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Human Toll like receptor 4 gene expression of PBMCs in diabetes mellitus type 2 patients

Z. Sepehri[1], Z. Kiani[2], A. A. Nasiri[1], M. A. Mashhadi[3], F. Javadian[4]✉, A. Haghighi[5], F. Kohan[1], A. Bahari[6], A. Sargazi[1]

[1] Zabol University of Medical Sciences, Zabol, Iran
[2] Kerman University of Medical Sciences, Kerman, Iran
[3] Zahedan University of Medical Sciences, Zahedan, Iran
[4] Zabol Medicinal Plant Research Center, Zabol University of Medical Sciences, Zabol, Iran
[5] Harvard Medical School, Department of Genetics, Boston, USA
[6] Assistant professor of Biotechnology, Research Institute of Modern Biological Techniques, University of Zanjan, Zanjan, Iran

Corresponding author: Fereshteh Javadian, Zabol Medicinal Plant Research Center, Zabol University of Medical Sciences, Zabol, Iran. E-mail: fer.javadian@gmail.com

Abstract

Toll like receptor 4 (TLR4) is one of the most pivotal pathogen recognition receptors (PRRs) in innate immune systems. In this study, we evaluate the expression of the TLR4 in patients with diabetes mellitus type 2 (DM2) in comparison to healthy controls. Expression of TLR4 in 32 human peripheral blood mononuclear cells (PBMCs) of patients with DM2 and 20 control samples was assessed using Real-Time PCR technique. For each patient, body mass index (BMI) and blood glucose levels were measured. The results of Real-Time PCR showed a 5-folds increase in expression of TLR4 on the PBMCs of DM2 patients in comparison to controls. No correlation was observed between the TLR4 expression and sex or BMI. Our results confirmed that DM2 can increase TLR4 expression independent from sex, blood glucose concentrations and BMI.

Key words: Pathogen recognition receptors, TLR4, Diabetes mellitus type 2.

Introduction

Diabetes mellitus type 2 (DM2), as a metabolic disorder, is characterized by high blood glucose, insulin resistance and also relative insulin deficiency. The disease is a major cause of cardiovascular mortality in developing countries like Iran (1, 2). Several intrinsic and extrinsic factors involve in insulin resistance. Hypoxia and circulating cytokines are considered as dominant extrinsic pathways which participate in modulation of peripheral insulin signaling. Innate immune response is the first line of defense of the body against the pathogens including bacteria, viruses, protozoa and fungi (3). Recently our understanding about DM2 has been evolved as human and animal models proposed that there is a close relationship between innate immune and DM2 (4). One major component of innate immune molecules, is pattern recognition receptors (PRRs). More than 10 members of toll like receptors (TLRs) are the most important of PRRs which can detect particular structural patterns of viruses and bacteria as pathogen associated molecular patterns (PAMPs) and also internal ligands as damage associated molecular patterns (DAMPs) (5). Lipopolysaccharide (LPS) of gram positive bacteria is a major ligand of TLR4 (3, 6). TLR4 proteins are predominantly expressed in innate immune cells particularly monocytes (7). Activation of TLR4 in monocytes and lymphocytes lead to NF-κB activation through both myd88 and TRIF signaling pathways which results in production of pro-inflammatory cytokines such as interleukin 1 beta (IL-1β) (8, 9). NF-κb is not only involved in inflammatory response but plays a role in glucose metabolism and insulin response (10). Accordingly, it seems that some PRRs like TLR4 may be involved in the pathogenesis of DM2. To the best of our knowledge, there are not related studies which have evaluated the expression of TLR4 on the PBMCs of DM2 patients. Therefore, the main objective of this study was to evaluate the expression of TLR4 on the peripheral blood mononuclear cells (PBMCs) of DM2 patients from South-East of Iran.

Materials and methods

Isolation of PBMCs

All reagents were purchased from Sigma-Aldrich (Sigma, St Louis, MO). To isolate the PBMCs, 52 blood samples (from 20 healthy volunteers and 32 DM2 patients) were collected into heparinized tubes. Blood samples were immediately used for PBMCs isolation using previously described method (11), with slight modifications. Blood samples were diluted in 1/2 ratio with PBS (phosphate-buffered solution) without $Mg2^{+}$ and $Ca2^{+}$ and layered onto a 50-ml Ficoll-Paque plus (Lympholyte®, Zierikzee, The Netherlands). After centrifugation at 800 ×g and 12°C for 30 min, the layer of PBMCs was collected. The purified PBMCs were washed once at 450×g and 4°C for 10 min with washing solution containing 98% DPBS (Dulbecco`s phosphate buffered saline), 1% penicillin/streptomycin (P/S) and 1% inactivated FCS (fetal calf serum). The pellets were resuspended and washed at 180 ×g and 4°C for 5 min twice with incomplete RPMI (RPMI 1640, 10% inactivated FCS, 1% P/S) and finally washed with complete RPMI.

RNA extraction and cDNA synthesis

Total RNA from diabetic and non-diabetic PBMCs

was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Quantity and quality of RNA was determined by NanoDrop (NanoDrop, Thermo Scientific, Wilmington, DE) and non-denaturing gel electrophoresis. The isolated RNA was stored at -80°C for further analyses. First strand cDNA was synthesized from 1µg total RNA in a 20 µl final volume using RevetAidTM First Strand cDNA synthesis kit (Fermentas,Burlington, ON, Canada).

Real Time qPCR

Real time PCR was performed with identical reaction conditions for all samples and standards, using Rotor-Gene 6000 Real-Time PCR machine (QIAGEN, Hilden, Germany). Primers used for TLR4 were 5`-ATGCCCCATCTTCAATTGTC-3` as forward and 5`-AGTGAGGATGATGCCAGGAT-3` as reverse with 143bp expected amplicon. Primers for the internal control, glyceraldehyde phosphate dehydrogenase (GAPDH), were 5`-GGATTTGGTCGTATTGGG-3` (forward) and 5`-TGGGTGGAATCATATTGG-3` (reverse) with 131bp expected amplicon size (Figure1). Briefly, cDNA (~500 ng) was mixed with 1× of 5x HOT FIREPol® EvaGreen®qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) in a 25 µl final reaction volume. qPCR conditions were as follows, 1 cycle of 94°C for 5 min followed by 45 cycles of 20 sec at 94°C, 57°C for20 sec and 72°C for 30 sec. Also a melting was constructed by ramping from 50 to 99 °C, rising by 0.5 °C in each step every 10 seconds. In melting curve analysis for each PCR product reaction, a single peak was obtained. As controls, we also used the reaction mixtures without cDNA (RT-minus samples) and non-template control (NTC). All CT values were first normalized based on the cDNA amount which was measured using Nanodrop. CT values were averaged between duplicates in each qPCR run and two experimental repeats after interplate calibration. Relative differences in Real-Time PCR among each plots of experiments were determined using the Pfaffl method (12). Optimization experiments were also performed to ensure that the efficiency of the target and the internal control gene (GAPDH) was approximately equal. Analysis of data was performed using Relative Expression Software Tool (REST) 2009 (Qiagen, Hilden, Germany). This study has been approved by the appropriate ethics committee and has been performed in accordance with the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study.

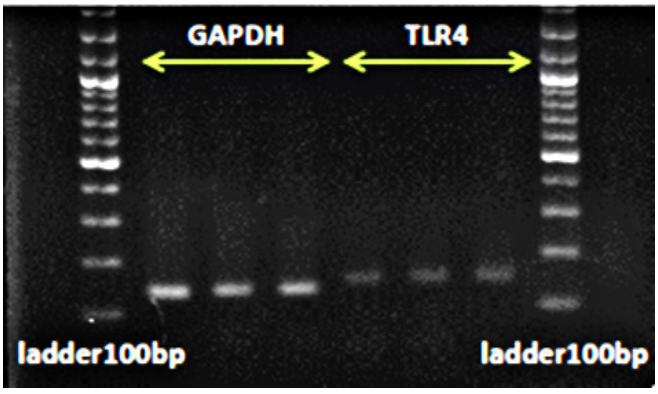

Figure1. The qPCR products on a 1.5% agarose electrophoresis gel. Size of a single product compared to the expected PCR size.

Results

Table 1 depicted calculated characteristics including age, sex, body mass index and blood sugar content. Student's t-test comparison did not reveal significant differences for age and body mass index between both control and DM2 groups. As expected glucose rate in DM2 patients was significantly higher than control groups (p= 0.05) (Table 1). After PCR optimization, a standard curve was obtained for the primers (GAPDH and TLR4) based on a serial dilution (10 folds). R square and efficiency were 95 and 88 percent in TLR4 and 99 and 100 percent in GAPDH, respectively. Threshold cycles were determined using data of real time PCR of GAPDH and the result of healthy sample data (Figure 2). TLR4 gene expression in PBMCs from DM2

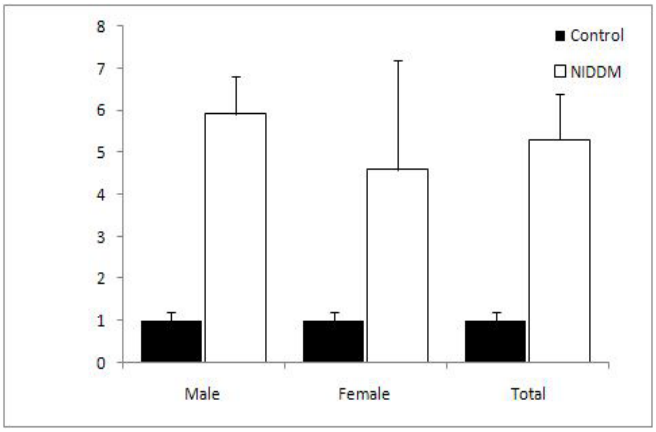

Figure 2. Relative gene expression of TLR4 for male, female and the combined groups of diabetes mellitus type 2 patients related to healthy samples using Pfaffle methods (explained in material and methods section). For each group control TLR4 gene expression one was assumed. Error bars show standard deviation for each group.

Table1. Characteristics of patients and control subjects.

		Type 2 Diabetic patients (n=32)	Healthy controls(n=20)	P value
Numbers	male	18	10	p>0.1
	female	14	10	
Age (years)	male	49.41	51.12	p>0.1
	female	48.37	46.35	
BMI (kg/m²)	male	28.3±1.0	25.6±0.9	p>0.1
	female	27.5±0.4	24.1±1.2	
Blood glucose (mg/dl)		132.7±9.2	107.3±2.7	p= 0.05

patients showed significant up-regulation compared to the healthy individuals (Student's t-test, p= 0.05) (Figure 2). TLR4 gene expression was statistically similar in male and female groups in patients and controls. Samples with different glucose did not display a correlation with TLR4 expression. In addition, there was no significant correlation between BMI and TLR4 gene expression.

Discussion

Diabetes mellitus type 2 comprises about 90% of worldwide diabetic cases. This condition which is comorbid with obesity poses a huge burden to the societies (13). Development of DM2 has two major causes, lifestyle and genetic predisposing factors (14). In addition, different factors like increasing age and female gender can increase the risk of developing DM2. Moreover, researches have shown that glucose metabolic pathway (using internal receptor for advanced glycation end-products (RAGE)) leads to activation of NF-κB (15); the most important transcription factors in innate immune system. NF-κB binds the promoters of innate immune system genes, particularly pro-inflammatory cytokines such as IL-1β and TNF-α (tumor necrosis factor-alpha) (16, 17). Also in binding specific TLR4 ligand (LPS), myd88 and TRIF signaling pathways are activated and finally these kind of cytokine are produced which are a bridge between innate and acquired immune system (3). Secretion of these cytokines exerts positive feedback on TLR4 expression. Thus, in DM2, TLR4 expression may increase due to activate NF-κB pathway. In the current study, 5.21-fold increase in TLR4 gene expression was identified in 32 blood samples from DM2 patients compared to 20 control samples (Figure 2). According to the results, it appears that overexpression of TLR4 on the PBMCs of DM2 patients can be considered as important candidate which results in inflammation in the DM2 and consequently leads to DM2 complications such as nephropathy (18). However, an activated innate immune system, probably via up-regulation of TLR4, also leads to increased pro-inflammatory cytokines such as IL-1β and TNF-α, and may promote insulin resistance. In parallel with our results, Nackiewicz et al., reported that TLR4-activated macrophages play significant roles in islet inflammation and cause impair beta cell functions (19). Previous studies demonstrated that endogenous ligands (DAMPs) (such as heat shock protein 60, high-mobility group box-1, hyaluronan and free fatty acids) are released during DM2 (20) and it can describe the roles played by TLR4 in induction of inflammation in DM2. Additionally, it has been demonstrated that alteration in gut microbiota in the DM2 results in increasing the plasma LPS levels (21). LPS is a main ligand for activation of TLR4 and increase the inflammation mediated insulin resistance (22). The important roles played by TLR4 in the pathogenesis of DM2 has also confirmed by Uchimura and colleagues which demonstrated that modulating TLR4 signaling leads to regulates hepatic insulin sensitivity (23). Additionally, it has been also documented that TLR4 also participates in induction of several DM2 complications including atherosclerosis (24), nephropathy (25) and so on. It suggests that the TLR4 response might be a part of chronic process of DM2.

Despite the fact that, the prevalence of diabetes is higher in women, results from this study revealed that the there is no significant difference between TLR4 expression in 14 females (4.69-fold mean increase) and 18 males (5.87-fold mean increase) (Figure 2). Moreover, a positive but not significant correlation observed between blood glucose content and increase in TLR4 in individuals, suggest that higher blood sugar may lead to induction of TLR4 expression, because we chose a uniform experimental field and standard deviation of glucose was less than 10 mg/dl (Table1). Overexpression of PRRs, particularly TLRs, may be either beneficial or damaging as they play a major role in tissue repair and tumor-genesis. In the physiological state, TLR4 up-regulation in DM2 may ultimately be beneficial to the organism but in disease conditions, overexpression of TLRs may cause more angiogenesis and TLR-dependent tumor progress. Some reports have shown that obesity may cause deregulation of fatty acid homeostasis which can mimic bacterial LPS and activate and up-regulate TLR4 (26). Genetic deletion of TLR4 in mice leads to protection against diet-induced insulin resistance (27) which indicates close relationship between TLR4 and DM2.

Overexpression of TLR4 due to DM2 can be a potential response of the immune system to prepare the organism for damage caused by diabetes. However, an activated innate immune system also leads to increased pro-inflammatory cytokines such as IL-1β and TNF-α, and may promote insulin resistance. In the disease state, it can be exacerbated damage from diabetes.

In this current study, we showed that TLR4 has been up-regulates on the PBMCs of DM2 patients in an independent manner of sex, blood glucose concentration and BMI. And it can be concluded that TLR4 can be considered as important candidate for developing DM2 and its related complications.

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