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High glucose induces inflammatory cytokine through protein kinase C-induced toll-like receptor 2 pathway in gingival fibroblasts

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ABSTRACT

Toll-like receptors (TLRs) play a key role in innate immune response and inflammation, especially in periodontitis. Meanwhile, hyperglycemia can induce inflammation in diabetes complications. However, the activity of TLRs in periodontitis complicated with hyperglycemia is still unclear. In the present study, high glucose (25 mmol/l) significantly induced TLR2 expression in gingival fibroblasts ($p < 0.05$). Also, high glucose increased nuclear factor kappa B (NF-κB) p65 nuclear activity, tumor necrosis factor-α (TNF-α) and interleukin-lβ (IL-1β) levels. Protein kinase C (PKC)-α and δ knockdown with siRNA significantly decreased TLR2 and NF-κB p65 expression ($p < 0.05$), whereas inhibition of PKC-β had no effect on TLR2 and NF-κB p65 under high glucose ($p < 0.05$). Additional studies revealed that TLR2 knockdown significantly abrogated high-glucose-induced NF-κB expression and inflammatory cytokine secretion. Collectively, these data suggest that high glucose stimulates TNF-α and IL-1β secretion via inducing TLR2 through PKC-α and PKC-δ in human gingival fibroblasts.

1. Introduction

Hyperglycemia, as one of the key abnormalities in diabetic mellitus, plays an important role in the development of inflammation in diabetes complication. It has been demonstrated that high glucose induced not only inflammatory cytokines and chemokines, but also p38 mitogen-activated protein kinase (p38 MAPK), protein kinase C (PKC), and nuclear factor-κB (NF-κB) activity in patients and animal models [1–4]. Although periodontitis has been considered as one of the complications of diabetes mellitus, the regulatory mechanism of hyperglycemia on the development of periodontitis in diabetic mellitus still remain unclear.

In the innate immune system, cells sense endogenous ligands and bacterial products mainly through toll-like receptors (TLRs). TLRs can induce innate immune responses via recognizing conserved pathogen-associated molecular patterns and delivering the inflammatory signal, which is essential for host defenses [5]. TLRs activation triggers a signaling cascade, resulting in cytokine secretion, innate immune response and adaptive immune response initiation [6]. Also, TLRs play important roles in humans and animals models with diabetes mellitus. It has been shown that TLRs' levels increased in inflammatory disorders, including diabetes mellitus [7,8]. In animal model exposed to a high-fat diet, inhibition of TLR2 function led to improved insulin sensitivity and decreased activation of pro-inflammatory pathways [9]. Furthermore, polymorphisms in TLR2 predict the development of type 1 diabetes [10]. Mohammad et al. [11] reported that TLR2 expression and pro-inflammatory cytokines increased in type 1 diabetic non-obese diabetic mice, accompanied with increased nuclear factor kappa B (NF-κB) activation in response to endotoxin. Kim et al. [12] demonstrated that TLR2 induced β-cell death and contributed to the instigation of autoimmune diabetes using TLR2, TLR4 knockout mice. Devaraj et al. [8] showed that TLR2 and TLR4 expression, intracellular signaling and TLRs-mediated inflammation increased in monocytes from type 1 diabetic patients. Also, Creely et al. [13] showed that TLR2 expression increased in the adipose tissue of type 2 diabetic patients, with strong correlation to endotoxin levels. Taken together, these observations suggest that TLR2 has a potential role in the pathology of diabetes and promotes the inflammation in diabetes mellitus.

Also, TLR2 is expressed widely in gingiva, including periodontal ligament cells, gingival epithelium and fibroblasts [14–17]. According to some researches, TLR2 level increased with the severity of periodontal inflammation, especially related with bone resorption in clinical patients [14,16,17]. In periodontitis, gingival fibroblasts could increase TLR2 and TLR4 levels after stimulated by *Porphyromonas gingivalis* (*p.g*) lipopolysaccharide (LPS), resulting in inflammatory cytokine increase [18]. Zhou et al. [19] demonstrated that *p.g* and *p.g* LPS induced inflammatory cytokines through TLR2-NF-κB pathway. Burns et al. [20] used *p.g* LPS to stimulate TLR2 and TLR4 knockout mice, and found that interleukin-l (IL-1) β, tumor necrosis factor-α (TNF-α) and Interferon-γ (INF-γ) were

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induced in TLR4 knockout and wild mice. However, these cytokines were absent in TLR2 knockout mice [20]. Especially, no bone resorption occurred in TLR2 knockout mice [20]. All these data support that TLR2 plays an essential role in the development of periodontitis.

Gingival fibroblasts, as the main cells in periodontal tissue, are involved in inflammation in periodontitis. Although inflammation and hyperglycemia interact clearly in human monocytes [8], human adipose tissue [9], bone marrow-derived macrophages [11], the relationship of hyperglycemia and TLR2 is still unclear in human gingival fibroblasts. Therefore, this study aimed to test the ability of high glucose to induce TLR2 expression in gingival fibroblasts and its relevant signal pathway of inducing inflammatory cytokines.

2. Materials and methods

2.1. Subjects and cell culture

Approval for human tissue specimens was obtained from the Committee of Ethics in Tianjin Medical University. After informed consent was obtained from every dental patient who was undergoing oral surgical procedure, discarded gingiva were collected. All five donors (age from 18 to 25) were non-smokers and almost in normal condition without systemic disease and overt clinical signs of inflammation in the periodontal tissue. The gingival explants were immersed in sterile phosphate balanced saline (PBS) (Gibco BRL, MD, USA), washed with sterile PBS and placed in 60 mm culture plates (Corning Inc., NY, USA) with modified essential medium (DMEM with normal glucose) that was supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, MD, USA), antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin sulfate) (Gibco BRL, MD, USA). When the cells migrated out of the explants and reached about 80% confluence, these cells were collected and passaged serially.

2.2. Treatments

Gingival fibroblasts were cultured in normal glucose (5.5 mmol/l) as the control. At indicated time points cells were treated with 25 mmol/l glucose. 14.5 mmol/l mannitol was added along with normal glucose as an osmotic control.

2.3. RNA extraction and real-time polymerase chain reaction (PCR)

RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture protocol. cDNA reverse transcription was performed using GoldScript cDNA synthesis system (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instruction. qPCR SuperMix-UDG ROX kit (Invitrogen, Carlsbad, CA, USA) was used to detect the mRNA expressions of TLR2, NF-κB p65 and β-actin as the manufacture's instruction. The real-time PCRs were performed in triplicates. Data were calculated using the $2^{-\Delta\Delta CT}$ method and are presented as the *x*-fold induction of transcripts for the TLR2 and NF-κB p65 gene normalized to β-actin over the control. The primers were as followings: TLR2 (5′-GCCAAAGTCTTGATGATTGG-3′/5′-TTAAGTTCTCCAGCTCCTG-3′), NF-κB p65 (5′-GCTGGCAGCTCTCCAGTCAGG-3′/5′-GAGCTCGTGCCGTCCAGCTT-3′) and β-actin as an internal control (5′-CCTGTACGCCAACACAGTGC-3′/5′-ATACTCCTGCTTGCTGATCC-3′).

2.4. Enzyme-linked immunosorbent assay (ELISA)

IL-1β and TNF-α were measured in the supernatants of gingival fibroblasts by ELISA (R&D Systems, MN, USA), according to the manufacture's instruction. In brief, each well was incubated at room temperature after standard, control, and samples were added. After three washes, TNF-α or IL-1β conjugate was added to each well respectively and incubated at room temperature. Then, the wells were washed three times and substrate solution was added to each well. After the stop solution was added to stop the enzyme reaction, the absorbance of each micro-well was read on a spectro-photometer using 450 nm (Bio-tek, VT, USA).

2.5. siRNA transfection assays

Prevalidated siRNAs of PKC subunits α, β, δ and TLR2 were obtained from RiboBio (Guangzhou, China), and transfection assays were performed with X-tremeGENE HP (Roche, Mannheim, Germany) following the manufacturer's instructions. Briefly, after PKC-α siRNA, PKC-β siRNA, PKC-δ siRNA or TLR2 siRNA was incubated with tranfection reagent for 20 min at room temperature, respectively, the mixture was added to each well. After transfected with siRNA, cells were subsequently treated with normal glucose (5.5 mmol/l) or high glucose (25 mmol/l) for 24 h. Cells treated with suitable vehicle and scrambled siRNA were taken as the controls.

2.6. Nuclear NF-κB p65 activity assay

Nuclear fraction was collected from gingival fibroblasts treated with high glucose, using Nuclear extract kit (Active Motif, Carlsbad, CA). In brief, after washing with ice-cold PBS/phosphatase inhibitors, the cells were removed from dish, transferred to a pre-chilled tube and centrifuged. Cell pellet was resuspended in Hypotonic buffer, incubated on ice, added Detergent and centrifuged to obtain the nuclear pellet. The nuclear pellet was resuspended in Complete lysis buffer with vortex at highest setting, incubated on ice. After centrifugation, the supernatant was transferred into a pre-chilled microcentrifuge tube, aliquoted and stored at −80 °C. NF-κB p65 DNA binding activity in the nuclear extracts of gingival fibroblasts was determined using the non-radioactive TransAM transcription factor assay (Active Motif, Carlsbad, CA) according to the manufacturer's instruction. NF-κB activity was described as the x-fold expression over the control.

2.7. Statistical analysis

Each experiment was repeated at least twice. Data were reported as means ± standard deviation (SD). Differences were statistically analyzed using analysis of variance (ANOVA) and appropriate post hoc method. A probability value of $p < 0.05$ was considered significant.

3. Results

3.1. High glucose induces TLR2 mRNA expression in gingival fibroblasts

As depicted in (Fig. 1), TLR2 mRNA level was significantly increased ($p < 0.05$) under hyperglycemic conditions compared to normal glucose control. At 25 mmol/l glucose concentration, the TLR2-to-β-actin mRNA ratio was significantly highest at 24 h (Fig. 1). Addition of 14.5 mmol/l mannitol to normal glucose did not result in the increase of TLR2 level, suggesting that osmotic increase had no effect on high glucose-induced increase in TLR2 expression.

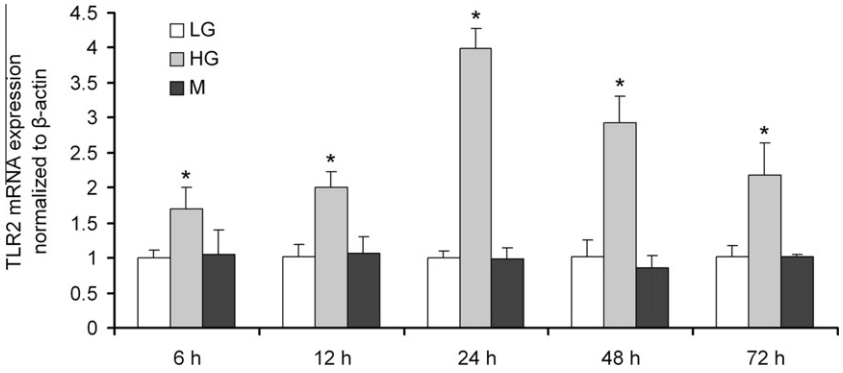

Fig. 1. TLR2 mRNA expression in gingival fibroblasts after glucose challenge for indicated time points by real-time PCR. Glucose (5.5 mmol/l) was added to mannitol. Values are normalized to β-actin and expressed as mean ± SD. $^{*}p < 0.05$ vs. LG or M. LG, low glucose; HG, high glucose; M, mannitol.

3.2. High glucose increases NF-κB p65 nuclear activity, TNF-α and IL-1β levels in gingival fibroblasts

To determine the TLR2 downstream factors, we detected the effects of high glucose on NF-κB p65 nuclear activity, TNF-α and IL-1β using ELISA techniques. High glucose significantly induced the nuclear NF-κB p65 activity in gingival fibroblasts at 24 h ($p < 0.05$) when compared with normal glucose (Fig. 2A). Addition of 14.5 mmol/l mannitol to normal glucose did not increase NF-κB p65 nuclear activity, which showed that high glucose-induced increase in NF-κB p65 nuclear activity was not an osmotic effect. High glucose significantly increased TNF-α levels at 6 h and 24 h when compared with normal glucose ($p < 0.05$) (Fig. 2B). Although high glucose did not increase the IL-1β level at 6 h, IL-1β secretion significantly increased at 24 h when compared with normal glucose ($p < 0.05$) (Fig. 2C). Addition of 14.5 mmol/l mannitol to normal glucose did not result in TNF-α and IL-1β levels increase, suggesting that high glucose-induced increase in TNF-α and IL-1β expression was not an osmotic effect.

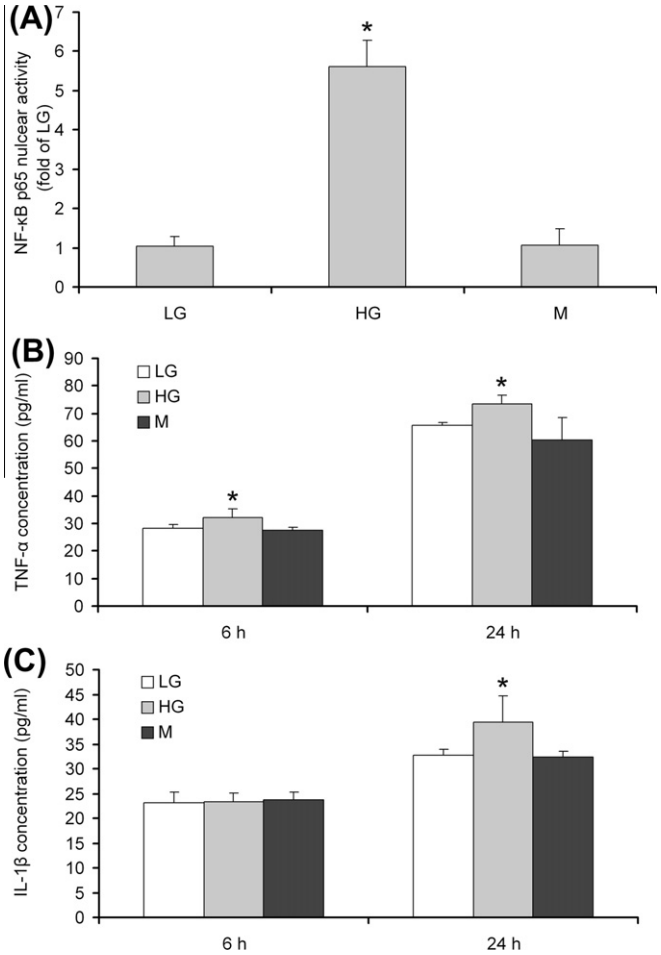

Fig. 2. High glucose induces NF-κB p65 nuclear activity, IL-1β and TNF-α levels in gingival fibroblasts via ELISA. A: NF-κB p65 nuclear activity at 24 h; B: TNF-α levels at 6 h and 24 h; C: IL-1β levels at 6 h and 24 h. $^{*}p < 0.05$ vs. LG or M. LG, low glucose; HG, high glucose; M, mannitol.

3.3. PKC-α and PKC-δ are involved in high-glucose-induced TLR2 and NF-κB p65

We investigated the mechanistic events of high-glucose-induced TLR2 activation in gingival fibroblasts using siRNA technology. To determine which isoform of PKC mediates TLR2 expression in gingival fibroblasts under high glucose, we used isotype gene-specific siRNAs to PKC-α, PKC-β, and PKC-δ. Scramble siRNA had no effects on TLR2 and NF-κB p65 mRNA expression ($p > 0.05$). PKC-α and PKC-δ siRNA significantly decreased TLR2 and NF-κB p65 mRNA expression in gingival fibroblasts ($p < 0.05$) when compared with scrambled controls in high glucose, whereas PKC-β siRNA had no effect on TLR2 and NF-κB p65 mRNA levels ($p > 0.05$) (Fig. 3).

3.4. High-glucose-induced NF-κB p65, TNF-α and IL-1β levels are abrogated in the absence of TLR2 expression

To further investigate TLR2-mediated inflammation under high glucose in gingival fibroblasts, we used specific siRNA to knock down TLR2 gene. After transfection with TLR2 siRNA, lack of TLR2 in gingival fibroblasts resulted in an obvious decrease in NF-κB p65 mRNA level when compared with scrambled controls in high glucose ($p < 0.05$, Fig. 4A). Next, we measured TNF-α and IL-1β release in the supernatants. Inhibition of TLR2 resulted in significant decreases in TNF-α and IL-1β, compared with scrambled controls in high glucose ($p < 0.05$, Fig. 4B and C). Scramble siRNA had no significant effects on NF-κB p65 mRNA expression, TNF-α and IL-1β release ($p > 0.05$).

4. Discussion

In this study, we observed that the expression of TLR2, one of key innate immune system sensors, increased in human gingival fibroblasts under high-glucose conditions. Also, we delineated

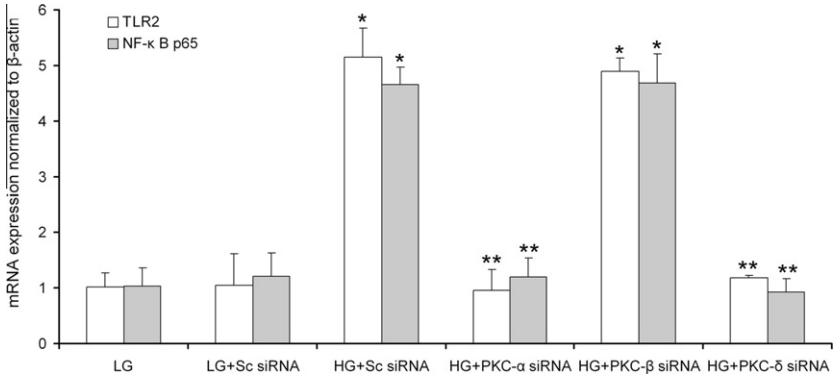

Fig. 3. Inhibition of PKC isoforms using siRNAs effects high-glucose-induced TLR2 and NF-κB p65 mRNA expression of gingival fibroblasts by real-time RT-PCR. Values are expressed as mean ± SD. $^{*}p < 0.05$ vs. Sc siRNA + LG; $^{**}p < 0.05$ vs. Sc siRNA + HG. LG, low glucose; HG, high glucose; Sc, scramble control.

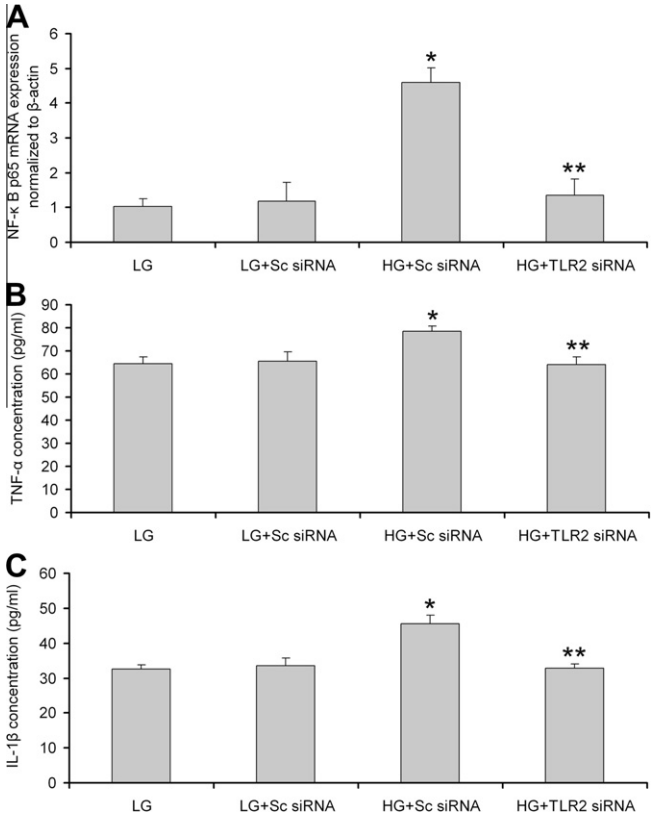

Fig. 4. NF-κB p65 mRNA expression, TNF-α and IL-1β concentration after high-glucose treatment in the absence of TLR2. A: NF-κB p65 mRNA expression via real-time RT-PCR; B: TNF-α levels via ELISA; C: IL-1β levels via ELISA. $^{*}p < 0.05$ vs. Sc siRNA + LG; $^{**}p < 0.05$ vs. Sc siRNA + HG. LG, low glucose; HG, high glucose; Sc, scramble control.

the related signaling pathway and its functional activation in human gingival high glucose-treated fibroblasts.

In previous researches, hyperglycemia could induce inflammation via TLRs pathway [7–13]. Also, Ghanim et al. [21] found that the inhibition of TLRs in insulin-administrated patients with diabetes resulted in the decrease of inflammation when compared with saline-administrated patients with diabetes. Our results showed that high glucose induced a marked increase in TLR2 mRNA expression in gingival fibroblasts, which was not an osmotic effect because mannitol had no effect. Increase in TLR2 expression observed here is consistent with those reported in human monocytes [8,22], human adipose tissue [9,13], bone marrow-derived macrophages [11] related with diabetes.

TLR2 plays a critical role in an innate immunity, via activating NF-κB transcription factor, resulting in the activation of downstream signaling pathways [5]. In animal model, inhibition of TLR2 function led to improved insulin sensitivity and decreased activation of pro-inflammatory pathways [9]. In this study, we found that NF-κB p65 nuclear activity increased under high glucose condition. Meanwhile, inhibition of TLR2 in cells resulted in an obvious decrease of NF-κB p65 level. These data indicate that TLR2 could regulate NF-κB p65 expression and activity under high glucose in gingival fibroblasts, which is consistent with other researches [4,5].

Inflammatory cytokines are involved in the pathological process of periodontitis. Hyperglycemia elevates the levels of inflammatory cytokines to stimulate inflammatory reaction. Albacker et al. [23] confirmed that high-dose insulin administration attenuates TNF-α secretion in patients. According to other researches, high glucose could induce IL-1β expression in human monocytes [8,24]. Also, Devaraj et al. [8] found that TNF-α and IL-1β levels increased via TLR2, 4-NF-κB pathway under high glucose in monocytes. In this study, we showed that TLR2 activated NF-κB p65, accompanied with the increased secretion of inflammatory cytokines TNF-α and IL-1β. Furthermore, we blocked TLR2 and found the decrease in TNF-α and IL-1β. These data suggest that TLR2 increase under high glucose in gingival fibroblasts could induce the periodontal inflammation in diabetes. From above, NF-κB p65, TNF-α and IL-1β levels are almost completely inhibited by TLR2 siRNA in gingival fibroblasts exposed to high glucose. Based on these results, it is considered that TLR2 is specific for the secretion of high glucose-induced inflammatory cytokines in gingival fibroblasts. Here, although our study focused on TLR2 has a little limitation, we showed that TLR2 plays a crucial role in inducing inflammation in gingiva under high glucose. Whether other TLRs are changed in gingival fibroblasts under high glucose and their functions have yet to be further clarified.

However, the mechanism of high glucose-induced TLR2 activation in the gingival fibroblasts is still unclear. It has been demonstrated that PKC pathway mediated widely cellular events and activates NF-κB [25]. Published data indicate that PKC-induced TLR2 exists under different conditions, especially in diabetes patients and cells under high glucose [4,26]. Dasu et al. [22] showed that TLR2 was regulated via PKC-α under high glucose condition in human monocytes, not via PKC-β and PKC-δ. Also, Asehnoune et al. [26] demonstrated that TLR2 was activated by PKC-α/β in neutrophils. However, in our present study, the results showed that TLR2 is regulated via PKC-α/δ under high glucose in gingival fibroblasts, not via PKC-β. We guess that the discrepancy is due to the different cell types used in these researches. Meanwhile, we confirmed that the blockade of PKC prevented the expression of NF-κB p65, which

is consistent with other researches [25]. Furthermore, in our study, TLR2 inhibition decreased the expression of NF-κB p65 when compared with high glucose-treated fibroblasts, which indicates that TLR2 regulates the expression of NF-κB p65. Taken together, the inflammatory signaling pathway is PKC-α/δ-TLR2-NF-κB p65 in gingival fibroblasts in high glucose environment.

Recently, TLR2 has been demonstrated to be heterodimerizated with TLR1 or TLR6 on the cell surface to recognize the specific ligands and transmit the signals [27,28]. TLRs are a family of transmembrane glycoproteins, containing a 16–18 diverse leucine-rich-repeat (LRR) ectodomain which is responsible for pathogen recognition. To date, a great diversity of TLR2 ligands have been found, including molecules with diacyl and triacylglycerol structure, proteins and polysaccharides [27]. However, Zähringer et al. demonstrated that only lipoproteins/lipopeptides (LPs) were the real TLR2 ligands [28]. Meanwhile, Beutler et al. showed that TLR2/TLR1 recognized triacyl LPs, however, diacyl LPs were recognized by TLR2/TLR6 [29]. Although the ligands of TLR2 are still controversial, based on these researches [27,28], glucose itself is not the ligand of TLR2. Also, from our research data, high glucose indirectly regulates TLR2 expression through endogenous PKC-α/δ in gingival fibroblasts. Whether high glucose has an influence on the heterodimerization of TLR1/2 or TLR2/6 still remains to be further explored.

In conclusion, these data suggest that high glucose could stimulate inflammatory cytokines TNF-α and IL-1β secretion, possibly via inducing TLR2 through PKC-α and PKC-δ in human gingival fibroblasts, in which NF-κB p65 is the downstream of TLR2.

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