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High-Glucose-Derived Oxidative Stress-Dependent Heme Oxygenase-1 Expression from Astrocytes Contributes to the Neuronal Apoptosis

Chuen-Mao Yang[1] · Chih-Chung Lin[2] · Hsi-Lung Hsieh[3]

Abstract An elevated level of glucose has been found in the blood of hyperglycemia and diabetes patients associated with several central nervous system (CNS) complications. These disorders may be due to the up-regulation of many neurotoxic mediators by host cells triggered by high glucose (HG). Moreover, heme oxygenase (HO-1) plays a crucial role in tissue pathological changes such as brain injuries. However, the molecular mechanisms underlying HG-induced HO-1 expression in brain cells remain poorly defined. Thus, we use the rat brain astrocytes (RBA-1) as a model to investigate the signaling mechanisms of HO-1 induction by HG and its effects on neuronal cells. We demonstrated that HG induced HO-1 expression via a reactive oxygen species (ROS)-dependent signaling pathway. NADPH oxidase (Nox)- and mitochondrion-dependent ROS generation led to activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun-N-terminal kinase (JNK) and then activated the downstream transcriptional factors nuclear factor-kappaB (NF-κB) and c-Fos/activator protein 1 (AP-1), respectively. Subsequently, the activated NF-κB and AP-1 turned on transcription of HO-1 gene. These results indicated that in brain astrocytes, activation of MAPK-mediated NF-κB and c-Fos/AP-1 cascades by Nox/ROS and mitoROS-dependent events is essential for HO-1 up-regulation induced by HG. Moreover, we found that HG-induced extracellular ROS increase and HO-1 expression from astrocytes resulted in neuronal apoptosis. These results offers new insights into the mechanisms and effects of the action of HG, supporting that HG may cause brain disorders in the development of diabetes- and hyperglycemia-induced CNS complications such as neurodegenerative diseases.

Keywords High glucose · Astrocytes · Reactive oxygen species · Heme oxygenase-1 · Neuronal apoptosis

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Introduction

Increasing studies have reported that persistent raised levels of glucose (a phenomenon referred to as hyperglycemia) in diabetes tend to cause a neuronal damage due to excessive neuronal glucose levels and consequently metabolic disturbance of glucose. These cause glucose neurotoxicity, a serious complication of long-term diabetes, ultimately leading to loss of protective sensation and insensible trauma [1]. Moreover, hyperglycemia is known to aggravate in vivo ischemic brain damage such as vasogenic brain edema [2]. Recent report indicates that patients with diabetes show inflammation in the central nervous system (CNS) and cognitive impairments [3]. Instead of resulting from diabetic neuropathy, these early deficits are derived from hyperglycemia and constitute the earliest functional marker of glucose neurotoxicity [4]. As the statistics shown in clinics, a significant number of patients with diabetes suffer neuropathic pain, and hyperglycemia is one of the main risk factors [5]. In diabetic neuropathy, both degenerating and regenerating axons are found in peripheral

nerve biopsies, and as the disease progresses, the number of regenerating axons declines [6]. Thus, high glucose (HG) has been recognized as a risk factor in CNS disorders.

In the CNS, neurons and neuroglia are two main types of cells. Among neuroglia, astroglia (astrocytes) are specialized glial cells that are ubiquitous throughout all regions of the CNS in the adult human brain. Astrocytes exert a wide range of functions including guidance of the development of neurons during brain development, production of growth factors, maintenance of the integrity of the blood–brain barrier (BBB), and participating in the immune and repairing responses to CNS injury [7]. Following injury and infection to the human CNS, astrocytes become reactive and respond in a stereotypical manner termed reactive astrogliosis [8]. The reactive astrogliosis is regulated by specific signals that mediate numerous beneficial functions, but under certain circumstances can lead to harmful effects, either through change of normal functions or through release of detrimental factors [8]. For instance, the reactive astrocytes may release inflammatory mediators, produce oxidative stress, and the glial scar could impede axonal growth [9]. These neurotoxic factors released by reactive neuroglia are important contributors to neuronal dysfunction and death in most neurological disorders. Recent study has demonstrated that bradykinin can induce expression or release of several mediators, including reactive oxygen species (ROS) or heme oxygenase (HO)-1 in astrocytes, which further cause neuronal death [10], suggesting that these astrocytic responses may be involved in the CNS pathological processes. However, very little is known about the mechanisms underlying HG-derived various mediators from astrocytes, which may play a key role in the neurotoxicity and initiation of neurodegenerative disease.

The HO is the rate-limiting enzyme that metabolizes heme molecules to biliverdin, ferrous iron, and carbon monoxide (CO) and is involved in both CNS physiological and pathological processes [11]. In normal mature rat brain, HO-2 isozyme is responsible for most of HO constitutive activity and widely distributed throughout the mammalian neuraxis; HO-1, a stress protein, expression is low and restricted to small groups of scattered neurons and neuroglia [12]. Several studies have shown that brain HO-1 expression is markedly induced by diverse factors including subarachnoid hemorrhage, trauma, and ischemia [12]. Moreover, HO-1 gene is induced by noxious stimuli (e.g., $A\beta$, H_2O_2, and cytokines) in brain diseases such as Alzheimer disease (AD), Parkinson disease (PD), and other neuropathological conditions [13]. In AD and mild cognitive impairment, oxidative stress is measured as the percentage of astrocytes expressing HO-1 in postmortem temporal cortex and hippocampus after dual HO-1/GFAP immunohistochemistry [14]. In PD brain, astroglia overexpressing HO-1 may predispose nearby neuronal cells to oxidative injury [15]. Recently, HO-1 has been reported to act as an important endogenous protective mechanism against many

biological stresses under various pathologic conditions, such as inflammatory processes, atherosclerosis, and brain disorders [11]. These results suggest that up-regulation of astrocytic HO-1 may play a critical role in the pathogenesis of brain disorders.

Oxidative stress has been shown to play an important role in the progression of various diseases [16, 17]. Under pathological conditions, many proinflammatory mediators induce expression of inflammatory genes during brain injury by increasing ROS production [16, 17]. The ROS are produced by mitochondria, microsomes, and enzymes, including superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($\cdot$OH), and hydrogen peroxide (H_2O_2) [18]. Molecular mechanisms associated with ROS production (e.g., mitochondrial dysfunction and NADPH oxidase (Nox) activation) and its influences have been investigated in various models of chronic neurodegenerative disorders [19] and the Nox2 subunit as a critical source of the neurovascular oxidative stress mediating the deleterious cerebrovascular effects associated with increasing age [20]. Moreover, increasing evidence attributes the neurodegenerative diseases such as AD to ROS generation that leads to brain inflammation and pathogenesis [16, 17]. In the CNS, ROS also exert as a signaling factor mediated astroglial activation induced by several proinflammatory mediators [21]. Hyperglycemia can induce inflammatory reaction in several organs and tissues in vivo under diabetes conditions [22]. Moreover, HG can induce ROS production and expression of proinflammatory cytokines and chemokines in various cell types [21, 23]. Although the effects of HG associated with ROS generation have been reported in several organ diseases [24], HG-induced ROS-dependent responses in rat brain astrocytes (RBA)-1 remain unclear.

Therefore, HG may play a critical role in regulation of ROS signal and ROS-related genes like HO-1 in the CNS. The experiments were performed to investigate the molecular mechanisms of ROS-mediated pathways involved in HG-induced HO-1 expression in brain astrocytes and its effects on neuronal functions. Herein, we found that HG stimulates ROS generation via the NADPH oxidase (Nox)- and mitochondrion-mediated cascade linking to activation of mitogen-activated protein kinases (MAPKs). The ROS-dependent MAPK signals contribute to activation of NF-κB and c-Fos/AP-1, which are essential for up-regulation of HO-1 in RBA-1 cells associated with neuronal apoptosis.

Experimental Procedures

Materials

Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, fetal bovine serum (FBS), and TRIzol reagent were from Invitrogen (Carlsbad, CA). Hybond C membrane, enhanced

chemiluminescence (ECL), and Western blotting detection system were from GE Healthcare Biosciences (Buckinghamshire, UK). HO-1 antibody was from Enzo (no. SPA895). PhosphoPlus ERK1/2 (Thr^{202}/Tyr^{204}), p38 MAPK (Thr^{180}/Tyr^{182}), and JNK1/2 (Thr^{183}/Tyr^{185}) antibody kits were from Cell Signaling (Danver, MA). Actinomycin D, cycloheximide, diphenyleneiodonium chloride (DPI), mito-TEMPO, U0126, SB202190, SP600125, Bay11-7082, tanshinone IIA (TSIIA), and z-DEVD-fmk were from Biomol (Plymouth Meeting, PA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Biogenesis (Bournemouth, UK). Bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL). D-(+)-glucose, lucigenin (10,10′-dimethyl-9,9′-biacridinium dinitrate), N-acetylcysteine (NAC), hemoglobin (Hb), enzymes, and other chemicals were from Sigma (St. Louis, MO).

Cell Cultures and Treatment

The RBA-1 originated from a primary cultured astrocyte of neonatal rat cerebrum and was naturally developed through successive cell passages and used throughout this study. Cells were cultured and treated as previously described [25]. The cells were plated onto 12-well culture plates and made quiescent at confluence by incubation in serum-free DMEM/F-12 for 24 h and then incubated with HG (10 to 30 mM) at 37 °C for the indicated time intervals. When the inhibitors were used, cells were pretreated with the inhibitor for 1 h before exposure to HG (20 mM). The SK-N-SH cells, a human neuroblastoma cell line, were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM/F-12 supplemented with 10 % FBS [26].

Preparation of Cell Extracts and Western Blot Analysis

Growth-arrested RBA-1 cells were incubated with HG at 37 °C for the indicated time intervals. The cells were washed with ice-cold phosphate-buffered saline (PBS), scraped, and collected by centrifugation at 45,000×g for 1 h at 4 °C to yield the whole cell extract, as previously described [25]. Samples were denatured, subjected to SDS-PAGE using a 10 % (w/v) running gel, and transferred to nitrocellulose membrane. Membranes were incubated overnight at 4 °C using an anti-HO-1, phospho-ERK1/2, phospho-p38, phospho-JNK1/2, or GAPDH antibody, which were diluted at 1:1000 in Tween 20 in Tris-buffered saline (TTBS) (100 ng/ml). Membranes were washed with TTBS four times for 5 min each and incubated with a 1:2000 dilution of anti-rabbit horseradish peroxidase antibody for 1 h. The immunoreactive bands were detected by ECL reagents.

Total RNA Extraction and Real-Time PCR Analysis

Total RNA was extracted from RBA-1 cells [25]. The complementary DNA (cDNA) obtained from 0.5-μg total RNA was used as a template for PCR amplification. Oligonucleotide primers were designed on the basis of GenBank entries for rat HO-1 and β-actin. The primers were

HO-1
5′-CACGCATATACCCGCTACCT-3′ (sense)
5′-TCTGTCACCCTGTGCTTGAC-3′ (antisense)
β-actin
5′-GAACCCTAAGGCCAACCGTG-3′ (sense)
5′-TGGCATAGAGGTCTTTACGG-3′ (anti-sense)

The amplification was performed in 30 cycles: 1 cycle at 94 °C for 3 min, 30 cycles at 94 °C denaturation for 30 s, 55 °C annealing for 30 s, and 72 °C extension for 1 min. PCR fragments were analyzed on 2 % agarose 1X TAE gel containing ethidium bromide, and their size was compared with a molecular weight marker. Amplification of β-actin, a relatively invariant internal reference RNA, was performed in parallel, and cDNA amounts were standardized to equivalent β-actin messenger RNA (mRNA) levels. These primer sets specifically recognize only the genes of interest as indicated by amplification of a single band of the expected size (209 bp for HO-1 and 557 bp for β-actin) and direct sequence analysis of the PCR product.

Measurement of Intracellular ROS Generation

The peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) was used to assess the generation of intracellular ROS [27] with minor modifications. RBA-1 cells on monolayers were incubated with 5 μM DCF-DA in Roswell Park Memorial Institute (RPMI) 1640 at 37 °C for 45 min. The supernatant was removed and replaced with fresh RPMI 1640 medium before exposure to HG (20 mM). For dihydroethidium (DHE) staining, cells were stimulated with HG (20 mM) for the indicated time periods and then incubated with DMEM/F-12 medium containing 10 μM DHE for 10 min. Fluorescent images were captured immediately using a fluorescence microscope (ZEISS, Axiovert 200M).

Determination of NADPH Oxidase Activity by Chemiluminescence Assay

The Nox activity in intact cells was assayed by lucigenin chemiluminescence [28]. After incubation, the cells were gently scraped and centrifuged at 400×g for 10 min at 4 °C. The cell pellet was resuspended in a known volume (35 μl/ well) of ice-cold RPMI 1640 medium, and the cell suspension was kept on ice. To a final 200 μl of prewarmed (37 °C) RPMI 1640 medium containing either NADPH (1 μM) or lucigenin (20 μM), 5 μl of cell suspension (2 × 10^4 cells) was added to

initiate the reaction followed by immediate measurement of chemiluminescence using an Appliskan luminometer (Thermo®) in an out-of-coincidence mode. Appropriate blanks and controls were established, and chemiluminescence was recorded. Neither NADPH nor NADH enhanced the background chemiluminescence of lucigenin alone (30–40 c/min). Chemiluminescence was continuously measured for 12 min, and the activity of Nox was expressed as counts per million cells.

Measurement of Mitochondrial ROS Generation

The production of mitochondrial ROS was measured by a mitochondrial superoxide indicator MitoSOX™ Red (a fluorogenic dye specifically targeted to mitochondrial superoxide in living cells), and the mitochondria were simultaneously labeled with MitoTracker® Green (a mitochondrial tracker). The cells were stimulated with HG (20 mM) for the indicated time intervals and then incubated with DMEM/F-12 medium containing 2.5 μM MitoSOX™ Red and 1 μM MitoTracker® Green for 10 min. The fluorescent images were monitored immediately using a fluorescence microscope (ZEISS, Axiovert 200M).

Measurement of Extracellular ROS Generation

RBA-1 cells in monolayers were incubated with HG (20 mM) for the indicated time intervals, and the conditioned media were collected and analyzed extracellular ROS levels by lucigenin chemiluminescence assay. The conditioned media (100 μl) were incubated with 100 μM lucigenin (100 μl) for 30 min at 37 °C. Relative luminescent intensity was recorded over time (5–60 min) by using a luminescent plate reader at an excitation wavelength of 485 nm, and emission was measured at a wavelength of 530 nm (Thermo, Appliskan).

Transient Transfection with Small Interfering RNAs

Transient transfection of small interfering RNA (siRNA) duplexes corresponding to rat ERK2, JNK1, p38, p65, c-Fos, and scrambled siRNAs (100 nM) was performed using a Lipofetamine™ RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence Staining

Growth-arrested RBA-1 cells were incubated with HG for 60 min. After washing twice with ice-cold PBS, the cells were fixed, permeabilized, and stained using an anti-p65 or c-Fos antibody as previously described [25]. The images were observed using a fluorescence microscope (ZEISS, Axiovert 200M).

Plasmid Construction, Transfection, and Luciferase Reporter Gene Assays

The rat HO-1 promoter (accession no. J02722.1; −766 to +20) was constructed (sense primer GGTACC CAGGAAGTCACAGTGTGGCC and antisense primer CCCGAGCTCGTCGAGCTGTGGGCGCTCCAT; 63 °C) and cloned to the pGL3-basic vector containing the luciferase reporter system. All plasmids were prepared by using QIAGEN plasmid DNA preparation kits. These constructs were transfected into RBA-1 cells by using a Lipofectamine reagent according to the instructions of manufacturer. The transfection efficiency (~60 %) was determined by transfection with enhanced GFP. After incubation with HG, cells were collected and disrupted by sonication in a lysis buffer (25 mM Tris, pH 7.8, 2 mM EDTA, 1 % Triton X-100, and 10 % glycerol). After centrifugation, aliquots of the supernatants were tested for promoter activity using a luciferase assay system (Promega, Madison, WI). Firefly luciferase activities were standardized for β-galactosidase activity.

Preparation of HG-Mediated Astrocytic-Conditioned Culture Medium

For collection of conditioned media, RBA-1 cells were plated and incubated with HG for 24 h (HG-CM). Cell-free supernatant fractions were applied to human neuroblastoma SK-N-SH cells to evaluate the changes in cell viability and related parameters in the study.

Cytotoxicity Assay

Cells were grown in 24-well culture plates at a concentration of 5×10^4 cells/well followed by proper treatment. Five-microliter Cell Counting Kit-8 (CCK-8) solution (Sigma) in 500-μl growth medium was added to each well. After incubation with CCK-8 at 37 °C for 2 h, cultured medium was collected. The water-soluble tetrazolium salt, a product of CCK-8 by the action of mitochondrial dehydrogenases, was solubilized in cultured medium and quantified spectrophotometrically at 450 nm, reference at 650 nm.

Apoptosis Assay

Hoechst 33342 staining was performed as previously described [10], with some modifications. In brief, attached cells were washed with PBS and stained with 1 μg/ml Hoechst 33342 in the dark for 10 min. Following rinsing with PBS and mounting on glass slides, at least 300 cells per condition were observed using a fluorescence microscope (ZEISS, Axiovert 200M). The two cell populations (normal and apoptotic cells) were counted for Hoechst 33342 staining with chromatin. The Hoechst 33342, a kind of blue fluorescent

dye, which stains the condensed chromatin in apoptotic cells, was brighter than that of normal cells. Apoptotic cells were also identified on the basis of morphology and condensation and fragmentation of nuclei.

Analysis of Data

All data were estimated using GraphPad Prism Program (GraphPad, San Diego, CA). Quantitative data were analyzed by one-way ANOVA followed by Tukey's honestly significant difference tests between individual groups. Data were expressed as mean ± SEM. A value of $P < 0.05$ was considered significant.

Results

HG Induces HO-1 Expression via the De Novo mRNA and Protein Synthesis in Brain Astrocytes

To investigate the effects of HG on HO-1 gene expression in brain astrocytes, RBA-1 cells were treated with 20 mM glucose for the indicated time intervals. The HO-1 protein expression was determined by Western blotting. As shown in Fig. 1a, HG induced HO-1 protein expression in a time-dependent manner. There was a significant increase within 6 h and sustained over 24 h. In addition, we further determined whether HG-induced HO-1 expression is a concentration-dependent manner; cells were treated with various concentrations of glucose (0, 10, 20, or 30 mM) for 16 h. The data showed that treatment with various concentrations significantly induced HO-1 expression in a concentration-dependent manner as compared with that of the basal level (Fig. 1b). Next, the HO-1 mRNA level was detected by reverse transcription (RT) PCR analysis. The results showed that HG significantly induced HO-1 mRNA expression in a time-dependent manner (Fig. 1c). These results suggested that HG induces HO-1 expression via up-regulation of HO-1 mRNA and protein in RBA-1 cells. Next, to confirm whether the effect of HG on HO-1 up-regulation is dependent on de novo protein synthesis, the transcription inhibitor actinomycin D and the translation inhibitor cycloheximide were used. As shown in Fig. 1d, e, pretreatment with actinomycin D (Act. D, 0.1 μM) significantly attenuated HG-induced HO-1 expression, including protein and mRNA. Moreover, pretreatment with cycloheximide (CHX, 1 μM) attenuated HG-induced HO-1 protein expression but not mRNA. These results suggested that up-regulation of HO-1 by HG is mediated through transcription and translation pathways in RBA-1 cells.

Role of Intracellular ROS Signal in the Induction of HO-1 by HG

Several studies have demonstrated that ROS contributes to HO-1 expression in various cell types [29]. To determine whether ROS participated in HG-induced HO-1 expression, a ROS scavenger NAC was used. Pretreatment with NAC (10 mM) markedly attenuated the HG-induced HO-1 protein and mRNA expression (Figs. 2a, b), implying that ROS signal may play a potential role in HG-induced HO-1 expression in RBA-1 cells. To further evaluate whether HG stimulates intracellular ROS production in these cells, two ROS-sensitive probes, DCF-DA or DHE, were used. Cells were incubated with HG (20 mM) for 10 min after loading with DCF-DA or DHE. As shown in Fig. 2c, the fluorescence images showed that HG increased the intracellular ROS generation, which was attenuated by pretreatment with NAC (an ROS scavenger), suggesting that ROS signals (i.e., superoxide anion and hydrogen peroxide) are involved in HG-induced HO-1 expression in RBA-1 cells. The NADPH oxidase (Nox) is considered to be a major source of ROS in several physiological and pathological processes [17, 30]. Moreover, there are extensive literatures supporting a role for mitochondrial dysfunction and oxidative damage in the pathogenesis of AD [19, 31]. Therefore, to investigate the sources of intracellular ROS generation (e.g., Nox and mitochondria) by HG in RBA-1 cells, the Nox activity and mitochondrial ROS were detected. The Nox activity data showed that HG stimulated Nox activity in a time-dependent manner with a maximal response within 10 min and sustained over 60 min (Fig. 2d). Subsequently, to detect whether HG stimulates mitochondrial ROS production in RBA-1 cells, the MitoSOX™ Red reagent and MitoTracker® Green were used. As shown in Fig. 2e, we found that HG time dependently stimulated an increase of MitoSOX Red flourescence intensity in RBA-1 cells. Moreover, the merge data showed that MitoSOX Red colocalized with MitoTracker Green, indicating that the superoxide anion was produced in mitochondria by HG. These data indicated that intracellular ROS signals, including Nox- and mitochondrion-mediated ROS, may play a critical role in HG-induced HO-1 expression in RBA-1 cells.

HG Stimulated Extracellular ROS Production via Nox- and Mitochondria-Derived ROS Release

Here, we also detected whether HG stimulates extracellular ROS increase in these cells; the conditioned media (HG-CM) were collected and analyzed by lucigenin chemiluminescence assay. The results showed that HG also time dependently stimulated an increase of extracellular ROS level in the HG-CM with a maximal response within 15 min (Fig. 3a). To further determine the sources of extracellular ROS increase by HG, the Nox inhibitor (DPI) and mitochondrion-targeted
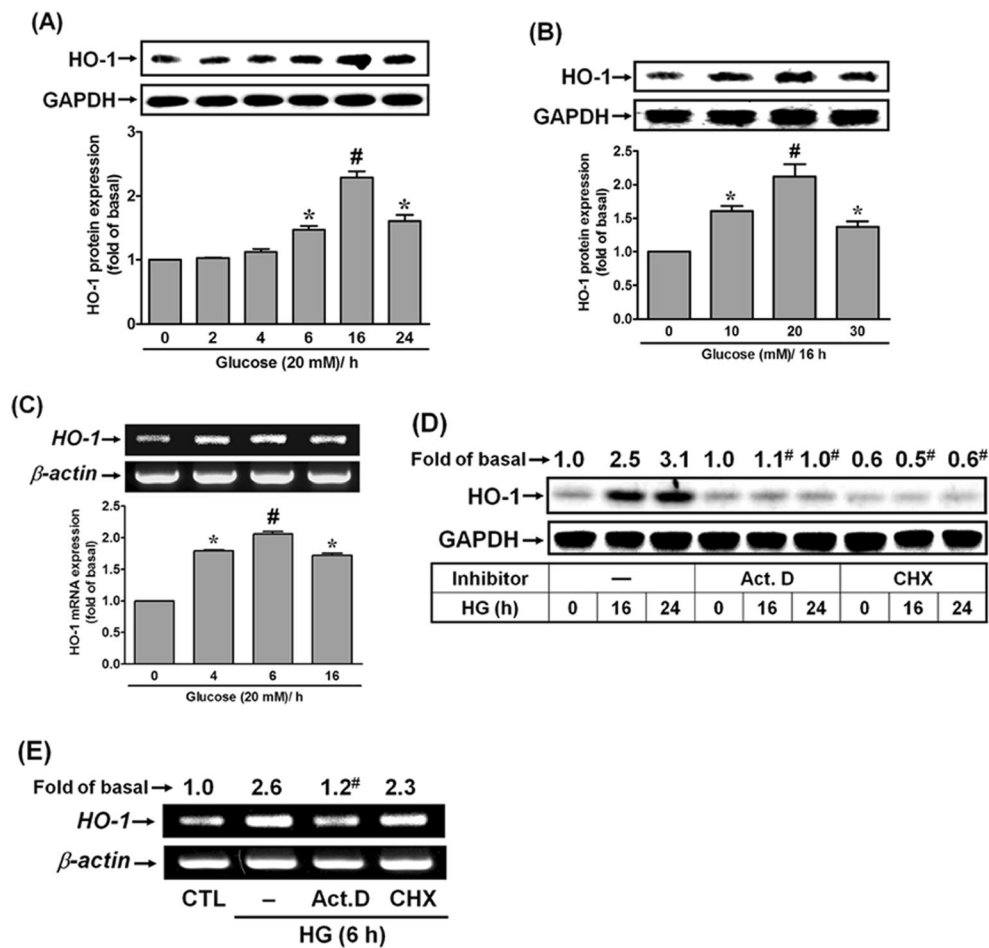
Fig. 1 High glucose (HG) induces HO-1 gene expression in RBA-1 cells. **a** Time dependence of HG-induced HO-1 protein expression, cells were incubated with 20 mM of glucose for the indicated time intervals. **b** Cells were treated with various concentrations of glucose for 16 h. **c** Time dependence of HG-induced HO-1 mRNA expression, cells were incubated with 20 mM glucose for the indicated time intervals. **d** Cells were treated with or without actinomycin D (Act. D, 0.1 μM) or cycloheximide (CHX, 1 μM) for 1 h before exposure to 20 mM glucose for the indicated times. **e** Cells were pretreated with Act. D

(0.1 μM) or CHX (1 μM) for 1 h and then exposure to 20 mM glucose for 6 h. The cell lysates (**a**, **b**, **d**) were prepared and analyzed by Western blot using an anti-HO-1 or anti-GAPDH (as an internal control) monoclonal antibody. The total RNA (**c**, **e**) was extracted and analyzed by RT-PCR analysis. Data are expressed as mean or mean ± SEM of three independent experiments ($n = 3$). *$P < 0.05$, #$P < 0.01$, as compared with the cells exposed to vehicle (**a**, **b**, **c**) or HG (**d**, **e**) alone. The figure represents one of three independent experiments ($n = 3$)

antioxidant (mito-TEMPO) were used. Pretreatment with DPI (1 μM) or mito-TEMPO (Mito, 20 nM) significantly attenuated extracellular ROS production by HG (Fig. 3b), suggesting that HG-stimulated extracellular ROS production is mediated through Nox- and mitochondrion-derived ROS releasing in RBA-1 cells.

Involvement of ROS-Dependent MAPK Pathways in HG-Induced HO-1 Expression

Recent reports have demonstrated that ROS-dependent activation of MAPKs contributes to HO-1 expression induced by various stimuli in brain astrocytes [25]. Therefore, we first determined the role of MAPK-dependent pathways in HG-induced HO-1 expression; three inhibitors of MAPKs, including U0126 (ERK1/2), SB202190 (p38 MAPK), and

SP600125 (JNK), were used. As shown in Fig. 4a, pretreatment with U0126 (1 μM) and SP600125 (1 μM) markedly attenuated HG-induced HO-1 protein expression but not SB202190 (10 μM). Similarly, HG-induced HO-1 mRNA expression was attenuated by pretreatment with U0126 and SP600125 but not SB202190 (Fig. 4b). The results suggested that ERK1/2 and JNK1/2 may be involved in HG-induced HO-1 expression in RBA-1 cells. We further demonstrated that HG stimulated phosphorylation of ERK1/2, p38 MAPK, and JNK1/2, which were significantly attenuated by their respective inhibitor (Fig. 4c). The data demonstrated that MAPKs (i.e., ERK1/2 and JNK1/2) play a critical role in HG-induced HO-1 expression in RBA-1 cells. To confirm this note, p38, ERK2, or JNK1 siRNA was used. Transfection with ERK2 or JNK1 siRNA, but not p38 MAPK, markedly attenuated HG-induced HO-1 expression (Fig. 4d). These results
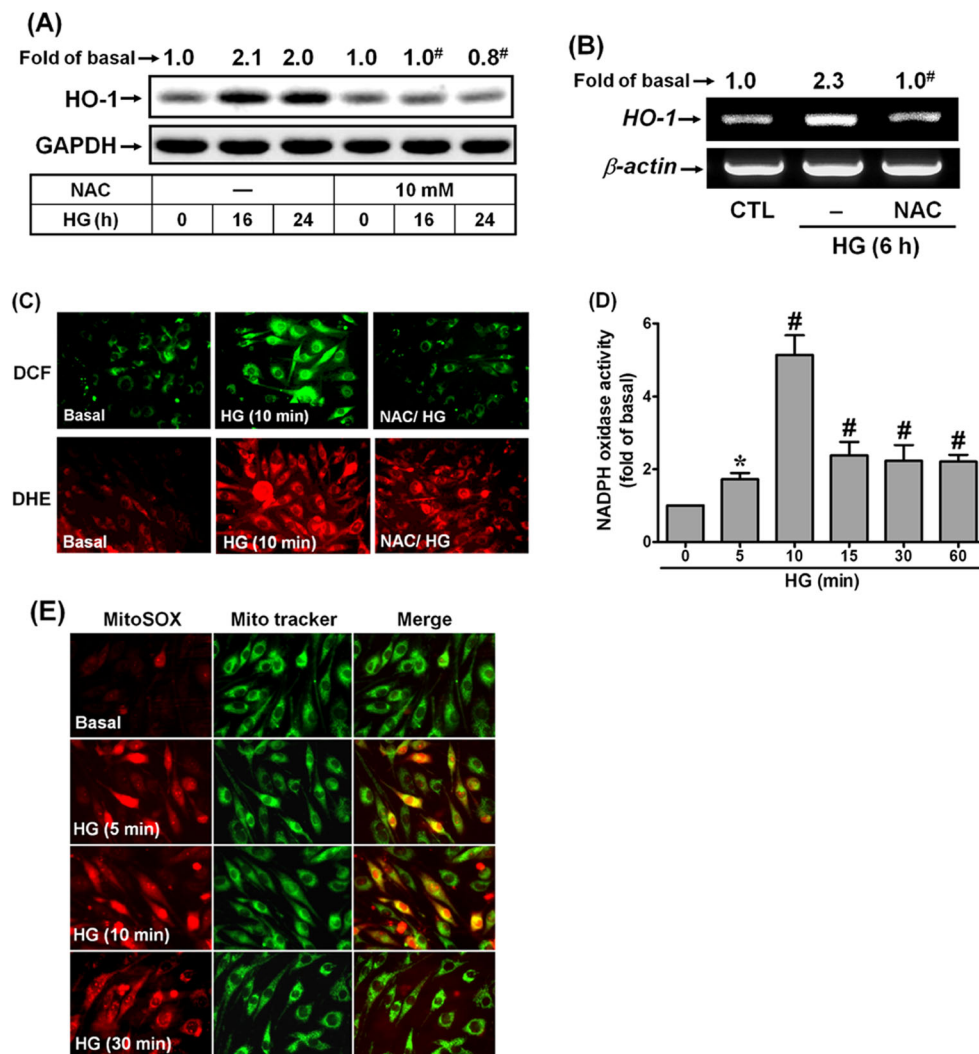
Fig. 2 ROS signal is involved in HG-induced HO-1 expression in RBA-1 cells. **a** Cells were treated with or without N-acetylcysteine (NAC, 10 mM) for 1 h before exposure to 20 mM glucose for the indicated times. The cell lysates were prepared for Western blotting analysis of HO-1 expression as described in Fig. 1. **b** Cells were pretreated with NAC (10 mM) for 1 h and then exposure to 20 mM glucose for 6 h. The total RNA was extracted and analyzed by RT-PCR analysis. **c** HG stimulates intracellular ROS generation; cells were incubated with the peroxide-sensitive fluorescent probe DCF-DA (5 μM) for 45 min or DHE (10 μM) for 10 min, followed by stimulation with 20 mM glucose for 10 min in the presence or absence of NAC (10 mM). The fluorescence intensity of cells was determined as described under "Experimental Procedures" section. Time dependence of HG-stimulated Nox activation (**d**) and mitochondrial ROS generation (**e**), cells were stimulation with 20 mM glucose for the indicated time intervals. The Nox activity (**d**) and mitochondrial ROS (**e**) were analyzed as described under "Experimental Procedures" section. Data are expressed as mean or mean ± SEM of three independent experiments ($n = 3$). *$P < 0.05$, #$P < 0.01$, as compared with the values of cells stimulated with vehicle (**d**) or HG (**a**, **b**) alone. The figure represents one of three independent experiments ($n = 3$)

confirmed a link between activation of MAPKs and induction of HO-1 by HG in RBA-1 cells. The previous data have shown that ROS signal is involved in HG-induced HO-1 expression (Fig. 2). Thus, we next confirm the role of ROS signal in HG-stimulated activation of MAPKs; the ROS scavenger (NAC, 10 mM), Nox inhibitor (DPI, 10 μM), or mito-TEMPO (Mito, 20 nM) was used. As shown in Fig. 4e, pretreatment with NAC, DPI, or mito-TEMPO markedly attenuated HG-stimulated phosphorylation of MAPKs including ERK1/2, p38 MAPK, and JNK1/2, indicating that HG stimulated phosphorylation of ERK1/2, p38 MAPK, and JNK1/2

via ROS-mediated pathway. These results suggested that HG-stimulated activation of MAPKs (i.e., ERK1/2 and JNK1/2) is mediated through a ROS-dependent cascade leading to up-regulation of HO-1 in RBA-1 cells.

NF-κB and AP-1 Are Required for HG-Induced HO-1 Expression via Enhancing HO-1 Promoter Transcription Activity

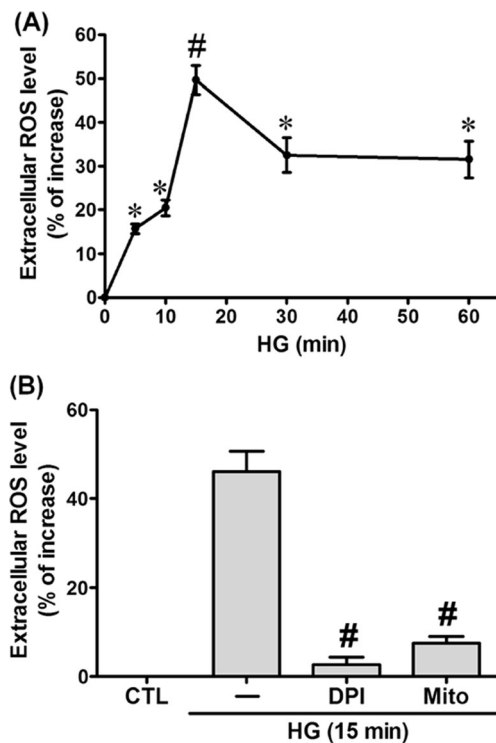Many transcription factors (e.g., NF-κB and AP-1) are well known to be modulated during oxidative stress associated

Fig. 3 HG stimulates extracellular ROS generation via NADPH oxidase (Nox) activation and mitochondrial ROS release in RBA-1 cells. **a** Time dependence of high-glucose-stimulated extracellular ROS increase, cells were stimulation with 20 mM glucose for the indicated times. **b** Cells were incubated with HG for 15 min in the absence or presence of DPI (1 μM) or mito-TEMPO (Mito, 20 nM) for 1 h. The conditioned media were collected and analyzed by lucigenin chemiluminescence assay as described under "Experimental Procedures" section. Data are expressed as mean ± SEM of three independent experiments ($n = 3$). *$P < 0.05$, #$P < 0.01$, as compared with the values of cells stimulated with vehicle (**a**) or HG (**b**) alone

with physiological and pathological events [32]. Among the factors, NF-κB and AP-1 have been shown to involve in HO-1 expression in various cell types [25]. To determine whether NF-κB and AP-1 are essential for HO-1 induction by HG, a NF-κB inhibitor (Bay11-7082) or an AP-1 inhibitor (tanshinone IIA) was used. As shown in Fig. 5a, HG-induced HO-1 expression was attenuated by pretreatment with Bay11-7082 (1 μM) or TSIIA (10 μM). Moreover, the similar results were observed by RT-PCR analysis (Fig. 5b), suggesting that NF-κB and AP-1 are required for HG-induced HO-1 protein and mRNA expression. HG-stimulated activation of NF-κB and AP-1 were further supported by the results obtained using immunofluorescence staining against a p65 NF-κB and c-Fos/AP-1 antibody, respectively. The results showed that HG-stimulated translocation of p65 NF-κB (Fig. 5c, upper panel) and expression and accumulation (in nucleus) of c-Fos/AP-1 (Fig. 5c, lower panel) were attenuated by pretreatment with their respective inhibitor. To further confirm the role of p65 NF-κB and c-Fos/AP-1 in HG-induced HO-1 expression, the p65 or c-Fos siRNA was used. The results

showed that transfection with p65 or c-Fos siRNA both significantly attenuated HG-induced HO-1 expression (Fig. 5d). These results demonstrated that NF-κB and AP-1 play a critical role in HG-induced HO-1 expression in RBA-1 cells.

Moreover, to examine whether HG induced HO-1 gene up-regulation via initiating its promoter activity, the HO-1 promoter was constructed and its activity was evaluated using a promoter-luciferase activity assay. The HO-1 promoter was constructed as illustrated in Fig. 5e (upper part), which may contain several putative recognition elements for a variety of transcriptional factors such as NF-κB or AP-1. To determine the effect of HG on the HO-1 promoter activity, as shown in Fig. 5e, HG increased the HO-1 promoter activity in a time-dependent manner with a maximal response within 4 h, suggesting that HG induced HO-1 expression via enhancing HO-1 promoter activity. Next, we determined whether these signaling components mentioned above are involved in HG-enhanced HO-1 promoter activity. As shown in Fig. 5f, HG-enhanced HO-1 promoter activity was significantly inhibited by pretreatment with NAC, DPI, mito-TEMPO, U0126, SP600125, Bay11-7082, or TSIIA. These results confirmed that the induction of the HO-1 promoter activity by HG is mediated through ROS-dependent MAPK (ERK1/2 and JNK1/2) cascades leading to NF-κB and c-Fos/AP-1 activation in RBA-1 cells.

Up-Regulation of Astroglial ROS and HO-1 by HG Causes Neuronal Apoptosis

Based on the present data, we demonstrated that HG can induce ROS release and HO-1 expression in brain astrocytes. Here, we further explored whether the induction of ROS and HO-1 from astrocytes is harmful to neuronal cells. The CM were collected as HG-CM for the following experiments. First, to determine the effects of CM, including control culture medium (CTL, BM + HG (20 mM)) and HG-CM, on neuronal cell survival, a human neuroblastoma SK-N-SH cell line was used. As shown in Fig. 6a, treatment with HG-CM markedly decreased cell viability (~40 % reduction at 24 h, $n = 3$) by an XTT assay kit. Next, we determined whether HG-CM-reduced SK-N-SH cell survival is mediated through ROS signals. The data showed that pretreatment with NAC (10 mM) significantly blocked HG-CM-induced SK-N-SH cell death (Fig. 6a), suggesting that the ROS released from HG-challenged astrocytes may act as a risk factor in SK-N-SK cell survival. Recent studies suggest that neuroglial HO-1 expression may play a critical role in the pathogenesis of brain disorders. As previous report [10], the HO-1-derived CO form astrocytes may induce neuronal cell death. Here we demonstrated that HG also induced HO-1 expression in brain astrocytes (Fig. 1). Hence, we further evaluated whether HO-1-derived CO by HG in astrocytes is involved in these responses; pretreatment with the CO scavenger hemoglobin
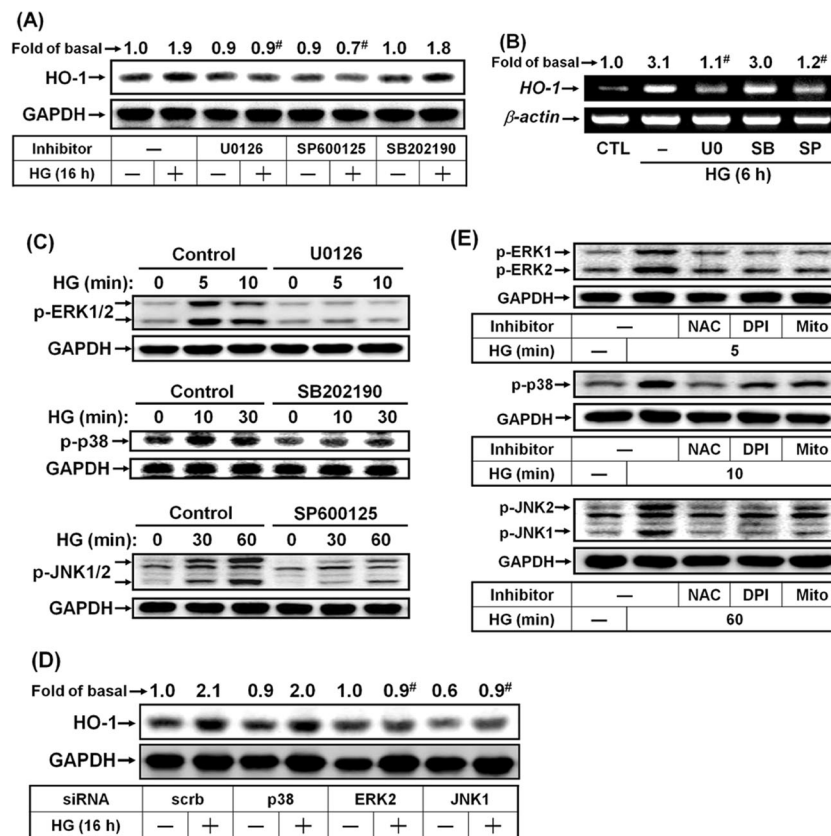
Fig. 4 ROS-dependent activation of MAPKs is involved in HG-induced HO-1 expression in RBA-1 cells. Cells were pretreated with or without U0126 (1 μM), SB202190 (10 μM), or SP600125 (1 μM) for 1 h before exposure to 20 mM glucose for 16 (**a**) or 6 h (**b**). The cell lysates and total RNA were prepared and analyzed by Western blot (**a**) and RT-PCR (**b**) for HO-1 expression as described in Fig. 1. **c** Cells were pretreated with U0126 (1 μM), SB202190 (10 μM), or SP600125 (1 μM) for 1 h and then exposure to 20 mM glucose for the indicated times. **d** Cells were transfected with scramble (scrb), p38, ERK2, or JNK1 siRNA for 24 h, followed by incubation with HG (20 mM) for 24 h. The cell lysates were analyzed by Western blot to determine the levels of HO-1 and GAPDH. **e** Cells were pretreated with or without NAC (10 mM), DPI (1 μM), or mito-TEMPO (Mito, 20 nM) for 1 h, followed by stimulation with 20 mM glucose for the indicated times. Cells were harvested for Western blotting analysis of phospho-ERK1/2, phospho-p38 MAPK, or phospho-JNK1/2, respectively, as described under "Experimental Procedures" section. Data are expressed as mean of three independent experiments ($n = 3$). $^{\#}P < 0.01$, as compared with the values of cells incubated with HG (**a**, **b**, **d**) alone. The figure represents one of three independent experiments ($n = 3$)

(Hb) significantly attenuated the effects of HG-CM on SK-N-SH cell viability, suggesting that up-regulation of HG-induced HO-1/CO system in astrocytes leads to neuronal (SK-N-SH) death. To determine whether HG-CM induces SK-N-SH cell death via a caspase-3-dependent apoptosis, a caspase-3 inhibitor z-DEVD-fmk was used. As shown in Fig. 6a, cells (SK-N-SH) that were pretreated with z-DEVD-fmk (z-DEVD, 10 μM) significantly blocked HG-CM-induced cell death, suggesting that caspase-3-mediated apoptosis is involved in HG-CM-induced SK-N-SH cell death. Moreover, to confirm this note, a DNA dye Hoechst 33342 was used after treatment with HG-CM for 24 h. The statistical data of apoptotic nuclei counting were shown in Fig. 6b, indicating that HG-CM significantly induced an increase of SK-N-SH cell apoptotic nuclei. Pretreatment with NAC, Hb, or z-DEVD-fmk that significantly attenuated HG-CM induced the response, suggesting that HG-CM-induced SK-N-SH cell apoptosis is mediated through ROS and HO-1/CO. These results suggested that

HG-CM from astrocytes may contain some damaging factors to induce neuronal apoptosis.

Discussion

Hyperglycemia, persistent raised levels of glucose, is one of the main risk factors in diabetes [2, 5], which may cause several complications such as retinopathy, heart disease, and neuropathy [22, 24]. Moreover, HG in diabetic patients may lead to neuronal damages [1]; these early deficits are derived from hyperglycemia and constitute the earliest functional marker of glucose neurotoxicity [4], ultimately leading to neurodegenerative disorders [1]. Recent report indicates that patients with diabetes show CNS inflammation and cognitive impairments [3]. Therefore, HG has been recognized as a dangerous factor in diabetes-associated CNS disorders. In the CNS, chronic inflammation is a prominent feature which contributes to
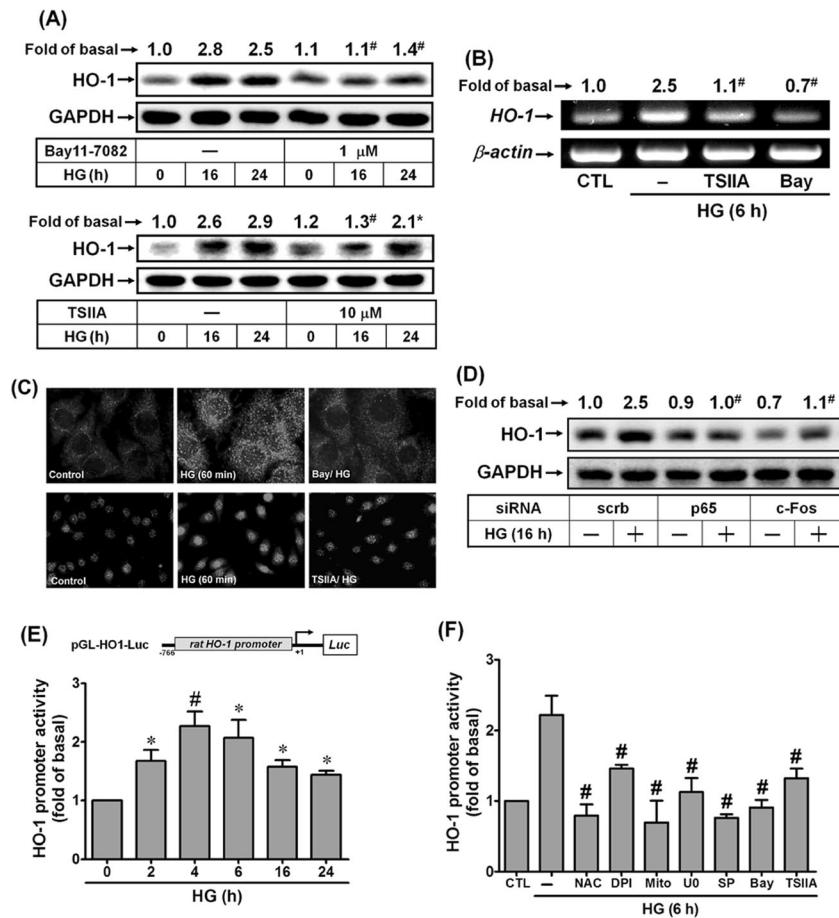
Fig. 5 NF-κB and AP-1 are required for HG-induced HO-1 expression and promoter transcription activity. Cells were pretreated with Bay11-7082 (1 μM) or TSIIA (10 μM) for 1 h and then incubated with 20 mM glucose for the indicated times. The cell lysates and total RNA were prepared and analyzed by Western blot (**a**) and RT-PCR (**b**) for HO-1 expression as described in Fig. 1. **c** HG-stimulated p65 NF-κB nuclear translocation and c-Fos/AP-1 accumulation were determined by immunofluorescence staining. Cells were pretreated with Bay11-7082 (1 μM) or TSIIA (10 μM) for 1 h and then stimulated with 20 mM glucose for 60 min. Cells were fixed and labeled with anti-p65 or anti-c-Fos antibody and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Individual cells were imaged as described in "Experimental Procedures" section. **d** Cells were transfected with scramble (scrb), p65, or c-Fos siRNA for 24 h, followed by incubation with HG (20 mM) for 24 h. The cell lysates were analyzed by Western blot to determine the levels of HO-1 and GAPDH. **e** Time dependence of HG-enhanced HO-1 promoter activity. Schematic representation of a 5′-promoter portions of the rat *HO-1* gene fused to the pGL-luciferase reporter gene (pGL-HO1-Luc). The translational start site (+1) of the *luciferase* reporter gene is indicated by the *arrow*. RBA-1 cells were transiently cotransfected with 0.9 μg of pGL-HO1-Luc or with 0.1 μg of pGal coding for β-galactosidase. After overnight transfection, cells were treated with HG (20 mM) for the indicated time intervals. **f** Cells were treated with HG for 6 h in the absence or presence of NAC (10 mM), DPI (1 μM), mito-TEMPO (Mito, 20 nM), U0126 (U0, 1 μM), SP600125 (SP, 1 μM), Bay11-7082 (Bay, 1 μM), or TSIIA (10 μM) for 1 h before being harvested for measuring the luciferase and β-galactosidase activities as described in "Experimental Procedures" section. Data are expressed as mean or mean ± SEM of three independent experiments ($n = 3$). *$P < 0.05$, #$P < 0.01$, as compared with the respective values of cells stimulated with vehicle (**e**) or HG (**a**, **b**, **d**, **f**) alone. The figure represents one of three independent experiments ($n = 3$)

various brain neurodegenerative pathologies such as AD [33–35]. It is important to understand the mechanisms by which inflammation is involved in the neurodegenerative diseases. Here, we investigated the mechanisms underlying HG-induced expression of the stress mediators such as HO-1 in cultured RBA-1 and its effects on neuronal cell (SK-N-SH) viability and apoptosis. Our results indicated that in brain astrocytes, HG-induced HO-1 expression is mediated through the Nox2- and mitochondrion-derived ROS signal linking to activation of MAPK cascade and leading to NF-κB and AP-1

pathways (Fig. 6c). Moreover, we demonstrated that HG-triggered HO-1/CO system and ROS release from brain astrocytes can induce neuronal apoptosis. These findings suggested that these astrocyte responses may exacerbate diabetes- and hyperglycemia-derived neuroinflammation or neurodegeneration via HG-induced ROS-dependent HO-1 expression.

Oxidative stress (e.g., ROS generation) exerts an important role in the normal physiological functions and the pathological responses [16, 17]. Imbalance in the level of ROS has been shown to play a causative role in several pathologies of
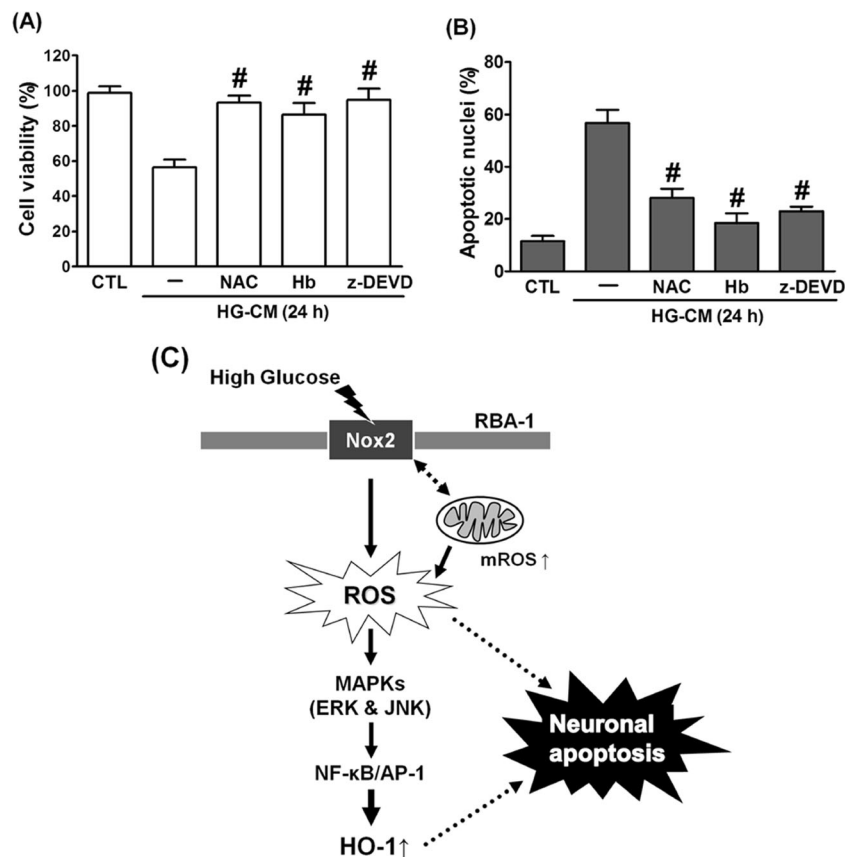
Fig. 6 The high-glucose-conditioned medium (HG-CM) induces SK-N-SH cell apoptosis via up-regulation of ROS and HO-1/CO system from HG-challenged RBA-1 cells. Cells were pretreated with or without NAC (10 mM), Hb (0.2 mg/ml), and z-DEVD-fmk (10 μM) for 1 h before exposure to HG-CM for 24 h. The cell survival was analyzed (**a**) and the number of apoptotic nuclei was counted (**b**), and the percentage was calculated as described in "Experimental Procedures" section. Data are expressed as mean ± SEM of three independent experiments ($n = 3$).

[#]$P < 0.01$, as compared with the values of cells stimulated with HG-CM alone. **c** Schematic presentation of the summary of this work. In brain astrocytes (RBA-1 cells), HG-mediated signaling pathways linked to up-regulation of ROS and HO-1/CO system in the conditioned medium (HG-CM). Exposure of cultured neuronal cells (SK-N-SH) to HG-CM results in cell apoptosis. The astrocyte-derived mediator (i.e., ROS and HO-1/CO) causes neuronal apoptosis via a caspase-3-dependent pathway

degenerative diseases [16, 17]. In the brain, ROS also extend to the control of vascular tone which is tightly modulated by metabolic activity within neurons. Moreover, increasing ROS generation by diverse stimuli can regulate the expression of inflammatory mediators in pathogenesis of brain disorders [16]. Previously, the cellular damage in neurodegenerative disorders such as AD is attributed to oxidative stress [16, 33], suggesting that oxidative stress (e.g., ROS) is increased in the brain of diabetic patients as biomarkers for oxidative damages. It has been shown that HG stimulates ROS production and expression of proinflammatory cytokines and chemokines in diabetic vascular, renal, and brain diseases [22, 23, 36]. In this study, our data demonstrated that HG induces ROS-dependent HO-1 expression via Nox2- and mitochondrion-mediated pathways in brain astrocytes, since pretreatment with Nox activity inhibitor DPI, mitochondrion-targeted antioxidant mito-TEMPO, and ROS scavenger NAC attenuated HG-induced responses. Herein, we are the first

group to establish that Nox2- and mitochondrion-derived ROS signal contributes to up-regulation of HO-1 by HG in brain astrocytes.

The Nox is considered to be a major source of ROS in several physiological and pathological processes [37]. However, the major studies of high glucose associated with ROS are reported in leukocytes and diabetic nephropathy [38, 39]. In the study, we first demonstrated that Nox activity and ROS generation were involved in HG-induced responses by using Nox inhibitor DPI and DCF and DHE staining (Fig. 2). Moreover, based on our recent data, it is demonstrated that Nox, Nox2 especially, contributes to HG-induced responses [40], suggesting that HG-induced HO-1 expression may be predominantly mediated through activation of Nox2. These results are consistent with previous studies showing that Nox2 is expressed in astrocytes and contributes to ROS generation [25, 41, 42] and Nox is involved in hyperglycemia-induced HO-1 expression in the diabetic retina [43]. Recently,

there is extensive literatures supporting a role for mitochondrial dysfunction and oxidative damage in the pathogenesis of AD [31], and ROS are associated with neuroinflammatory and neurodegenerative processing [44]. Mitochondrial oxidative stress not only alters the ability of the cells to generate energy but also affects cellular redox signaling [31, 44]. Herein, we found that mitochondrial ROS generation was involved in HG-induced responses by using a mitochondrion-targeted antioxidant mito-TEMPO and MitoSOX staining (Figs. 2 and 3). These results are consistent with previous studies emphasizing the role of mitochondrial dysfunction and mitochondrial ROS in diabetes [45]. Moreover, we also demonstrated that HG-stimulated extracellular ROS increase was mediated through Nox- and mitochondrion-dependent manner in astrocytes (Fig. 3), which may be a risk factor for neuronal cell survival. These results demonstrate that HG induced neuronal cell death in part through extracellular ROS generation via Nox and mitochondria, suggesting its potential to generate oxidative stress via mitochondrial damage and its involvement in oxidative stress-related neurodegenerative disease. In addition, several recent reports of cross talk between mitochondrion- and Nox-derived ROS have been reported [46]. Interplay between mitochondria and Nox is mediated by the level of mitochondrial ROS both in vitro and in vivo [47]. A report suggests that production of ROS by Nox leads to mitochondrial dysfunction [48]. These issues need to be further investigated.

Abnormal regulation of MAPKs might be implicated in the CNS diseases. Moreover, redox signal is a critical link among MAPKs and degenerative diseases [49]. Previous reports have implicated that HG acts as a risking factor through activation of MAPK cascades in the development and progression of diabetic complications [50]. Herein, the role of Nox- or mitochondrion-derived ROS generation in HG-induced phosphorylation of these three MAPKs was further confirmed by pretreatment with NAC, DPI, or mito-TEMPO, respectively (Fig. 4e), suggesting that Nox/ROS- and mitochondrion/ROS-dependent activation of MAPKs (i.e., ERK1/2 and JNK1/2) is involved in HG-induced HO-1 expression in RBA-1 cells. These results are consistent with several studies showing that MAPKs are the downstream signaling molecules regulated by Nox/ROS or mitochondrial ROS [51] and the induction of pro-inflammatory mediators by LPS via ROS-mediated MAPK pathways in BV-2 and primary microglial cells [52]. This study is the first to establish that activation of MAPKs by Nox2- and mitochondrion-derived ROS generation contributes to HG-induced HO-1 expression in brain astrocytes (RBA-1 cells).

Cells utilize multiple signaling pathways and transcription factors to regulate their responses to a specific stimulation. The excessive increase of oxidative stress during injuries can modulate the pattern of gene expression through various transcription factors. Among these factors, the members of NF-κB and AP-1 families are arguably the most important regulators

of the cellular stress response in vertebrates [53]. NF-κB is one important and widely investigated transcription factor which is a major participant in signaling pathways governing cellular responses to environmental (oxidative) stresses [54]. Moreover, NF-κB exerts as a positive regulator of many inflammatory gene expressions involved in chronic inflammatory diseases [55]. In astrocytes, we have also demonstrated that up-regulation of HO-1 by BK is mediated through a MAPK-dependent activation of NF-κB and AP-1 [25]. More recent report demonstrates that high glucose can induce matrix metalloproteinase (MMP)-9 expression via ROS-mediated activation of MAPK-dependent NF-κB in brain astrocytes [56]. Accumulating evidence has shown that HO-1 is up-regulated via an AP-1-dependent manner in various cell types [25]. Thus, the transcription factors NF-κB and AP-1 may play a key role in the regulation of several gene expressions like HO-1 by HG in brain astrocytes. However, the mechanistic connection between the HO-1 expression and the ROS-dependent NF-κB and AP-1 pathways induced by HG has not been established in RBA-1 cells. In this study, we demonstrated that NF-κB and AP-1 were essential for HG-induced HO-1 expression which was inhibited by NF-κB inhibitor Bay11-7082 or AP-1 inhibitor TSIIA (Fig. 5). The involvement of NF-κB and AP-1 in HG-induced responses was further confirmed by determining p65 NF-κB translocation and expression of c-Fos/AP-1 (Fig. 5c). The roles of p65 NF-κB and c-Fos/AP-1 in HG-induced HO-1 expression were further confirmed by transfection with p65 aor c-Fos siRNA (Fig. 5d). Next, we found that HG stimulated HO-1 promoter activity, which was attenuated by NAC, DPI, mito-TEMPO, U0126, SP600125, Bay11-7082, or TSIIA (Fig. 5f), indicating that HG induced HO-1 promoter activity via ROS signals (i.e., Nox and mitochondrion)-dependent activation of MAPKs (e.g., ERK1/2 and JNK1/2) cascades linking to NF-κB and AP-1 pathways in RBA-1 cells (Fig. 5). These results are consistent with the reports indicating that angiotensin II regulates a variety of gene expression including HO-1 through a ROS-dependent activation of NF-κB in the heart [57], and alpha-lipoic acid induces HO-1 expression through the production of ROS and subsequent activation of the p44/42 MAPK pathway and AP-1 in vascular smooth muscle cells [58].

In the study, we first demonstrated that HG induces HO-1 expression via ROS-dependent activation of MAPK (i.e., ERK1/2 and JNK1/2) cascades linking to transcription factor NF-κB and AP-1 pathways in RBA-1 cells. Our recent report has demonstrated that HG induced MMP-9 expression via the similar signaling cascade linking to NF-κB pathway in RBA-1 cells [56]. Next, we further found that HG induces extracellular ROS and HO-1/CO released from brain astrocytes as a neurotoxic effector in the HG-CM. The effector may exacerbate SK-N-SH cell apoptotic death through a caspase-3-dependent manner. Based on the observations from literatures

and our findings, Fig. 6c depicts a model for the mechanisms underlying HG-induced HO-1 up-regulation and HG-derived ROS and HO-1/CO increase in astrocytes which may cause neuronal apoptosis. These findings concerning HG-derived ROS-mediated up-regulation of HO-1/CO system in brain astrocytes imply that HG might play a critical role in the processes of diabetes-induced CNS complications. Among brain cells, astrocytes may play an important role in high-glucose-induced neurodegeneration. The clinic application of this study hinted that pharmacological approaches targeting HG-induced HO-1 and their signaling components in astrocytes could provide useful therapeutic strategies for diabetes- and hyperglycemia-induced neurodegeneration in the future.

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