HIGH GLUCOSE-INDUCED EXPRESSION OF INFLAMMATORY CYTOKINES AND REACTIVE OXYGEN SPECIES IN CULTURED ASTROCYTES

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Abstract—Astrocyte activation plays important roles both in physiological and pathological process in the CNS. In the latter, the process is further aggravated by hyperglycemia, leading to diabetes complications of CNS. We report here that high glucose (HG) treatment stimulated astrocytic morphological alteration coupled with changes in glial fibrillary acidic protein (GFAP) and vimentin expression. Additionally, HG upregulated the expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-4 (IL-4), and vascular endothelial growth factor (VEGF) expression; however, its effects on transforming growth factor-β (TGF-β) expression were not evident. HG treatment induced increased production of reactive oxygen species (ROS) as well as activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator transcription 3 (STAT 3). HG-induced expression of TNF-α, IL-6, IL-1β, IL-4, and VEGF was blocked by ROS scavenger and inhibitors specific for NF-κB and STAT 3, respectively. The results suggest that the aforementioned multiple inflammatory cytokines and mediators that may be linked to the pathogenesis of the diabetes complications of CNS are induced by HG via the key signaling pathways. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: high glucose, astrocytes, cytokines, ROS, NF-κB, STAT 3.

Diabetes mellitus, both insulin deficiency (type 1 diabetes) and insulin resistance (type 2 diabetes), causes progressive severe complications affecting the retina (Qiu et al., 2008), kidney (Drummond et al., 2002), muscle (Sreekumar et al., 2002), and blood vessels (Duh and Aiello, 1999), as well as the nervous system (Di Mario et al., 1995; Little et al., 2007; DCCT/EDIC et al. 2007). An elevated blood glucose level typically ranging from 7.0 mM to 50 mM is believed to be responsible for most diabetes complications (Burge et al., 2001; Song et al., 2007), which are alleviated when the blood glucose is properly controlled. In diabetes brain, hyperglycemia increases neuronal death and activates astrocytes to cause gliosis (Sarac et al., 2005; Hoffman et al., 2009, 2010; Northam et al., 2009). Neuronal death has been demonstrated to contribute to the neuropathy associated with diabetes (Greene et al., 1999). The role of activated astrocytes in diabetes-induced injury in the CNS, however, has remained uncertain.

Astrocytes play a crucial role both in physiological and pathological process in the CNS (Dong et al., 2001; Biesels et al., 1999). They respond swiftly to subtle changes in the microenvironment, including that from the blood supply because their end feet are tightly attached to the blood vessels, which contribute and maintain the functional integrity of the blood–brain barrier (BBB). Furthermore, they secrete an array of pro-inflammatory and anti-inflammatory cytokines, chemokines, and trophic factors to modify the ambient microenvironment (Ridet et al., 1997; Lau and Yu, 2001). Astrocyte-derived factors are important in neuronal survival, maturation, and neurogenesis (Song et al., 2002; Emsley et al., 2004). Under diabetes condition, hyperglycemia causes inflammatory reaction in other organs and tissues in vivo (Wei et al., 2010; Bellenger et al., 2011). It has been reported that high glucose (HG) in vitro can cause reactive oxygen species (ROS) production and expression of pro-inflammatory cytokines and chemokines in a variety of cells (Shanmugam et al., 2003; Zong et al., 2010; Quan et al., 2011). In the light of the aforementioned, it was surmised that astrocytes might also take part in the pathological process of diabetes in the CNS through expression and secretion of various inflammatory mediators.

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The present study was designed to examine whether HG would alter expression of inflammatory cytokines as well as ROS production in astrocytes. We report here that HG treatment significantly increased the expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1 β (IL-1β), interleukin-4 (IL-4), and vascular endothelial growth factor (VEGF); in contrast, transforming growth factor-β (TGF-β) expression remained relatively unaffected. Additionally, HG treatment induced ROS production, as well as activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT 3). Furthermore, the involvement of ROS, NF-κB, and STAT 3 in HG-induced cytokine expression was confirmed by using an ROS scavenger, namely, N-acetyl-5-methoxytryptamine (melatonin) and specific inhibitors pyrrolidine dithiocarbamate (PDTC) and cucurbicatin-1 (JST-124) targeting NF-κB and STAT 3, respectively. Our data strongly suggest that HG can induce the expression of multiple inflammatory cytokine-related genes via key signaling pathways in astrocyte activation, which may be the underlying cause of diabetes complications in the CNS.

EXPERIMENTAL PROCEDURES

Animals

BALB/C mice were obtained from the Laboratory Animal Center, Shandong University. All animals were kept under controlled light/dark conditions (12/12 h), temperature (23 °C), and humidity (60%). In the handling and care of all animals, the International Guiding Principles for Animal Research, as stipulated by the World Health Organization (1985) and as adopted by the Laboratory Animal Center, Shandong University were followed. All efforts were made to minimize pain and the number of animals used.

Isolation of astrocytes and culture

Primary astrocytes were prepared from 1- to 2-day-old BALB/C mice as described before (Wang et al., 2009) with modifications. Briefly, the cortical tissues freed of meninges and blood vessels were mechanically dissociated, and the cell suspension was seeded at density of $1 \times 10^6$ cells/ml in Dulbecco's modified Eagle (5.5 mmol/L glucose) medium (DMEM, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). When the culture was reaching confluency, the microglia and oligodendrocytes were removed by an orbital shaker; the remaining cells, a majority of them being astrocytes, were replated. The astrocytes were passaged for at least three times to further purify the cells, especially to avoid contamination from microglia and neurons. Astrocytes were left to recover for 7 days after each passage. By this method, cultures containing more than 95% astroglial cells, as determined by immunostaining for glial fibrillary acidic protein (GFAP), were obtained. All experiments were conducted using 80%–85% confluent cells. Before each experiment, the plated cells were incubated with serum-free DMEM medium for 1 h. After this, the medium was replaced with serum-free DMEM containing either HG (15 mM), very high glucose (VHG) (30 mM) (Sigma-Aldrich), lipopolysaccharide (LPS, 1 μg/ml, Sigma-Aldrich), and hydrogen peroxide (H$_2$O$_2$, 100 μM, Sigma-Aldrich) or melatonin (100 nM, Sigma-Aldrich) for indicated times.

Cell viability assay

Cell viability was determined using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were plated into 96-well culture plates at a density of $1 \times 10^4$ cells/well with 200 μl culture medium per well in triplicate. When reaching 80% confluency, the cells were incubated, respectively, in a medium containing 5.5 mM glucose, 15 mM glucose, 30 mM glucose, 5.5 mM glucose+9.5 mM mannitol, or 15 mM glucose+15 mM mannitol for 24 and 48 h. Then, 20 μl MTT solution (5 mg/ml, Sigma-Aldrich) was added to each well and incubated at 37 °C for 4 h. The culture medium was aspirated and followed by addition of 200 μl dimethyl sulfoxide (DMSO). The absorbance value was measured in a microplate reader (Bio-Rad Laboratories, Shanghai, PR China) at 490 nm. Values were expressed as a percentage relative to those obtained in controls.

TUNEL assay

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay was performed by using an in situ cell detection kit (FITC) following the manufacturer’s instructions (Chemicon, Temecula, CA, USA). In brief, cells grown on glass cover slips were fixed in 4% parafomaldehyde for 20 min at room temperature. Cover slips were then washed with phosphate-buff ered saline (PBS) and incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. Then, 50 μl of TUNEL reaction mixture was added on cover slips and incubated in a humidified chamber for 1 h at 37 °C in the dark. Finally, cells were washed and double stained with 4’, 6-diamidino-2-phenylindole (DAPI, 1 μg/ml, Sigma-Aldrich) at room temperature for 5 min, then examined by microscopy. TUNEL-positive (apoptotic) cells were stained bright green with a blue fluorescent nucleus. Images of TUNEL-positive cells were captured with a fluorescence microscope (IX71, Olympus, Tokyo, Japan), and the proportion of TUNEL-positive cells was expressed as a percentage over the total cells counted.

ROS assay

Intracellular H$_2$O$_2$ and superoxide (O$_2^-$) levels were measured by 2’, 7’-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) and dihydroethidium (DHE) assays, respectively. H$_2$DCFDA or DHE is a membrane-permeable dye that is oxidized by intracellular H$_2$O$_2$ or O$_2^-$ to the fluorescent product DCF or ethidium, respectively. The fluorescence excitation and emission wavelengths were 490/520 nm for H$_2$DCFDA and 510/595 nm for DHE. Briefly, astrocytes were treated with HG, H$_2$O$_2$, HG+melatonin, HG+PDTC, and HG+JST-124 for 24 h. For HG+melatonin, HG+PDTC, and HG+JST-124, the culture medium was aspirated and followed by addition of 200 μl dimethyl sulfoxide (DMSO). The absorbance value was measured in a microplate reader (Bio-Rad Laboratories, Shanghai, PR China) at 490 nm. Values were expressed as a percentage relative to those obtained in controls.

Table 1. Primers for real-time RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences (5'-3')</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Sense: CATGGAATCCGTGTCTTCC</td>
<td>200</td>
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<tr>
<td></td>
<td>Antisense: GAAGCTGTGCTGTCATTACG</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>Sense: GTTGGAGTTAACAGACATC</td>
<td>248</td>
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<tr>
<td></td>
<td>Antisense: ACGTACTCTGGTGGC</td>
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</tr>
<tr>
<td>IL-6</td>
<td>Sense: ATCCAGTGGCCTTCTGGAGACTGA</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>Antisense: TAAGCTCTGGTATGGTGTGG</td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>Sense: GAAGGAGAGTTGAGGTTTCTT</td>
<td>217</td>
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<tr>
<td></td>
<td>Antisense: GTTGGCATGTGTTAGTGTAACAGCAG</td>
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</tr>
<tr>
<td>TNF-α</td>
<td>Sense: CATCTTCTCAAAAATCTGGATGAAACA</td>
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<tr>
<td></td>
<td>Antisense: TGGGAGTAGACAAGCAAGTAAAACCC</td>
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<tr>
<td>VEGF</td>
<td>Sense: GTGAGCCAGGGTCAGAAAG</td>
<td>206</td>
</tr>
<tr>
<td></td>
<td>Antisense: GAATGGCGTTGCGGAGTCGT</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense: AAAGGCACACGGTAAAAGAT</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Antisense: GTGGTACGACCAGGCGCATA</td>
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</table>
HG + JSI-124, the cells were pretreated with melatonin, PDTC, or JSI-124, respectively, for 24 h, and then incubated with either 10 μM H2DCFDA or 2 μM DHE (Molecular Probes, Eugene, OR, USA) for 20 min at 37 °C. The immunofluorescence images were taken with a fluorescence microscope (IX71, Olympus) using DP controller and DP manager (Olympus). The fluorescence was measured with a fluorescence plate reader (Fluroskan Ascent II, Labsystems, Helsinki, Finland). Results in arbitrary units were expressed as a ratio to the fluorescence signal of untreated cells (control) set at 1.0.

**Immunocytochemistry**

Astrocytes were plated on poly-L-lysine (PLL)-treated cover slips. Following each treatment for 24 h, cells were fixed in 4% paraformaldehyde for 20 min and blocked with 10% goat serum in PBS. Slides were incubated overnight in a humidified chamber at 4 °C with the primary antibodies: anti-GFAP (1:500, mouse monoclonal, Millipore Corporation, Billerica, MA, USA), anti-vimentin (1:100, rabbit polyclonal, Santa Cruz Biotechnology, Inc., CA, USA), anti-NF-κB (1:100, rabbit polyclonal, Santa Cruz Biotechnology, Inc., CA, USA), and anti-signal transducer and activator transcription 3 (STAT 3, 1:100, rabbit polyclonal, Santa Cruz Biotechnology, Inc., CA, USA). After primary antibody incubation, samples were washed again and incubated in the appropriate fluorescent-conjugated secondary antibody (goat anti-mouse/rabbit IgG, 1:200, Sigma-Aldrich, MO, USA) for 1 h. The cells were counterstained by DAPI. Images were captured with a fluorescence microscope (IX71, Olympus).

**RNA extraction and real-time RT-PCR assay**

Total RNA was isolated using TRIzol reagent (Takara, Otsu, Shiga, Japan). Identical amounts of RNA (0.5 ng) were reverse transcribed into complement DNA (cDNA) by using a commercial RT-PCR kit (Fermentas, Vilnius, Lithuania) according to the manufacturer’s instructions. For real-time polymerase chain reaction (real-time PCR), the LightCycler 2.0 instrument (Roche Applied Science, Mannheim, Germany) and the LightCycler FastStart DNA Master SYBR Green I Reagent Kit (Roche Molecular Biochemicals, Mannheim, Germany) were used according to the protocol provided by the manufacturer. The primers and PCR conditions for amplification were used as described previously (Goldberg et al., 2008; Peairs et al., 2009; Lavalette et al., 2011; Kwon et al., 2011) and shown in Table 1. Melting curve analysis was used to confirm amplification specificity. The quantification data were analyzed with LightCycler analysis software version 4.0 (Roche Applied Science, Mannheim, Germany). The relative expression was normalized on the basis of β-actin. At least three independent experiments for each condition were conducted.

![Fig. 1. HG increases cell viability of astrocytes. (A) GFAP (green) was expressed by more than 95% of cultured cells; nuclei were counterstained by DAPI (blue). (B) HG (15 mM glucose) significantly increased cell viability of cultured astrocytes compared with normal glucose (NG). VHG (30 mM glucose) significantly decreased cell viability of cultured astrocytes. Osmotic changes were not responsible for the glucose effect on astrocytes, as mannitol showed no significant influence on cell viability of cultured astrocytes. (C) VHG induced apoptosis of astrocytes, while it did not induce apoptosis at NG (5.5 mM) and HG. * P<0.05, # P<0.01, compare with those incubated with NG. At least three independent experiments were conducted. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.](image)
Western blot analysis

Equal amount of total proteins was loaded onto a 4%–20% gradient polyacrylamide gel, electrophoretically transferred to polyvinylidene difluoride membrane, and probed with primary antibodies: anti-NF-κB (1:800, rabbit polyclonal, Abcam, Cambridge, MA, USA), anti-STAT 3 (1:500, mouse monoclonal Santa Cruz), and anti-phospho-STAT 3 (Tyr705) (1:1000, rabbit polyclonal, CST, Danvers, MA, USA); anti-proliferating cell nuclear antigen (PCNA) (1:800, rabbit polyclonal, Abcam) was used as an internal control. Secondary antibodies were horseradish peroxidase conjugated to goat/mouse anti-rabbit IgG (1:5000, Sigma-Aldrich). The membranes were developed using an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA).

Statistical analysis

Quantitative data were presented as the mean±SD of at least three independent experiments. Statistical analysis of data was done by Student’s t-test or by one-way ANOVA using Dunnett’s test in multiple comparisons of means. Differences were considered statistically significant if the P-value was <0.05 (+) or <0.01 (#).

RESULTS

HG increased cell viability of astrocytes

Astrocytes were isolated and cultured following protocols described previously. After three passages, astrocytes appeared flat and polygonal grown in a monolayer. More than 95% of the cells were GFAP positive, and hence, they were identified to be astrocytes (Fig. 1A). To examine the effects of different concentrations of glucose on astrocytes, MTT and TUNEL assays were performed. MTT result showed that HG (15 mM) increased (24h: 1.53±0.12; 48h: 1.26±0.06 fold over NG, both P<0.05) the cell viability of astrocytes; however, VHG (30 mM) was deleterious (24 h: 61.33±12.50%; 48 h: 18.67±4.04% of NG, 24 h P<0.05; 48 h P<0.01) to the cultured astrocytes (Fig. 1B).

Fig. 2. HG activates cultured astrocytes. (A) HG and LPS treatment induced morphological changes of cultured astrocytes, which transformed from flat, polygonal cells to small, contracted, and highly branched cells. (B, C) HG treatment induced expression of vimentin and GFAP, as demonstrated by immunocytochemistry (B) and Western blot assay (C). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
This is consistent with the TUNEL assay, which showed that HG did not induce apoptosis of astrocytes, but the incidence of astrocytes undergoing apoptosis was drastically increased with VHG (44.50% ± 5.57%, P < 0.01) (Fig. 1C). To exclude the possibility that osmotic pressure changes might affect the cultured cells, mannitol was employed as an osmotic pressure-matched control. Astrocytes were cultured in NG (5.5 mM) with added mannitol or HG with added mannitol. Despite the change in osmotic pressure, there was no significant difference in cell viability compared with those with the counterpart medium (P > 0.05) (Fig. 1B). The results indicated that glucose at different concentrations exerts differential effects on astrocytes. Because increased cell viability indicated astrocytic activation, which was observed in diabetes brain, we had used 15 mM glucose for all subsequent experimental analyses for determination of HG on astrocytes.

**HG-stimulated morphology changes and vimentin expression in astrocytes**

Within hours of stimulation with HG, most astrocytes transformed from flat, polygonal cells to small, contracted, highly branched cells (Fig. 2A); similar changes were observed in astrocytes stimulated by LPS (Fig. 2A). Intermediate filaments were immunostained in astrocytes with or without HG stimulation. By immunocytochemistry, expression of vimentin, a marker for activated astrocytes, and GFAP was noticeably augmented in astrocytes stimulated with HG (1.95 ± 0.22 fold, P < 0.01) when compared with cells treated with NG (Fig. 2B). Immunoblot results confirmed enhanced expression of vimentin and GFAP in astrocytes following stimulation with HG (1.67 ± 0.15 fold over NG, P < 0.01) (Fig. 2C). The results demonstrated that HG could activate astrocytes.

**HG-induced expression of pro-inflammatory and anti-inflammatory cytokines**

It is well established that astrocytes participate in normal and abnormal processes of the CNS via release of cytokines (Ridet et al., 1997; Lau and Yu, 2001). We, therefore, evaluated the expression of pro-inflammatory and anti-inflammatory cytokines following HG stimulation. Real-time RT-PCR results showed that HG stimulation increased the mRNA expression levels of pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β, and anti-inflammatory cytokine IL-4 (P < 0.05 or P < 0.01), but the mRNA expression level of TGF-β remained unaltered (P > 0.05) (Fig. 3). It is noteworthy that the effect of HG on cytokine expression was time dependent (Fig. 3).

**HG-induced ROS production**

It has been reported that HG-induced ROS production participates in different pathological conditions (Jia et al., 2008; Pandey et al., 2011; Quan et al., 2011). It remained uncertain whether HG would induce ROS production in astrocytes. To ascertain this, the level of ROS and produc-
tion of $\text{H}_2\text{O}_2$ and $O_2^-$ in astrocytes following HG stimulation was measured. HG induced ROS production in a time-dependent manner, peaking at 6 h posttreatment (data not shown). Melatonin, a potent ROS scavenger (Korkmaz et al., 2009), was used to block HG-induced ROS production. Pretreatment with melatonin for 24 h significantly inhibited HG-induced $\text{H}_2\text{O}_2$ and $O_2^-$ production ($\text{H}_2\text{O}_2$: $2.83 \pm 0.31$ vs $4.60 \pm 0.56$ fold over NG; $O_2^-$: $2.63 \pm 0.25$ vs $4.30 \pm 0.40$ fold over NG, both $P<0.05$) (Fig. 4A, B), suggesting that ROS was involved in HG effect on astrocytes.

HG induced NF-$\kappa$B and STAT 3 activation

NF-$\kappa$B and STAT 3 are transcripts involved in cytokine expression in many human cells, including astrocytes (Wang et al., 2002; Quan et al., 2011). Because both NF-$\kappa$B and STAT 3 function as activated form that is translocated into nucleus, we measured NF-$\kappa$B and p-STAT3 expression in astrocytes. NF-$\kappa$B was detected in the cytoplasm in NG but was detected in the nucleus of astrocytes at 24 h after HG stimulation (Fig. 5A). p-STAT 3, which was undetected in astrocytes in NG, was also detected in the nucleus of astrocytes following HG stimulation (Fig. 5B). These results suggested that both NF-$\kappa$B and STAT 3 were involved in HG effects on astrocytes.

**HG activated NF-$\kappa$B and STAT 3 through a ROS-dependent pathway**

Further experiments revealed that inhibition of ROS by melatonin reduced HG-induced NF-$\kappa$B, p-STAT 3, and STAT 3 expression in the nucleus of astrocytes (Fig. 6A, B), whereas inhibition of NF-$\kappa$B by PDTC (100 $\mu$M) or inhibition of STAT 3 by JSI-124 (100 nM) did not affect HG-induced ROS production ($P>0.05$) (Fig. 6C). These results indicated that HG activated NF-$\kappa$B and STAT 3 through ROS production.

Inhibition of ROS, NF-$\kappa$B, or STAT 3 attenuated HG-induced upregulation of cytokines

To determine if ROS production as well as NF-$\kappa$B and STAT 3 activation were involved in HG-induced upregula-
tion of cytokines in astrocytes, the cells were incubated with melatonin (100 nM), PDTC (100 μM), or JSI-124 (100 nM) for 24 h, followed by treatment with HG for 24 h. The mRNA expression levels of TNF-α, IL-6, IL-1β, TGF-1β, IL-4, and VEGF were evaluated by real-time RT-PCR. The results showed that inhibition of ROS, NF-κB, or STAT 3 suppressed the HG-induced expression of cytokines at varying degrees \((P<0.05 \text{ or } P<0.01)\) (Fig. 7). Remarkably, TGF-1β mRNA expression was unaltered in various treatments \((P>0.05)\) (Fig. 7). Nonetheless, the results suggested that ROS, NF-κB, and STAT 3 were involved in HG-induced upregulation of cytokines in astrocytes.

**DISCUSSION**

The present study investigated the effect of HG on expression of various cytokines and chemokines and ROS production in astrocytes. Along with this, we sought to clarify the underlying regulatory molecular mechanisms. We have shown that high concentration of glucose (HG) can activate astrocytes in vitro, whereas very high concentration of glucose (VHG) induces apoptosis of astrocytes. HG treatment not only can induce the morphological changes and vimentin expression in astrocytes but also can upregulate expression of pro-inflammatory and anti-inflammatory cytokine and chemokine genes. Additionally, we have shown that oxidative stress pathways, such as ROS and NF-κB, and STAT 3 pathways are involved in the process. To this end, it is relevant to note that HG induces a significant increase in ROS production concomitant to NF-κB and STAT 3 activation, but more importantly, inhibition of these pathways with their respective inhibitors suppresses the HG-induced upregulation of cytokines; hence, it is suggested that astrocyte activation participates in diabetes and hyperglycemia-induced brain injuries via pro-inflammatory and anti-inflammatory cytokines as well as ROS.

Glucose is the primary energy source in the CNS and is required in the culture of astrocytes as well as neurons. Blood glucose varies widely in diabetes patients, with plasma glucose concentrations ranging between 7.0 mM and 50 mM (Burge et al., 2001; Song et al., 2007). Growth of cultured cells in HG concentrations is a pathophysiological condition relevant to diabetes. In the present study, both short-term and long-term incubation with 15 mM glucose significantly increased the cell viability of astrocytes, suggesting glial activation when compared with cells incubated with 5.5 mM glucose (Fig. 1B and data not shown); hence, 15 mM glucose was considered to be the “high glucose” when compared with the “normal glucose” at 5.5 mM. In view of the aforementioned, 15 mM glucose was used to mimic hyperglycemia condition in vivo in all subsequent experiments in vitro.
We have shown that HG, that is, 15 mM glucose could increase cell viability and induce increased expression of inflammatory cytokines in astrocytes. In contrast, VHG, that is, 30 mM glucose exerted an adverse effect because it increased the incidence of astrocyte apoptosis. Remarkably, the same concentration of glucose did not induce similar degenerative changes in cultured neurons (data not shown here). Although it has been reported that HG induces changes in the content and distribution of some exocytotic proteins and apoptosis in cultured neurons (Santiago et al., 2007; Gaspar et al., 2010), it could induce similar degenerative changes only at a longer exposure of more than 7 days coupled with a higher concentration of glucose (30 mM–50 mM) as observed in the astrocytes. It has been reported that astrocytes can protect neurons against excitotoxicity and oxidative insults through expression and secretion of a variety of neurotrophic factors and cytokines (Mattson et al., 1995), but in some instances, the mediators may be toxic to neurons. For example, astrocyte-derived S100B increases survival and neurite extension in neurons and glial cells at physiology level; however, it is toxic to neurons at very high concentration (Donato, 2001). In uncontrolled diabetes, reactive gliosis invariably involving astrocytes exacerbates diabetes-associated neurodegenerations (Baydas et al., 2003). In the light of the aforementioned and taking into consideration of present results, it is suggested that robust astrocyte activation in the early stage in diabetes may lead to subsequent neurodegeneration in diabetes-induced complications in the CNS.

Inflammation plays a pivotal role in many CNS diseases (Chavarria and Alcocer-Varela, 2004). A hallmark of inflammation in the CNS is activation of glial cells and production of cytokines that trigger neural damage (McGeer and McGeer, 2010). In this connection, microglia is well known to be a primary inflammatory cell type in the

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**Fig. 6.** HG activates NF-κB and STAT 3 through a ROS-dependent pathway. (A, B) HG elevated nuclear NF-κB (A), p-STAT 3, and STAT 3(B) levels; melatonin inhibited HG elevation of nuclear NF-κB (A), p-STAT 3 and STAT 3 (B). PCNA was used as the internal control. (C) NF-κB inhibitor PDTC or STAT 3 inhibitor JSI-124 did not affect HG-induced ROS production. * P<0.05, # P<0.01, in comparison with those incubated with HG. At least three independent experiments were conducted.
CNS. Many studies have suggested that astrocytes may act as a regulator of microglial inflammatory responses (Aloisi et al., 1997; Vincent et al., 1997; Pyo et al., 2003; Min et al., 2006), especially in brain injury in which astrocytes may act as a controller to rapidly suppress microglial activation (Kim et al., 2010). However, the underlying mechanisms have remained to be fully elucidated. A possibility for this would be that the anti-inflammatory cytokines, such as IL-4, released by activated astrocytes as demonstrated in this study by HG stimulation, might suppress microglial activation via paracrine mode in neuroinflammation. The interplay or cross talk between the two glial cell types in diabetes condition, however, awaits further investigation.

Cytokines participate in inflammation and play different roles. Overexpression of TNF-α or IL-6 in astrocytes resulted in neurodegeneration, gliosis, and progressive neurological disease in transgenic mice (Campbell et al., 1993; Akwa et al., 1998). Overexpression of IL-1β is cytotoxic to astrocytes and will induce cellular degeneration and death (Moynah, 2005). In contrast, anti-inflammatory cytokines such as IL-4 and TGF-β can protect neurons from traumatic, ischemic, and other injuries (Mahesh et al., 2006; Clarke et al., 2008). VEGF is a potent mitogen for endothelial cells and is rapidly produced in the brain in response to both hypoxia and cytokines. There is evidence indicating that activated astrocytes are involved in VEGF-mediated angiogenesis following CNS injury. It has been shown that VEGF mRNA expression, VEGF protein, and one of the VEGF receptors, flt-1, are increased in activated astrocytes after glioma implantation, stab wounds, and neural grafting (Krum and Rosenstein, 1998). These results underscore the diverse or even opposing roles of astrocytes, which are evidently implicated in the diabetic complications of the CNS as shown in the present study.

Oxidative stress is increased in diabetes brain as markers for oxidative damages were present (Hoffman et al., 2010). ROS, whether endogenously produced or exogenously added, has been shown to activate critical signal pathways to promote cell activation or growth responses in many cell types (Dröge, 2002; Martindale and Holbrook, 2002). As shown in this study, HG treatment induces endogenous ROS generation, followed by activation of NF-κB and STAT 3 pathways. These results suggest that ROS may be responsible for HG-induced astrocyte activation as evidenced by the increase in cell viability in vitro. It has been reported that low levels of ROS have a physiological effect on neuronal plasticity (Kishida and Klann, 2007); however, in excess, ROS causes oxidation of lipids, proteins, and nucleic acids, resulting in neuronal cell death. Oxidative stress-mediated toxicity may be closely related to the pathogenesis of neurodegenerative
diseases such as Alzheimer’s disease, Parkinson disease, and Huntington disease (Andersen, 2004). In the present study, we found that HG-induced ROS production, which mediated astrocyte activation and cytokine production, suggesting that ROS may be involved in diabetes complications of CNS.

HG activates oxidative stress pathway in many types of cells, including neural stem cells (Jia et al., 2008; Pandey et al., 2011; Quan et al., 2011). NF-κB is a transcription factor that is translocated into nucleus when activated; its subsequent binding to DNA initiates many transcriptions (Tiligada, 2002). NF-κB is one of the main pathways that regulate cytokine expression (Shanmugam et al., 2003); other pathways, such as STAT 3, MAPK, and AMPK are also involved in regulation of cytokines (Agrawal et al., 2011). In the present study, we have found that both oxidative stress pathway and STAT 3 were activated by HG. It would appear that HG-induced ROS production activates NF-κB and STAT 3, which regulate various cytokines and chemokines. This is evident by the fact that inhibition of ROS with melatonin decreased activation of NF-κB and STAT 3. Conversely, inhibition of NF-κB and STAT 3 with their respective inhibitors did not affect HG-induced ROS production. Additionally, we have shown HG-induced expression of cytokines was blocked by melatonin, a ROS scavenger, and inhibitors of NF-κB and STAT 3, namely, PDTC and JSI-124; thus, further strengthening the view that ROS, NF-κB, and STAT 3 are involved in HG-induced expression of cytokines.

CONCLUSIONS

We concluded from the present results that HG can induce astrocyte activation, which triggers a cascade of genes regulating the pathways linked to the production of various pro-inflammatory cytokines and chemokines as well as ROS. The astrocyte derived inflammatory mediators are linked to the pathogenesis of diabetic complications of the CNS.

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