The Effect of Induced Hyperglycemia (Diabetes Type 1) on the mRNA Level of Toll-Like Receptor 4 Gene in the Diabetic Heart of Wistar Rats.

Ali Dehghani Firoozabadi[1,4], Samaneh Shojaeii[2,3*], Alireza Haghparast[2,3], Hamidreza Dehghani Firoozabadi[5]

1. Yazd Cardiovascular Research Center, Shahid Sadooghi University of Medical Sciences, Yazd, Iran.
2. Immunology Section, Department Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad-Iran.
3. Biotechnology Section, Department of Pathology, Faculty of Veterinary Medicine, Ferdowsi University of Maashhad, Mashhad-Iran.
4. Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad-Iran.
5. Department of Biology, Faculty of Science, Isfahan University, Isfahan, Iran.

*Correspondence:
Samaneh Shojaeii, Immunology Section, Department Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad-Iran.

Email: ali.dehghani.molmed@gmail.com
Tel: (98) 353 724 0171

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Abstract

Objective: The previous studies indicated that the TLRs especially Toll-like receptor 4 (TLR4) are involved in the process of diabetes. The aim of the present research was to investigate the time course expression pattern of TLR4-a main component of innate immune system- in mRNA levels in the heart tissue of diabetic male wistar rats.

Materials and Methods: In this research, adult Wistar rats (6-8 weeks old and 200-250 g weight) were mated and the pups were grown to 6-8 weeks old. Heart tissues were collected and analyzed by real-time PCR to evaluate the mRNA levels of TLR4. The mRNA levels of TLR4 in total heart tissue were evaluated in STZ-treated control rats in various times after diabetes induction.

Results: Results showed that the up-regulation of TLR4 during the time course after diabetes induction as compared to the control. The abundant expression of TLR4, a major component of innate immunity system, provides strong evidence that TLRs play important roles in the pathogenesis and expansion of diabetes and it is possible that the expression of TLRs eventually lead to cardiovascular disease.

Conclusion: The abundant expression of TLR4 which is major component of innate immunity system, provides strong evidence that TLRs play important roles in innate immunity and the pathogenesis and expansion of diabetes. It is possible that the expression of TLRs eventually lead to cardiovascular disease. The mechanisms for the increase in TLR expression and activity in diabetes have not been studied in depth. Modulating these TLRs could be beneficial in prevention of diabetic complications.

Keywords: mRNA levels, Wistar rats, diabetic heart, Toll-like receptor 4

Introduction

Toll-like receptors are the key components of the innate immune system. The receptors recognize conserved pathogen-associated molecular patterns (PAMPs) and these genes have an early evolutionary origin (1). So far, 11 TLRs (TLR1 to TLR11) have been identified in humans (2). Many animal species express the various types of TLRs too (2-4). The PAMPs and damage associated molecular patterns

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(DAMPs) trigger the TLRs expression (3,5,6). While many of TLRs are expressed on the surface of cell membrane, but TLR3, TLR7, TLR8, and TLR9 are expressed on the endosomal surfaces (7,8). TLR1, 2, 4, 5 and 6 are commonly identified as bacterial products because they are produced by bacteria and cannot be synthesized by host cells. The bacterial products include bacterial outer cell membrane lip polysaccharides (LPS) that is recognized by TLR4, lipoproteins that is detected by TLR2 and flagellin that is identified by TLR5 (9-11). TLR3, 7, 8 and 9 are responsible for the detection of viruses and nucleic acids. Also, the ligands such as unmethylated cytosine/guanine (CpG) dinucleotides, double-stranded such as polyI. polyC, analogues of adenosine and guanosine, single-stranded viral RNAs and other RNA and DNA, and imidazole quinolines are identified by TLR9, TLR3, TLR7 and TLR8, respectively (12). In addition to classical molecular patterns that are recognized as PAMPs, recently it is known that TLRs also identified the types of DAMPs that act as endogenous ligands for TLRs. For example, it has been found that TLRs especially TLR4 identify the endogenous molecules such as cellular fibronectin, fibrinogen, oxidized lipids, surfactant protein A and heat shock proteins (HSPs) (13-16). The previous studies indicated that there are molecular linkage between innate immunity, sterile inflammation, and a several types of disease. Although several studies noted the role of innate immunity in induction of diabetes and cardiovascular diseases, however, studies are limited on the time course expression of TLRs following diabetes induction and hyperglycemic condition in heart tissue. Identification of these genes and determine their expression levels at different times of the development of cardiovascular disease, and ultimately, determine their role in the pathogenesis of cardiovascular diseases will help the development of innovative therapeutic targets in this area. The aim of the present research was to investigate the time course expression pattern of TLR4-a main component of innate immune system- in mRNA levels in the heart tissue of diabetic male wistar rats.

Materials and Methods

Animals

In this research, adult Wistar rats (6-8 weeks old and 200-250 g weight) were mated and the pups were grown to 6-8 weeks old. They were housed in constant temperature of 20-22º C with a relative humidity of 55% and standard 12:12 h light-darkness cycles and had free access to standard rat chow ad libitum and tap water and they allowed 1 wk acclimatizing to the laboratory conditions before experiments. All the procedures including diabetes induction and sacrifice operation were in strict accordance with Iranian legislation on use and care of laboratory animals.

Experimental Design

Rat given a single intraperitoneal (i.p) injection of freshly 55 mg/kg STZ (STZ; Sigma-Aldrich, Germany) dissolved in 10 mM (0.1 M) sodium citrate buffer (pH 4.5) in a dark environment, while control animals were received only citrate buffer. On 2th day after STZ induction, the blood was obtained from the rat tail vein and random glucose level was measured using blood glucose monitoring system (Bionime, USA). For the present study, diabetes were defined as a blood glucose measurement of 20 mM (250 mg/dl) or higher. These model diabetic rats were further divided into 5 groups (n=5): diabetes control treated with water and groups of 5 diabetes rats were sacrificed at 4,6,8 (early phase of diabetes progression) and 20 (late phase of diabetes progression).

General condition and blood sampling:

In order to analyses the blood glucose and insulin, the tail blood sample was collected for further analysis.

The evaluation of the Body weight:

At the end of the experiment in different groups, all animals were denied of food until the next morning, but had free access to water. Rats were weighted to measure the Body

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Weight. Then, Animals were sacrificed by the decapitation.

Evaluation of mRNA levels by RT-PCR:

Oligonucleotide primers were designed for the specific PCR amplification of an amplified fragment of rat TLR4 among all copies of transcripts converted to cDNA. For selected genes, to amplification of the related sequences and in order to non-specific amplification does not happen the gene sequence and transcripts as well as the unpredictable pseudo genes in the rat genome were investigated in the National Center for Biotechnology Information (NCBI) and Bioinformatics Institute Europe site (Ensembl). Also, to determine primer specificity, they investigated the Basic Local Alignment Search Tool (BLAST). All sequences of primers were checked in Gen Bank database to avoid sequence similarity. The TLR4 primers were 5'-GGCATCATCTTCATTGTCCTTG-3', (sense primer) and 5'-AGCATTGTCCTCCCACTCG -3'(antisense primer), yielding a 111-bp fragment. And β-Actin gene as Housekeeping (5'-GTGCTATGTTGCCCTAGACTTCG-3', (sense primer) and 5'-GATGCCACAGGATTCCATACCC -3'(antisense primer), yielding a 175-bp fragment. the primers were designed using Primer Premier software (version 6.0) and synthesized by Bioneer Biotechnology (Korea).For RT-PCR total RNA was isolated from rat heart with the Total RNA isolation system (Roche) and treated with RNase-Free DNAse I (Roche), following the manufacturer's protocols. After RNA extraction, quality and concentration of extracted RNA were assessed with a Nano Drop spectrophotometer. So that, RNA concentration according ng/µl, 260/280 and 260/230 ratio were measured with Nano drop. Also, the quality of the extracted RNA was determined by agarose gel electrophoresis. Total RNA was reverse transcriped to cDNA using oligo (dT) primer and Revert Aid TM M-MuLV reverse transcriptase (Thermo Scientific) according to the supplier's recommendations.

Real-time PCR:

Real-time PCR reactions were prepared using SYBR Green PCR Master mix (Applied biosystems) and 80 nM of forward and reverse primers as defined above. The PCR reaction conditions were 95°C for 10 min, 95°C for 30 sec, 58°C for one min and 72°C for 30 sec (40 cycles). Amplification was performed according to the manufacturer's protocol and done in triplicate. Gene expression was assessed as $2^{-\Delta\Delta(CT)}$ (13), where Ct is cycle threshold, ΔΔ(Ct) = sample 1Δ (Ct) -sample 2Δ(Ct); Δ(Ct) = GAPDH (Ct) – testing gene (Ct) (17). Data was analyzed using Applied Biosystem software Microsoft Excel 2003, and Graph Pad Prism software.

Statistical Analysis

Comparison between means were conducted by Analysis of variance (ANOVA). The p-value less than 0.05 was regarded as statistically significant.

Results

Biochemistry Parameters of STZ-treated Rats

STZ-treated rats showed typical manifestation of diabetes, including significant hyperglycemia and slow weight gain compared to STZ-insulin treated and normal controls (Figure 1).

Effects of increased glucose following diabetes type 1 on TLR4 gene expression:

Levels of heart TLR4 mRNA were increased in animals sacrificed at 4 weak after diabetes type 1 induction. TLR4 mRNA increased after 6 weak rapidly. From the sixth to the eighth week after induction of diabetes, the level of mRNA is detectable. From the 8 to 20 week after induction of diabetes, the amount of TLR4 expression was enhanced when compared to 4, 6 weeks diabetes and controls. The molecular results for TLR4 mRNA levels at 4,6,8 and 20 weeks after induction of diabetes are depicted in figure 2. At weeks

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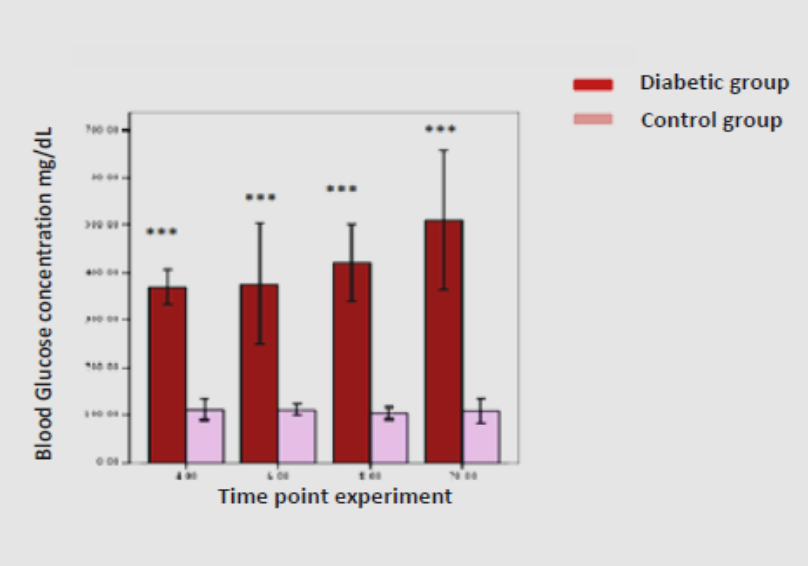


Figure 1. Time point blood glucose concentration in non-diabetic and diabetic groups at specific time after diabetes type 1 induction.
Comparison (Mean±Standard Deviation) with the control group.
*****p<0.001, Sample size:5 male rats, Statistical methods: ANOVA and student T-test.**

4,6,8 and 20 after diabetes induction, a significant increase of both TLR4 mRNA was detected in heart (Figure 2).

Heart TLR4 mRNA was evaluated by specific QRT-PCR amplification of heart cDNA samples of healthy control and diabetic rats. cDNA's were standardized for β-actin content. Quantitative data were gotten by quantitative RT-PCR assessment products which were compared to a standard curve obtained by amplification of a serial dilution of highly concentrated cDNA. Mean ± Standard deviation are shown in all graphs and representative for 3 independent RT-PCR

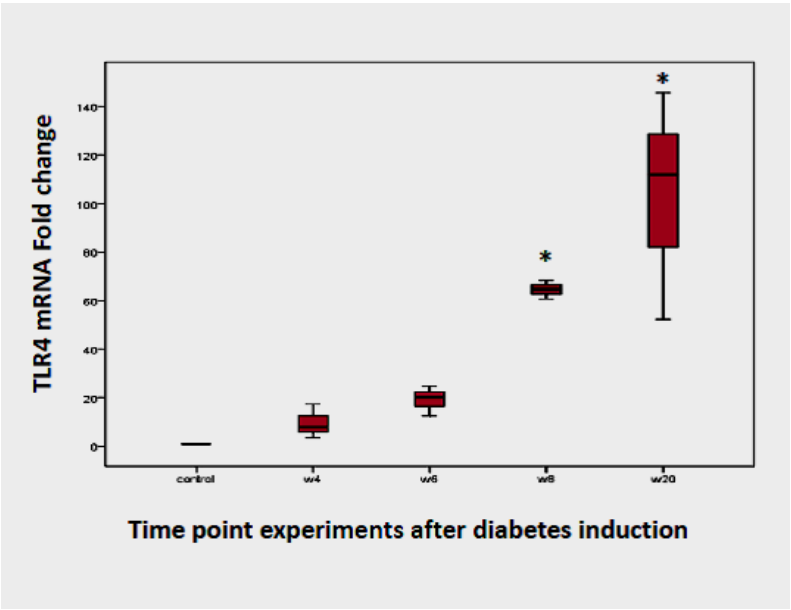


Figure2.Type 1 diabetes changes in TLR4 mRNA syntheses during the early and late phases of diabetes.

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reactions on heart cDNA samples of 5 Rats in each treatment group. Statistical significance was considered at a p value less than 0.05.

Discussion

Rats were exposed to diabetes type 1, a process that leads to tissue damage and a subsequent sterile inflammatory reaction. Many studies show that diabetes can lead to many cardiovascular diseases such as stroke, coronary heart disease (CHD), peripheral atrial disease, atherosclerosis and cardiomyopathy (18-24). In fact, in type 1 diabetes, heart constantly is exposed to a variety of pathological disorders such as accelerated atherosclerosis, cardiac autonomic neuropathy and cardiomyopathy (25). For example, myocardial ischemia due to coronary atherosclerosis usually occurs in diabetic patients. Thus, diabetes with other major risk factors such as obesity and unhealthy life style are major causes of cardiovascular disease in diabetic patients. It is not exaggerated to say that diabetes is a cardiovascular disease (26). Type 1 diabetic patients are10 times higher than healthy individuals at risk of heart disease. So that, heart attacks in diabetic patients about 60% can lead to death, while the death rate from stroke is 25%. Although many studies show the mortality rate of diabetic patients due to cardiovascular disease is very high, however, the molecular mechanisms of cardiovascular disease in diabetic patientsare not known.

Recent studies show that low and moderate inflammation increase the risk and progression of diabetes (27). For example, it is accepted that the chronic inflammation is a part of the insulin resistance and strongly associated with metabolic syndromes (28,29). The mechanisms of chronic inflammation which lead to diabetes are not properly identified. But it is clear that innate immunity and inflammation are involved in pathogenesis of diabetes (27,30). So, pro-inflammatory cytokines and acute phase reactants are involved in several metabolic pathways related to insulin resistance such as insulin regulation and ROS and lipoprotein lipase action (27). The question is whether inflammation plays a role in the development of diabetes and delayed complications of diabetes such as heart failure or not. Recent studies show that inflammation and pro-inflammatory cytokines play an important role in the progression of microvascular and macrovascular diabetic complications (31).

Our experimental animal data have indicated that after induction of diabetes type 1, mRNA expression of TLR4 is increased in the heart tissue in early phase in the course of diabetes. Recently, it has been indicated that chronic inflammation plays main role in the development of cardiovascular disease. However, the underlying mechanisms involved in the development of inflammatory responses are not clear. The heart has a unique endogenous immune system and this immune system can specially activated by various form of tissue damage (32,33). Recent evidence indicates that the trigger of cardiac immune system and release of pro-inflammatory mediators in response to cardiac tissue damage act an early warning system (34). Although the initial and short-term activation of the innate immune system and expression of pro-inflammatory cytokines is necessary to a rapid adaptive response following the tissue damage, however, sustained activation and over expression of inflammatory signaling for a long time can be detrimental and may lead to reverse results (35,36). In addition to immune cells such as leukocytes and macrophage and several organs such as spleen, lung, kidney, TLRs are expressed in cardiomyocytes and endothelial cells indicating the functional importance of these genes in the cardiovascular system (37). So far, many studies have been conducted to understand the role of TLRs in the development of cardiovascular disease in the cardiovascular system.

Conclusion

The abundant expression of TLR4 which is major component of innate immunity system,

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provides strong evidence that TLRs play important roles in innate immunity and the pathogenesis and expansion of diabetes. It is possible that the expression of TLRs eventually lead to cardiovascular disease. The mechanisms for the increase in TLR expression and activity in diabetes have not been studied in depth. Modulating these TLRs could be beneficial in prevention of diabetic complications.

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