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Original Article

P38 MAPK signaling pathway mediates the induction effect of LPS on human intrahepatic bile duct cell MUC5AC

Jie Xiong, Yuan Liu, Zhongyong Jiang, Gang Dan

Department of Clinical Laboratory, Chengdu Military General Hospital, Chengdu Military District, Chengdu 610083, Sichuan, China

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Abstract: Bile duct inflammation is an important influencing factor of the pathogenesis of intrahepatic bile duct stone. MUC (mucins protein) excessive secretion may cause intrahepatic bile duct inflammation. Previous studies showed that inhibiting p38 MAPK mediated signaling pathway can reduce LPS induced mucin synthesis, thereby reducing gastric epithelial cell apoptosis. This study explored the protective role of p38 MAPK mediated signaling pathway in intrahepatic bile duct inflammation. Human intrahepatic biliary epithelial cells (HIBEC) were randomly divided into four groups: control group, LPS group, LPS+NC group, and LPS+P38si group. Agarose gel electrophoresis was used to test MUC5AC gene expression. qRT-PCR was applied to detect MUC5AC mRNA expression. Western Blot was performed to determine MUC5AC, p38, and p-p38 protein expression levels. ELISA was used to detect IL-1β and TNF-α. Compared with the normal control, LPS significantly induced MUC5AC mRNA and protein expression in HIBEC. P38 protein level also elevated significantly after LPS induction. Under LPS effect, p38 interference obviously reduced MUC5AC protein expression, indicating that LPS induced MUC5AC overexpression in HIBEC through p38 phosphorylation. LPS elevated IL-1β and TNF-α level, while p38 interference markedly declined inflammatory markers expression. The above results showed that inhibiting p38 MAPK signaling pathway alleviated LPS induced HIBEC inflammation by reducing MUC5AC expression. Suppressing p38 MAPK signaling pathway alleviated LPS induced HIBEC inflammation through decreasing MUC5AC expression.

Keywords: p38 MAPK, HIBEC, MUC5AC, bile duct inflammation, LPS

Introduction

Intrahepatic bile duct stone, a type of gallstones, refers to the stone in the branch bile duct before left and right hepatic duct confluence. As an important cause of death in benign biliary tract disease, it can cause severe complications [1]. Thus, searching for effective methods for control and treatment is extremely important. Bile duct inflammation is an important influencing factor of intrahepatic bile duct stone, while lipopolysaccharide (LPS) is considered to be the main inducing factor of systemic inflammatory response [2]. Studies found that LPS can increase mucin MUC5AC level, thus exacerbated the inflammatory response [3]. Therefore, MUC5AC might be an important target for inhibiting bile duct inflammation.

Mucin (MUC) widely expressed on various tissue epithelium with lubricate and protective effect [4]. MUC is rich in intrahepatic bile duct mucosa, including MUC1, MUC2, MUC4, MUC5 (MUC5AC and MUC5B), and MUC6 [5]. It was reported that MUC5AC expression was extremely rare in normal intrahepatic bile duct tissues. However, its level significantly upregulated in intrahepatic bile duct stone [6], which further promotes us to investigate the role of MUC5AC in bile duct inflammation.

As one of the subtypes of mitogen-activated protein kinase (MAPK), p38 MAPK widely exists in mammalian cells. P38MAPK was found mainly involved in apoptosis and cell cycle related pathophysiological process in early studies, thus play an important role in liver cancer, ovarian cancer, non-Hodgkin's lymphoma [7, 8]. Recent studies revealed that suppressing p38 MAPK signaling pathway can reduce MUC synthesis induced by LPS, thus alleviate gastric

Table 1. p38 and normal control interference sequence

Name	Sequence (5'-3')	Base number
p38 siRNA sense-strand	AGUGUGUGCUAACCGUUACCU	21
p38 siRNA anti-sense-strand	ACUGCCCAGUUCGUUUCAGUG	21
Control siRNA sense-strand	UUCUCCGAACGUGUCACGUUU	21
Control siRNA anti-sense-strand	ACGUGACACGUUCGGAGAAUU	21

Table 2. Experimental grouping

Grouping	1	2	3	4
100 μg/ml LPS for 24 h	-	+	+	+
NC siRNA	-	-	+	-
P38 siRNA	-	-	-	+

epithelial cell apoptosis [9]. It was still unclear about whether p38 MAPK signaling pathway affected LPS induced bile duct inflammation through MUC5AC.

This research mainly focused on MUC5AC mRNA and protein expression changes under LPS stimulation, to clarify MUC5AC as target in bile duct inflammation treatment. We further explored the role of p38 MAPK regulating MUC5AC in bile duct inflammation. This research was helpful to provide new target for biliary tract disease prevention and treatment.

Materials and methods

Experimental cell line

Human intrahepatic biliary epithelial cells (HIBEC) [10] were bought from the Chinese Academy of Sciences.

Main reagents

LPS was bought from Sigma (St. Louis, MO, USA). Si p38 and si normal control (NC) were designed and synthesized by Sigma. DMEM-F12 medium, fetal bovine serum, penicillin-streptomycin, PBS, and potassium phosphate buffer were provided by Epitomics (Burlingame, CA, USA). GAPDH was bought from Beijing Liuyi Instrument Factory (Beijing, China). Mucin 5AC antibody and p38 antibody were got from Tongren (Japan). HRP tagged goat anti-rat IgG was from Shanghai BestBio Biology. Opti-MEM medium and Lipofectamine 2000 were bought from Abcam (USA). Human IL-β ELISA kit was purchased from RayBio (Georgia, USA). SYBR Green PCR Master Mix was from GenePharma Biotech (Shanghai, China).

Cell transfection

SiRNA/miRNA working concentration was 50 nM diluted by lipofectamine 2000 and Opti-MEM medium. The cells received transfection at 30-50% fusion degree. The transfection solution was prepared as follows: 1.25 μl siRNA was solved in 100 μl Opti-MEM medium as fluid A, and 1 μl lipofectamine 2000 was dissolved in Opti-MEM medium as fluid B. A and B were mixed after 5 min. 20 min later, the transfection solution was added to the well for 4 h. After 6 h, the cells were photographed under fluorescence microscope to observe the transfection efficiency. SiRNA sequences were shown in **Table 1**.

Grouping and modeling

HIBECs were randomly divided into four groups: control group, LPS group, LPS+NC group, and LPS+P38si group (**Table 2**). 100 μg/ml LPS treated for 24 h was used for modeling.

RNA extraction

The cells were collected and added with 1 ml Trizol for 5 min. Then 0.2 ml chloroform was added at room temperature for 5 min. After centrifuged at 12000 g and 4°C for 15 min, RNA existed in the upper aqueous phase. 500 μl aqueous phase was moved to another EP tube and added with equal volume of isopropanol at room temperature for 20 min. After centrifuged at 12000 g and 4°C for 10 min, the supernatant was removed and 1 ml 75% ethanol (0.1% DEPC water based) was added to wash RNA precipitation. After centrifuged at 7500 g and 4°C for 5 min twice, the RNA was dried and dissolved in 30 μl DEPC water stored at -80°C.

qRT-PCR primer design and synthesis

The primers used were listed as follows: MUC5AC-F, 5' CAGCATCATCAACAGCGAAAC; MUC5AC-R, 5' TAGTCACAGAACAGTGGGCAGA; GAPDH-F, 5' ATGGGGAAGGTGAAGGTGG; GAPDH-R, 5' GGGGTCATTGAGGCAACAATA.

qRT-PCR

RNA was quantified by UV-2100 ultraviolet and visible spectrophotometer. Equal amount of RNA was applied for reaction as follows: 50°C

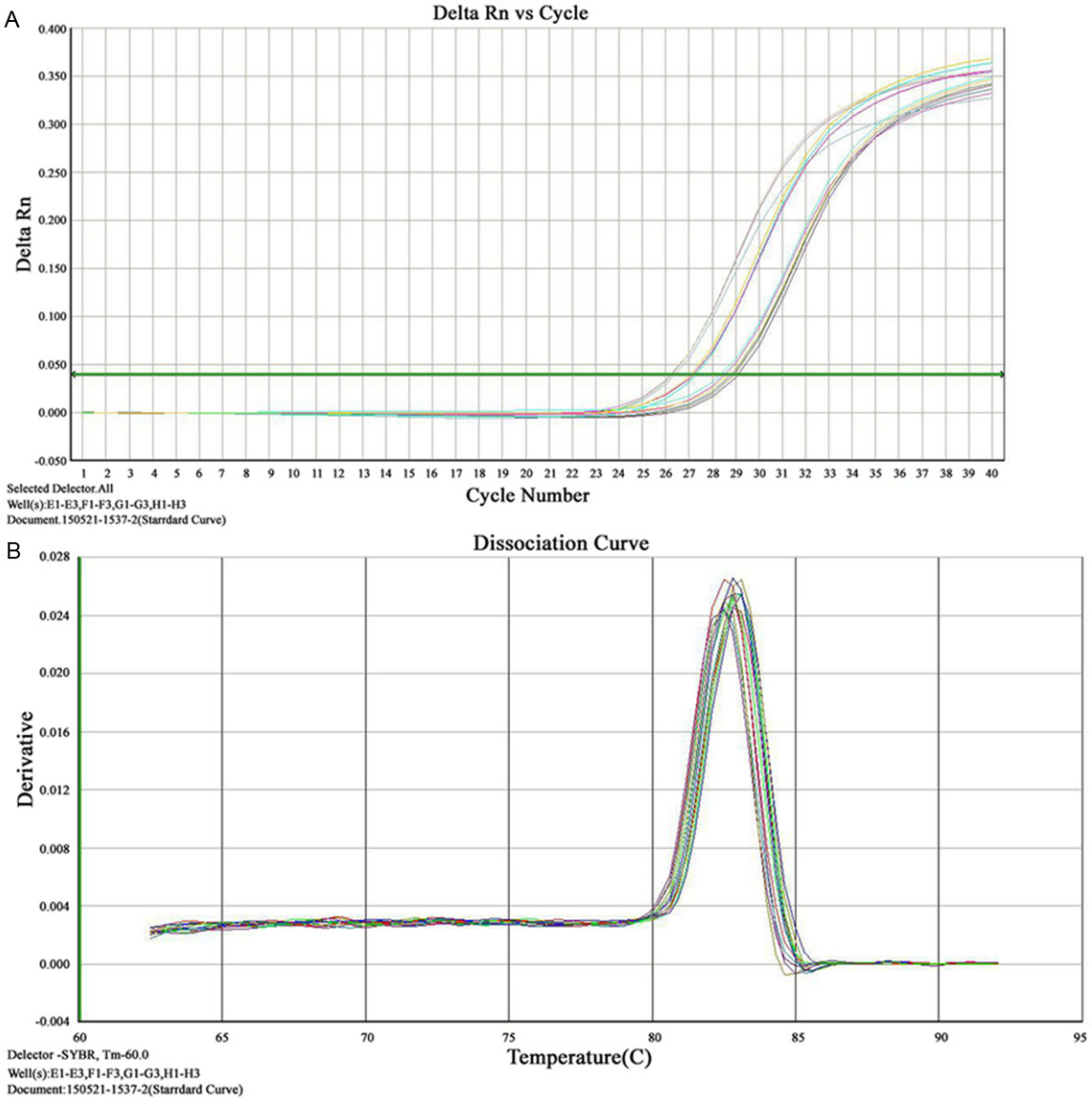

Figure 1. A. MUC5AC amplification curve. B. MUC5AC melting curve.

for 30 min; 30 cycles of 95°C for 5 min, 95°C for 30 s, 55°C for 30 s, and 72°C for 50 s; at last, 72°C for 10 min. $2^{-\Delta\Delta Ct}$ method was performed to calculate gene expression based on melting curve and amplification curve. Quantitative product was analyzed using Applied Biosystems 7300 System.

Western blot

Protein extraction: PMSF was added to RIPA to make the final concentration to 1 mM. Medium was removed and the plate was washed by PBS for three times. RIPA was added to the cells at 4°C for 15 min, and then the fluid was centrifuged at 15000 rpm for 15 min. The supernatant was moved to a new precooled EP tube. Protein concentration was determined by BCA method, and diluted to the same level. After degenerated at 100°C for 5 min, the protein was stored at -80°C.

Western blot: Different concentration of separation gel was prepared based on protein molecular weight. 60 μg protein was separated by SDS-PAGE at 100 V. Then the protein was transferred to PVDF membrane at 300 mA. The membrane was blocked at 5% skim milk at 37°C for 2 h and incubated in primary antibody at 1:1000 at 4°C overnight. After washed by TTBS for three times (10 min/time), the membrane was then incubated in secondary antibody (1:1000) at 37°C for 2 h. TMB substrate

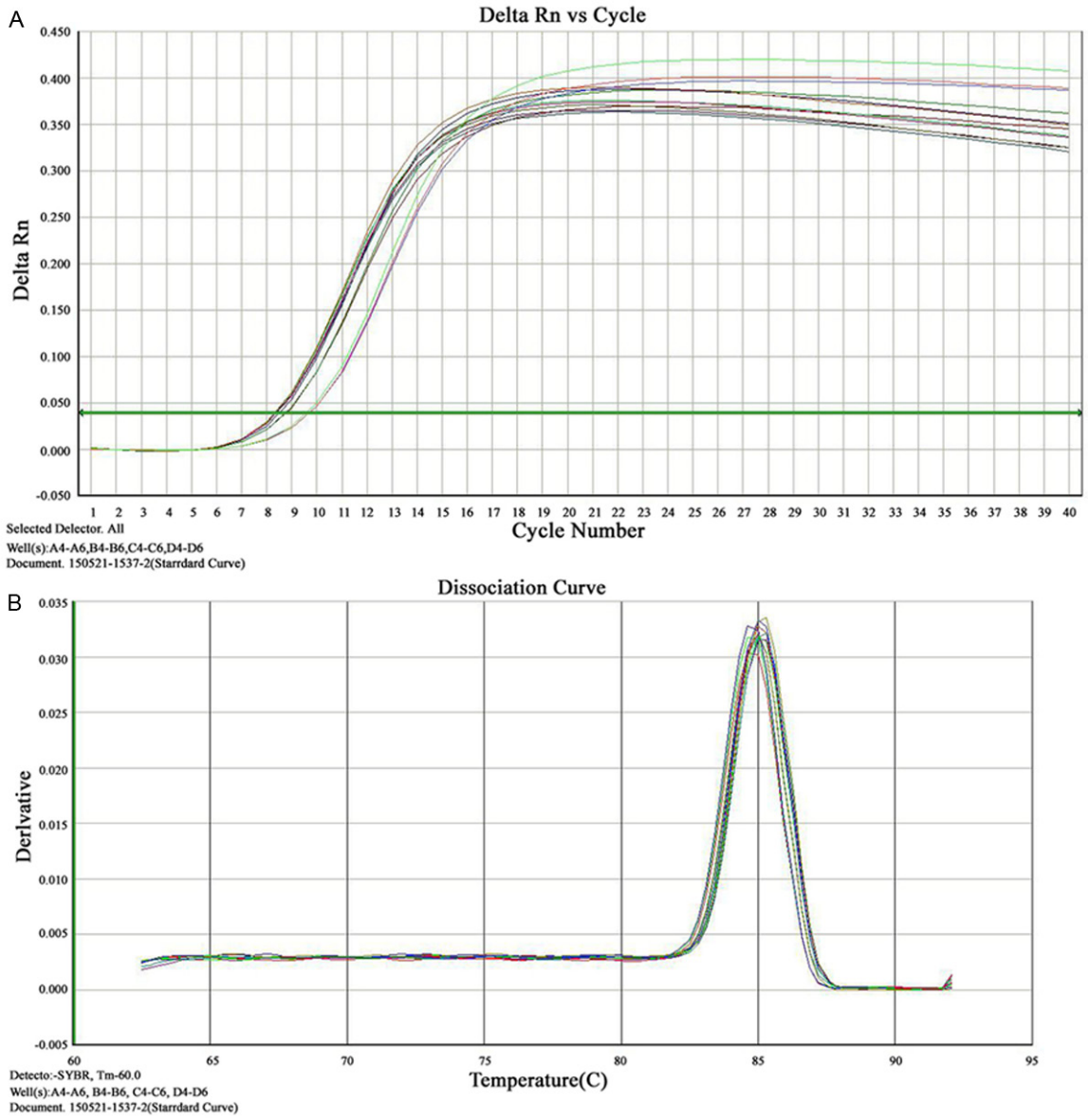

Figure 2. A. GAPDH amplification curve. B. GAPDH melting curve.

was prepared according to the ECL kit instruction. After added with TMB substrate away from light for 2 min, protein bind was analyzed using Gel-Pro-Analyzer 5.0 software. All the experiments were repeated for three times.

ELISA

100 μl standard substance or sample was added to each well overnight. After removing the fluid, the plate was washed by buffer for 4 times and added 100 μl antibody solution for 1 h. After washed by buffer for 4 times, the plate was added with HRP-Streptavidin solution for 45 min. After washing, the plate was added with 100 μl TMB-substrate reagent (Item H) for 30 min. Then 50 μl stop buffer (Item I) was added to each well and the plate was read at 450 nm to calculate concentration based on standard curve.

Statistical analysis

All the statistical analyses were performed on SPSS19.0. The data was presented as mean ± SD. ANOVA or LSD test was used for comparison. $P<0.05$ was considered as significant difference.

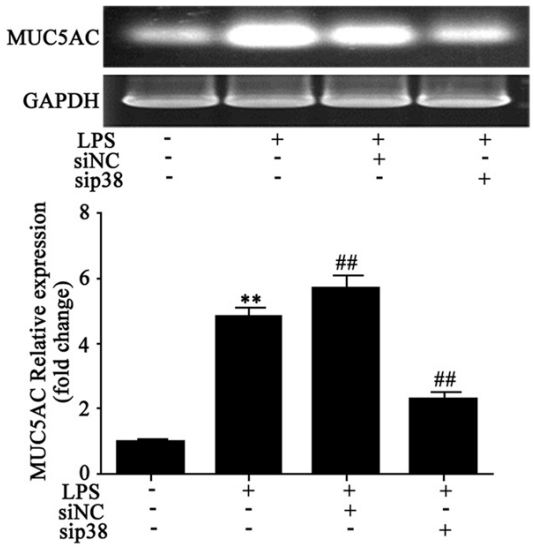

Figure 3. Agarose gel electrophoresis detection of MUC5AC gene expression. **P<0.05, compared with normal control; ##P<0.05, compared with siNC.

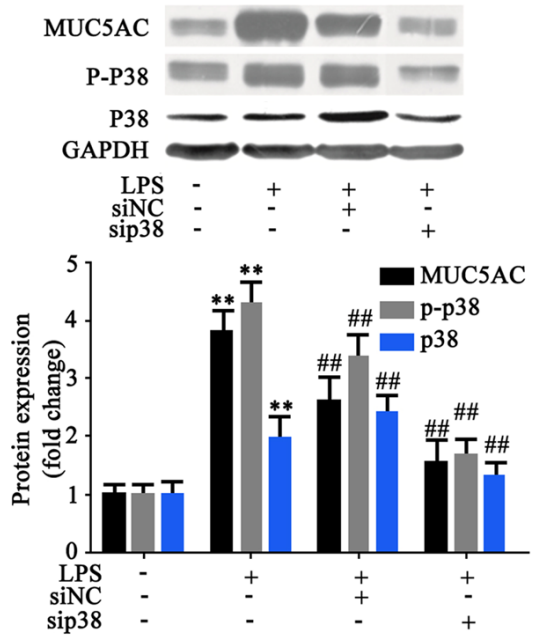

Figure 4. LPS effect on MUC5AC, p38, and p-p38 protein expression. **P<0.05, compared with normal control; ##P<0.05, compared with siNC.

Results

MUC5AC gene detection

MUC5AC expression upregulated significantly in intrahepatic stone compared with normal control [6]. LPS was considered as the main induction factor of systemic inflammatory reaction. Studies found that LPS can elevate MUC5AC level in mice, thus aggravate inflammation [3]. In this study, we tested MUC5AC gene expression in normal and LPS stimulation. As shown in **Figure 1A** and **1B**, MUC5AC melting curve presented single peak and amplification curve presented amplification phenomena under LPS stimulation, while NC did not showed such phenomena (**Figure 2A** and **2B**). It indicated that MUC5AC mRNA elevated under LPS stimulation, and was an important target for intrahepatic stone.

p38 MAPK regulation on MUC5AC

It was confirmed that MUC5AC and NF-κB were important regulatory factors mediating inflammatory reaction [11]. Increased MUC5AC can cause various inflammatory reactions, while p38 signaling pathway can activate NF-κB [11]. To explore p38 expression impact on MUC5AC synthesis, we detected phosphorylated p38 and MUC5AC mRNA and protein expression under normal or interference p38. As shown in **Figure 3**, LPS stimulation significantly raised MUC5AC level (P<0.05). After p38 interference, p38 reduction obviously decreased LPS induced MUC5AC overexpression. We then used Western blot to determine MUC5AC, p38, and p-p38 protein expression. LPS elevated MUC5AC protein content, increased p38 level, and promoted p38 phosphorylation level (**Figure 4**). P38 interference inhibited such trend, suggesting that LPS may cause MUC5AC overexpression through p38 signaling pathway.

p38 MAPK impact on inflammation through MUC5AC

It was showed that MUC5AC was one of the important regulatory factors mediating a variety of inflammatory responses [11]. To investigate whether p38 MAPK signaling pathway can regulate MUC5AC and impact bile duct inflammation, we tested IL-1β and TNF-α content in the medium. As shown in **Table 3**, LPS raised IL-1β and TNF-α level, whereas sip38 obviously reduced IL-1β and TNF-α expression. It revealed that p38 interference can decline inflammatory cytokine production, thus inhibiting LPS induced inflammation.

Discussion

Intrahepatic bile duct stone is a type of gallstones that is an important death cause of benign biliary tract disease. Multiple factors

Table 3. IL-1β and TNF-α level detection

Group	IL-1β (pg/ml)	TNF-α (pg/ml)
Control	16.81±3.43	28.17±7.07
LPS	111.26±3.19**	157.06±2.08**
LPS+Ncsi	109.78±5.52	158.17±3.6
LPS+P38si	59.04±1.05##	109.83±1.36##

**P<0.05, compared with normal control; ##P<0.05, compared with siNC.

can affect disease progression, such as nutritional status, environmental factor, biliary infection, cholestasis, and bile duct variation [12, 13]. Researches showed that bile duct inflammation is a critical influence factor of inducing intrahepatic biliary calculi [14]. Thus, searching for effective method to control and treat bile duct inflammation became an effective way to prevent and treat intrahepatic bile duct stone. In this study, we clarified that: 1) MUC5AC played an important role in regulating LPS induced inflammatory response. 2) p38 MAPK signaling pathway impact LPS induced inflammation through regulating MUC5AC. 3) Inhibiting p38 expression can alleviate LPS induced inflammation by reducing MUC5AC level, thus becoming an important target for biliary tract disease.

LPS was considered to be the main inducing factor of systemic inflammatory response. It was found that LPS can cause mice MUC5AC expression elevation, thus exacerbating inflammatory response. Excessive secreted MUC5AC may adhere to the bile duct wall or fill in the bile duct cavity, triggering biliary obstruction and cholestasis. It further induced inflammation, and inflammatory reaction further caused MUC5AC overexpression [15, 16]. Therefore, MUC5AC might become an important target of inhibiting bile duct inflammatory response. To prove the important role of MUC5AC in bile duct inflammation, we established HIBEC inflammation model induced by LPS. It was found that LPS obviously upregulated IL-1β and TNF-α levels, together with MUC5AC mRNA and protein. These results revealed that LPS induced HIBEC inflammation was related to MUC5AC abnormal expression. Inflammation aggravation followed by MUC5AC overexpression, thus MUC5AC was an important target for inhibiting intrahepatic bile duct inflammation.

MAPK family was conservative serine/threonine protein kinase in the regulation of a series of physiological and pathological processes [17]. P38 was an important molecular of MAPK family in controlling inflammation [18]. Recent studies showed that p38 MAPK can affect airway inflammation and gastric epithelial cells apoptosis by regulating MUC5AC synthesis [19-21]. To confirm whether p38 MAPK signaling pathway can impact biliary tract inflammation through regulating MUC5AC, we interfere with p38 MAPK to detect MUC5AC level and inflammatory cytokine content. The results showed that LPS can increase p38 MAPK and MUC5AC expression, and aggravate inflammation. P38 MAPK interference significantly reduced LPS induced MUC5AC elevation and alleviated inflammation. MUC5AC overexpression induced by LPS was mediated by p38 signal pathway, and associated with p38 MAPK phosphorylation.

Conclusion

Reducing MUC5AC expression can decline the incidence of biliary obstruction and cholestasis, so as to alleviate inflammatory reaction. Our results proved that p38 MAPK signaling pathway can influence LPS induced bile duct inflammation by regulating MUC5AC expression. This pathway could provide target for drug treatment of bile duct inflammation and intrahepatic bile duct stone.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jie Xiong, Department of Clinical Laboratory, Chengdu Military General Hospital, Chengdu Military District, 270 Rongdu Road, Jinniu District, Chengdu 610083, Sichuan, China. Tel: +86-28-86570452; Fax: +86-28-86570452; E-mail: xiongjieli@126.com

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