory response serves a critical role in the progression of SAP (12,13). The pathogenesis of SAP may be associated with excessive activation of the NF-κB signaling pathway, leading to a large number of inflammatory mediators (14,15). Once the cytokines are produced, they not only activate themselves, but also promote the production of other cytokines, causing interlocking and amplification effects that impair the structure and function of the pancreas (16). The inflammatory process is important in the development of pancreatitis (17). In a healthy pancreas, the NF-κB signaling is inactive. However, in the early stages of pancreatitis, NF-κB signaling is activated and enhances the inflammation process through activation of anti-apoptotic and inflammatory genes (18). Long periods of activated signaling in pancreatic cells lead to pancreatic damage and fibrosis (19).

SAP is essentially a systemic inflammatory response syndrome, and the endotoxin/Toll-like receptor 4 (TLR4)/NF-κB signaling pathway may be important in mediating the AP inflammatory response (20). An experimental study demonstrated that the expression of TLR4 in SAP rats increased in the pathogenesis of this disease, which indicates that the expression of TLR4 directly reflects the severity of SAP (21). TLR4 is also involved in mediating pancreatic cell apoptosis in mice with AP (22). Taken together, TLR4 serves a vital role in the pathogenesis of SAP, and its correlation with NF-κB signaling is also important for studying the mechanism of SAP. Thus, to fully understand the pathogenesis of SAP and identify a novel target for SAP treatment, it is necessary to examine the function of TLR4/NF-κB in SAP.

The present study focused on the effect of TLR4 and inflammatory signaling activation on the generation of oxidative stress in pancreatic cells. The results revealed that the activation of inflammatory signaling increased the expression level of TLR4. In order to prove that the increased ROS levels in pancreatic cells were caused by TLR4 overexpression, pancreatic acinar cells were then treated with TLR4 antagonist, and the effect caused by inflammatory stimulation was partially reversed. The current study indicates that the lipopolysaccharide (LPS)-induced TLR4/NF-κB pathway is critically involved in the initiation of inflammation, oxidative stress and decreased pancreatic cell viability.

Materials and methods

Chemicals and materials. Fetal bovine serum (FBS) was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, USA). The Cell Counting Kit-8 (CCK-8) assay kit was obtained from KeyGen Biotech Co., Ltd. (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Antibodies against β-actin (sc-58673), p65 (sc-71675), phosphorylated (p)-p65 (sc-136548), B-cell lymphoma 2 (Bcl2; sc-509), Bcl2-associated X protein (Bax; sc-20067), PMAIP1 (sc-515840) and TLR4 (sc-293072) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). LPS was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Primary culture of pancreatic acinar cells and treatment. Pancreatic cells were isolated from 100 healthy adult male (to avoid the effects of estrogen) 4-6-weeks-old C57BL/6J mice (25-30 g, Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), according to the procedure described in previously published study (22). The animal experiments of the present study were approved by the Ethics Committee of Xi'an Jiaotong University (Xi'an, China). Briefly, the pancreas was immediately removed from the sacrificed mouse and incubated in buffer solution (containing 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4 and 0.2% bovine serum albumin (Thermo Scientific Fisher, Inc.) at 37˚C for 10 min. Then the cell suspension was centrifuged at 30 x g for 5 min at 4˚C. Next, the acinar cell pellets were resuspended in HEPES buffer without collagenase and centrifuged at 30 x g for 5 min at 4˚C, following which the supernatant was removed. Primary pancreatic cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), 2 mM L-glutamine and 25 μg/ml gentamicin at 37˚C in a humidified atmosphere with 5% CO_2.

To investigate the potential protective effect of TAK-242, an inhibitor of TLR4, primary pancreatic acinar cells were treated with TAK-242 (1 μM) following LPS treatment (100 ng/ml) for 24 h at the temperature of 37˚C. The concentration of TAK-242 was selected according to previous published data.

Transfection. For transfection, pancreatic cells (80% confluence) were seeded into 6-well culture plates; cells were transfected with 3 μg of the TLR4 overexpression plasmid and pCDNA3.0 (Invitrogen; Thermo Fisher Scientific, Inc.) as the control using Lipofectamine® 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The sequence of TLR4 cloned into the pCDNA3.0 plasmid was obtained by RT-qPCR; the primers employed for plasmid construction were: Forward, 5'-GACGAGCTCATGATGCCTCCCTGG CTCCT-3' and reverse, 5'-TACCCGTCAGGTCCAAGTTGC CGTTTCT-3'. After 2 days following transfection, cells were harvested for RNA and protein isolation.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). After pancreatic cells were treated with LPS for 2 h and transfected with plasmids, total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). All the procedures were conducted according to the manufacturer's protocol. Briefly, 1 μg total RNA was reverse transcribed using an PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The RT reaction program was as follows: 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 min. qPCR was then conducted using the SYBR-Green for Real-Time PCR kit and the ROCHE Light 480 detection system (Roche Diagnostics, Basel, Switzerland), and the amplification conditions were 95˚C for 10 min, followed by 50 cycles of denaturation at 95˚C for 15 sec, and annealing at 62˚C for 1 min. The primers for qPCR were: TLR4 forward, 5'-AGCCATTGCTGCCAACATCA-3' and reverse, 5'-GCCAGAGCTACTCAGAAAC-3'. Bcl2 forward, 5'-GGTGGGGTCATGTGTGTGG-3' and reverse, 5'-CGG TTCAGGTACTCAGTCATCC-3'; Bax forward, 5'-CCCGAG AGGTCTTTTTCCGAG and reverse, 5'-CCAGCCCATGAT

GGTTCTGAT-3'; PMAIP1 forward, 5'-ACCAAGCCGGAT TTGCGATT-3' and reverse, 5'-ACTTGCACTTGTTCCTCG TGG-3' and β-actin forward, 5'-CATGTACGTTGCTATCCA GGC-3' and reverse, 5'-CTCCTTAATGTCACGCACGAT-3'. All reactions were repeated for three times, and the relative mRNA expression levels of target genes were normalized to β-actin. Results were expressed as fold differences relative to the level of β-actin using the $2^{-\Delta\Delta Cq}$ method (23).

Western blot analysis. Following treatment with LPS and transfection with plasmids, pancreatic cells were harvested for protein isolation. Briefly, cell pellets were lysed in 200 μl of ice-cold lysis buffer (pH 7.4; 50 mmol/l HEPES, 5 mmol/l EDTA, 100 mmol/l NaCl, 1% Triton X-100, protease inhibitor cocktail). Protein samples (20 μg) were separated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% defatted milk in Tris-buffered saline/Tween-20 (TBST) at room temperature for 1 h and then incubated with primary antibodies overnight at 4˚C. Antibody against TLR4 (1:200), p65 (1:400), p-p65 (1:200), Bcl2 (1:200), Bax (1:500) and PMAIP1 (1:100) were used. On the following day, the PVDF membranes were washed with TBST buffer and then incubated with a mouse peroxidase-conjugated secondary antibody (1:2,000; sc-2005, Santa Cruz Biotechnology, Inc.) with agitation for 1 h. Finally, an enhanced chemiluminescence solution (Thermo Scientific Fisher, Inc.) was prepared in a dark room, and the exposure time was determined according to the fluorescence intensity. The results were quantified according to the intensity of the bands by GraphPad 6.0c Software, Inc. (La Jolla, CA, USA).

MTT assay. LPS was added at the concentration of 100 ng/ml following cell attachment to the plates for 24, 48 and 72 h at the temperature of 37˚C. Then after overnight culture, baseline values were obtained by an MTT assay (Thermo Fisher Scientific, Inc.), a colorimetric assay to determine cell viability by measuring the formazan reduced from MTT. After 20 min of incubation with MTT at the temperature of 37˚C, formazan was diluted with DMSO and tested the optical density at 540 nm. MTT assay was performed at different time points (at 24, 48 and 72 h, respectively). Experiments were conducted in triplicate.

Cell proliferation. Pancreatic cells were suspended in complete RPMI-1640 medium, the cell concentration was adjusted to $5x10^6$ cells/ml, and cells were grown in 96-well cell culture plates with 100 μl in each well. For cell proliferation detection, cells were harvested in medium at 24 h after transfection. Cell proliferation was detected by a CCK-8 assay kit according to the manufacturer's protocol for the duration of 1 h. Cell viability was calculated according to the absorbance detected at 450 nm by a microplate reader.

Cell apoptosis assay. A total of $5x10^5$ pancreatic cells were seeded in a 6-well cell culture dish and complete RPMI-1640 medium was added. Following the addition of medium containing 100 ng/ml LPS, the cells were cultured for 24 h and collected by digestion with 0.25% trypsin. Pancreatic cells were washed twice with pre-cooled PBS and re-suspended in 1 X binding buffer to a final concentration of 10^6 cells/ml. Next, 100 μl of cell suspension was placed in a flow tube, and 5 μl Annexin-V-FITC (A211-01/02, Vazyme, Piscataway, NJ, USA) and 5 μl propidium iodide (A211-01/02, Vazyme) were added for a 15-min incubation in the dark. At 1 h after addition of 400 μl binding buffer to each well, the fluorescence intensity was measured by flow cytometry.

ROS measurement. Pancreatic cells were seeded in a 6-well cell culture dish at 60% confluence, and RPMI-1640 complete medium was added. Subsequent to treatment with medium containing different concentrations of LPS, cells were cultured for 24 h and collected by digestion with 0.25% trypsin. Next, cells were washed with pre-cooled PBS, lysed with chemical buffer and the protein concentration was determined by the Bradford method. ROS concentration was determined based on MDA and GPx levels (24). Following the manufacturer's protocol of the malondialdehyde (MDA) (cat. no. A003-1) and glutathione peroxidase (GPx) (cat. no. A005) determination kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the concentration of MDA and the activity of GPx was detected with a spectrophotometer. The wavelength for MDA detection was 532 and 412 nm for Gpx.

Statistical analysis. Each experiment was repeated at least in triplicate. The results are presented as the mean value ± standard deviation. Statistical analysis for comparison of two groups was conducted using Student's t-test, while analysis of variance was used for multiple group comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LPS induces high-level oxidative stress in pancreatic cells. To investigate the biological effects of inflammatory stimuli on primary pancreatic cells, the cells were treated with 100 ng/ml LPS to activate inflammatory signaling. The MDA level was measured, which is an indicator of oxidative damage and reflects the ROS level in cells. According to the results shown in Fig. 1A, the MDA level was significantly increased in LPS-treated pancreatic cells, as compared with the control cells. In addition, the GPx level was markedly decreased in the LPS-treated group (Fig. 1B). Thus, these results indicate that LPS increased ROS levels and thus induced oxidative stress in pancreatic cells.

Pancreatic cell viability is decreased by LPS treatment. To identify whether the increased ROS levels influenced the cell viability, CCK-8 and MTT assays were applied to detect the cell viability. Subsequent to the addition of 100 ng/ml LPS, pancreatic cells were harvested for the detection of cell viability. The results demonstrated that LPS significantly decreased the pancreatic cell viability and proliferation ability compared with the control cells (Fig. 1C and D). Furthermore, the percentage of cell apoptosis was detected by flow cytometry. The results demonstrated that LPS significantly increased the percentage of apoptotic cells compared with that in the control cells (Fig. 1E and F).

Bcl2, Bax and PMAIP1 levels in LPS-stimulated pancreatic cells. To identify how oxidative stress induces apoptosis

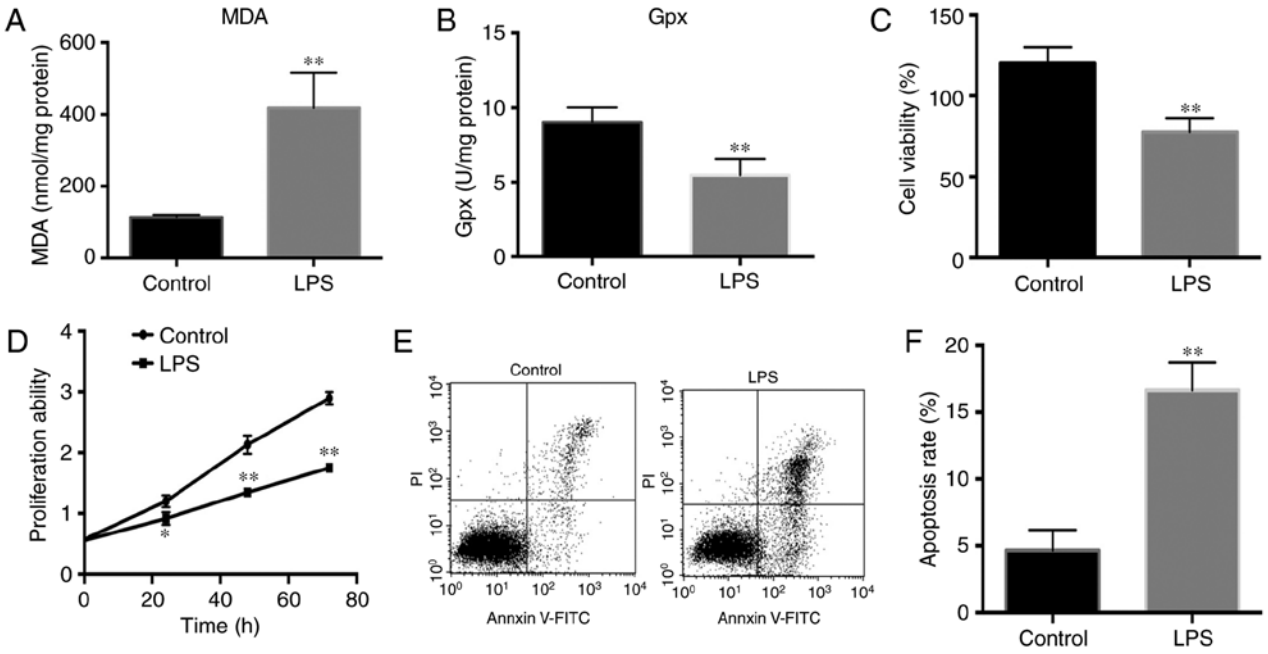

Figure 1. LPS reduced the viability of primary pancreatic cells by increasing ROS and the apoptosis rate. (A) MDA and (B) GPx concentration detection was performed after primary pancreatic cells were treated with 100 ng/ml LPS for 24 h, compared with the negative control. MDA and GPx were two markers used to represent ROS level in the cells. (C) Cell Counting Kit-8 assay was used to analyze the cell viability. (D) MTT assay was used to analyze the proliferation ability of primary pancreatic cells treated with 100 ng/ml LPS for 0, 24, 48 and 72 h. (E) Flow cytometry was used to detect the percentage of apoptotic cells. (F) Statistical analysis of the percentage of apoptosis cells. $^{**}P<0.01$ vs. control group (n=3). LPS, lipopolysaccharide; ROS, reactive oxygen species; MDA, malondialdehyde; GPx, glutathione peroxidase.

in primary pancreatic cells the expression levels of Bcl2, Bax and PMAIP1 were detected. These three genes reflect the apoptosis rate, while they also regulate cell apoptosis. Pancreatic cells were treated with 100 ng/ml LPS for 2 h, and then the mRNA expression levels of Bcl2, Bax and PMAIP1 were detected by RT-qPCR. As shown in Fig. 2A-C, the mRNA expression level of Bcl2 was significantly decreased in LPS-treated pancreatic cells compared with that in the controls, while the expression levels of Bax and PMAIP1 were significantly increased in LPS-treated pancreatic cells. The protein levels of Bcl2, Bax and PMAIP1 were also detected at 24 h after LPS stimulation, and changes in these levels were consistent with those observed for the mRNA levels (Fig. 2D-G).

LPS induces TLR4 expression and activation of NF-κB signaling in pancreatic cells. LPS is often used to activate inflammatory signaling. In the present study, the activation of inflammatory signaling increased the ROS level and induced cell death, Next, the study verified the activity of inflammatory signaling by detecting the protein levels of p-p65. According to the result shown in Fig. 3A, p-p65 levels were notably increased after pancreatic cells were treated with LPS. Since TLR4 is the upstream molecule that activates NF-κB signaling, the current study next detected the expression levels of TLR4 in LPS-treated pancreatic cells. The results revealed that both the mRNA and protein levels of TLR4 were increased following LPS exposure (Fig. 3B and C).

TLR4 regulates ROS levels and pancreatic cell viability. TLR4 serves a critical role in the pathogenesis of pancreatitis and activates inflammatory signaling in via the myeloid differentiation primary response 88-dependent and -independent pathways (25). To elaborate the mechanism of increased TLR4 in pancreatic cells in response to LPS stimulation, a TLR4 overexpression plasmid was constructed and used for cell transfection. Subsequent to confirming the overexpression effect induced by the plasmid using western blot analysis (Fig. 4A), the cell viability and ROS level in pancreatic cells were detected. CCK-8 and MTT assays demonstrated that cell viability and the proliferation ability were significantly decreased in cells with overexpression of TLR4 (Fig. 4B and C). The apoptosis rate was significantly increased, which was consistent with the change in cell viability (Fig. 4D). Finally, the ROS level we determined following the transfection of pancreatic cells with the TLR4 overexpression plasmid for 24 h. According to the results shown in Fig. 4E and F, TLR4 promoted the generation of oxidative stress in pancreatic cells.

TLR4 inhibitors reverse the LPS-induced cellular damage to pancreatic cells and the activation of NF-κB signaling. To confirm the contribution of NF-κB activation to the increased ROS levels and decreased cell viability, a TLR4 inhibitor was used to inhibit the function of TLR4. The concentration of TLR4 inhibitor used in the current study did not affect the cell viability (data not shown). Primary pancreatic cells were co-treated with LPS and TLR4 inhibitor. The cell viability and ROS levels were reversed in cells co-treated with LPS and TLR4, as compared with cells treated with LPS alone (Fig. 5A-C). Furthermore, as shown in Fig. 5D and E, the TLR4 inhibitor partially restored the apoptosis rate of pancreatic cells

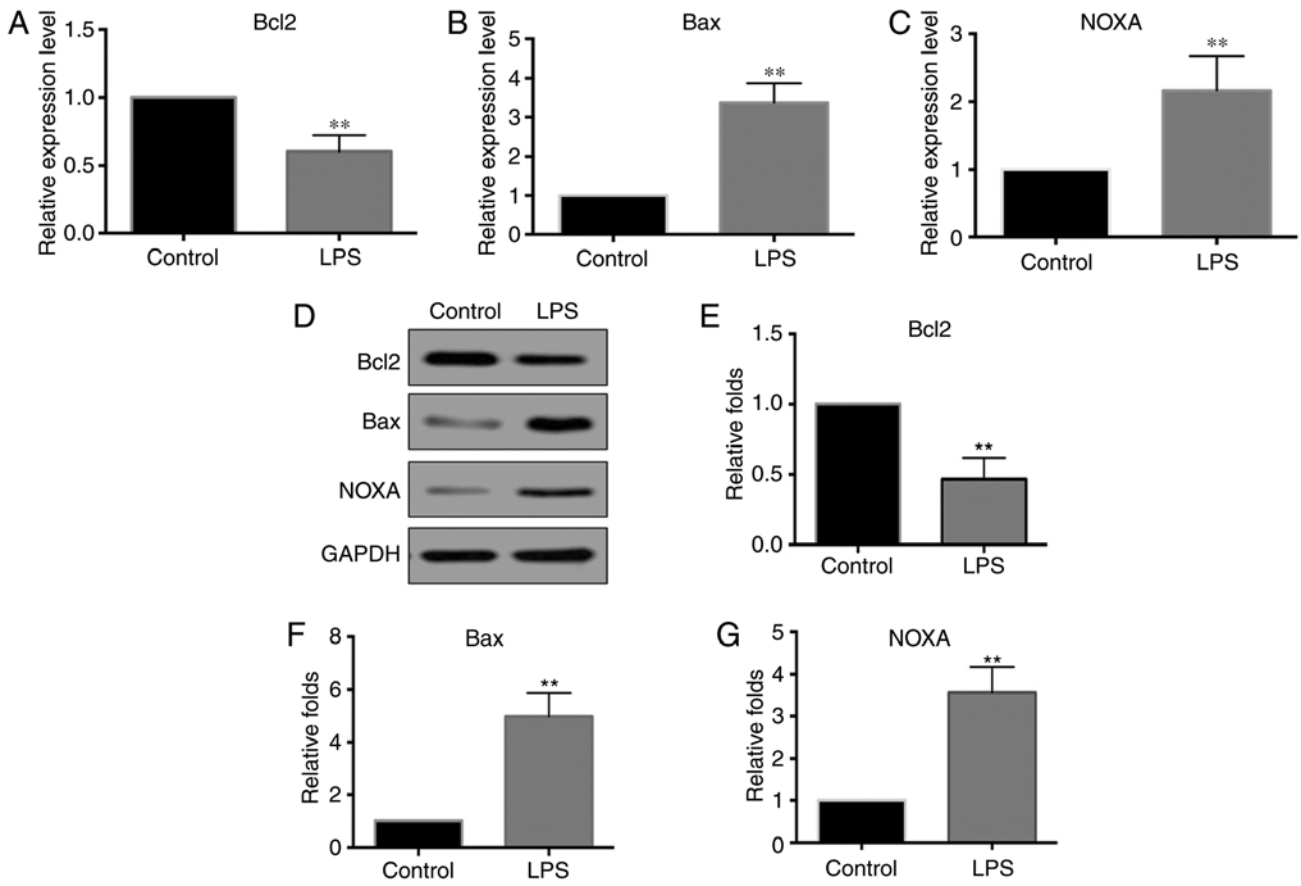

Figure 2. LPS altered the expression of Bcl2 family proteins. Following treatment of primary pancreatic cells with 100 ng/ml LPS for 2 h, RNA was isolated and the mRNA levels of (A) Bcl2, (B) Bax and (C) PMAIP1 was detected by reverse transcription-quantitative polymerase chain reaction. (D) Protein levels of Bcl2, Bax and PMAIP1 were detected by western blot assay in primary pancreatic cells treated with 100 ng/ml LPS for 24 h. Gray analysis of the protein levels of (E) Bcl2, (F) Bax and (G) PMAIP1. $^{**}P<0.01$ vs. control group. LPS, lipopolysaccharide; Bcl2, B-cell lymphoma 2; Bax, Bcl2-associated X protein.

treated by LPS for 24 h. These results suggested that TLR4 may serve a central role in LPS-induced oxidative stress and cell apoptosis.

TLR4 inhibitor reverses the changes in Bcl2, Bax and PMAIP1 expression levels. As indicated earlier, the levels of Bcl2, Bax and PMAIP1 were changed in LPS-stimulated pancreatic cells. In order to further confirm whether the expression of these three genes was affected by TLR4, the expression levels were tested in primary pancreatic cells co-treated with LPS and TLR4 inhibitor. As shown in Fig. 6A-D, the TLR4 inhibitor partially reversed the LPS-induced changes in the expression levels of Bcl2, Bax and PMAIP1 at both the mRNA and protein levels. Further experiments revealed that the inhibition of TLR4 by the selective inhibitor prevented the activation of NF-κB signaling (Fig. 6E). The increased p-p65 level by LPS stimulation was inhibited in TLR4 inhibitor treated cells.

Discussion

SAP is an acute inflammation process caused by the digestion of trypsin in the pancreas and its surrounding tissues. At present, the pathogenesis of SAP is not fully understood, resulting in a lack of appropriate treatment strategies for this disease. In the present study, increased expression of TLR4 in LPS-treated pancreatic cells was observed, while increased ROS level led to pancreatic cell damage, which was caused by TLR4. The current study provided the specific mechanism of the TLR4/NF-κB pathway in causing inflammation-stimulated cell death in the pancreas, indicating a possible target for pancreatitis treatment.

Recently, several studies have identified the importance of TLRs in the mechanism of anti-inflammatory immunity (26,27). TLRs are able to recognize bacterial LPS molecules in pathogenic microorganisms and are closely associated with clinical inflammatory diseases (27). Among these receptors, TLR4 was the first to be identified, and its activation can lead to changes in multiple inflammatory mediators and cytokines, which is regulated by NF-κB signaling (28). It has been reported that NF-κB is able to act as a messenger in the same inflammatory response syndrome in the body. At the early onset stage of local inflammation in an organism, inflammatory cytokines can be activated through NF-κB signaling (29). Thus, the TLR/NF-κB pathway serves an important role in the progression of inflammation.

Functional studies on LPS, TLR4 and NF-κB, and their interaction in inflammatory signaling have increased the awareness on anti-inflammatory immunity. Due to the importance of the LPS/TLR4/NF-kB pathway in the anti-inflammatory immune response, a number of studies have focused on its underlying mechanism. At present, research on TLR/NF-κB has mainly

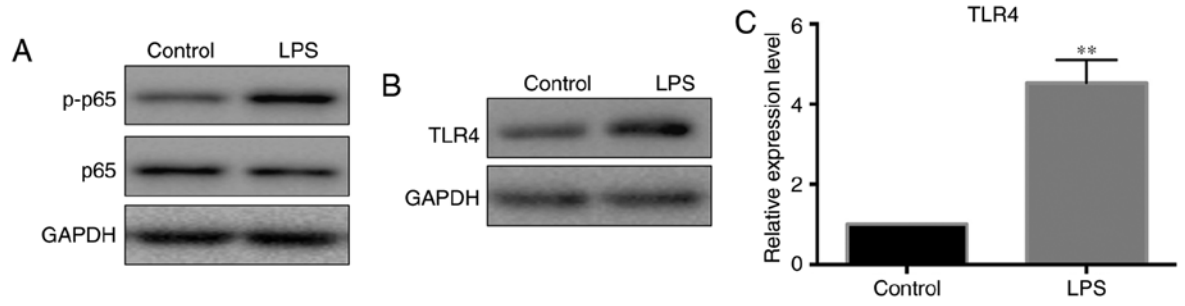

Figure 3. LPS induced TLR4 expression and the activation of NF-κB signaling in pancreatic cells. Primary pancreatic cells were treated with 100 ng/ml LPS for 2 h, and then harvested for RNA and protein isolation. (A) Activity of NF-κB signaling was analyzed based on the protein levels of p65 and p-p65. (B) Protein level of TLR4, detected by western blot assay. (C) mRNA expression level of TLR4, detected by reverse transcription-quantitative polymerase chain reaction. **P<0.01 vs. control group. LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; NF-κB, nuclear factor-κB; p-p65, phosphorylated p65.

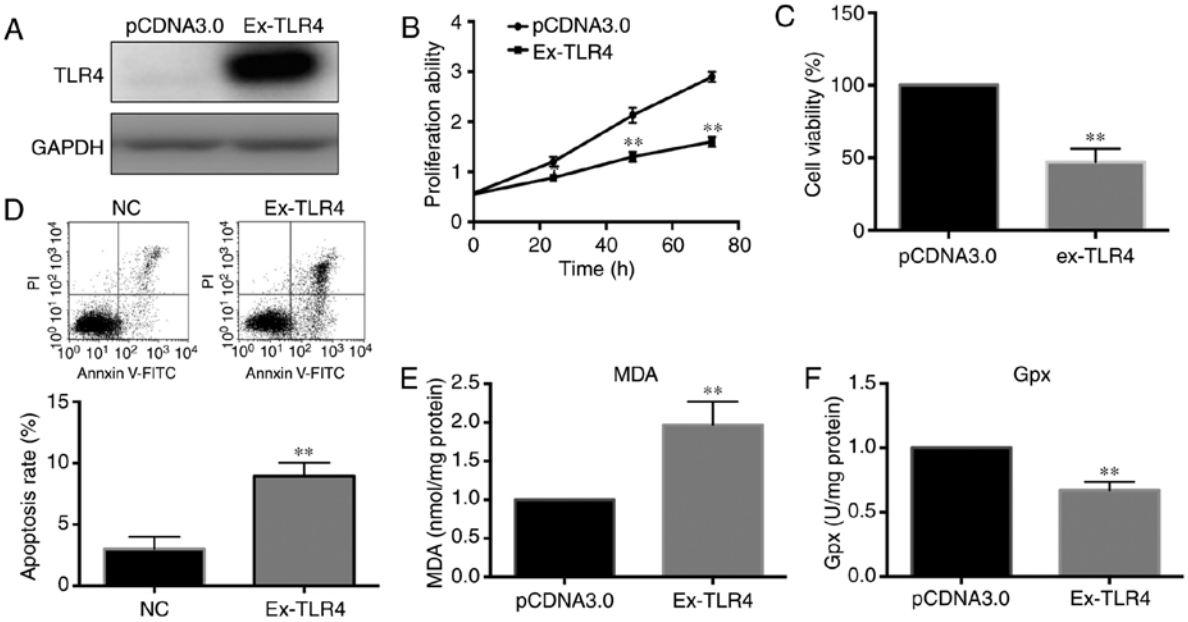

Figure 4. TLR4 regulated the reactive oxygen species level and pancreatic cell viability. Primary pancreatic cells were transfected with TLR4 overexpression plasmid and pCDNA3.0 plasmid. (A) The overexpression effect of TLR4 plasmid was confirmed by western blot analysis. (B) Cell proliferation ability and (C) cell viability following transfection with TLR4 overexpression plasmid or pCDNA3.0 plasmid were detected by MTT and Cell Counting Kit-8 assay, respectively. (D) Percentage of apoptotic cells was detected by flow cytometry. (E) MDA and (F) GPx concentrations were measured in transfected cells. *P<0.05 and **P<0.01 vs. control (pCDNA3.0) group. TLR4, Toll-like receptor 4; MDA, malondialdehyde; GPx, glutathione peroxidase; NC, negative control; ex-TLR4, TLR4 overexpression plasmid.

focused on the identification of TLR structure, the regulation mechanism of NF-κB activation and the nature of TLR-oriented anti-infective drugs (30,31). The present study focused on the regulatory mechanism of NF-κB activation and observed that this involved the generation of cellular ROS. In addition, the regulatory role of TLR4 in pancreatic cells may serve as a new target for pancreatitis treatment. However, attention must also be paid to other pathways in anti-inflammatory immunity and their impacts. On-going studies on these problems will broaden our understanding of the complexities of the roles of pathogens and anti-inflammatory immunity, and provide a theoretical and experimental basis for the further development of drugs targeting the LPS/TLR4/NF-kB pathway. For clinical studies, interventions in certain aspects of the LPS/TLR4/NF-kB pathway may be effective strategies for clinical treatment and provide a new approach for the prevention of certain infectious diseases.

In the current study, decreased cell viability was also observed, which may be caused by increased cell apoptosis. Thus, the expression levels of Bcl2, Bax and PMAIP1 in LPS-treated pancreatic cells were then examined. Bcl2 is one of the most important anti-apoptotic genes, while Bax and PMAIP1 are two important members of apoptotic genes. The expression levels of these Bcl2 family genes reflect the percentage of apoptosis. One possible mechanism through which LPS causes increased ROS level may involve the induction of decreased cell viability, which then leads to a change in the expression levels of Bcl2 family genes. The expression levels of anti-apoptotic gene (Bcl2) were decreased and the pro-apoptotic genes (Bax and PMAIP1) were increased. Another possible mechanism is regulation of the three genes by TLR4. According to the results of the present study, the expression levels of Bcl2, Bax and PMAIP1 exhibited the same changes when pancreatic cells were treated with LPS or transfected with TLR4 overexpression plasmid.

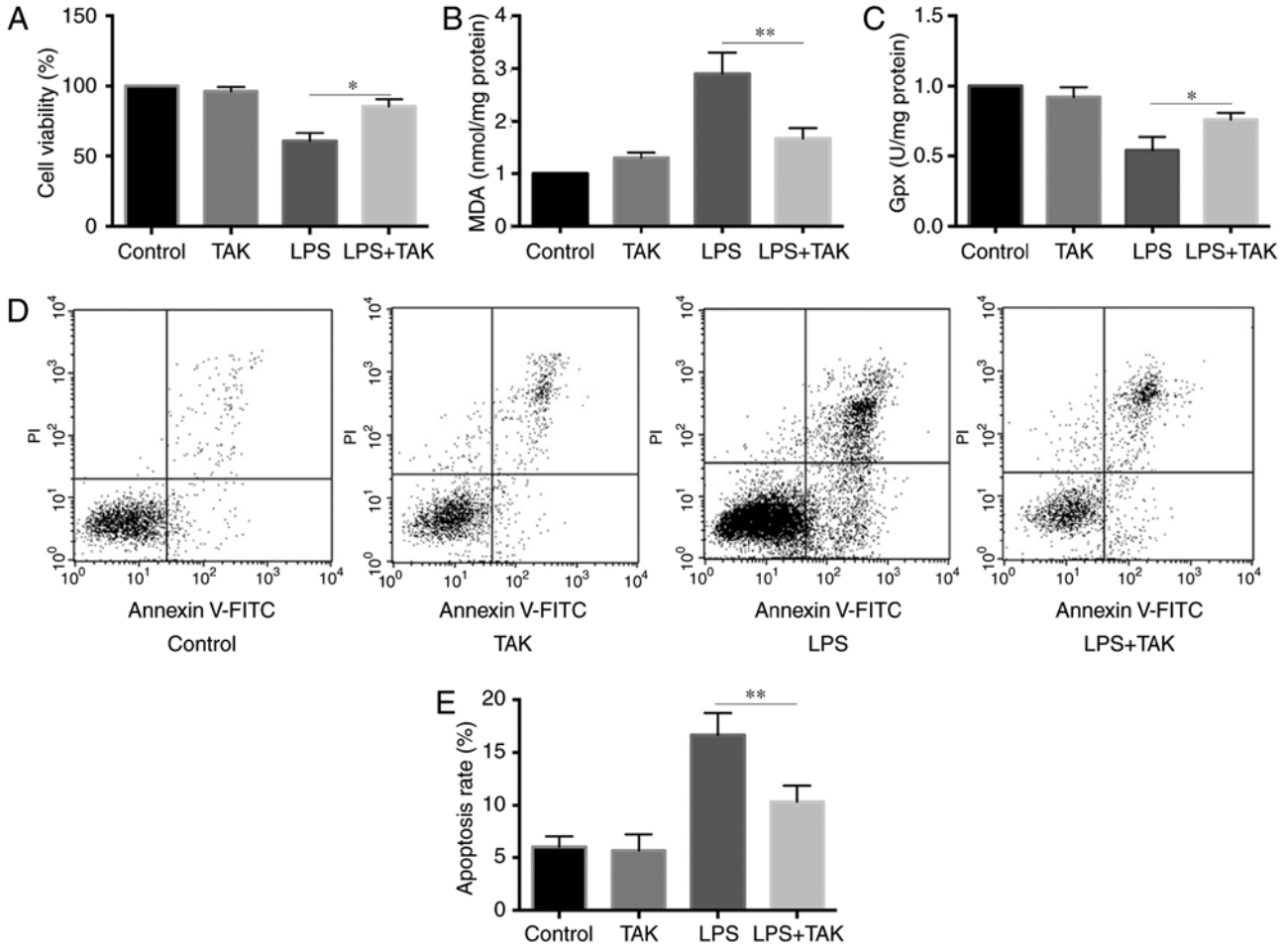

Figure 5. TLR4 inhibitor reversed the LPS-induced cellular damage to pancreatic cells. Primary pancreatic cells were divided into four groups, as follows: Control, TAK-242 (1 μM), LPS and LPS + TAK-242. After 24 h of treatment, cells were harvested for reactive oxygen species level and cell viability ability examination. (A) Cell viability of the four cell groups, detected by Cell Counting Kit-8 assay. (B) MDA and (C) GPx concentrations were measured in cells treated with LPS and/or TAK-242. (D) Flow cytometry results and (E) percentage of apoptotic cells. *P<0.05 and **P<0.01. LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; MDA, malondialdehyde; GPx, glutathione peroxidase.

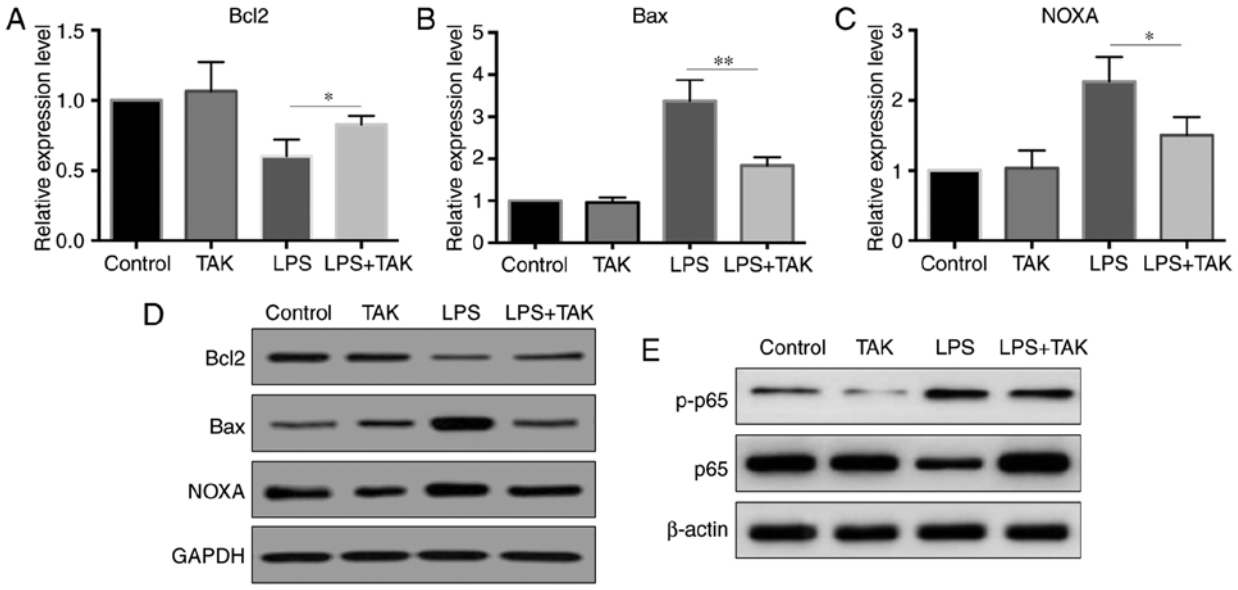

Figure 6. TLR4 inhibitor reversed the LPS-induced changes in Bcl2, Bax and PMAIP1 expression levels. Primary pancreatic cells were divided into four groups [control, TAK-242 (1 μM), LPS and LPS + TAK-242] and treated for 2 h, after which cells were harvested for RNA or protein isolation. (A) Bcl2, (B) Bax and (C) PMAIP1 mRNA expression levels were detected by reverse transcription-quantitative polymerase chain reaction. (D) Protein levels of Bcl2, Bax and PMAIP1, detected by western blot analysis. (E) Protein levels of p65 and p-p65, detected by western blot analysis. *P<0.05 and **P<0.01. LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; Bcl2, B-cell lymphoma 2; Bax, Bcl2-associated X protein; p-p65, phosphorylated p65.

In conclusion, the data obtained in the present study indicated that the LPS-induced TLR4/NF-κB pathway serves a central role in the initiation of inflammation, oxidative stress and pancreatic cell death. Furthermore, TLR4 activation was considered necessary for LPS-induced cell damage. This mechanism has the potential to be a novel therapeutic target in the clinical treatment of pancreatitis.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

HP and LP designed and initiated this study. LP, LY and LW performed the experiments of primary culture of pancreatic acinar cells. JH and JS performed reverse transcription-quantitative polymerase chain reaction of lipopolysaccharide (LPS)-stimulated cells and plasmid-transfected cells. HW and HF performed western blotting in LPS-stimulated and plasmid-transfected pancreatic acinar cells. ZB performed cell proliferation and viability assays. XW measured the reactive oxygen species level in LPS-stimulated pancreatic acinar cells. HP, LP and HF wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the Xi'an Jiaotong University (Xi'an, China) approved the present study. Analysis was performed in accordance with the ethical standards of the hospital and the tenets of the Declaration of Helsinki/Declaration of Istanbul.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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