Role of TLR4/MyD88/NF-κB signaling in heart and liver-related complications in a rat model of type 2 diabetes mellitus

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Abstract
Aims: To analyze expression of members of the Toll-like receptor (TLR)4/myeloid differentiation primary response 88 (MyD88)/nuclear factor (NF)-κB signaling pathway in the heart and liver in a rat model of type 2 diabetes mellitus (T2DM). Our overall goal was to understand the underlying pathophysiological mechanisms.

Methods: We measured fasting blood glucose (FBG) and insulin (FINS) in a rat model of T2DM. Expression of members of the TLR4/MyD88/NF-κB signaling pathway as well as downstream cytokines was investigated. Levels of mRNA and protein were assessed using quantitative real-time polymerase chain reaction and western blotting, respectively. Protein content of tissue homogenates was assessed using enzyme-linked immunosorbent assays.

Results: Diabetic rats had lower body weights, higher FBG, higher FINS, and higher intraperitoneal glucose tolerance than normal rats. In addition, biochemical indicators related to heart and liver function were elevated in diabetic rats compared with normal rats. TLR4 and MyD88 were involved in the occurrence of T2DM as well as T2DM-related heart and liver complications. TLR4 caused T2DM-related heart and liver complications through activation of NF-κB.

Conclusions: TLR4/MyD88/NF-κB signaling induces production of tumor necrosis factor-α, interleukin-6, and monocyte chemoattractant protein-1, leading to the heart- and liver-related complications of T2DM.
Keywords
Type 2 diabetes mellitus, Toll-like receptor 4, nuclear factor-κB, tumor necrosis factor-α, insulin resistance, heart, liver

Date received: 11 January 2021; accepted: 21 January 2021

Introduction
Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by hyperglycemia and insulin deficiency. T2DM is accompanied by a variety of complications affecting the heart, kidney, liver, retina, and nervous system. The prevalence of T2DM in China is the highest in the world. Incidence is increasing year by year along with economic improvements and changing diets. T2DM has become a major public health issue of global concern. The pathogenesis of T2DM is complex and is influenced by genetic predisposition, inflammatory responses, insulin resistance, oxidative stress, and microcirculation abnormalities. Inflammation can lead to insulin resistance and T2DM, while long-term inflammation can also aggravate hyperglycemia and promote the occurrence of diabetic complications. Long-term chronic inflammation can cause abnormal adipocyte function, leading to changes in the insulin sensitivity of adipose tissue. Expression of Toll-like receptor (TLR) 2 and TLR4 in peripheral blood mononuclear cells, as well as expression of TLR2 and myeloid differentiation primary response 88 (MyD88) in abdominal subcutaneous fat, was significantly higher in patients with T2DM compared with normal subjects. TLRs may also contribute to dysfunction of islet β cells and development of T2DM. Increasing attention has been paid to the role of immune factors in the pathogenesis of T2DM.

TLR4 is a type I transmembrane protein and the first TLR to be well characterized. TLR4 can recognize the pathogen-associated molecular patterns of different pathogens including exogenous ligands such as lipopolysaccharide (LPS), peptidoglycan, unmethylated cytosine and guanine phosphate. Endogenous TLR4 ligands include heat shock protein and hyaluronic acid. TLR4 is widely expressed in various cells and tissues including the liver, adipose tissue, islet β cells, and vascular tissue. Following ligand binding to TLR4, various signaling transduction pathways are activated among which the TLR4/nuclear factor (NF)-κB axis is the most important. Activation of this pathway can lead to downstream production of inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, which are involved in immune responses. NF-κB signaling can be activated by TLR4 via two pathways: the MyD88-dependent signaling pathway and the Toll/IL-1R domain-containing adaptor-inducing interferon-β (TRIF)-dependent signaling pathway. Diets high in fat and sugar are associated with increased levels of LPS, TLR4, TNF-α, and oxidative stress and inflammation; all of these factors contribute to activation of the TLR4 signaling pathway. Expression of TLR4, MyD88, phosphorylated IL-1R-associated kinase 1 and other inflammatory factors (TNF-α, macrophage chemotactic protein [MCP]-1, IL-6 and IL-8) in monocytes from patients with T2DM was significantly higher compared...
with healthy individuals. These studies suggested that TLR4-associated inflammatory signaling may be closely related to the pathogenesis of T2DM. Cardiac, vascular, and kidney-associated diabetic complications are the most harmful. TLR4 signaling can activate the transcription and translation of TNF-α, IL-6, and IL-12, causing inflammatory responses and inducing liver and kidney injuries. Activation of NF-κB in the tissues and organs of diabetic rats resulted in up-regulation of TNF-α and IL-1 expression. Overactivation of NF-κB can cause abnormal expression of inflammatory response-related genes, promoting inflammatory responses and tissue damage. The above studies indicated that the TLR4 signaling pathway is involved in T2DM, although the specific mechanisms remain unknown.

In the present study, we investigated the role of TLR4 in heart- and liver-associated complications in a rat model of T2DM.

**Materials and methods**

**Animals**

Thirty-five male specific pathogen-free Sprague–Dawley rats (body weight 221 ± 9.34 g) were obtained from Vital River Laboratories (Beijing, China). The rats were raised in a well-ventilated laboratory with a 12-hour/12-hour light/dark cycle at 20°C to 25°C and 40% to 70% humidity. All rats had free access to water and food.

After 1 week of adaptive feeding, the animals were randomly divided into control and experimental groups (n = 25 rats each). The rats in the control group were fed regular feed, while the rats in the experimental group were fed a high-fat diet (feed supplemented with 20% sugar, 2.5% cholesterol and 15% lard). After 4 weeks of feeding, rats in the experimental group and the control group were intraperitoneally injected with 30 mg/kg streptozotocin (STZ; prepared in 0.1 M citric acid, pH 4.5) for 2 weeks to induce diabetes. Rats in the experimental group with fasting blood glucose (FBG) ≥12.8 mmol/L and decreased insulin sensitivity index were selected as the T2DM model group (n = 18). Over the entire duration of the experiment, urine volumes and body weights of all rats were recorded.

Prior to blood sample collection, rats were anesthetized with 10% chloral hydrate (3.5 mL/kg body weight). FBG and fasting insulin (FINS) levels were determined and an intraperitoneal glucose tolerance test (IPGTT) was administered. Biochemical indicators related to heart and liver function were also measured. Heart and liver tissues were collected, weighed, and stored at −80°C. All animal experiments were performed in accordance with the ethical guidelines of the PLA Rocket Force Characteristic Medical Center. Written informed consent for publication of any associated data and accompanying images were obtained from all patients or their parents, guardians or next of kin.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted with TRIzol and reverse-transcribed into cDNA using SuperScript™ III reverse transcriptase according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed to amplify target genes using an iQ5 instrument (Bio-Rad, Hercules, CA, USA). The amplification protocol was as follows: 94°C for 3 minutes; 40 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s. The primer sequences were as follows: TLR4, forward 5’-TCAGTGTGCTTGTGGTAG-3’ and reverse 5’-TCTGCTAAGAAGCT-3’; NF-κB, forward 5’-ACCTGGATGAGAGTGGATGGA-3’ and reverse 5’-CGTGAAGTATTTCCCCAGGTTT-3’; β-actin, forward 5’-CCCATCTATGAGG-3’.
GTTACGC-3' and reverse 5'-TITAA TGTCACGCACGATTTC-3'; and MyD88, forward 5'-GGCATCTCCACCCGAGTTA C-3' and reverse 5'-TTGGCGATTTAGG TGTCCG-3'. Relative expression levels of target genes were calculated using the 2^-ΔΔCT method.16 β-actin was used as an internal reference.

**Western blotting**

Cells or tissues were lysed using radioimmunoprecipitation assay buffer. Protein concentrations were determined and then protein samples were separated by SDS-PAGE. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk, the membranes were incubated with primary antibodies (all from Cell Signaling Technology, Danvers, MA, USA) against TLR4 (1:1000 dilution; Cat. No. 14358), NF-κB (1:1000 dilution; Cat. No. 50010S) and β-actin (1:1000 dilution; Cat. No. 3700). After washing, the membranes were treated with a horseradish peroxidase-conjugated secondary antibody (1:1000; Cell Signaling Technology). Blots were developed using enhanced chemiluminescence (Abcam, Cambridge, UK). Band density was assessed using Image Lab v3.0 software (Bio-Rad). β-actin was used as an internal control.

**Enzyme-linked immunosorbent assay (ELISA)**

In a microplate, 50 μL of sample or standard was added to each well. After shaking, the plate was incubated at room temperature for 2.5 hours. After washing, the plate was incubated with biotin-labeled antibodies at room temperature for 1 hour. After another wash, horseradish peroxidase-conjugated streptavidin was added to the plate and incubated at room temperature for 45 minutes. After washing, tetramethylbenzidine substrate was added to each well and incubated at room temperature for 30 minutes, then 50 μL of stop solution was added to stop the reaction. Absorbance at 450 nm was measured within 15 minutes. Standard curves were plotted and the protein concentrations were calculated.

**Hematoxylin and eosin (HE) staining**

Islet tissues were removed and fixed in 10% formalin. The tissues were dehydrated in a series of graded alcohol solutions. After embedding in paraffin, tissues were sectioned to 3- to 4-μm thickness and then stained with HE for histopathological evaluation.

**Statistical analysis**

Data were expressed as means ± standard deviations. Statistical analysis was conducted using SPSS 22.0 (IBM, Armonk, NY, USA). Pairwise comparisons were performed using the paired Student’s t-test. Multiple comparisons were performed using one-way analysis of variance followed by a Student–Newman–Keuls post-hoc test. Values of P < 0.05 were considered statistically significant.

**Results**

**Diabetic rats have lower body weights, higher FBG, higher FINS, and higher IPGTT results than normal rats**

After 4 weeks of feeding a high-fat diet, rats in the experimental group were intraperitoneally injected with 30 mg/kg STZ for 2 weeks to induce diabetes (T2DM rats). After 4 weeks, the body weights of T2DM rats were significantly higher than those of control rats (P < 0.05). After 6 weeks, the body weights of T2DM rats were significantly lower than those of control rats (P < 0.05). The body weights of T2DM rats at week 6 were significantly lower
than at week 4 ($P < 0.05$) (Figure 1a). Moreover, FBG and FINS in T2DM rats were significantly higher than those of control rats at week 4 ($P < 0.05$). FBG in T2DM rats at week 6 was significantly higher than at week 4 ($P < 0.05$), while FINS in T2DM rats at week 6 was significantly lower than at week 4 ($P < 0.05$) (Figure 1b and c). According to IPGTTs, glucose levels in T2DM rats peaked at 15 minutes and maintained elevated until 120 minutes, and were significantly higher than those of control rats from 30 minutes until 120 minutes ($P < 0.01$ for all time points) (Figure 1d). HE staining showed that in T2DM rats, only a small number of islet

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Body weights, fasting blood glucose (FBG), fasting insulin (FINS), intraperitoneal glucose tolerance test (IPGTT) results, and islet structure of normal rats and type 2 diabetes mellitus (T2DM) rats. (a) Body weight, (b) FBG, and (c) FINS of control and T2DM rats at the end of week 4 and week 6. *$P < 0.05$ and **$P < 0.01$ compared with control rats in the same week; #*$P < 0.05$ and ###$P < 0.01$ compared with T2DM rats at week 4. (d) IPGTT results of control and T2DM rats at the end of week 6. **$P < 0.01$ compared with control rats at the same time point. (e) Islet structures of control and T2DM rats at the end of week 6 as shown by hematoxylin and eosin staining. Islets were circled using red lines. Magnification, $\times 200$. $N = 12$. 
cells were observed in islet tissue; the islet structures were atrophic, scattered, and damaged, and the number of cells was reduced compared with control rats (Figure 1e). Thus, T2DM rats had lower body weights, higher FBG, higher FINS, and higher IPGTT results than normal rats.

**T2DM rats have elevated biochemical indicators related to heart and liver function compared with normal rats**

To evaluate the effects of T2DM on heart and liver function, sera were collected at the end of week 6 for biochemical analysis of indicators of heart and liver function.

Serum levels of aspartate aminotransferase, alanine aminotransferase, triglycerides, creatinine kinase, total cholesterol, and low density lipoprotein-C in T2DM rats were significantly elevated compared with control rats ($P < 0.05$) (Figure 2a–f). These results indicated that T2DM rats had higher levels of biochemical indicators related to heart and liver function than normal rats.

**TLR4 and MyD88 may be involved in regulating the occurrence of T2DM and its complications in the heart and liver**

The heart and liver express other TLR family members in addition to TLR4.

![Figure 2. Biochemical indicators related to heart and liver function.](image)

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- **TLR4 and MyD88 may be involved in regulating the occurrence of T2DM and its complications in the heart and liver**

The heart and liver express other TLR family members in addition to TLR4.
Following a literature search, we selected TLR2, TLR3 and TLR9 as potentially related to T2DM and evaluated the expression of these molecules in liver and heart by qRT-PCR. Levels of mRNA encoding these TLRs in T2DM rats did not differ from those of control rats. However, TLR4 mRNA levels in T2DM rats were significantly higher than those of control rats ($P < 0.01$) (Figure 3a). To investigate the expression levels of TLR4 and MyD88 in rat heart and liver tissue at week 6, qRT-PCR and western blotting was performed. Levels of TLR4 and MyD88 mRNA in the hearts and livers of T2DM rats were significantly elevated compared with those of control rats ($P < 0.05$) (Figure 3b and c). Similarly, levels of TLR4 and MyD88 protein in the hearts and livers of T2DM rats were significantly elevated compared with those of control rats ($P < 0.05$) (Figure 3d and e). Together, these results suggested that TLR4 and MyD88 may be involved in regulating the occurrence of T2DM and its complications in the heart and liver.

**TLR4 may cause heart and liver-related complications in T2DM rats through activation of NF-κB**

The expression of NF-κB and its downstream inflammatory factors (TNF-α, IL-6 and MCP-1) in heart and liver tissue homogenates was measured using ELISA. Levels of NF-κB, TNF-α and IL-6 in the heart tissues of T2DM rats were significantly elevated compared with those of control rats ($P < 0.05$) (Figure 4a–d). However, levels of MCP-1 were similar in the two groups. In addition, levels of NF-κB, TNF-α, IL-6 and MCP-1 in the liver tissues of T2DM rats were significantly elevated compared with those of control rats ($P < 0.05$) (Figure 4e–h). These results indicated that TLR4 may induce heart- and liver-related complications in T2DM rats through activation of NF-κB.

**Discussion**

Diabetes can affect many tissues and organs, leading to a series of complications. As disease progresses, complications can affect the heart, liver, kidneys, blood vessels, nervous system, and skin. Diabetic heart disease and liver injury can seriously threaten the lives and health of patients with diabetes. The pathogenesis of T2DM involves development of insulin resistance and decreased number or function of islet β cells. It is generally believed that heredity, diet, viral infections, and inflammation are key factors in development of T2DM.

In patients with T2DM, levels of acute phase inflammatory markers such as IL-6, C-reactive protein, cortisol, and serum amyloid A are significantly higher compared with healthy individuals. Inflammation can lead to insulin resistance and T2DM. Long-term inflammation can also aggravate hyperglycemia and promote diabetic complications. Long-term chronic inflammation can alter the functions of adipocytes, leading to altered insulin sensitivity of adipose tissues.

TLR4 plays important roles in orchestrating innate immune responses and in regulating insulin resistance and diabetic complications. Levels of TLR4 mRNA in the monocytes of patients with T2DM were higher than those of healthy individuals. Animal experiments showed that LPS-induced insulin resistance was mediated by TLR4 and could induce diabetes. MyD88 is one of the most important adaptor proteins in TLR signal transduction, activating downstream molecules such as NF-κB that trigger inflammatory pathways. The risk of diabetes in MyD88-knockout mice was significantly higher than that in wild-type mice. When MyD88-knockout mice were fed a high-fat diet, levels of insulin and cholesterol increased and liver dysfunction developed. These factors are significantly
Figure 3. Relative expression of Toll-like receptor (TLR) 4 and myeloid differentiation primary response 88 (MyD88) in rat heart and liver tissue. (a) Relative levels of TLR2, TLR3, TLR4 and TLR9 mRNA in heart and liver tissues of control and type 2 diabetes mellitus (T2DM) rats at the end of week 6. (b, c) Relative levels of TLR4 and MyD88 mRNA in (b) heart and (c) liver tissues of control and T2DM rats at the end of week 6. (d, e) Relative expression of TLR4 and MyD88 proteins in (d) heart and (e) liver tissues of control and T2DM rats at the end of week 6. *P < 0.05 and **P < 0.01 compared with control rats. N = 8.
correlated with the occurrence of diabetes.\textsuperscript{36} NF-κB is a key factor in inflammatory signaling, and both TLR4 and NF-κB are generally recognized as inflammatory markers.\textsuperscript{37} Increased activation and expression of NF-κB leads to the secretion and release of IL-1β and promotes apoptosis of islet β cells.\textsuperscript{38} In this study, expression levels of TLR4 and NF-κB in T2DM rats were higher than those of normal rats, leading to heart- and liver-related complications. Adding palmitic acid to human microvascular

\begin{figure}
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\caption{Levels of nuclear factor (NF)-κB and downstream inflammatory factors in rat heart and liver homogenates. (a–d) levels of (a) NF-κB, (b) tumor necrosis factor (TNF)-α, (c) interleukin (IL)-6 and (d) macrophage chemotactic protein (MCP)-1 in heart tissue from control and type 2 diabetes mellitus (T2DM) rats at the end of week 6. (e-h) Levels of (e) NF-κB, (f) TNF-α, (g) IL-6 and (h) MCP-1 in liver tissue from control and T2DM rats at the end of week 6. *P < 0.05 and **P < 0.01 compared with control group. N = 8.}
\end{figure}
endothelial cells activated NF-κB signaling, suppressed activation of protein kinase β, and led to insulin resistance. These data suggested that TLR4/NF-κB signaling might be related to T2DM. 39

In conclusion, the present study demonstrated that TLR4/MyD88/NF-κB signaling triggered upregulation of TNF-α, IL-6 and MCP-1, subsequently leading to heart- and liver-related complications of T2DM.

Acknowledgements
The authors wish to thank their departments and research teams for their efforts and dedication.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

Funding
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions
JT and LL designed the study. JT, YZ and LW performed the experiments. JT and LW analyzed the data. JT, YZ and LL interpreted results and prepared the manuscript. All authors read and approved the final version of the manuscript.

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