Activation of the Nuclear Factor-kappa B Signaling Pathway Damages the Epithelial Barrier in the Human Pancreatic Ductal Adenocarcinoma Cell Line HPAF-II

Zhou Su, MA, Yahui Gong, MA, Huiyang Yang, MD, Dehai Deng, MD, and Zhihai Liang, MD

Objectives: Injury of the pancreatic duct epithelial barrier plays a critical role in the development of acute pancreatitis. The activity of the nuclear factor-kappa B (NF-κB) pathway is involved in the disruption of the pancreatic duct epithelial barrier. This study investigated how NF-κB impacts the dysfunction of the pancreatic duct epithelial barrier.

Methods: A human pancreatic ductal adenocarcinoma cell line was treated with tumor necrosis factor-alpha (TNF-α) and pyrrolidine dithiocarbamate. The expression levels of p65 and p-p65 were detected to evaluate NF-κB activity. Tricellulin (TRIC) expression levels were measured to assess the change in tight junction (TJ)-related proteins. The expression and localization of myosin light chain kinase (MLCK) were investigated. The structure of TJs and monolayer permeability were also examined.

Results: NF-κB was activated by TNF-α and suppressed by pyrrolidine dithiocarbamate. Activation of NF-κB upregulated the expression levels of TRIC and MLCK. Broadened TJs were observed after NF-κB was activated. Lower monolayer permeability was observed when NF-κB was suppressed.

Conclusions: Activation of the NF-κB pathway induced by TNF-α leads to increased TRIC and MLCK expression, resulting in broadened TJs and high permeability, which contribute to damage to the pancreatic duct epithelial barrier.

Key Words: NF-κB, myosin light chain kinase, tight junctions, pancreatic duct epithelial barrier, acute pancreatitis, mechanism

The pancreatic duct epithelial barrier consists of pancreatic duct epithelial cells and secreted mucus. Pancreatic duct epithelial cells transport the enzymes produced by acinar cells into the duodenum. In addition, they secrete fluid that is rich in HCO₃⁻ to neutralize gastric acid from the stomach. Thus, the pancreatic duct epithelial barrier can prevent bile and trypsin from refluxing into the pancreatic parenchyma and prevent pancreatic juice rich in HCO₃⁻ from refluxing into blood. Tight junctions (TJs) apically located in intercellular junction complexes play an important role in barrier function. In addition, the disruption of TJs in pancreatic duct epithelium is an early event in acute pancreatitis (AP). Our previous studies showed that myosin light chain kinase (MLCK) was upregulated and TJs were broadened in a rat model of AP. Myosin light chain kinase might promote the damage of pancreatic tissue via TJ regulation. However, the mechanisms of TJ disruption and the pathogenesis of AP remain unknown in human pancreatic duct epithelial cells.

Myosin light chain kinase is one of the key substances in the regulation of TJs in epithelial cells. This protein has two main functional domains: calmodulin and immunoglobulin G. Myosin light chain kinase combines Ca²⁺ with calmodulin and then combines MLC with immunoglobulin C2, resulting in MLC phosphorylation. The phosphorylation of MLC shrinks myosin driven by ATP, which broadens TJs, eventually leading to barrier dysfunction. The damage to TJs was observed in an intestinal cell model in which MLCK was highly expressed in the cell membrane. Furthermore, both MLCK gene knockout and MLCK inhibitor treatment could relieve the damage to TJs. However, the relationship between MLCK and TJs in pancreatic duct epithelial cells is rarely reported.

Studies have shown that the effect of MLCK on TJs is closely related to the inflammatory response mediated by the nuclear factor-kappa B (NF-κB) signaling pathway. Nuclear factor-kappa B is a critical regulator of gene expression in inflammation. Nuclear factor-kappa B induced by the inflammatory factor tumor necrosis factor-alpha (TNF-α) was associated with increased epithelial permeability and TJ dysfunction. In a recent study, NF-κB was activated by TNF-α, increasing the expression of MLCK and leading to TJ disruption and high permeability in intestinal epithelial cells. Significantly, NF-κB binding motifs were observed on the MLCK promoter region, acting as a regulatory site to promote MLCK gene expression. Together, NF-κB activation induced by TNF-α promoted MLCK expression, leading to TJ dysfunction and hyperpermeability in intestinal epithelial cells. The mechanism of how NF-κB affects TJ disruption in pancreatic duct epithelial cells remains to be clarified. We hypothesize that activation of NF-κB damages the pancreatic duct epithelial barrier and triggers the onset of AP.

In this study, we investigate the role of the NF-κB pathway in pancreatic duct epithelial barrier damage in AP.

MATERIALS AND METHODS

Cell Culture and Preparation

The human pancreatic duct adenocarcinoma cell line, HPAF-II, was obtained from the American Type Culture Collection (ATCC, Manassas, Va). The HPAF-II cell line is useful as a model for studies aimed at understanding epithelial polarity, regulation of junctional complexes, and disease processes in the pancreas. The cell line was maintained in ATCC-formulated Eagle’s Minimum Essential Medium supplemented with 10% fetal calf serum (FCS, Invitrogen, ...
Real-Time Quantitative Polymerase Chain Reaction

TRizol reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, Mass) was used to extract total RNA from the cells. Reverse transcription was carried out. Gene expression levels were measured by a RT-PCR kit (Qiagen, Inc., Valencia, Calif), according to the manufacturer's instructions. The primers were designed using the software Primer Premier 5.0 (Premier Bio soft International, Inc., Palo Alto, Calif). The primer sequences were as follows: P65, forward 5′-ACAACACCTCTCAGAAGACG-3′, reverse 5′-GCC TGGTCCCCGTGAAATACA-3′; tricellulin (TRIC), forward 5′-GAT GCCGCAGCTATGTGGCACA-3′; reverse 5′-CTCTCATGGTCCTGTGG CGCCTTGC-3′; MLCK, forward 5′-GGGGAGCTTCTCAAGCTGGTG-TG A-3′; reverse 5′-ACATTCTCCCTGACGCAAGT-3′; GAPDH, forward 5′-CAATATCAGGACGCTCA-3′; reverse 5′-GACT CCACGAGTACTCAGC-3′. The conditions for quantitative analysis were as follows: 95°C for 2 minutes, 95°C for 5 seconds, and 60°C for 30 seconds. Expression levels were calculated using the 2−ΔΔct method. The internal control was the GAPDH gene.

Western Blot Analysis

A radioimmunoprecipitation assay was utilized to extract total proteins from the HPAF-II cells. The concentrations of the extracted proteins were measured by a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Fifty micrograms of proteins for each sample were loaded into each lane of 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, separated by gel electrophoresis, and transferred to polyvinylidene fluoride membranes. After blocking, blots were incubated with primary antibodies, including NF-κB p65 mAb (3033, CST), anti-MLCK antibody (ab76092; Abcam, Cambridge, UK), and anti-TRIC antibody (Ab203567; Abcam), overnight at room temperature. After that, incubation was carried out with horseradish peroxidase-conjugated secondary antibodies (ab97023; Abcam) at room temperature for 1 hour and then detected by ECL reagents (BioRad, Shanghai, China) using X-ray film. Band density was measured by ImageJ (version 1.8; National Institutes of Health, Bethesda, Md). Protein expression of MLCK was detected by WES (ProteinSimple, San Francisco, Calif), using the Wes 66 to 440 kDa Mouse Master kit (PS-ST03-8; ProteinSimple) and Wes 12 to 230 kDa Master kit (SM-W002; ProteinSimple), according to the manufacturer's instructions.

Enzyme-linked Immune Sorbent Assay Assays

The protein levels of MLCK in HPAF-II cells were detected by a MLCK enzyme-linked immune sorbent assay (ELISA) assay kit (CUSABIO, Wuhan, China) following the manufacturer's instructions. Protein standards were always utilized for MLCK quantification.

Immunofluorescence Staining

Cells were cultured on slides overnight. The cultures were fixed with 4% paraformaldehyde for 15 minutes and then permeabilized with 0.5% Triton X-100 for 15 minutes. After blocking, the cells were incubated with secondary antibody Alexa Fluor 488-conjugated Affinipure Goat Anti-Rabbit IgG and then detected by ECL reagents (BioRad, Shanghai, China) for 1 hour. The slides were stained with DAPI (C1005; Beyotime, Shanghai, China) for 30 minutes. The slides were then removed and dried after washing. Images were captured by laser scanning fluorescence microscopy (Nikon Eclipse 80i; Nikon Corporation, Tokyo, Japan) at 400 × magnification. ImageJ was used to analyze fluorescence intensity.

Immunohistochemistry Assays

The slides were prepared as described above for immunofluorescence. After blocking, the slides were treated with 3% hydrogen peroxide at room temperature for 10 minutes. Anti-MLCK antibodies (ab76092; Abcam) were added and incubated overnight. Then, cells were incubated with HRP-conjugated secondary antibodies (ab97023; Abcam) at room temperature for 1 hour. Slides were dyed with diaminobenzidine and counterstained with hematoxylin afterward. The slides were visualized using a fluorescence microscope (CX71; Olympus Corporation, Tokyo, Japan) at 400 × magnification.

Observation of Intercellular TJs by Transmission Electronic Microscopy

Three groups of cells were fixed in 4% phosphate-buffered glutaraldehyde and then fixed with 1% osmium tetroxide. Cells were put into a graded series of ethanol and acetone for dehydration and immersed in Epon12 epoxy resin overnight. After embedding, the embedded, polymerized blocks were sliced by an LKB-V ultrathin slicer with a thickness of 60 nm and stained with uranyl acetate and lead citrate. Changes in TJs between epithelial cells were observed by transmission electronic microscopy (H-7650; Hitachi, Ltd., Tokyo, Japan) at ×20,000 magnification.

Measurement of Paracellular Marker Fluorescein Isothiocyanate-Dextran Flux

Human pancreatic duct adenocarcinoma cells were resuspended at a density of 3 × 105 cells/mL. One hundred microliters of cell suspension was added to each upper transwell chamber, whereas 600 μL of complete medium was added to each lower transwell chamber, and incubation was carried out at 37°C overnight. The culture medium was changed every 24 hours. After the cells grew for 4 days, 100 μL fluorescein isothiocyanate-dextran (FITC-D) (1 mg/mL) was added into each upper chamber, and 600 μL polybutylene succinate was added into each lower chamber. The incubation was conducted at room temperature for 1 hour. The fluorescence of the culture medium in the lower chambers was detected by a fluorescence spectrophotometer. The relative permeability of FITC-D = Fluorescence value of TNF-α or PDTC group/fluorescence value of control group.

Statistical Analysis

Quantitative data are presented as the mean ± standard error (SE) of the mean. The difference was assessed by analysis of variance (ANOVA) using SPSS 17.0 software (SPSS, Inc., Chicago, Ill). P less than 0.05 was considered statistically significant.

RESULTS

TNF-α Activated the NF-κB Signaling Pathway, and PDTC Inhibited NF-κB in HPAF-II Cells

After treatment with TNF-α for 6 hours, the expression of p65 mRNA detected by qPCR was upregulated compared with the controls (Fig. 1A). Although p65 protein detected by Western blotting was downregulated, p-p65 protein was upregulated compared...
The expression levels of MLCK are positively correlated with the group compared with the controls. These results indicated that both Western blotting and ELISA was decreased in the PDTC results shown are representative of three similar experiments.

expression levels detected by qPCR were opposite to the results of PDTC (Fig. 2). These results showed that changes in TRIC mRNA were tested by Western blotting and ELISA, and there was a significant increase in MLCK protein expression detected by qPCR was downregulated compared with the TNF-α group (Fig. 1A). Protein levels of p65 and p-p65 were also downregulated (Fig. 1B). Thus, PDTC inhibited the expression and phosphorylation of p65. These results indicated that the NF-κB signaling pathway was involved in this experiment.

NF-κB Activation Increased TRIC Expression, and the Opposite Effect Was Observed When NF-κB Was Inhibited

In HPAF-II cell lines, TRIC mRNA and protein were all upregulated by treatment with TNF-α, whereas TRIC mRNA expression increased and TRIC protein decreased by treatment with PDTC (Fig. 2). These results showed that changes in TRIC mRNA expression levels detected by qPCR were opposite to the results of Western blotting. However, Chen et al. also showed that measurement of the mRNA response for many genes was not predictive of the protein response. The level of mRNA is an indicator of gene transcription, but it is not the only indicator of protein production. Since the protein, not the RNA, is the effector molecule of gene, the expression levels of TRIC were evaluated by Western blotting in this study.

NF-κB Activation Induced by TNF-α Increased the Transcription and Expression of MLCK, Whereas MLCK Was Suppressed by Inhibiting NF-κB

Myosin light chain kinase mRNA detected by qPCR was upregulated in response to TNF-α stimulation in HPAF-II cells compared with the controls (Fig. 3A). The protein levels of MLCK were tested by Western blotting and ELISA, and there was a significant increase in MLCK protein expression in the TNF-α group compared with the controls (Fig. 3B and Fig. 3C). On the other hand, after treatment with PDTC, MLCK mRNA was downregulated compared to the TNF-α group. The expression of MLCK in both Western blotting and ELISA was decreased in the PDTC group compared with the controls. These results indicated that the expression levels of MLCK are positively correlated with the activity of the NF-κB signaling pathway. To observe the distribution of MLCK, immunofluorescent staining and immunohistochemistry were performed in HPAF-II cell lines. As expected, TNF-α strongly induced MLCK protein staining compared with the controls (Fig. 3D and Fig. 3E).

NF-κB Activation Widened Intercellular TJs, and NF-κB Inhibition Downregulated Permeability

Cellular structure and intercellular TJs were observed in HPAF-II cells. To detect the structure of the treated cells, we observed the TJs by electron microscopy. Intercellular TJs in the control group showed no significant change. Broadened TJs were observed in the TNF-α group, and tighter TJs were observed in the PDTC group (Fig. 4A). The monolayer permeability of the HPAF-II cell line was evaluated by FIFC-D flux. After being exposed to TNF-α, the permeability was increased indistinctly. However, the permeability was obviously decreased after treatment with PDTC compared with the control group (P < 0.05) (Fig. 4B). These results indicated that activation of NF-κB had a disruptive effect on TJs and permeability of HPAF-II cells, and the inhibition of NF-κB induced tighter conjunction.

DISCUSSION

Acute pancreatitis is a disease with high morbidity, but its pathogenesis has not been clarified. Previous studies have shown that activation of the NF-κB pathway damages the pancreas in experimental pancreatitis. Tumor necrosis factor-alpha can activate the NF-κB signaling pathway, causing increased NF-κB-mediated MLCK expression and MLCK phosphorylation, resulting in altered permeability and TJ disruption in the intestinal epithelium. To investigate whether impairment of pancreatic duct epithelium is consistent with the mechanism above, this study was carried out.

First, NF-κB activity was detected in pancreatic duct epithelial cells treated with TNF-α and PDTC. In this study, TNF-α upregulated the expression and phosphorylation of p65, which means that TNF-α can activate the NF-κB signaling pathway. We all know that PDTC is a specific inhibitor of NF-κB. This study confirmed that PDTC downregulates the expression and phosphorylation of p65, leading to the suppression of the NF-κB signaling pathway. With the altered activity of the NF-κB pathway, a series of changes takes place in the barrier function of pancreatic duct epithelial cells.
FIGURE 2. The expression of TRIC was upregulated by the activation of the NF-κB pathway, which was opposite when the NF-κB pathway was inhibited. TRIC mRNA levels were increased in the TNF-α and PDTC groups (A). The expression of TRIC protein was increased when NF-κB was activated and decreased after NF-κB was suppressed (B). The results shown are representative of three similar experiments. *P < 0.05 vs group control.

FIGURE 3. The activity of the NF-κB pathway was positively correlated with MLCK expression in HPAF-II cells. MLCK mRNA and protein expression levels determined by qPCR and Western blotting were upregulated by the activation of NF-κB and downregulated by the inhibition of NF-κB compared with the controls (A, B). The level of MLCK protein was also determined by ELISA. The production of MLCK was increased by the activation of the NF-κB pathway and was decreased by the inactivation of NF-κB (C). HPAF-II cell monolayers were stained for MLCK by immunofluorescence and immunohistochemistry (magnification, 400×) (D). The optical density of immunohistochemistry images was calculated (E). The results shown are representative of three similar experiments. *P < 0.05 vs group control; #P < 0.05 vs TNF-α group.
The TJ barrier may be one of the mechanisms of AP. Further study on regulating the pancreatic duct epithelial barrier is needed. This study demonstrates that the NF-κB signaling pathway affected the barrier function of pancreatic duct epithelial cells by altering TJ-related proteins, upregulating MLCK expression and widening TJs. The damage to the pancreatic duct epithelial barrier may be one of the mechanisms of AP. Further study on regulating the pancreatic duct epithelial barrier is needed. This study provides some theoretical basis for the pathogenesis of AP.

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