Original Paper

Enhancement of LPS-Induced Microglial Inflammation Response via TLR4 Under High Glucose Conditions

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Key Words

High glucose • Microglia • JAK2/STAT3 • TLR4

Abstract

Background: Microglia activation mediated by toll-like receptor 4 (TLR4) plays an important role in neuroinflammation and postoperative cognitive dysfunction (POCD). Diabetes mellitus (DM) has been recently suggested as an independent risk factor for POCD. In this study, we investigate the potential exacerbation of the inflammatory response in primary microglia due to high glucose conditions. **Methods:** Primary microglial cells were exposed to normal glucose (25 mmol/L) and high glucose (35 mmol/L) levels alone or with lipopolysaccharide (LPS 0, 2, 5, 10 ng/mL). The pro-inflammatory response of the cells was assessed by measuring changes in cytokine levels and the evaluation of associated signaling pathways. **Results:** Neither high glucose nor low LPS (≤ 5 ng/ml) alone had an effect on TNF- α and IL-6 levels, but the combination of low LPS and high glucose stimulated the inflammatory response. Analyses of the associated signaling pathways demonstrated that high glucose enhanced the LPS-induced microglial activation via the TLR4/JAK2/STAT3 pathway. **Conclusion:** This study demonstrates that high glucose, one of the key abnormalities characteristic of DM, can augment LPS-induced microglial activation and inflammatory cytokine levels through the TLR4/JAK2/STAT3 pathway, offering new insight into the pathophysiological relationship between DM and POCD.

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Introduction

Microglia, the resident immune cells in the brain, plays a pivotal role in the immune surveillance of the central nervous system (CNS). Consequently, these cells are expected to play an important role in the development of protective immune responses and the

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progression of damaging inflammation during CNS disease states [1-4]. Activated microglia carry out several immune effector functions typically associated with macrophages. When subjected to abnormal stimulation, such as neurotoxins, neuronal debris, injury, microglia are gradually activated and produce numerous inflammatory mediators, including tumor necrosis factor- α (TNF- α), prostaglandin E2 (PGE2), interleukin-6 (IL-6), nitric oxide (NO), and reactive oxygen species (ROS). The accumulation of these pro-inflammatory and cytotoxic mediators is deleterious to the neurons and induces further activation of microglia, resulting in a vicious cycle [5, 6]. Thus, the inhibition of microglial activation and the subsequent inflammatory process may help identify novel therapeutic strategies to eliminate these harmful effects [4].

Toll-like receptor 4 (TLR4) is a pattern-recognition receptor (PRR) that recognizes distinct pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), a bacterial cell-wall component, and cytokines [7]. Upon ligand binding, TLR4 recruits signaling adaptors and initiates a series of signaling cascades that result in the activation of NF- κ B and the release of inflammatory cytokines [8]. Microglia activation can occur through a TLR4-mediated pathway. The presence of functional TLR4 has been shown to have deleterious effects in neurodegenerative and stroke models and plays an integral part in microglial signaling in some disease processes [9].

Diabetes mellitus (DM) has long been recognized as an independent risk factor for postoperative cognitive dysfunction (POCD) [10-13], but the mechanistic connection between the pathologies is not understood. It is well-known that the neuroinflammatory response induced by surgery is a key factor in the occurrence and development of POCD [14], which is partially attributed to the activation of the immune system and is commonly observed in the elderly. Microglia are the resident immune cells in the CNS and mediate the neuroinflammatory and neurodegenerative disease processes, TLR4 contributes to the activation of microglia specifically.

However, the effects of high glucose, a factor implicated in the inflammatory process in both DM and POCD, on microglia via TLR4 have not been studied. Because microglia contributes to the neuroinflammatory response, resulting in POCD, we investigated whether high glucose conditions sensitize microglia to the LPS stimulus in vitro using primary cultured microglia.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), 0.25% Trypsin-EDTA solution and fetal calf serum (FCS) were purchased from Gibco-BRL (Grand Island, NY, USA). LPS (Coli 0111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). WST-8 dye, RIPA buffer and the BCA kit were purchased from Beyotime (Shanghai, China). The specific mouse anti-rat ED8 (anti-CD11b/CD18) monoclonal antibody (a marker for complement receptor 3 of activated microglia) was purchased from AbD Serotec (Raleigh, NC, USA). Fluoroshield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) and rabbit anti-TLR4 polyclonal antibody were purchased from Abcam (Hongkong, China). Rat IL-6 Immunoassay Kit and Rat TNF- α Immunoassay Kit were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Specific rabbit monoclonal antibodies against p-JAK2, p-STAT3, JAK2, STAT3 and GAPDH, and secondary anti-rabbit and anti-mouse antibodies were all purchased from Cell Signaling (Boston, MA, USA). FITC-conjugated goat anti-mouse IgG antibody was purchased from Santa Cruz (Santa Cruz Biotechnology, CA, USA).

Primary microglia cultures and treatments

Primary microglia cells were cultured according to a previously described protocol with slight modification [15]. In brief, after decapitation, cerebral hemispheres of newborn Sprague Dawley (SD) rats were dissociated mechanically. The meninges and blood vessels

were removed aseptically. Next, the brains were dissociated mechanically and suspended in 0.25% Trypsin-EDTA solution for 10 minutes at 37°C, followed by addition of an equal volume of culture medium to stop the trypsinization. The digested cells were filtered through a 100-µm pore mesh, pelleted and resuspended in DMEM containing 10% FBS. The cells were cultured on poly-D-lysine pre-coated flasks, and medium was replenished 3-4 days after initial seeding. The microglial cells were separated by shaking at 150 rpm for 5 hours after a confluent monolayer formed (10 to 14 days), seeded into 12-well cultured plates at a density of 10^5 cells/cm². After exposure to serum-free medium for 24 h, the microglial cells were exposed to different concentrations of LPS (0, 2, 5, 10 ng/mL) and/or glucose (25, 35, 50 mmol/L) for 24 h. Cells were also incubated in equal levels of mannitol to those of glucose to provided an osmotic control. The purity of the microglia was >98% as determined by OX-42-IR.

Cell viability assay

Cell viability was measured by the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-8) dye according to the manufacturer's instructions. Briefly, cells were collected and seeded in a 96-well plate at a density of 3×10^4 cells/well. Following treatment with glucose and/or LPS, the WST-8 dye was added to each well, then the plate was incubated for 2 hours at 37°C, the absorbance of each well was determined at 450 nm using a microplate reader.

TNF-α and IL-6 assay

The concentrations of TNF-α and IL-6 in the culture supernatants were measured with a commercial ELISA kit from R&D Systems.

Immunofluorescence

To determine the levels of the microglial activation, the cells were fixed with 4% paraformaldehyde for 30 minutes; unspecific binding was blocked by incubating cells in a 5% BSA and 0.1% Triton X-100 solution at room temperature for 1 hour. The microglial cells were incubated with mouse anti-ED8 monoclonal antibody (1:300) in the blocking solution overnight at 4°C. After three washes with PBS, the microglial cells were incubated with the corresponding FITC-conjugated goat anti-mouse IgG (1:200) at room temperature for 2 hours and the nuclei were stained with DAPI. Fluorescent images were acquired using a confocal microscope.

Flow cytometry analysis

The microglial cells were pelleted by centrifugation at 1,500 rpm for 10 minutes and then fixed in 4% paraformaldehyde for 30 minutes. After 3 washed with PBS, the cells were resuspended in PBS. To determine the level of microglial activation, the cells were incubated with PE-conjugated mouse anti-rat ED8 monoclonal antibody or isotype control (1:200) at 37°C for 1 hour. Cells were finally resuspended in PBS and analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA, USA).

Western blotting

Cells were collected and homogenized in ice-cold lysis buffer. After incubation for 20 min on ice, cell lysate was centrifuged and protein concentration in the extracts was measured using a BCA kit. Proteins (50 µg) in the cell extracts were denatured with sodium dodecyl sulfate (SDS) sample buffer and separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore, Bedford, MA, USA), which was then blocked with 5% skim milk at room temperature for 1 h. This was followed by incubation with primary antibody overnight at 4°C. The following antibodies were used: rabbit monoclonal anti-TLR4, anti-p-JAK2, anti-p-STAT3, anti-JAK2, anti-STAT3, and mouse monoclonal anti-GAPDH (1:1000). After incubation with the anti-rabbit or anti-mouse secondary antibody (1:1000) for 1 hour, the protein bands on the membranes were detected with an enhanced chemiluminescence kit.

Statistical analysis

All experimental results were from at least three separate experiments. The data were represented as the mean $\pm$ s.e.m. The significance of the difference between the control and samples treated with various compounds was determined by one-way ANOVA followed by the post-hoc least significant difference test. A probability value of $P < 0.05$ was considered significant.

Results

Effects of glucose and lipopolysaccharide (LPS) on viability of microglial cells

The WST-8 assay was used to evaluate the toxicity of LPS and/or glucose levels on microglia. The microglial cells were incubated with or without LPS (10 ng/ml) at several glucose levels (25, 35 or 50 mmol/L) for 24 hours, and then cell viability was detected by WST-8 assay. Our results indicate that none of the tested conditions exert obvious toxic effects on microglia and use of mannitol as an osmotic control showed no effect on cell viability (Fig. 1).

Effects of lipopolysaccharide (LPS) and glucose on cytokines secretion in microglia

The incubation of microglia with high glucose (35 mmol/L) for 24 h did not induce changes in levels of TNF- α or IL-6 release. However, the exposure of microglia to 50 mmol/L of glucose significantly increased the concentration of TNF- α and IL-6 in the culture supernatants (Fig. 2A-B). Mannitol as an osmotic control showed no effect on TNF- α and IL-6 levels.

Under normal glucose condition (25 mmol/L), high concentration of LPS (10 ng/ml) also increased the concentrations of TNF- α and IL-6 (Fig. 2C-D). Low concentration of LPS (≤ 5 ng/L) alone showed no obvious effect on TNF- α and IL-6 release, but it could stimulate the inflammatory response under high glucose conditions (35 mmol/L), suggesting that microglia may be more sensitive to LPS under high glucose conditions (Fig. 2E-F). Thus, we selected 5 ng/ml of LPS and 35 mmol/L of glucose as the following treatment concentrations.

Enhancement of lipopolysaccharide (LPS)-induced microglia activation under high glucose conditions

Activated microglial cells were detected with monoclonal antibody ED8, which recognizes complement receptor 3 (CR3). The microglia cells were treated with a low concentration of LPS (5 ng/ml) and/or high concentration of glucose (35 mmol/L) for 24 hours. The normal level of glucose (25 mmol/L) plus 10 mmol/L mannitol were used as osmotic control. Flow cytometry analysis showed that high glucose (35 mmol/L) alone dose not induce microglial activation, LPS (5 ng/ml) activates microglial cells slightly, and co-incubation with high glucose and LPS increased microglial activation significantly (Fig. 3A-B). Meanwhile, high glucose remarkably increased LPS-induced CR3 expression (green Fig.

Fig. 1. The effects of glucose and lipopolysaccharide (LPS) on cell viability in microglia. The microglia cells were exposed to different concentrations of glucose (25, 35 and 50 mmol/L) with and without LPS (10 ng/ml) for 24 hours. Cell viability was tested using a colorimetric method. Each data point represents the mean $\pm$ s.e.m. of at least three separate experiments in which treatments were performed in quadruplicates.

Fig. 2. The effects of LPS and glucose levels on cytokine secretion in microglia. (A–B) Effect of different concentrations of glucose on TNF- α and IL-6 release * $P < 0.05$, ** $P < 0.001$ vs normal glucose group (25 mmol/L). (C–D) Effect of different concentrations of LPS on TNF- α and IL-6 release. \$\$ $P < 0.001$ vs control group (LPS 0 ng/ml). (E–F) Effect of LPS levels on TNF- α and IL-6 release under high glucose conditions. # $P < 0.05$, ## $P < 0.001$ vs normal glucose group (25 mmol/L) with the same LPS concentration. The data are presented as the mean $\pm$ s.e.m. of three independent experiments.

3C). These results suggest that high glucose can enhance LPS-induced microglial activation.

Effect of LPS on TLR4 expression and JAK2/STAT3 activation in microglia under high glucose conditions

Compared with normal glucose levels, high glucose (35 mmol/L) alone increased the TLR4 expression but did not activate the microglia. Treatment with LPS (5 ng/ml) alone increased the TLR4 expression and activation of microglia slightly, but did not influence cytokine levels. In contrast, co-incubation with high glucose (35 mmol/L) and LPS (5 ng/ml) induced TLR4 expression significantly along with microglial activation and cytokine secretion (Fig. 4).

Meanwhile, LPS (5 ng/ml) up-regulates phosphorylated JAK2 and phosphorylated STAT3 levels significantly under high glucose (35 mmol/L) conditions in parallel with TLR4 (Fig. 4).

Role of JAK2/STAT3 in LPS-induced microglia activation under high glucose conditions

Our data showed that high glucose (35 mmol/L) enhances the LPS-induced microglia activation and activates JAK2/STAT3 signaling. It is known that the JAK2/STAT3 pathway plays a role in regulating the expression of TLR4 in macrophages [16]. Our previous study demonstrated that TLR4 can regulate microglia activation and inflammatory response [15].

Fig. 3. Enhancement of LPS-induced microglia activation under high glucose conditions. Primary microglia cells were incubated with LPS (5 ng/ml) and/or high glucose (35 mmol/L) for 24 hours. (A-B) For flow cytometric analysis, the cells were incubated with PE-conjugated ED8 antibody at 37°C for 1 hour. **P < 0.001 vs the response to medium alone. #P < 0.05, ###P < 0.001 vs LPS group. The data are presented as the mean $\pm$ s.e.m. of three independent experiments. (C) The cells were stained with ED8 antibody and increased ED8-immunopositive expression (green) on the activated microglia was observed using confocal scanning. The blue staining represents DAPI. Scale bar 50 μ m.

Thus, we next tested the hypothesis that high glucose could accelerate LPS-induced microglia inflammation via the JAK2/STAT3 pathway.

To investigate the role of JAK2/STAT3 signaling in LPS-induced microglia activation under high glucose conditions, we used AG490 (30 μ mol/L), a JAK2-specific inhibitor, to block the JAK2/STAT3 pathway. The inhibition of JAK2 abolished the LPS-induced microglia activation under high glucose conditions (Fig. 5C). Consistently, the IL-6 and TNF- α production were markedly increased by co-induction of high glucose (35 mmol/L) and LPS (5 ng/ml), and this effect was abolished by the JAK2 inhibitor AG490 (Fig. 5D). Additionally, STAT3 phosphorylation was abolished by AG490, suggesting that the LPS-induced STAT3 activation is mediated by JAK2 signaling (Fig. 5A-B). These results suggest that the JAK2/STAT3 pathway is involved in LPS-induced microglia activation under high glucose conditions.

Discussion

The inflammatory responses of the CNS, particularly microglia activation, play a vital role in POCD. Though DM promotes the development and progression of POCD [10-13], the

Fig. 4. The effect of LPS on TLR4 expression and JAK2/STAT3 activation in microglia under high glucose conditions. The cells were incubated with LPS (5ng/ml) and/or glucose (35 mmol/L) for 24 hours. (A) Western blotting analysis of TLR4, p-JAK2 and p-STAT3 levels in LPS-stimulated primary microglia under high glucose conditions. (B) The levels of TLR4, p-JAK2 and p-STAT3 were quantified and normalized to the respective GAPDH, total JAK2, or STAT3 levels. Each value was then expressed relative to the one treated with medium, which was set as 1. *P < 0.05, **P < 0.001 versus the response to medium alone. The data are presented as the mean $\pm$ s.e.m. of three independent experiments.

Fig. 5. Role of JAK2/STAT3 in lipopolysaccharide (LPS)-induced microglia activation under high glucose conditions. In the absence or presence of AG490 preconditioning (10 μ mol/L) for 30 minutes, the cells were co-cultured with LPS (5ng/ml) and glucose (35mmol/L) for 24 hours. Western blotting analysis was performed using corresponding antibodies (A) and the TLR/GAPDH, p-JAK2/JAK2 and p-STAT3/STAT3 ratio was determined by densitometric analysis (B). These results are representative of three independent experiments. Data are mean $\pm$ s.e.m. **P < 0.001 versus the response to medium alone. (C) The cells were stained with ED8 antibody and upregulated ED8-immunopositive expression (green) on activated microglia was observed using confocal. The blue staining represents DAPI. Scale bar 50 μ m. (D-E) Quantification of IL-6 and TNF- α production in media, **P < 0.001 versus the response to medium alone. ##P < 0.001 versus LPS+Glucose groups. The data are presented as the mean $\pm$ s.e.m. of three independent experiments.

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cellular and molecular mechanisms whereby DM accelerates POCD remain to be elucidated. In the present study, we aimed to explore whether microglia showed increased sensitivity *in vitro* to LPS stimulus under high glucose relative to normal glucose conditions. We found that a low concentration of LPS had no obvious effect on microglia activation or cytokine secretion alone, but low concentration of LPS was capable of stimulating the inflammatory response under high glucose conditions, suggesting that high glucose enhances the inflammatory response in microglia.

Microglia activation is an early sign that often precedes and triggers neuronal death in chronic neurodegenerative diseases [4, 5, 17]. Therefore, the inhibition of microglia activation and subsequent neuroinflammation may offer prospective clinical therapeutic benefits for neuroinflammation-related neurodegenerative disorders. However, the LPS-induced microglia activation under high glucose conditions *in vitro* has remained undefined. The results presented in our study revealed that neither low concentrations of LPS (5 ng/L) nor high glucose (35 mmol/L) stimulate activation of primary microglia, but co-incubation with low LPS and high glucose not only induces microglia activation, but also increases IL-6 and TNF- α production, suggesting that high glucose enhances the LPS-induced inflammatory response in microglia.

TLR4, a receptor of LPS, serves as the primary mediator of innate immune responses to pathogens by activating a cascade of pro-inflammatory events [18]. The activation of TLR4 by LPS triggers signaling via downstream signaling factors such as adaptor myeloid differentiation protein 88 (MyD88), leading to the activation of NF- κ B and ultimately inducing the expression of inflammation-related genes [19]. Meantime, TLR4 can also transduce signals via MyD88-independent pathways through its association with other adaptors such as TIR domain-containing adaptor protein and TIR domain-containing adaptor inducing IFN- β that lead to the induction of IFN-inducible gene products [20, 21]. Thus, the inhibition of TLR4 signaling can reduce pro-inflammatory downstream signaling pathways by suppressing target gene expression and cellular responses. It has been shown that inhibiting TLR4 expression can suppress LPS-stimulated production of IL- β and TNF- α in the BV-2 microglia cell line [22]. Our previous study showed that pre-treatment with lithium inhibits LPS-induced TLR4 expression and microglial activation through the PI3K/Akt/FoxO1 signaling pathway, which is in accordance with the results in this study [15]. We found that co-incubation with glucose and LPS increased the expression of TLR4 (Fig. 4A-B), inducing microglia activation and cytokine production, suggesting that TLR4 was involved in the enhancement effect of high glucose on microglia activation. In addition, we found that LPS (5 ng/mL) up-regulate phosphorylated JAK2 and phosphorylated STAT3 levels under high glucose conditions (Fig. 4A-B).

JAK-STAT signaling pathways have been reported to be involved not only in the immune response of numerous cytokines but also in the activity of growth factors and hormones. The JAK-STAT cascade is an essential inflammatory signaling pathway that mediates immune responses. The binding of ligand to its receptor induces assembly of an active receptor complex and consequent phosphorylation of the receptor-associated JAK proteins (JAK1, JAK2, JAK3 and TYK2). Phosphorylation of JAKs provides the docking sites for STAT, which in turn are phosphorylated on tyrosine and serine residues; the phosphorylation of both amino acids is required for full STAT activity. Phosphorylated STAT proteins are released from the receptor complex and form dimers. These dimers are translocated to the nucleus where they directly bind to the promoter region and regulate transcription of specific target genes, many of which are involved in immune responses [23, 24]. A previous study has indicated that JAK2, as the downstream signal of TLR4, plays an essential role in phagocytosis by macrophages [16]. The JAK2-STAT3 pathway is known to cause a pro-inflammatory response in activation of microglial cells [25, 26]. Our results showed that co-cultured with LPS (5 ng/ml) and high glucose (35 mmol/L) markedly augmented the activation of primary microglia and the cytokine production, and this effect can be suppressed by pre-treatment with AG490, a specific inhibitor of JAK2 (Fig. 5C-E). Thus, we hypothesize that the JAK2-STAT3 pathway is also involved in the enhancement of LPS-induced microglia inflammation under

high glucose conditions. Furthermore, AG490 inhibited the expression of JAK2 specifically and ameliorated the following inflammatory response, but had no obvious effect on TLR4 expression, suggesting that JAK2-STAT3 is downstream of TLR4 and that high glucose enhances LPS-induced microglial activation via the TLR4/JAK2/STAT3 pathway.

Many studies have shown that the proinflammatory phenotype in diabetes is characterized by elevated plasma levels of C-reactive protein (CRP), cytokines, chemokines, adhesion molecules, and monocytic activity [27, 28]. Hyperglycemia contributes to complications from diabetes. High glucose has been shown to induce inflammatory cytokines, chemokines, p38 mitogen-activated protein kinase, reactive oxygen species (ROS), protein kinase C (PKC), and NF- κ B activity in both clinical and experimental systems [29-32]. It is known that high glucose can enhance the intracellular Ca^{2+} response triggered by purinergic stimulation in neurons and microglia [33] and can induce TLR2 and TLR4 expression via PKC- α and PKC- γ , respectively in human monocytes [34]. Our study provides new information on the combined impact of LPS and hyperglycemia in microglia. The originality of this manuscript consists in showing *in vitro* data on the potential role of high glucose in increasing LPS-induced TLR4 and inflammatory cytokines in primary microglia. It should be noted that the concentrations of glucose *in vivo* do not need to reach the levels required *in vitro* to observe the pro-inflammatory effects. This is a limitation of all *in vitro* work, which must be carried out at supra-pharmacological concentrations.

Conclusion

In conclusion, the present study indicates that high glucose and LPS act in concert to augment pro-inflammatory cytokine expression in primary microglia, which has important implications for the development and progression of POCD in DM.

Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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