

## Original Article

# High glucose stimulates TNF $\alpha$ and MCP-1 expression in rat microglia via ROS and NF- $\kappa$ B pathways

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**Aim:** To investigate whether high glucose stimulates the expression of inflammatory cytokines and the possible mechanisms involved.

**Methods:** ELISA and real-time PCR were used to determine the expression of the inflammatory factors, and a chemiluminescence assay was used to measure the production of reactive oxygen species (ROS).

**Results:** Compared to low glucose (10 mmol/L), treatment with high glucose (35 mmol/L) increased the secretion of tumor necrosis factor (TNF) $\alpha$  and monocyte chemoattractant protein-1 (MCP-1), but not interleukin (IL)-1 $\beta$  and IL-6, in a time-dependent manner in primary cultured rat microglia. The mRNA expression of TNF $\alpha$  and MCP-1 also increased in response to high glucose. This upregulation was specific to high glucose because it was not observed in the osmotic control. High-glucose treatment stimulated the formation of ROS. Furthermore, treatment with the ROS scavenger NAC significantly reduced the high glucose-induced TNF $\alpha$  and MCP-1 secretion. In addition, the nuclear factor kappa B (NF- $\kappa$ B) inhibitors MG132 and PDTC completely blocked the high glucose-induced TNF $\alpha$  and MCP-1 secretion.

**Conclusion:** We found that high glucose induces TNF $\alpha$  and MCP-1 secretion as well as mRNA expression in rat microglia *in vitro*, and this effect is mediated by the ROS and NF- $\kappa$ B pathways.

**Keywords:** high glucose; microglia; tumor necrosis factor  $\alpha$ ; monocyte chemoattractant protein-1; reactive oxygen species; NF- $\kappa$ B

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## Introduction

Inflammation plays a key role in central nervous system diseases. As the immune cells in the brain, microglia play an important role in the pathogenesis of central nervous system. In 2000, using nonradioactive *in situ* hybridization, Gregersen reported that microglia were one of the major sources of TNF after the induction of stroke in mice<sup>[1]</sup>. Glutamate was released by the oxygen and glucose deprivation-stressed neuron-astrocyte cultures, and then activated microglia by mGluRIIs to produce and release tumor necrosis factor (TNF)  $\alpha$ , which induced neurotoxicity in this stroke model<sup>[2]</sup>. These results show that cytokines, such as TNF $\alpha$ , released by microglia contribute to the development of neuronal damage during a stroke. Other studies have suggested that microglia might be involved in the pathogenesis of Parkinson's disease. In the mesencephalic neuron-glia cultures, extracellular aggregated human  $\alpha$ -synuclein activated microglia, and the activation of

microglia enhanced dopaminergic neurodegeneration<sup>[3]</sup>. A loss of  $\alpha$ -synuclein expression altered the morphology and inflammatory response of microglia and impaired microglial phagocytic ability<sup>[4]</sup>. Increased TNF $\alpha$  production from amyloid  $\beta$ -stimulated microglia led to elevated iNOS in astrocytes and neuronal apoptosis, illustrating that an increase in microglia-derived TNF $\alpha$  in response to amyloid  $\beta$  may be a contributor to neuron loss in Alzheimer's disease<sup>[5]</sup>. Moreover, the suppression of microglial derived TNF $\alpha$  and interleukin (IL)-1 $\beta$  by copolymer-1 diminished neurodegeneration in HIV-1 encephalitis<sup>[6]</sup>. Thus, inflammation induced by activated microglia is one of the major contributors to the pathogenesis of brain related diseases.

Diabetes causes many complications, including retinopathy, nephropathy, and peripheral neuropathy. In addition, diabetes has a central nervous system complication, diabetic encephalopathy, which leads to direct neuronal damage<sup>[7, 8]</sup>. Apoptosis-induced neuronal loss and damage in type 1 diabetes is associated with cognitive impairment<sup>[9]</sup>. Although the mechanisms of neuronal apoptosis in diabetic encephalopathy have not been clearly studied, hyperglycemia is thought to be

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one of the most important factors<sup>[10-12]</sup>. An increased level of glucose in the brain induced by hyperglycemia leads to neuronal apoptosis and impaired cognition<sup>[9, 12-15]</sup>. However, the effect of hyperglycemia on the activation of microglia and the role of inflammation in the pathogenesis of diabetic encephalopathy is still unclear. We previously reported that high glucose can activate microglia and significantly increase the secretion and mRNA expression of growth-regulated oncogene (GRO), a member of the IL-8 family, in rat microglia *in vitro*<sup>[16]</sup>. This finding suggests that in the central nervous system of patients with diabetes mellitus, high concentrations of glucose may induce microglial activation and the secretion of IL-8, thus contributing to the development of diabetic encephalopathy. In this study, we investigated the effect of high glucose on the inflammatory function of microglia. We found that compared with low glucose (10 mmol/L), high glucose (35 mmol/L) increased the secretion and mRNA expression of TNF $\alpha$  and monocyte chemoattract protein-1 (MCP-1) in rat microglia *in vitro*. Reactive oxygen species (ROS) and nuclear factor kappa B (NF- $\kappa$ B) pathways were involved in this process. However, there were no effects of high glucose on the secretion of IL-1 $\beta$  and IL-6.

## Materials and methods

### Reagents

The OX-42 antibody was purchased from Serotec (Oxford, UK). The MCP-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The glial fibrillary acidic protein (GFAP) antibody was purchased from Zhongshan Goldenbridge Biotechnology (Beijing, China). Poly-L-lysine, *N*-acetyl-L-cysteine (NAC), MG132 and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma Chemical Co (St Louis, MO, USA). Culture medium RPMI-1640 was obtained from Hyclone (Logan, UT, USA). *D*-glucose was obtained from Beihua Fine Chemicals (Beijing, China). Mannitol was obtained from DCPC (Beijing, China). A TNF $\alpha$  ELISA kit was obtained from Jingmei Biotech Co (Beijing, China).

### Rat microglia primary culture

The treatment of laboratory animals and experimental protocols adhered to the guidelines of the Health Science Center of Peking University and were approved by the Institutional Authority for Laboratory Animal Care. Rat microglial cells were isolated as previously described<sup>[16]</sup>. In brief, cortical tissue was carefully freed from the blood vessels and meninges, minced, mechanically agitated, and washed. Single cortical cells were cultured in RPMI-1640 containing 10% fetal bovine serum (Invitrogen, Karlsruhe, Germany), 2 mmol/L glutamine, 100 U/mL penicillin G sodium and 100  $\mu$ g/mL streptomycin sulfate (medium was changed every 4 d). After 14 d, the microglia were separated from the underlying astrocytic monolayer by gentle agitation and spun down. The cell pellet was resuspended and plated on uncoated Costar culture dishes. Non-adherent cells were removed after 30-60 min by changing the medium, and the adherent microglia were incubated for 24 h in culture medium before use. The purity of the

microglial cultures was assessed by fluorescence immunocytochemistry with antibodies against OX-42 (microglial marker) and GFAP (astrocyte marker). This procedure produced pure primary microglial cultures that were more than 95% microglia (data not shown).

### Cytokine and chemokine secretion analysis

After being seeded for 24 h, the cells were stimulated with low glucose (LG; 10 mmol/L) or high glucose (HG; 35 mmol/L). The treatment was stopped at different times, and samples of the supernatants were collected and frozen at -40 °C. MCP-1 secreted into the medium was detected using ELISA, as previously described<sup>[16]</sup>. TNF $\alpha$  and IL-6 were measured with an enzyme-linked immunosorbent assay (ELISA) kit (Jingmei Biotech Co, Beijing, China). IL-1 $\beta$  was quantified with a commercial kit (R&D Systems, Minneapolis, MN, USA).

### Real-time quantitative RT-PCR

Total RNA was isolated using RNAtip from Applygene (Beijing, China) and converted into first-strand cDNA with AMV reverse transcriptase, 40 U/ $\mu$ L of RNase inhibitor and 500 ng/ $\mu$ L Oligo (dT)<sub>15</sub> primer (Promega, Madison, WI, USA). Real-time PCR was performed using 1-3  $\mu$ L of the reverse transcription product, 0.5  $\mu$ L HotStart Taq DNA Polymerase (TIANGEN, China), SYBR Green I (Molecular Probes, Eugene, OR, USA), 1 mmol/L dNTPs, and 0.2  $\mu$ mol/L of each primer. Cycling conditions were 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, and a final incubation for 5 min at 72 °C. The primers for TNF $\alpha$  were 5'-TCC CAA CAA GGA GGA GAA GT-3' and 5'-TGG TAT GAA GTG GCA AAT CG-3'. The primers for MCP-1 were 5'-GGC CTG TTG TTC ACA GTT GCT-3' and 5'-TCT CAC TTG GTT CTG GTC CAG T-3'. The  $\beta$ -actin primers were 5'-GAG ACC TTC AAC ACC CCA GCC-3' and 5'-TCG GGG CAT CGG AAC CGC TCA-3'. To assure the specificity of the primers, the amplicons underwent melting-curve analysis. Quantification of the PCR product was carried out using the relative quantification method. Results are expressed as arbitrary units and normalized against  $\beta$ -actin mRNA expression, as previously described<sup>[16]</sup>.

### Detection of reactive oxygen species

The production of ROS, especially H<sub>2</sub>O<sub>2</sub>, in rat microglia was detected with luminol plus horseradish peroxidase-derived chemiluminescence in a light tight box using a luminescence analyzer (BPCL Ultra-weak, Beijing, China) at 37 °C<sup>[17, 18]</sup>. Photon counts were integrated over 1 s and digitized onto a computer monitor. Preparation of the reaction buffer was described previously<sup>[18]</sup>. Briefly, 10 mg/mL horseradish peroxidase, 0.5 mmol/L luminol and HG or NG were dissolved in a total volume of 2 mL.

### Statistical analyses

The results are expressed as means $\pm$ SD. The differences between two groups were analyzed by Student's *t*-test. Multiple comparisons were made using a one-way ANOVA as

appropriate with Bonferroni *post-hoc* test. Statistical analysis was carried out using the Prism program from GraphPad Software.

## Results

### High glucose induces the inflammatory reaction of rat microglia

We used ELISA to examine the level of TNF $\alpha$  in the culture medium. Treatment with high glucose increased TNF $\alpha$  secretion from rat microglia in a time-dependent manner beginning at 6 h and continuing to 24 h. The final concentration was 2-fold higher than that observed in cells treated with low glucose (Figure 1A). Incubation with mannitol for 24 h had no effect on TNF $\alpha$  secretion (Figure 1B). These results demonstrate that the high glucose-induced TNF $\alpha$  secretion was not due to osmotic pressure.

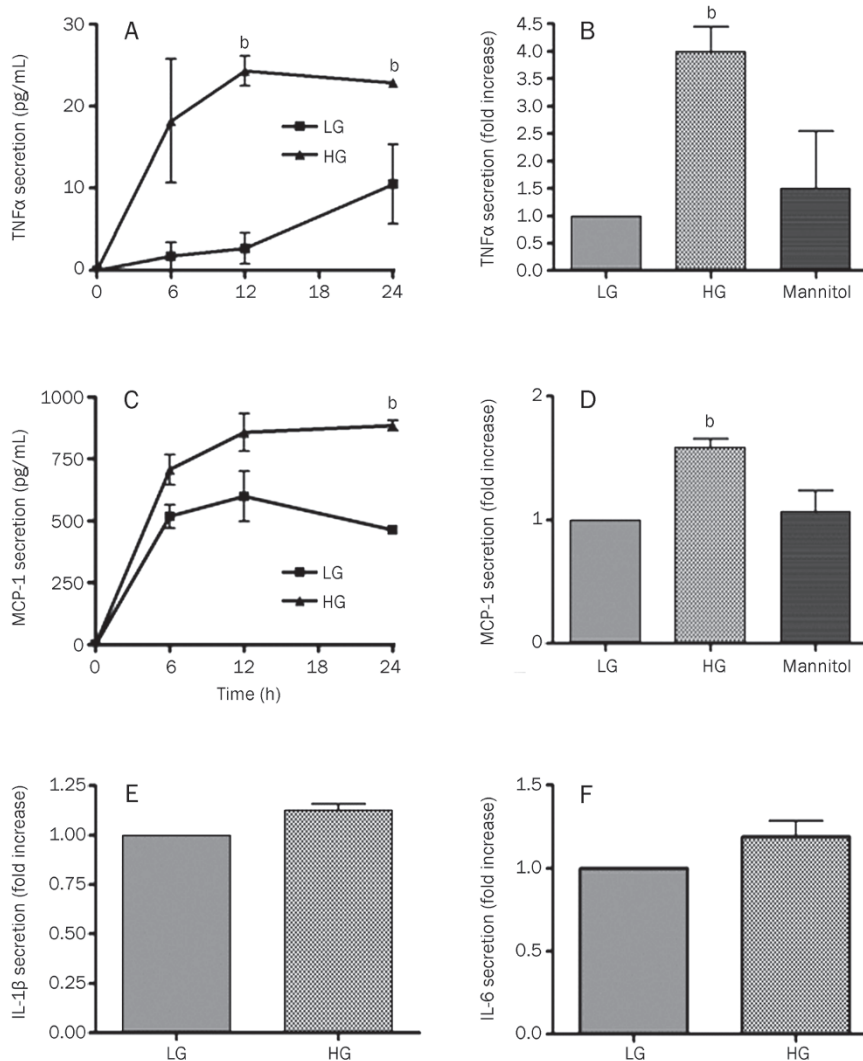
The level of MCP-1 in the culture medium was also determined by ELISA. MCP-1 secretion from high glucose-treated rat microglia increased in a time-dependent manner, with a final 1.5-fold increase compared to treatment with low glucose (Figure 1C). Incubation with mannitol for 24 h had no effect on MCP-1 secretion (Figure 1D). However, treatment

with high glucose for 24 h had no effect on either IL-1 $\beta$  or IL-6 (Figure 1E, 1F) secretion compared with low glucose.

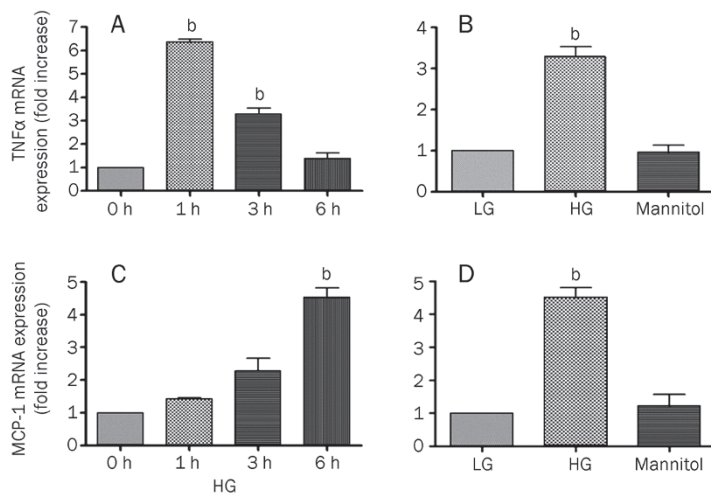
### High glucose elevates TNF $\alpha$ and MCP-1 mRNA expression

We also investigated whether high-glucose treatment could upregulate the mRNA expression of TNF $\alpha$ . Using real-time PCR, we found that high glucose treatment induced a 6-fold increase in TNF $\alpha$  mRNA expression after 1 h and a 3-fold increase after 3 h compared with low glucose (Figure 2A). However, there was no difference in TNF $\alpha$  mRNA expression between low glucose and high glucose treatment after 6 h. Similarly, the induction of TNF $\alpha$  mRNA by high glucose incubation was independent of hyperosmolality, since equimolar amounts of mannitol had no effect (Figure 2B).

Treatment with high glucose also induced MCP-1 mRNA expression in a time-dependent manner (Figure 2C). Rat microglia treated with high glucose for 3 h showed a 2-fold increase in MCP-1 mRNA expression compared to low glucose and an even higher increase after 6 h. Equimolar amounts of mannitol did not increase MCP-1 mRNA expression; thus, the high glucose-induced MCP-1 mRNA expression was also



**Figure 1.** High glucose induces an inflammatory reaction in rat microglia. (A) Time course of TNF $\alpha$  secretion stimulated by high-glucose treatment. Rat microglia were cultured under low glucose (LG; 10 mmol/L) or high glucose (HG; 35 mmol/L), and the secretion of TNF $\alpha$  in the supernatant was measured with ELISA at the indicated time points. (B) The high-glucose treatment increased TNF $\alpha$  secretion independent of osmotic pressure. Rat microglia were cultured for 24 h in LG, HG, or mannitol (25 mmol/L+10 mmol/L glucose). (C) Time course of MCP-1 secretion stimulated by the high-glucose treatment. (D) The high-glucose treatment increased MCP-1 secretion independent of osmotic pressure. (E, F) High glucose had no effect on IL-1 $\beta$  and IL-6 secretion. The data represent the mean $\pm$ SD of three independent experiments. <sup>b</sup>*P*<0.05 vs LG.



**Figure 2.** High-glucose treatment promotes the expression of TNF $\alpha$  and MCP-1 mRNA in rat microglia. (A) Real-time PCR analysis of the upregulation of TNF $\alpha$  mRNA by HG at different time points. <sup>b</sup> $P$ <0.05 vs 0 h. (B) Treatment with high glucose elevated TNF $\alpha$  mRNA independent of osmotic pressure. Rat microglia was treated with LG, HG or mannitol for 3 h. <sup>b</sup> $P$ <0.05 vs LG. (C) Real-time PCR analysis of the upregulation of MCP-1 mRNA by HG at different time points. <sup>b</sup> $P$ <0.05 vs 0 h. (D) Treatment with high glucose increased MCP-1 mRNA independent of osmotic pressure. Rat microglia was treated with LG, HG or mannitol for 6 h. The data represent the mean $\pm$ SD of three individual experiments. <sup>b</sup> $P$ <0.05 vs LG.

independent of osmotic pressure (Figure 2D).

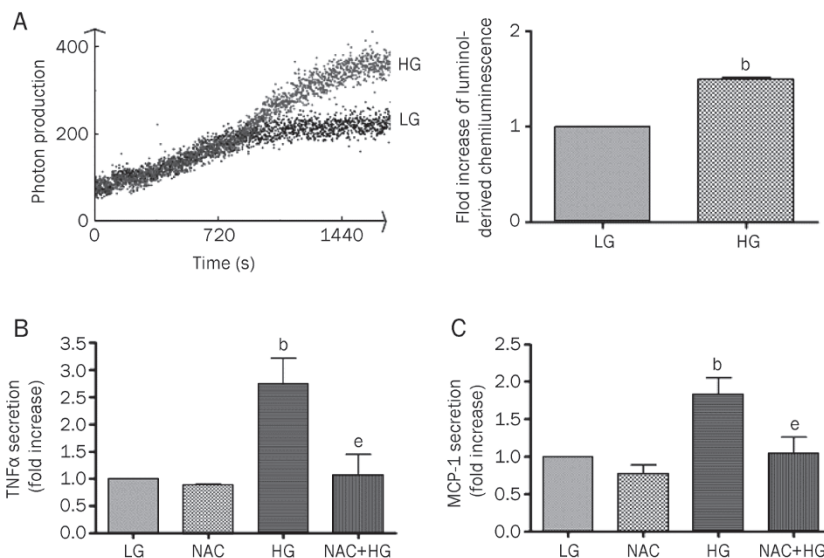
**Reactive oxygen species are involved in high glucose-induced secretion of TNF $\alpha$  and MCP-1**

The ROS pathway is one of the most important contributors to hyperglycemia-induced cell damage<sup>[19]</sup>. To examine whether high-glucose treatment induces ROS production in cultured rat microglia, we measured ROS levels using chemiluminescence. After incubation with low glucose and high glucose for 20 min, we observed ROS production. Compared with low glucose, high glucose stimulated the chemiluminescence photon production and the photon-signal peak for high glucose-treated cells occurred at 1500 s (Figure 3A). To detect the involvement of ROS in the TNF $\alpha$  and MCP-1 secretion induced by high glucose, we tested the effect of a non-specific ROS scavenger, NAC. Microglia were pretreated with 2 mmol/L NAC for 60 min and then exposed to high glucose treatment for 24 h. NAC significantly prevented the high glu-

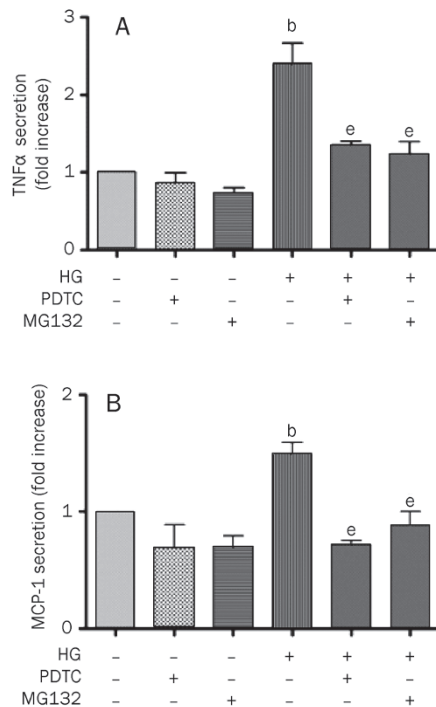
cose-induced secretion of TNF $\alpha$  and MCP-1 (Figure 3B, 3C), but NAC alone had no effect on TNF $\alpha$  and MCP-1 secretion.

**NF- $\kappa$ B plays a key role in high glucose-induced TNF $\alpha$  and MCP-1 secretion from rat microglia**

NF- $\kappa$ B is one of the most important transcription factors in the expression of inflammatory genes<sup>[20]</sup> and is associated with the gene expression of TNF $\alpha$  and MCP-1<sup>[20, 21]</sup>. High glucose incubation has been shown to significantly enhance NF- $\kappa$ B activation by increasing its DNA binding activity<sup>[16]</sup>. We investigated the role of NF- $\kappa$ B in high glucose-induced TNF $\alpha$  and IL-6 secretion at the transcriptional level. We pretreated rat microglia with the NF- $\kappa$ B inhibitors MG132 (10  $\mu$ mol/L) or PDTC (5  $\mu$ mol/L) for 60 min and then exposed them to low or high glucose for 24 h. Both MG132 and PDTC completely blocked the high glucose-induced TNF $\alpha$  and IL-6 secretion (Figure 4A and 4B), but neither had an effect on TNF $\alpha$  and IL-6 secretion alone.



**Figure 3.** ROS play a key role in the high glucose-induced secretion of TNF $\alpha$  and MCP-1 in rat microglia. (A) Effect of LG and HG on cellular ROS level. Microglia was stimulated with LG and HG for 20 min followed by a luminol-derived chemiluminescent assay. Cellular ROS levels were quantified. (B) The role of ROS in high glucose-induced TNF $\alpha$  secretion. (C) The role of ROS in high glucose-induced MCP-1 secretion. After treatment with 2 mmol/L of the ROS scavenger NAC for 1 h, microglia was cultured in LG or HG for 24 h. The medium was collected and the levels of TNF $\alpha$  and MCP-1 were analyzed by ELISA. Data represent the mean $\pm$ SD of three experiments. <sup>b</sup> $P$ <0.05 vs LG. <sup>e</sup> $P$ <0.05 vs HG.



**Figure 4.** NF- $\kappa$ B is essential for high glucose-induced TNF $\alpha$  and MCP-1 secretion in rat microglia. After treatment with 10  $\mu$ mol/L MG132 or 5  $\mu$ mol/L PDTC for 1 h, microglia was cultured in LG or HG for 24 h. The medium was collected and the secretion of TNF $\alpha$  and MCP-1 was measured by ELISA. (A) Inhibition of the NF- $\kappa$ B pathway blocked high glucose-mediated TNF $\alpha$  secretion. (B) Inhibition of the NF- $\kappa$ B pathway blocked high glucose-mediated MCP-1 secretion. Data represent the mean $\pm$ SD of three experiments. <sup>b</sup> $P$ <0.05 vs control. <sup>e</sup> $P$ <0.05 vs HG.

## Discussion

We have shown that high glucose increases the secretion and mRNA expression of TNF $\alpha$  and MCP-1 in rat microglia. These effects are specific to high glucose rather than hyperosmolality. ROS and NF- $\kappa$ B pathways mediate the high glucose-induced secretion of TNF $\alpha$  and MCP-1. However, high glucose has no effect on the secretion of IL-1 $\beta$  and IL-6 compared to low glucose.

TNF $\alpha$ , a proinflammatory cytokine, is synthesized and released by microglia and can activate the caspase pathway, leading to neural apoptosis through the TNF receptor<sup>[22, 23]</sup>. It has been reported that fewer sympathetic and sensory neurons died during the phase of naturally occurring neuronal death in TNF-deficient embryos, and neurons from these embryos also survived in the culture better than wild-type neurons<sup>[24]</sup>. In addition to its role in apoptotic events, TNF $\alpha$  acts as a regulator of synaptic plasticity. Using TNF $\alpha$  knockout mice, Golan *et al* showed that TNF $\alpha$  may specifically suppress hippocampal function in adult mice through the suppression of NGF expression and dentate gyrus development<sup>[25]</sup>. MCP-1, one of the most important chemokines, plays a critical role in the migration of bone marrow derived and resident cells to sites of inflammation<sup>[26]</sup>. Huang *et al* found that MCP-1 null mice

exhibited markedly reduced clinical and histological autoimmune encephalomyelitis (EAE) compared with wild-type animals. These results suggest that MCP-1 plays a crucial role in Th1 immune responses during EAE induction<sup>[27]</sup>. MCP-1 has been shown to inhibit HIV-tat and NMDA-induced apoptosis in mixed cultures of human neurons and astrocytes by reducing the extracellular levels of glutamate and regulating the intracellular trafficking of tat and NMDA receptor 1 expression in neurons. This finding suggests that MCP-1 may play a novel role as a protective agent against the toxic effects of glutamate and HIV-tat<sup>[28]</sup>. In the CNS of diabetic patients, high concentrations of glucose may induce microglial activation and the secretion of TNF $\alpha$  and MCP-1, thus contributing to the development of diabetic encephalopathy.

Oxidative injury induced by high glucose concentrations plays a central role in the pathogenesis of diabetic complications<sup>[29]</sup>. Recent observations suggest that hyperglycemia-triggered ROS contribute to the development of diabetic nephropathy<sup>[30, 31]</sup>. High glucose-induced ROS activated NF- $\kappa$ B and increased MCP-1 production, which may cause macrophage recruitment and activation, resulting in diabetic renal injury<sup>[32]</sup>. In addition, the formation of oxygen-derived radicals led to the activation of NF- $\kappa$ B, which increased the expression of vascular cell adhesion molecule 1 and MCP-1 in human aortic endothelial cells. Thus, ROS-mediated NF- $\kappa$ B activation may play a role in the onset of atherosclerosis in diabetic patients<sup>[33]</sup>. The involvement of ROS and NF- $\kappa$ B in high glucose-induced apoptosis has also been demonstrated in cultured human endothelial cells<sup>[34]</sup>. We found that treatment with a high concentration of glucose induced ROS formation and that the ROS scavenger NAC significantly reduced the high glucose-induced secretion of TNF $\alpha$  and MCP-1. Furthermore, the NF- $\kappa$ B inhibitors MG132 and PDTC completely prevented the high glucose-induced TNF $\alpha$  and MCP-1 secretion. Thus, ROS and NF- $\kappa$ B pathways mediate high glucose-induced secretion of TNF $\alpha$  and MCP-1 from rat microglia.

In conclusion, in our diabetic model, microglia are activated by high glucose treatment to produce ROS, which, in turn, activate the transcription factor NF- $\kappa$ B. Activation of NF- $\kappa$ B leads to the transcription of TNF $\alpha$  and MCP-1, resulting in an increased expression of TNF $\alpha$  and MCP-1. The release of inflammatory TNF $\alpha$  and MCP-1 by microglia may be involved in the pathogenesis of neuronal injury in diabetic encephalopathy. Overall, our study demonstrates that microglia might be a new target for the treatment of diabetic encephalopathy.

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## Author contribution

Most experiments were done by Yi QUAN and she also wrote the paper. Chang-tao JIANG, Bing XUE, and Shi-gong ZHU

did some of the experiments. Xian WANG and Yi QUAN designed the experiments together.

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