TLR2 mRNA Upregulation in Ischemic Lobes in Mouse Partial Hepatic Ischemia/Reperfusion Injury Model*

ZHANG Jinxiang (张进祥)[1], WU Heshui (吴河水)[1], WANG Lin (王 琳)[2], ZHANG Jinhui (张锦辉)[3], WANG Hui (王 慧)[4], ZHENG Qichang (郑启昌)[3]

[1] *Department of Emergency Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China*

[2] *Department of Pediatrics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

[3] *Department of General Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

[4] *Hereditary Department, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*

Summary: To investigate TLR2 (Toll-like receptor 2) mRNA expression in ischemic hepatic lobes under the condition of partial hepatic ischemia/reperfusion injury in BALB/c mice and its relationship with liver function impairment. A partial ischemia/reperfusion injury model was established. The portal vein and hepatic artery supply to the median and left lobes of the liver were obstructed by an atraumatic artery micro-clip, with the obstruction lasting for about 60 min. Then reperfusion was fulfilled by removal of the clip. The liver samples were collected at the 4th h after the restoration of blood inflow. Total RNA was extracted from the liver samples and analyzed quantitatively by method of real-time PCR. At the same time, portal vein serum and plasma were taken respectively for further detection of the level of endotoxin, tumor necrosis factor alpha (TNF-alpha) and plasmic alanine aminotransferase (pALT). The results indicated that TLR2 mRNA in ischemic lobe was up-regulated markedly in mice partial liver ischemia/reperfusion injury model compared to that in sham operation group (ΔCt: 1.05 ± 1.02 vs 5.08 ± 1.36, $P<0.001$). The level of portal vein pALT and TNF-alpha increased significantly (112.32 ± 17.56 pg/ml vs 6.07 ± 5.33 pg/ml, $P<0.01$; 890 ± 127 μ/L vs 30 ± 5 μ/L, $P<0.001$). However, the level of portal vein endotoxin remained below the normal line, suggesting a state of non-endotoxemia. TLR2 mRNA expression in ischemic lobe, as well as portal vein pALT and TNF-alpha, was up-regulated in the model of mice partial ischemia/reperfusion injury, suggesting the involvement of TLR2 in ischemia/reperfusion pathological process.

Key words: toll-like receptor 2; reperfusion injury; endotoxin; liver

This research was aimed to study the variation of TLR2 mRNA expression in ischemic hepatic lobes under a partial hepatic ischemia/reperfusion pathological process, and to elucidate the mechanism of toll-like receptors (TLRs) activation and the role played by TLRs signaling pathway in hepatic ischemia/reperfusion injury.

1 MATERIALS AND METHODS

1.1 Materials

The materials used included TRIzol reagent (Invitrogen Co., Hongkong, China), TLR2 primer: upstream 5′-GCCACCATTTCCACGGACT-3′, downstream 5′-GGCTTCCTCTTGGCCTGG-3′ (Invitrogen Hongkong, China), probe sequence: 5′ (FAM) - TGGTACCTGAGAATGATGTGGGCGTG-(TAMRA) 3′ (Shanghai Shenyou Bio. Co., China), internal control β-actin primers: upstream: 5′-GCTACAGCTTCACCACCACAG-3′, downstream: 5′-GGTCTTTACGGATGTCAACGTC-3′, probe: 5′(FAM) - ATGACCTGGCCGTCAGGCAGC-(TAMRA) 3′ (Shanghai Shenyou Bio. Co., China). M-MLV reverse transcriptase (Lot: #169410, Promega Co., USA), RNasin ribonuclease inhibitor (Huamei Bio. Co, China), recombinant Taq DNA polymerase (Lot: 4564, # EP0404, MBI Ferments, USA), Quantified limulus assay kit (Shanghai Yihua Medical Science and Technology Co., China). TNF-alpha enzyme-linked immunosorbent assay (ELISA) kit (Jingmei Bio Co., China).

1.2 Animal Modeling and Grouping

BALB/c mice were supplied by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. All the animals were male, with age ranging from 6 to 8weeks and weighing 20—25 g. The animals were fasted for 12 h with free access to water and randomly divided into ischemia/reperfusion (I/R) and sham operation groups (SH). Mice were anesthetized with pentobarbital (60 mg/

ZHANG Jinxiang, male, born in 1973, M. D., Ph. D.

* This project was supported by a grant from National Natural Sciences Foundation of China (No. 30200272).

kg). A midline laparotomy was performed and an atraumatic clip was placed across the hepatic hilus to interrupt blood supply to the left and median lobes of the liver. After 60 min of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion. Sham operation group mice underwent the same protocol but without vascular occlusion.

1.3 Samples Harvesting and Management

Mice were killed after 4 h of blood supply restoration. Blood and liver samples were taken for further analysis. The fresh liver samples (100 mg) from ischemic hepatic lobes were collected, kept in liquid nitrogen and homogenized with glass homodouncer in 0.5 ml TRIzol solution. Then total RNA was extracted by following the manufacturer's protocol. Blood samples from portal vein were taken respectively for assay of pALT, TNF-alpha and endotoxin according to the manufacturer's manual.

1.4 Quantitative Assay of TLR2 mRNA Expression

After retro-transcription of total RNA with primer oligodT, target TLR2 gene and internal control β-actin gene were duplicated by the technique of real-time PCR with touchdown method. The duplicating process was monitored with an automatic analysis software in type FTC-2000 Sequence Detection System (Shanghai Fengling Bio Co., China). The level of gene expression was expressed as the difference between the Ct value of TLR2 mRNA and that of β-actin mRNA, i.e. ΔCt. The less theΔCt, the more the gene expressed.

1.5 Statistical Analysis

All numeric data were expressed as $\bar{x} \pm s$. Differences between I/R and SH groups were analyzed with SPSS10.0 software.

2 RESULTS

2.1 The Expression of TLR2 mRNA in Ischemic Hepatic Lobe

The values of ΔCt in I/R group and SH group were 1.05±1.02 vs 5.08±1.36 respectively ($P<0.001$), indicating that the expression level of TLR2 mRNA in ischemic hepatic lobe in I/R group is higher than that in SH group.

2.2 Levels of Serum Endotoxin, TNF-alpha and pALT in Portal Veins

The levels of portal vein serum endotoxin in I/R and SH groups were 3.90±1.42 Eu/ml vs 3.85 ± 1.61 Eu/ml respectively. The difference between them was not significant ($P>0.05$). The level of serum TNF-alpha in portal vein in I/R group was higher compared to that in SH group 112.32±17.56 pg/ml vs 6.07±5.33 pg/ml). The difference between them was very significant ($P<0.001$). The level of pALT in portal vein, which is an indicator of liver function compromise, was higher in I/R group than in SH group (890.21 ±272.91 u/L vs 40.66±15.42 u/L). The difference between both groups was also very significant ($P<0.001$, table 1).

Table 1 Level of serum endotoxin, TNF-alpha and pALT in portal vein

Groups	I/R group	SH group
Endotoxin con ($\times10^{-2}$)(Eu/ml)	3.90±1.42*	3.85±1.61*
Serum TNF-alpha (pg/ml)	112.32±17.56△	6.07±5.33△
Plasma ALT (U/L)	890.21±272.91#	40.66±15.42#

* $P>0.05$, △ $P<0.001$, # $P<0.001$

3 DISCUSSIONS

TLR family members are type I transmembrane proteins containing repeated leucine-rich motifs in their extracellular portions, similar to other pattern recognition proteins of the innate immune system, and a cytoplasmic domain, which is homologous to the signaling domain of the IL-1 receptor [1]. TLRs recognize pathogen-associated molecular pattern molecules. TLR2 are widely present on the surface of macrophages residing in liver and spleen, endothelial cells, dendritic cells, and some gastroenterologic tract epithelia *et al*[2,3]. It recognizes lipoproteins from a variety of pathogens, lipoteichoic acid (LTA), lipoarabinomannan, and atypical LPS, *etc*. When activated, the cytoplasmic portion of TLR2 interacts with MyD88, which has the TIR (Toll/IL-1 receptor) domain in its C-terminal portion, and MyD88 recruits a death domain-containing serine/threonine kinase, the IL-1 receptor-associated kinase (IRAK). IRAK is activated by phosphorylation and is associated with TRAF6, which leads to the activation of two distinct signaling pathways, JNK and NF-κB, resulting in the proinflammatory cascade[4]. Hepatic ischemia/reperfusion (I/R) injury is a major complication of liver resection surgery, transplantation, and hypovolemic shock[5]. Although the detailed biochemical mechanisms are unclear, hypoxia-reoxygenation (ischemia/reperfusion) may play an important role in the generation of reactive oxygen species (ROS), as well as in the secretion of cytokines, such as tumor necrosis factor α (TNF-α), by Kuppfer cells. The stimulation of NF-κB and the following inflammatory cytokines cascade may play critical roles during the process[6]. We demonstrated that TLR2 mRNA expression in the ischemic hepatic lobe was up-regulated during partial hepatic ischemia/reperfusion injury process in mice model.

A mouse model of lobar, rather than total, hepatic ischemia/reperfusion was established to produce a severe hepatic ischemic insult without inducing mesenteric venous congestion. The blood supply to the median and left lobes were blocked, while the blood supply to the right and caudal

lobes, as well as venous outflow, remained intact, thereby preventing the development of intestinal venous hypertension and the resulting endotoxemia[7]. Under this non-endotoxemia condition, the mechanism of I/R could be clarified without the involvement of endotoxin, and the influence could be excluded of cytokines evoked by endotoxin on the expression of the interest gene[8]. Different from the previous studies, it was the process of I/R alone, not lipopolysaccharide[9], that stimulated the expression increase of TLR2 mRNA. It seems that the TLR2 could be activated by the procedure of ischemia and reperfusion, mimicking the phenomenon that TLR2 could be activated by a variety of cytokines[10]. Although the level of serum TNF-alpha and plasma ALT were elevated during simultaneously, we couldn't definitely define the relationship between the activation of TLR2 and the increased level of TNF-alpha and ALT. But our experiment indicates that the TLR2 may play a part in the pathological process of hepatic ischemia/reperfusion injury.

Few researches focused on the pathophysiological role of TLRs during the I/R injury. Previous study has proved that TLR4, another member of TLRs family, played a role in the process of I/R[11]. The well-being of the body could be monitored through recognition of tissue degradation by TLR4[12], even the TLR2 can be activated by LPS[10]. However, under the condition of non-endotoxemia, the mechanism of TLR2 activation and liver cells on which TLR2 is activated during I/R process remain unclear and need be further studied.

REFERENCES

1 Takeda K, Akira S. Microreview: Toll receptors and pathogen resistance. Cellular Microbiology , 2003, 5 (3):143

2 Frantz S, Kelly AR, Bourcier T. Role of TLR2 in the activation of nuclear factor-κB by oxidative stress in cardiac myocytes. J Biol Chem, 2001,276:5197

3 Cario E, Rosenberg I M, Brandwein S L *et al*. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. J Immunol, 2000,164:966

4 Yang R B, Mark M R, Gray A *et al*. Toll-like receptor-2 mediates lipopolysaccharide -induced cellular signaling. Nature, 1998,395:284

5 Constantino F, Ronald W B, Jerzy W K *et al*. Hepatic ischemia/reperfusion injury—a fresh look. Experimental and Molecular Pathology, 2003,74:86

6 Funaki H, Shimizu K, Harada S *et al*. Essential role for nuclear factor kappaB in ischemic preconditioning for ischemia-reperfusion injury of the mouse liver. Transplantation, 2002, 74:551

7 Colletti L M, Remick D G, Burtch G D *et al*. Role of tumor necrosis factor-alpha in the pathophysiologic alterations after hepatic ischemia/reperfusion injury in the rat. J Clin Invest, 1990,85:1936

8 Kojima Y, Suzuki S, Tsuchiya Y *et al*. Regulation of pro-inflammatory and anti-inflammatory cytokine responses by Kupffer cells in endotoxin-enhanced reperfusion injury after total hepatic ischemia. Transpl Int, 2003,16(4):231

9 Matsuguchi T, Musikacharoen T, Ogawa T *et al*. Gene expression of toll-like receptor 2, but not toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. J Immunol, 2000,165:5767

10 Matsumura T, Ito A, Takii T *et al*. Endotoxin and cytokine regulation of toll-like receptor (TLR) 2 and TLR4 gene expression in murine liver and hepatocytes. J Interferon Cytokine Res, 2000,20(10):915

11 Wu H S, Wang L, Tian Y *et al*. TLR4 is involved in hepatic ischemia/reperfusion injury in mice. Chin J Liver Dis (Chinese), 2003,11:424

12 Johnson G B, Brunn G J, Kodaira Y *et al*. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. J Immunol, 2002,168:5233.

(Received Oct. 11, 2003)