Toll-like receptor (TLR)-2/4 expression in retinal ganglion cells in a high-glucose environment and its implications

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Genet. Mol. Res. 15 (2): gmr.15026998
Received June 15, 2015
Accepted December 21, 2015
Published April 25, 2016
DOI http://dx.doi.org/10.4238/gmr.15026998

ABSTRACT. Diabetic retinopathy (DR), a major complication of diabetes mellitus, is the leading cause of adult blindness. The Toll-like receptor (TLR) family is believed to be involved in the pathogenesis and progression of DR. Here, we investigated the expression profiles of TLR-2 and TLR-4 in retinal ganglion cells (RGCs), in an attempt to elucidate the role of these molecules in the etiology of DR. *In vitro* cultured RGCs were divided into control and high-glucose groups. The mRNA and protein levels of TLR-2, TLR-4, and nuclear factor (NF)-κB were detected by real-time PCR and western blotting. RGCs were further transfected with specific siRNA targeting TLR2/TLR4; the proliferation of transfected RGCs and their tumor necrosis factor (TNF)-α and interleukin (IL)-8 secretory capacity were analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and enzyme-linked immunosorbent assays (ELISA), respectively. In a high-glucose environment, TLR-2/4 expression was significantly

upregulated in RGCs (while their viability decreased); additionally, NF-κB expression and secretion of TNF-α and IL-8 were significantly increased. Co-silencing of the *TLR-2* and *TLR-4* genes inhibited NF-κB expression and TNF-α/IL-8 secretion, while increasing the survival rate of RGCs. Therefore, a high-glucose environment can potentiate the expression of TLR-2 and TLR-4 in RGCs, activate the downstream signaling pathway, and increase the secretion of pro-inflammatory factors, thereby aggravating DR.

Key words: Diabetic retinopathy; Retinal ganglion cells; TLR-4; Toll-like receptor-2

INTRODUCTION

Diabetic retinopathy (DR), the most important diabetes mellitus (DM)-related complication, is the current leading cause of adult blindness in ophthalmological practice (Huang et al., 2015; Mysona et al., 2015). A WHO survey revealed a >30% incidence of DR in DM patients, leading to irreversible vision loss (Zhao et al., 2015). Tissue inflammation is correlated with DM, as multiple factors, including C-reactive protein, tumor necrosis factor (TNF)-α, and interleukin (IL)-8, are involved in the impairment of organ functions (Boynton et al., 2015; Garcia et al., 2015). During the pathogenesis of DR, injuries to the retinal ganglion cells (RGCs) precede micro-vessel injury, as supported by early-onset vision defects in DM patients with no observable retinal microvascular disease (Hao et al., 2015; Tamadon et al., 2015). RGCs, which are among the earliest differentiated neurons in the retina, are critical for visual perception. Therefore, death of RGCs is a major factor contributing to irreversible vision loss during the pathogenesis and progression of DR (Jia et al., 2014).

Toll-like receptors (TLRs) are a family of receptors that are specific for pathogen-related molecules and mainly participate in the innate immune response. TLR activation triggers a downstream signal cascade, which stimulates the secretion of large amounts of pro-inflammatory factors, thereby initiating an inflammatory response (Madhumitha et al., 2015; Xu et al., 2015). In total, 11 members of the TLR family have been identified so far (TLR-1-11), among which TLR-2 and TLR-4 have the widest distribution, broadest spectrum for pathogen recognition, and release the greatest variety of products. These two receptors play a role in the regulation of various auto-immune diseases, including atherosclerosis and rheumatoid arthritis (Tang et al., 2013; Rajamani and Jialal, 2014). Previous studies have shown that TLR expression is elevated in muscles, adipocytes, and B lymphocytes in DM patients. Further genetic studies in Chinese type II DM patients have revealed a correlation between polymorphisms in the *TLR-4* gene and disease progression, suggesting the involvement of TLR-2 and TLR-4 in the mediation of both type I and type II DM (Habib et al., 2015; Singh et al., 2015a,b). The function of these two factors in DR and the related mechanism, however, remains unclear. Therefore, the aim of this study was to elucidate the expression profiles of TLR-2 and TLR-4 in RGCs in a high-glucose environment, in order to elucidate their functional role (and the related mechanism) in the pathogenesis and progression of DR.

MATERIAL AND METHODS

RGC culture and transfection

Human RGC-5 cells [American-Type Cell Culture Association (ATCC), Manassas, VA, USA] were cultivated in Dulbecco's modified Eagle's medium (HyClone Laboratories Inc., Logan, UT, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (HyClone Laboratories Inc.), and 50 μg/mL ciliary neurotrophic factor (Sigma-Aldrich, St. Louis, MO, USA), at a concentration of 1 x 10^5 cells/mL at 37°C. The cells were passaged every 3 days. Log-phase cells were randomly divided into three groups: control, high-glucose, and siRNA-transfected groups. Glucose (25 mM) was used to mimic the DR model 24 h after the log-phase. siRNAs targeting both TLR-2 and TLR-4 were used to transfect the RGCs in the transfected group.

Specific siRNA targeting TLR-2 and TLR-4 were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: siRNA-TLR-2, 5'-CUAGA GUUCU CCUUG GAAA-3'; siRNA-TLR-4, 5'-CCUCC UAGAU UUGGA AGUA-3'. The siRNAs were used to co-transfect the cultured log-phase RGC-5 cells by using the Lipo2000 kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. In brief, log-phase RGC-5 cells (3 x 10^5 cells/mL) are cultured on a 6-well plate at 37°C in a 5% CO_2 chamber until attaining a confluence of 70%. The cells were initially incubated with serum-free medium containing the transfection buffer (2.5%) for 15 min; the cells were then incubated with the siRNA-containing buffer for 30 min. The supernatant was removed, the cells were rinsed gently, and fresh serum-free medium added. The cells were then cultivated in a 5% CO_2 chamber at 37°C for 6 h, before changing the medium again.

Real-time polymerase chain reaction (PCR)

Total mRNA was extracted from RGC-5 cells by using the TRIzol reagent (Invitrogen) according to the manufacturer protocols. cDNA was then synthesized by *in vitro* reverse transcription by using mRNA as the template. The target gene expression was quantified by real-time quantitative PCR by using specific primers (Table 1). The reaction conditions were set as follows: 38 cycles of denaturation at 90°C for 30 s, annealing at 58°C for 50 s, and elongation at 72°C for 35 s. The level of target gene expression was determined by a fluorescent PCR cycler; this was standardized against the expression of the internal reference β-actin by the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer sequences for real-time polymerase chain reaction.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	GCCAG CAGGT CTCAT ATCTC G	TTGAC GACTA GTGCC GTGGG T
TLR-2	GATCT CTCAG CGAAG AAACG T	ATTCT GCACG CGTAC TCCG
TLR-4	TCAGA AACTG CTCGG TCAGA	GCCTC AGGGG ATTAA AGCTC
NF-κB	GATCC AGGAT GAGGG GATTT	CAGGG TGTCT CCTGG TCTGT

Enzyme-linked immunosorbent assay (ELISA)

TNF-α and IL-8 expression in the cultured supernatant was quantified using standard ELISA kits (R&D Systems, McKinley Place, MN, USA), per the instructions of

the manufacturer. In brief, serial diluted standard samples were added to a 96-well plate in triplicate, along with equal volumes of the test samples. The samples were washed gently and the supernatant was removed; the samples were incubated with enzyme-linked reagents at 37°C for 30 min. The chromogenic substrates A and B were sequentially added to each well, and the color was developed by incubating for 10 min in the dark. The reaction was stopped by the addition of the quenching buffer. The optical density values of each well at 450 nm were obtained using a micro-plate reader (Bio-Rad, Hercules, CA, USA). A standard curve was plotted to deduce the sample concentrations.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cells were incubated for 48 h, digested, and inoculated on a 96-well plate (3000 cells per well). The cells in each well were incubated with the MTT reagent (5 g/L; Gibco, Life Technologies, Carlsbad, CA, USA) for 4 h. The supernatant was discarded, and dimethyl sulfoxide (HyClone) was added to each well. The plate was vibrated to ensure complete solvation. The absorbance of the cells at 570 nm was determined using a microplate reader, to calculate the rate of cell proliferation.

Western blot

Total proteins were extracted from RGC-5 cells by using a standard lysis buffer (Beyotime, Shanghai, China) and ultrasonic treatment. The cells were centrifuged at 10,000 *g* for 15 min, and the supernatant was retained. The proteins in the supernatant were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%). The proteins were then transferred to a polyvinylidene difluoride membrane (Pall Life Sciences, Port Washington, NY, USA) by using a semi-drying method. Non-specific binding sites were blocked by 5% defatted milk powder. The membrane was incubated overnight with rabbit anti-mouse TLR-2/TLR-4 monoclonal antibody (1:1000; Cell Signaling Technologies) or rabbit anti-mouse NF-κB monoclonal antibody (1:500; Cell Signaling Technologies) at 4°C. The cells were then incubated with goat anti-rabbit IgG (1:2000; Cell Signaling Technologies) for 30 min. Enhanced chemiluminescence chromogenic reagents (Amersham Biosciences, Piscataway, NJ, USA) were used to develop the membrane, which was then exposed to an X-ray. The images were captured and analyzed by the Quantity One software (BioRad). All studies were repeated four times (N = 4).

Statistical analysis

The collected data were analyzed using the SPSS v.16.0 platform software (IBM, Armonk, NY, USA); all data are reported as means ± standard deviation (SD). Multiple groups were compared by one-way analysis of variance (ANOVA). A statistical significance was defined when $P < 0.05$.

RESULTS

TLR gene expression in high-glucose-treated RGCs

The mRNA levels of both TLR-2 and TLR-4 were significantly increased under high-

glucose conditions compared to those in glucose-controlled cells (Figure 1; $P < 0.05$). The co-transfection of siRNA targeting TLR-2/4 effectively inhibited the target gene expression in a high-glucose environment.

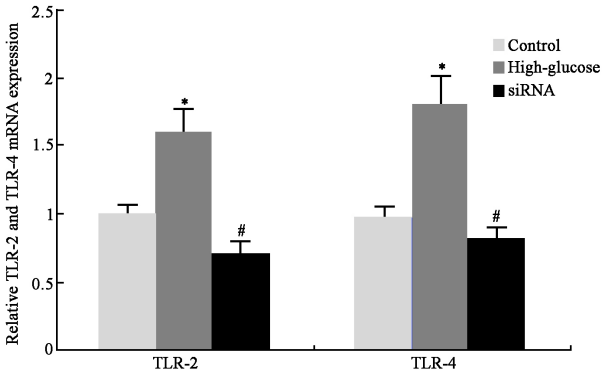

Figure 1. TLR-2 and TLR-4 mRNA levels in RGC-5 cells. *$P < 0.05$ compared to the control group. #$P < 0.05$ compared to the high-glucose group.

Furthermore, the results of western blotting (Figure 2) were consistent with those of RT-PCR; that is, compared to control cells, high-glucose-treated RGC-5 cells showed significantly elevated expression of TLR-2 and TLR-4 proteins ($P < 0.05$). siRNA transfection led to a significant overexpression of high-glucose-induced TLR-2/4. These two results collectively suggested that the *TLR*-2 and *TLR*-4 genes were significantly upregulated in a high-glucose environment.

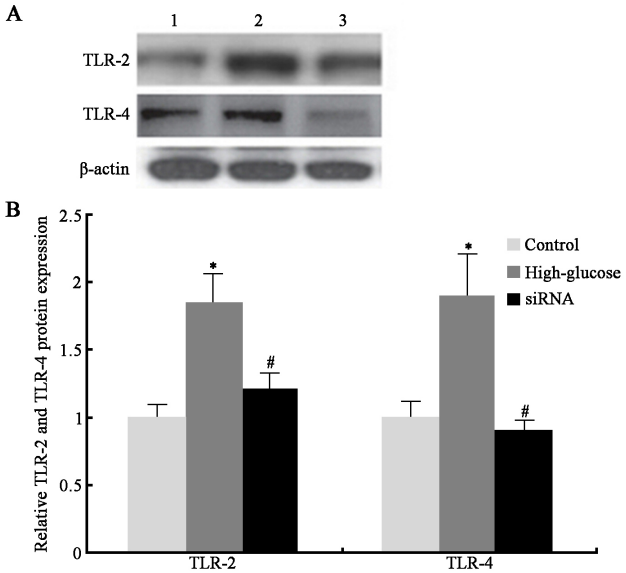

Figure 2. Protein expression levels of TLR-2 and TLR-4. **A.** Representative blotting bands of two proteins. *Lane 1*, control; *lane 2*, high-glucose treated; *lane 3*, siRNA-transfected cells. **B.** Quantitative results of protein expression levels. *$P < 0.05$ compared to the control group. #$P < 0.05$ compared to the high-glucose group.

Effect of siRNA transfection on RGC-5 cell survival

The MTT assay displayed the decreased viability of RGC-5 cells in a high-glucose environment (Figure 3; $P < 0.05$). Co-transfection of TLR-2/4 siRNA, however, led to a significant improvement in cell viability. These results indicated the correlation between TLR-2/4 over-expression and the inhibition of RGC survival.

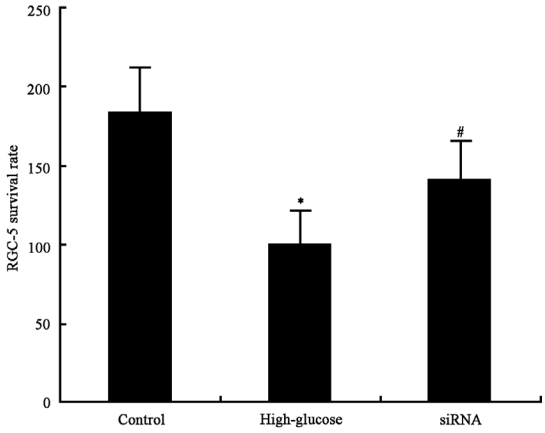

Figure 3. Cell survival rate of retinal ganglion cells (RGCs). *P < 0.05 compared to the control group. #P < 0.05 compared to the high-glucose group.

Regulation of NF-κB expression by siRNA transfection

The mRNA and protein levels of TLR-2 and TLR-4 in RGC-5 cells were detected by RT-PCR and western blot analyses, respectively. The results of these analyses (Figure 4) revealed that high-glucose-treated cells had elevated NF-κB expression, which was significantly downregulated by co-transfection with TLR-2/4 siRNA. These results suggested the potential involvement of NF-κB in the TLR-mediated pathway (regulating RGC survival).

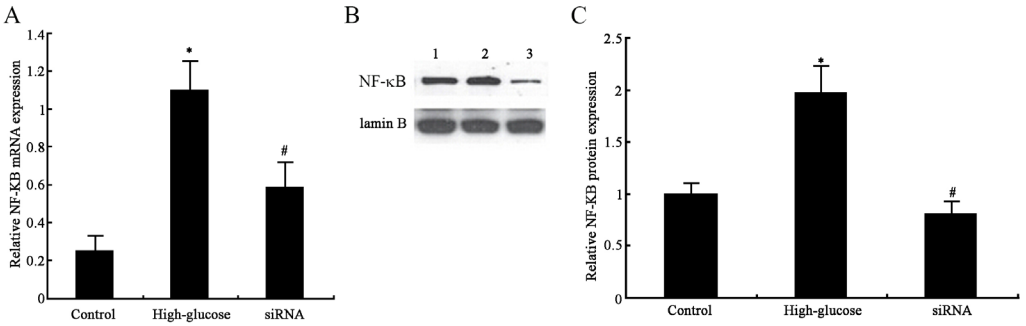

Figure 4. NF-κB expression in RGC-5 cells. **A.** mRNA levels. **B.** Representative blotting bands of proteins. *Lane 1*, control; *lane 2*, high-glucose treated cells; *lane 3*, siRNA-transfected cells. **C.** Quantitative results of protein expression levels. *P < 0.05 compared to the control group. #P < 0.05 compared to the high-glucose group.

Regulation of TNF-α and IL-8 expression by siRNA transfection

We investigated the expression of potential inflammatory factors involved in RGC pathology, such as TNF-α and IL-8, by ELISA. We observed significantly elevated concentrations ($P < 0.05$) of these two factors in a high-glucose environment (Figure 5). The co-transfection of TLR siRNA led to a significant decrease in the level of secretion of both factors. These results clearly indicated that the activation of TNF-α and IL-8 inflammatory response functioned as the downstream signal of TLR-2/4 activation, thereby damaging the RGCs. siRNA targeting TLR-2/4 may inhibit TNF-α and IL-8 secretion by downregulating NF-κB expression, thereby alleviating DR-related injury.

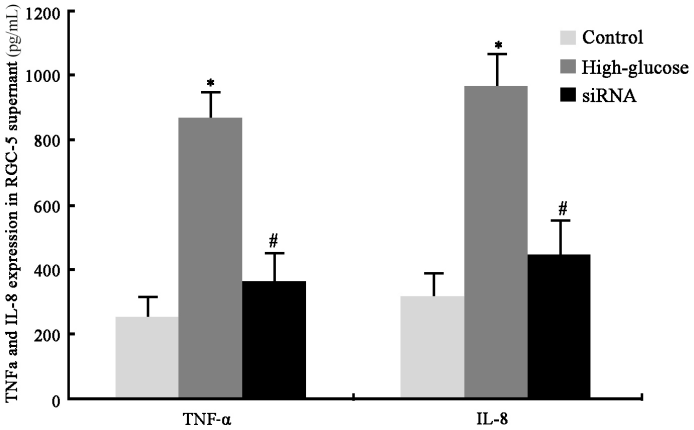

Figure 5. Secreted levels of TNF-α and IL-8. *$P < 0.05$ compared to the control group. #$P < 0.05$ compared to the high-glucose group.

DISCUSSION

DR is a common DM-related micro-vascular complication, which alters the permeability of retinal micro-vessels and increases the adhesion of leukocytes and secretion of cytokines and chemokines, leading to progressive damage of the micro-vessels (Kim et al., 2015). Approximately one-third of DM patients presented retinal complications, including diabetic macular edema and proliferative diabetic retinopathy, both of which may lead to severe vision impairment or even blindness (Price et al., 2015). RGCs are among the most widely distributed and earliest differentiated retinal neurons, and therefore play a role in vision signal transformation, transduction, and processing. RGCs are predisposed to injuries at their axons in various systemic diseases, including DM, which causes irreversible damage to the retina (Furukawa et al., 2015). Therefore, the protection of RGCs can ameliorate the progression of DR.

TLR-2 and TLR-4 are abundantly expressed in various neurons, including the cortical and dorsal neurons, and trigeminal nerves (Fernandez-Lizarbe et al., 2013; Cardoso et al., 2015). Bi et al. (2014) and Rosenberger et al. (2014) proposed that alternations in the expression of TLR-2/4 may regulate the proliferation and growth of neurons, and the inflammatory response. Therefore, we hypothesized that TLR-2/4 may regulate RGC survival and growth. In this study, we observed a significant elevation in the expression of TLR-2 and TLR-4, which could induce the intracellular signaling pathway to initiate the nuclear

transfer of NF-κB, thereby facilitating the secretion of the pro-inflammatory factors TNF-α and IL-8. These factors will, in turn, initiate the inflammatory response, induce the adhesion of leukocytes, and maturation/migration of immune cells, further accelerating the inflammation, leading to RGC damage and aggravation of DR. The co-transfection of siRNA targeting TLR-2/4 downregulated the expression of NF-κB, in addition to reducing the secretion of TNF-α and IL-8, thereby providing a novel means for the protection of RGCs in a high-glucose environment, and to alleviate DR.

In summary, high-glucose treatment may activate the expression of TLR-2 and TLR-4 in RGCs in order to upregulate the secretion of pro-inflammatory factors, thereby aggravating DR. siRNA targeting TLRs may therefore provide a new means to slow the progression of this disease.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the anonymous reviewers for reviewing this manuscript.

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