PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2006 January 21; 12(3): 485-488
World Journal of Gastroenterology ISSN 1007-9327
© 2006 The WJG Press. All rights reserved.

RAPID COMMUNICATION

Role of nitric oxide in Toll-like receptor 2 and 4 mRNA expression in liver of acute hemorrhagic necrotizing pancreatitis rats

Lei Zhang, He-Shui Wu, Yan Chen, Xing-Jun Guo, Lin Wang, Chun-You Wang, Jing-Hui Zhang, Yuan Tian

Lei Zhang, He-Shui Wu, Xing-Jun Guo, Lin Wang, Chun-You Wang, Center of Pancreatic Surgery, Affiliated Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China
Jing-Hui Zhang, Yuan Tian, Laboratory of General Surgery, Affiliated Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China
Yan Chen, Pediatric Department, Affiliated Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
Supported by the National Natural Science Foundation of China, No. 30200272
Correspondence to: He-Shui Wu, Center of Pancreatic Surgery, Affiliated Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China. whs1898@public.wh.hb.cn
Telephone: +86-27-85726273
Received: 2005-05-30 Accepted: 2005-06-11

Abstract

AIM: To investigate the role of nitric oxide (NO) in Toll-like receptor 2 (TLR2)/4mRNA expression in livers of acute hemorrhagic necrotizing pancreatitis (AHNP) rats.

METHODS: One hundred and ten SD male rats were randomly divided into sham-operated group ($n = 10$), AHNP group ($n = 30$), chloroquine (CQ)-treated group ($n = 30$) and L-Arg-treated group ($n = 40$). TLR2/4mRNA expression in the liver of AHNP rats was measured by RT-PCR.

RESULTS: Expression of TLR2/4mRNA could be detected in the liver of AHNP rats in sham-operated group (0.155E-5 ± 0.230E-6 and 0.115E-2 ± 0.545E-4), but was markedly increased at 3 h in AHNP group (0.197E-2 ± 0.114E-3 and 0.175 ± 0.349E-2) peaking at 12 h (0.294E-2 ± 0.998E-4 and 2.673 ± 2.795E-2, $P < 0.01$). Hepatic injuries were aggravated, TNF-α concentration in the liver was increased and NO concentration was decreased ($P < 0.05$ or $P < 0.01$). When TLR2/4mRNA expression was inhibited by CQ (3 h: 1.037E-4 ± 3.299E-6 and 0.026 ± 3.462E-3; 6 h: 1.884E-4 ± 4.679E-6 and 0.108 ± 6.115E-3; 12 h: 2.443E-4 ± 7.714E-6 and 0.348 ± 6.807E-3; $P < 0.01$), hepatic injuries were relieved, NO concentration in the liver was increased and TNF-α concentration was decreased ($P < 0.05$ or $P < 0.01$). When rats with AHNP were treated with L-Arg, TLR2/4mRNA expression in the liver could be effectively inhibited (50 mg-T: 0.232E-2 ± 0.532E-4 and 0.230 ± 6.883E-3; 100 mg-T: 0.210E-2 ± 1.691E-4 and 0.187 ± 0.849E-2; 200 mg-T: 0.163E-2 ± 0.404E-4 and 0.107 ± 0.195E-2; 400 mg-T: 0.100E-2 ± 0.317E-4 and 0.084 ± 0.552E-2; $P < 0.01$) and hepatic injuries were relieved. At the same time, NO concentration in the liver was markedly increased and TNF-α concentration was decreased ($P < 0.05$ or $P < 0.01$).

CONCLUSION: The expression of TLR2/4mRNA is increased and hepatic injuries are aggravated in the liver of AHNP rats. TLR2/4mRNA gene expression in the liver of AHNP rats can be markedly inhibited by NO, leading to the relief of hepatic injuries.

© 2006 The WJG Press. All rights reserved.

Key words: Toll-like receptors; Acute hemorrhage necrotizing pancreatitis; Liver; Nitric oxide; Chloroquine

Zhang L, Wu HS, Chen Y, Guo XJ, Wang L, Wang CY, Zhang JH, Tian Y. Role of nitric oxide in Toll-like receptor 2 and 4 mRNA expression in liver of acute hemorrhagic necrotizing pancreatitis rats. *World J Gastroenterol* 2006; 12(3): 485-488

http://www.wjgnet.com/1007-9327/12/485.asp

INTRODUCTION

Acute hemorrhagic necrotizing pancreatitis (AHNP) is a serious disease of human beings with a high mortality and morbidity. AHNP could cause multiple organ dysfunction syndrome (MODS). Unfortunately, the pathogenesis and mechanism of AHNP are still unclear. Many researches indicate that diverse inflammatory factors such as tumor necrosis factor (TNF-α), interleukin-1 (IL-1), IL-6 and reactive oxygen species result in systemic inflammatory response syndrome (SIRS) which might play an important role in the pathogenesis and development of AHNP[1-3]. It was reported that Toll-like receptor 2 (TLR2)/4 activated by stimulations can result in excessive production and release of cytokines[4]. In the present study, we have investigated the changes of TLR2/4 gene expression and the effect of NO on TLR2/4 gene expression in livers of AHNP rats.

Table 1 Serum amylase, ALT, and AST concentrations (mean±SD)

		n	Serum amylase (U/L)	ALT (U/L)	AST (U/L)
A	unit	10	985±159.68	74.0±4.47	176.6±4.52
B	unit	10	6 367±1 122.17[b]	101.8±4.11[a]	447.9±54.49[b]
C	unit	10	9 370±2 282.79[b]	232.9±24.01[b]	1055.9±41.57[b]
D	unit	10	13 189±3 365.14[b]	546.5±37.36[b]	1276.2±44.22[b]
E	unit	10	3 450±711.25[bd]	95.5±4.19[b]	370.3±19.67[b]
F	unit	10	4 165±1 005.31[b,c,e]	119.7±4.74[b,d]	784.6±68.93[b,d]
G	unit	10	5 540±1 274.81[b,c,e]	197.5±9.09[b,d]	982.7±46.22[b,d]
H	unit	10	6 793±1 414.78[b]	212.8±5.50[b]	854.3±53.26[b,d]
I	unit	10	6 518±246.13[b,c]	142.2±7.73[b,d]	405.9±62.62[b,d]
J	unit	10	5 462±822.44[b,c]	115.4±6.30[b,d]	385.4±6.72[b,c,d]
K	unit	10	4 789±826.59[b,c]	92.3±3.69[b,d]	309.7±15.90[b,d]

[a]$P<0.05$, [b]$P<0.01$ *vs* A unit; [c]$P>0.05$ *vs* ahead unit; [e]$P<0.05$, [d]$P<0.01$ *vs* unit at the same time point of AHNP group.

MATERIALS AND METHODS

Chloroquine (CQ) and sodium taurocholate (TAC) were purchased from Sigma (St. Louis, MO, USA). L-Arg was purchased from Cayman Chemical Company, USA. Trizol was purchased from Promega Co., Hong Kong, China. Reverse transcriptase RNase and DNA polymerase were purchased from TOYOBO CO., LTD, Japan. Serum-amylase and NO detection kits were provided by Jiancheng Biological Engineering Research Institute, Nanjing, China.

Groups and models

One hundred and ten SD male rats (weighing 180-200 g) were purchased from Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Rats were randomized into sham-operated group ($n=10$), AHNP group (3, 6, and 12 h, units B-D, $n=10$), CQ-treated group (3, 6, and 12 h, units E-G, $n=10$), L-Arg-treated group (50, 100, 200, and 400 mg; units H-K; $n=10$).

AHNP was induced by infusion of 5% TAC (1 mL/kg) into biliopancreatic duct. After models of AHNP were made, L-Arg (100 mg/kg) was immediately injected via inferior vena to make L-Arg-treated models. Sham-operated models were made by flipping ceca. Samples of liver and blood were taken for analysis.

Alanine aminotransferase, aspartate aminotransferase, AST, serum amylase, and NO concentrations in the liver

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were detected to assess the degrees of hepatic injuries. ALT and AST concentrations were measured using an automatic biochemistry analyzer. Concentrations of serum amylase and NO in the liver were measured spectrophotometrically.

TLR2/4 mRNA and TNF-α mRNA gene expression in the liver

The expression of TLR2/4mRNA and TNF-αmRNA in the liver was assayed by RT-PCR. The sequence of TLR2 primer was 5'-CGCTTCCTGA ACTTGTCC-3' (sense), 5'-GGTTGTCACCTGCTTCCA-3' (anti-sense) and 5'-ACTAAGAGGCGGAGCGGA-3' (fluorescent probe). The sequence of TLR4 primer was 5'-ATCATGGCATTGTTCCTTTCCT-3' (sense), 5'-CTGAGATTCTG ATCCATGCATTG-3' (anti-sense) and 5'-TCGGTAACG ACGGTTGTAG-3' (fluorescent probe). The sequence of TNF-α primer was 5'-CCCGTCG GAACAGGGAACTT-3' (sense), 5'-GGGTGTCCTTAGGGCAAG-3' (anti-sense) and 5'-CGAGGAGGCGAACCACCAA-3' (fluorescent probe). The sequence of β-actin primer 5'-GAACGGTGAAGGTGACAG-3' (sense), 5' -TAGA GAGAGTGGGGTGG-3' (anti-sense) and 5'-ACCACAGCACCTGCGG GAT-3' (fluorescent probe). Results were obtained using FTC-2000 real-time instrument (Fengling Biotechnology Limited Company, Shanghai, China).

Statistical analysis

The data were expressed as mean ± SD. The differences between the two groups were assessed by Student's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Serum amylase, ALT and AST concentrations

Serum amylase concentration was lower in CQ-treated and L-Arg-treated groups than in AHNP group (*t*: 2.087-2.195, $P<0.05$). ALT and AST concentrations were low in sham-operated group but significantly increased in AHNP group (*t*: 4.58-9.740, $P<0.01$). Administration of CQ or L-Arg significantly reduced ALT and AST concentrations in the liver (*t*: 1.074-8.765, $P<0.05$, Table 1).

NO, TNF-α concentrations and TLR2/4mRNA expression

TNF-α was low in sham-operated group but significantly increased in AHNP group (*t*: 6.848-9.959, $P<0.01$). Administration of CQ or L-Arg significantly reduced TNF-α concentration in liver (*t*: 3.946-8.997, $P<0.01$). NO concentration was markedly lower in AHNP group than in sham-operated group (*t*: 2.403–8.521, $P<0.05$, CQ-treated group and L-Arg-treated group (*t*: 2.138-9.597, $P<0.05$). TLR2/4mRNA expression was low in sham-operated group but markedly increased at 3 h in AHNP group and peaked at 12 h (*t*: 2.193-9.623, $P<0.01$). TLR2/4mRNA expression was inhibited by CQ (*t*: 2.294-8.382, $P<0.01$), and L-Arg (*t*: 3.880-8.995, $P<0.05$; Table 2).

DISCUSSION

Ten members have been identified from the mammalian TLR family[5,6]. TLRs belong to a wider superfamily, called IL-1 receptors/TLR superfamily, including receptors for the pro-inflammatory cytokines IL-1 and IL-18. All members possess cytoplasmic Toll/IL-1 receptor (TIR) domains. The TIR domain consisting of 160 amino acids is essential for signaling. TLRs are the key front-line sensors of invading microbes, responding to a wide range of microbial products through recognizing a different pathogen-associated molecular pattern (PAMP). At the same time, when TLRs are combined with PAMP, antigen presenting cells (APCs) are activated and produce co-

Table 2 NO and TNF-α concentrations, and TLR2/4mRNA expression in livers (mean±SD)

		n	NO (μmol/gprot)	TNF-α	TLR2	TLR4
A	unit	10	16.19±0.862	0.003±0.129E-3	0.115E-5±0.229E-6	0.1145E-2±0.545E-4
B	unit	10	12.91±1.058[a]	2.331±0.101[b]	0.197E-2±0.114E-3[a]	0.175±0.349E-2[b]
C	unit	10	9.53±0.344[b]	1.618±0.173[b]	0.275E-2±0.352E-4[b]	0.285±0.516E-2[b]
D	unit	10	4.52±0.356[b]	0.296±0.04[b]	0.294E-2±0.998E-4[b,c]	2.673±2.795E-2[b]
E	unit	10	27.78±0.542[b,d]	1.440±5.147E-2[b,d]	1.037E-4±3.299E-6[b,d]	0.026±3.462E-3[b,d]
F	unit	10	20.73±0.462[b,d]	0.862±3.197E-2[b,d]	1.884E-4±4.679E-6[b,d]	0.108±6.115E-3[b,d]
G	unit	10	13.70±0.734[a,d]	0.117±1.492E-2[b,d]	2.443E-4±7.714E-6[b,d]	0.348±6.807E-3[b,d]
H	unit	10	9.87±0.095[b]	0.676±2.092E-2[b,d]	2.324E-2±0.532E-4[b,d]	0.229±6.883E-3[b,d]
I	unit	10	11.69±0.954[b,c,d]	0.369±1.300E-2[b,d]	0.210E-2±1.691E-4[b,c,d]	0.187±0.085E-3[b,d]
J	unit	10	25.43±0.919[b,d]	0.115±0.587E-2[b,d]	0.163E-2±0.404E-4[b,e]	0.107±1.946E-3[b,d]
K	unit	10	32.99±0.382[b,d]	0.058±0.652E-2[b,d]	0.100E-2±0.316E-4[b,e]	0.084±5.522E-3[b,d]

[a]$P<0.05$, [b]$P<0.01$ *vs* A unit; [c]$P>0.05$ *vs* ahead unit; [d]$P<0.01$, [e]$P<0.05$ *vs* unit at the same time point of AHNP group.

stimulatory molecules and cytokines, activating specific immune responses. TLR2/4 plays the most important role in responding to bacterial infections. TLR2 is the major receptor for PAMPs of Gram-positive bacteria, such as peptidoglycan and lipoteichoic acids. TLR4, the major receptor for LPS, are highly susceptible to infections with Gram-negative bacteria and fungal pathogens, such as lipopolysaccharide (LPS) and heat shock protein (HSP)[7]. Mechanisms responsible for TLR-mediated protection, potentiation of cytokine release, mediation of neutrophil recruitment to the site of infection and release of oxygen and nitrogen radicals, and contribute to TLR activation[8]. Researches showed that $TLR2^{-/-}$ or $TLR4^{-/-}$ mice have an increased susceptibility to infections[9-12]. NO produced from L-Arg by catalysis of nitric oxide synthase (NOS) is the only resource of NO in body[13]. In our experiment, rats were injected with L-Arg so that NO concentration in the bodies of rats was elevated. At the same time, it was reported that TLR2/4mRNA expression can be inhibited by CQ[14]. In the present study, we investigated the changes of TLR2/4 gene expression and NO concentration in the liver when TLR2/4mRNA expression was inhibited by CQ and the effect of NO on TLR2/4 gene expression in the liver of AHNP rats.

Enteric bacteria and endotoxin can result in aggravation of AHNP. In the course, enteric bacteria and endotoxin enter the liver through portal vein and are deactivated. But when the function of the liver is damaged or the quantity of enteric bacteria and endotoxin exceeds the endurance of liver, bacteria and endotoxin enter the blood and result in sepsis. At the same time, AHNP can lead to the dysfunction of the liver and even the failure of liver, suggesting that liver may play an important role in the pathogenesis and development of AHNP.

Our study showed that TLR2/4mRNA expression could be detected in sham-operated group (0.155E-5±0.230E-6 and 0.115E-2±0.545E-4), but markedly increased at 3 h in AHNP group (0.197E-2±0.114E-3 and 0.175±0.349E-2) and peaked at 12 h (0.294E-2±0.998E-4 and 2.673±2.795E-2, $P<0.01$). Hepatic injuries were aggravated, while TNF-α concentration increased and NO concentration decreased ($P<0.05$). When TLR2/4mRNA expression was inhibited by CQ (3 h: 1.037E-4±3.299E-6 and 0.026±3.462E-3; 6 h: 1.884E-4±4.679E-6 and 0.108±6.115E-3; 12 h: 2.443E-4±7.714E-6 and 0.348±6.807E-3; $P<0.01$), hepatic injuries were relieved while NO concentration increased and TNF-α concentration decreased ($P<0.05$). When AHNP rats were treated with L-Arg, TLR2/4 mRNA expression was effectively inhibited (50 mg-T: 0.232E-2±0.532E-4 and 0.230±6.883E-3; 100 mg-T: 0.210E-2±1.691E-4 and 0.187±0.849E-2; 200 mg-T: 0.163E-2±0.404E-4 and 0.107±0.195E-2; 400 mg-T: 0.100E-2±0.317E-4 and 0.084±0.552E-2; $P<0.01$) and hepatic injuries were relieved. At the same time, NO concentration markedly increased and TNF-α concentration decreased ($P<0.05$).

When TLR2/4mRNA expression inhibited, synthesis and release of inflammatory factors decreased and hepatic injuries were relieved, suggesting that lower concentration of NO has the anti-inflammatory effect[15,16]. In our study, NO concentration in AHNP rats was decreased. When TLR2/4mRNA expression was inhibited by CQ, NO concentration increased, suggesting that TLR2/4mRNA expressions can be inhibited by NO. Our results suggest that synthesis and release of anti-inflammatory factors might be inhibited by TLR2/4mRNA expression and TLR2/4mRNA gene expression might play an important role in the pathogenesis and development of hepatic injury in AHNP.

Lower concentration of NO may exert its anti-inflammatory effect by reducing neutrophilic leukocytes, platelet and adhesion molecules, concentration of inflammatory factors in bronchoalveolar lavage, improving the pancreatic blood flow and pancreatic microcirculation, inhibiting the production of oxyradicals and cytokines, interaction of leukocytes and endotheliocytes.

NO may decrease TLR2/4mRNA expression by directly inhibiting production of cytokines, reducing differentiation of phagocytes and interaction between leukocytes and endotheliocytes. The results of our experiment provide evidence for the role of NO in TLR2/4mRNA expression in the liver of AHNP rats.

REFERENCES

1 **Morrison CP**, Teague BD, Court FG, Wemyss-Holden SA,

Metcalfe MS, Dennison AR, Maddern GJ. Experimental studies of serum cytokine concentration following pancreatic electrolytic ablation. *Med Sci Monit* 2003; **9**: BR43-BR46

2 **Zhang Q**, Ni Q, Cai D, Zhang Y, Zhang N, Hou L. Mechanisms of multiple organ damages in acute necrotizing pancreatitis. *Chin Med J (Engl)* 2001; **114**: 738-742

3 **Maki Sugimoto**, Tadahiro Takada, Hideki Yasuda, Hodaka Amano, Masahiro Yoshida, Takahiro Isaka, Naoyuki Toyota, Keita Wada, Kenji Takagi. Cytokine storm in acute pancreatitis: influence of inflammatory attack and arterial infusion therapy. *International Congress Series* 2003; **1255**: 183-186

4 **Frantz S**, Kelly RA, Bourcier T. Role of TLR-2 in the activation of nuclear factor kappaB by oxidative stress in cardiac myocytes. *J Biol Chem* 2001; **276**: 5197-5203

5 **Takeda K**, Akira S. TLR signaling pathways. *Semin Immunol* 2004; **16**: 3-9

6 **Zhang D**, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S. A toll-like receptor that prevents infection by uropathogenic bacteria. *Science* 2004; **303**: 1522-1526

7 **Netea MG**, Van der Meer JW, Kullberg BJ. Toll-like receptors as an escape mechanism from the host defense. *Trends Microbiol* 2004; **12**: 484-488

8 **Kaisho T**, Akira S. Toll-like receptors as adjuvant receptors. *Biochim Biophys Acta* 2002; **1589**: 1-13

9 **Echchannaoui H**, Frei K, Schnell C, Leib SL, Zimmerli W, Landmann R. Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation. *J Infect Dis* 2002; **186**: 798-806

10 **Koedel U**, Angele B, Rupprecht T, Wagner H, Roggenkamp A, Pfister HW, Kirschning CJ. Toll-like receptor 2 participates in mediation of immune response in experimental pneumococcal meningitis. *J Immunol* 2003; **170**: 438-444

11 **Woods JP**, Frelinger JA, Warrack G, Cannon JG. Mouse genetic locus Lps influences susceptibility to Neisseria meningitidis infection. *Infect Immun* 1988; **56**: 1950-1955

12 **Shahin RD**, Engberg I, Hagberg L, Svanborg Edén C. Neutrophil recruitment and bacterial clearance correlated with LPS responsiveness in local gram-negative infection. *J Immunol* 1987; **138**: 3475-3480

13 **Li GK**, Zhang ZHD, Liu XB, Guo YY. The role of L-arginine in the treatment of acute edematous pancreatitis in rats. *Chin Experimental Surgery* J 1999; **16**: 244-248

14 **Hong Z**, Jiang Z, Liangxi W, Guofu D, Ping L, Yongling L, Wendong P, Minghai W. Chloroquine protects mice from challenge with CpG ODN and LPS by decreasing proinflammatory cytokine release. *Int Immunopharmacol* 2004; **4**: 223-234

15 **Chen XJ,** Zhang QY, Chen BC. Experimental research of L-argignine in the treatment of acute hemorrhagic necrotizing pancreatitis in early stage. *J Hepatopancreatobiliary Surg* 2002; **14**: 220-222

16 **Masamune A**, Shimosegawa T, Satoh A, Fujita M, Sakai Y, Toyota T. Nitric oxide decreases endothelial activation by rat experimental severe pancreatitis-associated ascitic fluids. *Pancreas* 2000; **20**: 297-304

S- Editor Wang XL and Guo SY **L- Editor** Elsevier HK **E- Editor** Kong LH