Excessive nNOS/NO/AMPK signaling activation mediated by the blockage of the CBS/H_2S system contributes to oxygen-glucose deprivation-induced endoplasmic reticulum stress in PC12 cells

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Abstract. Hypoxic-ischemia stress causes severe brain injury, leading to death and disability worldwide. Although it has been reported that endoplasmic reticulum (ER) stress is an essential step in the progression of hypoxia or ischemia-induced brain injury, the underlying molecular mechanisms are and have not yet been fully elucidated. Accumulating evidence has indicated that both nitric oxide (NO) and hydrogen sulfide (H_2S) play an important role in the development of cerebral ischemic injury. In the present study, we aimed to investigate the effect of the association between NO signaling and the cystathionine β-synthase (CBS)/H_2S system on ER stress in a cell model of cerebral hypoxia-ischemia injury. We found that oxygen-glucose deprivation (OGD) markedly increased the NO level and neuronal NO synthase (nNOS) activity. 3-Bromo-7-nitroindaz ole (3-Br-7-NI), a relatively selective nNOS inhibitor, abolished the OGD-induced inhibition of cell viability and the increased expression of ER stress-related proteins, including glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP) and cleaved caspase-12 in PC12 cells, indicating the contribution of excessive nNOS/NO signaling to OGD-induced ER stress. Furthermore, we found that OGD increased the phosphorylated AMP-activated protein kinase (p-AMPK)/AMPK ratio, and the AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribo furanoside (AICAR), attenuated the effects on OGD-induced ER stress, suggesting that OGD-induced NO overproduction results in AMPK activation in PC12 cells. We also found that OGD induced the downregulation of the CBS/H_2S system, as indicated by the decreased H_2S level in the culture supernatant and CBS activity in PC12 cells. In addition, we found that treatment with NaHS (a H_2S donor) or S-adenosyl-L-methionine (SAM, a CBS agonist) mitigated OGD-induced ER stress, as well as the NO level, nNOS activity and AMPK phosphorylation in PC12 cells. On the whole, these results suggest that the inhibition of the CBS/H_2S system, which facilitated excessive nNOS/NO/AMPK activation, contributes to OGD-induced ER stress.

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Introduction

A number of empirical studies have demonstrated that hypoxic and ischemic stress play a role in multiple human central nervous system (CNS) diseases including ischemic stroke, which is becoming one of the leading causes of mortality worldwide (1). Although various pathogenic mechanisms associated with ischemic stroke, such as oxidative stress, apoptosis, inflammation, calcium overload, endoplasmic reticulum (ER) stress and the disruption of the blood-brain barrier (BBB) have been put forward (2,3), the underlying mechanisms of hypoxic/ischemic-induced cerebral injury are unclear and remain to be fully explored. According to recent studies, ER stress is an essential step in neuronal injury resulting from cerebral ischemia and the modulation of ER stress provides a remarkable protective function in the ischemic brain (4,5). Therefore, it is of realistic significance to further investigate the potential mechanisms associated with ER stress under conditions of cerebral ischemia. It is generally known that oxygen-glucose deprivation (OGD) induces neuronal cell injury, and this model is commonly used in research to examine cerebral ischemic injury (6). Thus, in this study, we used PC12 cells, which were induced to differentiate by nerve growth factor, and were then exposed to OGD to establish a cerebral hypoxia-ischemia model, in order to investigate the underlying mechanisms of OGD-induced ER stress.

It is well established that nitric oxide (NO) is a physiological intercellular messenger in the CNS, synthesized by the nitric oxide synthase (NOS)-catalyzed reaction (7,8). There are three isoforms of NOS characterized in brain cells, namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (9). Increasing evidence has

confirmed that particularly excessive NO production by nNOS mediates excitotoxicity by promoting a cascade reaction under energy depletion-induced neuronal brain injury (10,11). However, the mechanisms through which elevated levels of NO result in neuronal cell death in ischemic brain injury are still unclear. AMP-activated protein kinase (AMPK) is a serine threonine kinase, considered as a key metabolic and stress sensor/effector (12). AMPK is activated under pathological conditions, incuding nutrient deprivation, vigorous exercise, or heat shock. It has been demonstrated that AMPK plays an essential role in cerebral ischemia; however, its role remains controversial (13,14). It has been shown that AMPK activation is detrimental and the inhibition of AMPK activation is protective under conditions of cerebral ischemia (15). By contrast, others have suggested that the activation of AMPK leads to neuroprotection (16). Notably, increasing evidence indicates that NO is an important activator of AMPK, and the activation of AMPK is muted in mice lacking nNOS under conditions of cerebral ischemia (17,18). However, the association between AMPK and NOS/NO in OGD-induced PC12 cell injury remains unclear.

Hydrogen sulfide (H_2S) is a third signaling gaseous mediator followed by CO and NO, predominantly produced from L-cysteine in the CNS by cystathionine β-synthase (CBS), and has a variety of physiological and pathophysiological functions in the CNS (19). Emerging evidence indicates that H_2S is considered as not only a neuromodulator, but also a neuroprotectant (20). It has also been shown that *in vivo* and *in vitro* models of cerebral ischemia injury, H_2S treatment significantly reduces the infarct size and ameliorates neurological function via its antioxidant, anti-apoptotic and anti-inflammatory effects, implying the therapeutic role of H_2S in cerebral ischemic stroke (21,22). In addition, H_2S reduces ER stress induced by multiple neurotoxins, such as 6-hydroxydopamine (6-OHDA) (23) and homocysteine (24), resulting in neuroprotective effects. Thus, we wished to investigate whether the disruption of endogenous H_2S generation is involved in OGD-induced ER stress. Furthermore, it has been shown that the complex interaction between CBS/H_2S and NO signaling plays an important role in ischemia/reperfusion (I/R)-related brain damage (25). The activity of CBS can be suppressed by NO (26,27), while H_2S increases eNOS activation and NO generation (28). Hence, the associatoin between these in cerebral ischemic injury is worthy of research.

In the present study, we observed that OGD induced ER stress accompanied by the upregulation of nNOS/NO/AMPK signaling and the downregulation of the CBS/H_2S system in PC12 cells. Furthermore, NaHS or inhibitor of the nNOS pathway attenuated the ER stress induced by OGD. Simultaneously, the promotion of the CBS/H_2S system attenuated OGD-induced nNOS/AMPK activation. These results demonstrated that OGD induced ER stress through the activation of nNOS/NO/AMPK signaling as a result of CBS/H_2S system blockage.

Materials and methods

Reagents. 3-Bromo-7-nitroindazole (3-Br-7-NI) was purchased from Tocris Cookson, Ltd. (Avonmouth, UK). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was obtained from Toronto Research Chemicals, Inc. (North York, ON, Canada). NaHS and S-adenosyl-L-methionine (SAM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Hoechst 33258, penicillin/streptomycin, RIPA lysis buffer and enhanced chemiluminescence (ECL) reagents were supplied by Beyotime Biotechnology (Shanghai, China). Cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). The Nitric Oxide Assay kit and Total Nitric Oxide Synthase Assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The primary antibodies against glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), cleaved caspase-12 and phosphorylated AMP-activated protein kinase (p-AMPK) were supplied by Cell Signaling Technology, Inc. (Beverly, MA, USA). Specific antibody to AMPK was obtained from Santa Cruz Biotechnology, Inc. (San Diego, CA, USA). All reagents were of the purest commercial grade.

Cell culture and model of OGD-induced cell injury and treatment. Highly differentiated rat adrenal pheochromocytoma (PC12) cells, obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO_2/95% air. To establish the model of OGD-induced cell injury, PC12 cells were exposed to OGD for 12 h by incubation in deoxygenated glucose-free and serum-free DMEM at 37˚C in a humidified atmosphere with 5% CO_2, 94% N_2, and 1% O_2 for 12 h. To investigate the effect of nNOS/NO signaling on OGD-induced injury, the PC12 cells were pre-treated with 3-Br-7-NI (a relatively selective nNOS inhibitor, 10 μM) for 30 min and then co-incubated with OGD for 12 h. To investigate the effect of the AMPK pathway on 3-Br-7-NI-caused the inhibition of ODG-induced injury in PC12 cells, PC12 cells ware pretreated with AICAR (20 μM) for 30 min and then incubated with 3-Br-7-NI (10 μM) for 30 min followed by exposure to OGD for 12 h.

Measurement of cell viability. The viability of the PC12 cells was evaluated by CCK-8 assay according to the manufacturer's instructions. In brief, the PC12 cells in the logarithmic phase were seeded into 96-well plate at a density of approximately $1x10^4$ cells/well overnight. At the end of drug treatment, 10 μl of CCK-8 reagent were added to each well, followed by incubation for 4 h at 37˚C. The absorbance at 450 nm was measured using a microplate reader (Epoch; BioTek, Winooski, VT, USA). The viability of the cells was expressed as a percentage of that of the control cells. The assays were performed in duplicate for 3 times.

Determination of intracellular NO levels and NOS activity. PC12 cells at the logarithmic growth phase were seeded in 6-well plates a density of approximately $1x10^6$ cells/well overnight. Following the different interventions, total protein was extracted in PBS with Ultrasonic Cell Disruption System (5 sec, 15 times, 4˚C) and quantified using the BCA Protein Assay kit. The NO level and total NOS activity were assessed using the Nitric Oxide Assay kit and Total Nitric Oxide Synthase Assay kit following the instructions provided by the respective manufacturers. Data related to the NO level are expressed as pmol/mg protein. Data related to total NOS activity are expressed as U/mg protein. Each independent experiment was repeated at least in triplicate.

Measurement of H_2S concentration. The level of H_2S in the cell culture supernatant was measured using the methylene blue spectrophotometric method in that H_2S and zinc acetate were co-incubated to form zinc sulfide which then dissolved in hydrochloric acid solution supplemented with *N*,*N*-dimethyl-*p*-phenylenediamine sulphate yielding and ferric chloride ($FeCl_3$), resulting in the formation of methylene blue, which was quantified spectrophotometrically. Briefly, the culture supernatant of PC12 cells with different interventions was collected following centrifugation for 5 min at 1,000 rpm. A total of 500 μl of supernatant was combined with 250 μl of zinc acetate (1%), 250 μl of *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 mmol/l) and 200 μl of $FeCl_3$ (30 mmol/l) in 500 μl of hydrochloric acid solution (10%). Following incubation for 15 min at room temperature, the absorbance of at 670 nm was measured by spectrophotometry (LAS-3000; Fujifilm, Tokyo, Japan). The H_2S concentration was calculated based on the standard curve which was generated by serial dilution of NaHS and was expressed in μmol/l.

Measurement of CBS activity. The PC12 cells subjected to the different treatments were collected and homogenized in potassium phosphate buffer (50 mmol/l, pH 6.8). Following centrifugation at 14,000 x g for 60 min at 4˚C, the supernatant was collected for enzyme assays. A mixing system comprising L-cysteine (0.5 mol/l), enzyme protein (0-100 μg), 5-pyridoxal phosphate/potassium phosphate buffer solution (100 mmol/l) and potassium phosphate buffer (100 mM, pH 7.4) was used, transferring the Eppendorf tubes from ice to a shaking water bath at 37˚C. Following incubation for 60 min, the reaction was terminated by the addition of w/v zinc acetate (1%) to trap H_2S followed by v/v trichloroacetic acid (10%) to precipitate proteins. Subsequently, *N*,*N*-dimethyl-*p*-phenylenediamine-sulfate in HCl (7.2 M) was immediately added to the reaction system followed by addition of $FeCl_3$ in HCl (1.2 M). The absorbance of at 670 nm was measured by spectrophotometry (LAS-3000; Fujifilm) and the H_2S content was calculated against a calibration curve of standard NaHS solutions. The CBS activity was expressed as the amount of H_2S generated per mg of reaction samples, with a unit of nmol/mg protein.

Measurement of caspase-3 activity. The activity of capsase-3 was detected using a commercial kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. The absorbance at 405 nm was detected using a microplate reader (Epoch; BioTek). Data were expressed as the fold of the control cells.

Western blot analysis. The PC12 cells were homogenized in RIPA lysis buffer at 4˚C for 30 min and the supernatant was collected following centrifugation at 12,000 rpm for 10 min at 4˚C. The protein concentration was measured using the BCA Protein Assay kit. Equal amounts of proteins were separated by 10-15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). After blocking for 2 h in TBS with 0.01% Tween-20 (TBST) containing 5% skim milk at room temperature, the membranes were incubated with primary antibodies against CBS (1:2,000; ab96252; Abcam, Cambridge, UK), AMPK (sc-19128; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), p-AMPK (1:2,000; cat. no. #2537), GRP78 (1:2000; cat. no. #3183) and CHOP (1:2,000; cat. no. #2895) (all from Cell Signaling Technology, Inc), cleaved caspase-12 (1:1,000; ab62484; Abcam), and β-actin (1:1,000; cat. no. #4970; Cell Signaling Technology, Inc.) overnight at 4˚C, respectively. After washing with TBST for 3 times, the membranes were incubated with the appropriate diluted horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. Subsequently, the membranes were washed again and the protein bands were detected using the ECL system (ZsBio, Beijing, China). The integrated optical density was calculated using ImageJ 1.4 6i software. The amount of protein was represented as a percentage of that of the control cells.

Statistical analysis. Data are expressed as the means ± SEM. The analysis of significant differences groups was performed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. Differences were considered statistically significant at $P<0.05$.

Results

OGD induces cytotoxicity and ER stress by promoting nNOS/NO signaling in PC12 cells. As NOS/NO signaling is known to play a role in cerebral ischemic injury (10,29), in this study, we first investigated the alternations of intracellular NO generation in OGD-exposed PC12 cells. We found that the exposure of PC12 cells to OGD for 12 h caused an obvious increase in the level of NO in PC12 cells (Fig. 1A). To investigate the possible role of nNOS activity in mediating OGD-induced NO generation in PC12 cells, we detected the activity of nNOS and found an increase in the activity of nNOS following the exposure of the PC12 cells to OGD for 12 h (Fig. 1B). In addition, we examined the effect of the OGD-induced increase in nNOS/NO signaling on ER stress in PC12 cells. We found that incubation of the PC12 cells with 3-Br-7-NI (10 μM), a relatively selective nNOS inhibitor, for 30 min significantly abrogated the OGD-induced decrease in the viability of the PC12 cells (Fig. 1C). Simultaneously, pre-treatment with 3-Br-7-NI attenuated OGD-induced ER stress, as evidenced by the decreased expression of ER-related proteins, including GRP78 (Fig. 1D), CHOP (Fig. 1E) and cleaved caspase-12 (Fig. 1F) proteins in PC12 cells. Of note, 3-Br-7-NI treatment alone had no effect on cell viability and ER-related protein expression. Taken together, these results suggest that the OGD-mediated NO overproduction via the increased activity of nNOS, is in part, responsible for nerve cytotoxicity and ER stress during ischemia-induced cerebral injury.

nNOS/NO signaling is involved in OGD-induced AMPK activation in PC12 cells. A previous demonstrated the interaction between NO and AMPK activation in stroke (17). Thus, we wished to determine whether this interaction exists in OGD-exposed PC12 cells. We found that exposure to OGD for 12 h led to a marked increase in the expression of phosphorylated AMPK (p-AMPK Thr-172) and in the ratio of p-AMPK/AMPK in the PC12 cells (Fig. 2A), indicating that OGD induced AMPK activation. Subsequently, in order to determine whether the OGD-induced AMPK activation is dependent on the enhancement of nNOS/NO signaling, the PC12 cells were pre-treated

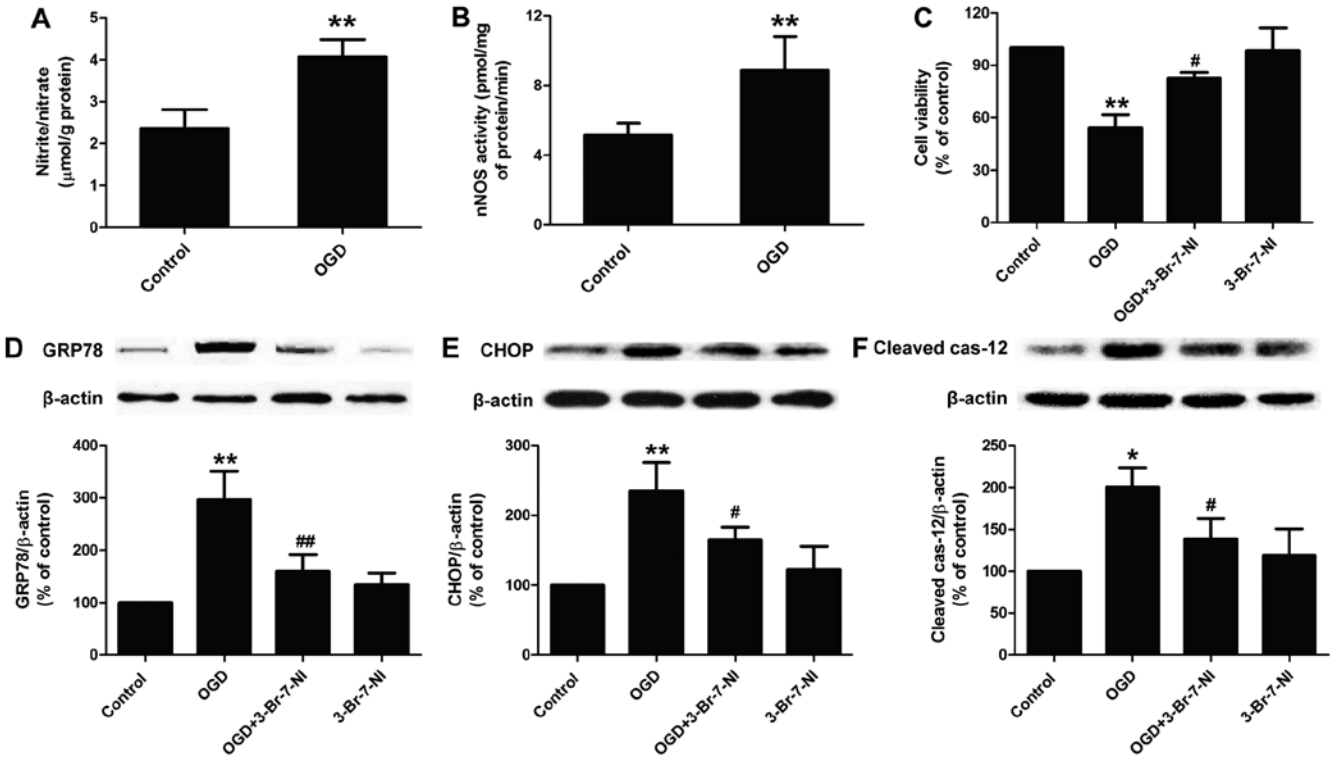

Figure 1. Effect of nNOS/NO signaling on OGD-induced cytotoxicity and ER stress in PC12 cells. PC12 cells were incubated with OGD for 12 h in the presence or absence of 3-Br-7-NI (a relatively selective nNOS inhibitor, 10 μM). (A) The NO levels were measured by Nitric Oxide Assay kit as described in the Materials and methods. (B) NOS activity was detected by Total Nitric Oxide Synthase Assay kit as described in the Materials and methods. (C) Cell viability was measured by CCK-8 assay. The expression levels of (D) GRP78, (E) CHOP, and (F) cleaved caspase-12 proteins were detected by western blot analysis. Data are presented as the means ± SEM from independent experiments performed in triplicate. *P<0.05, **P<0.01 as compared to untreated control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 as compared to the group exposed to OGD alone. nNOS, neuronal nitric oxide synthase; NO, nitric oxide; OGD, oxygen-glucose deprivation; ER, endoplasmic reticulum; NOS, nitric oxide synthase; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein.

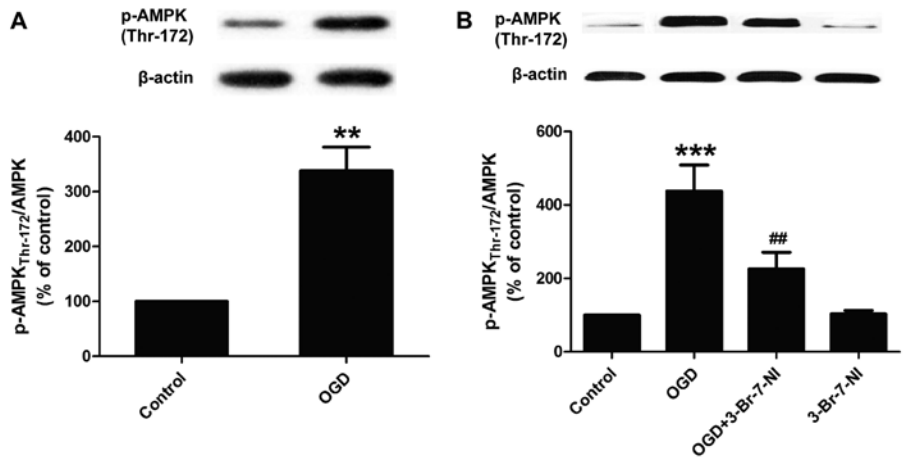

Figure 2. Effect of nNOS/NO signaling on the OGD-induced phosphorylation of AMPK in PC12 cells. PC12 cells were incubated under conditions of OGD for 12 h in the presence or absence of 3-Br-7-NI (a relatively selective nNOS inhibitor, 10 μM). The expression of p-AMPK (Thr-172) and AMPK were measured by western blot analysis. Data are presented as the means ± SEM from independent experiments performed in triplicate. **P<0.01, ***P=0.01 as compared to the untreated control group; $^{\#\#}$P<0.01 as compared to the group exposed to OGD alone. nNOS, neuronal nitric oxide synthase; NO, nitric oxide; OGD, oxygen-glucose deprivation; p-AMPK, phosphorylated AMP-activated protein kinase.

with 3-Br-7-NI (a nNOS inhibitor, 10 μM) for 30 min and then co-exposed to OGD for 12 h. We found that 3-Br-7-NI markedly abolished the OGD-induced increase in the phosphorylation levels of AMPK (Fig. 2B) in the PC12 cells. These results indicated that the OGD-induced activation of AMPK was dependent on nNOS/NO signaling; this in turn, may potentially promote neuronal injury during cerebral ischemia and anoxia.

Activation of AMPK signaling attenuates the inhibitory effects of 3-Br-7-NI on OGD-induced excessive ER stress in PC12 cells. To further demonstrate whether AMPK activation is involved in OGD-induced ER stress mediated by the nNOS/NO system, the effect of AICAR, an AMPK activator, on ER stress was investigated. As shown in Fig. 3A, pre-treatment with AICAR (20 μM) attenuated the promoting

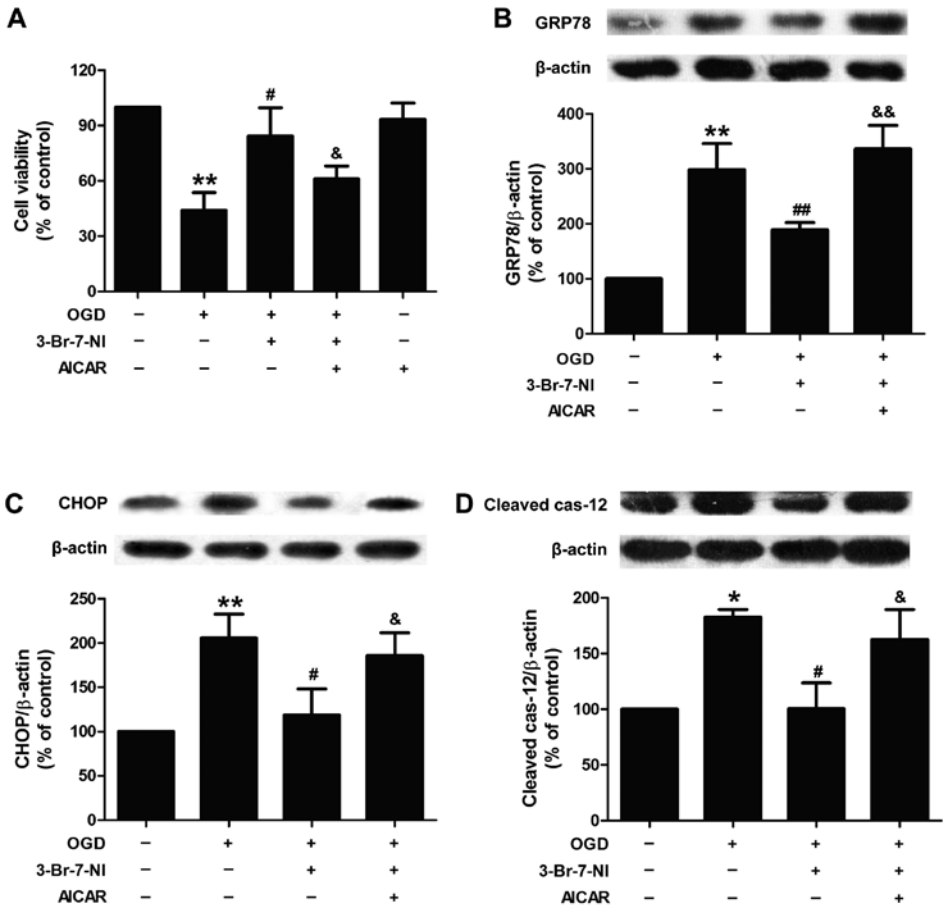

Figure 3. Effect of AMPK activator (AICAR) on the inhibition of ODG-induced ER stress in PC12 cells by a relatively selective nNOS inhibitor (3-Br-7-NI). PC12 cells were pre-treated with AICAR (20 μM) for 30 min and then incubated with 3-Br-7-NI (10 μM) for 30 min followed by exposure to OGD for 12 h. (A) Cell viability was measured by CCK-8 assay. The expression levels of (B) CRP78, (C) CHOP, and (D) cleaved caspase-12 proteins were measured by western blot analysis and β-actin was used as a loading control. Data are presented as the means ± SEM from independent experiments performed in triplicate. *P<0.05, **P<0.01 as compared to the untreated control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 as compared to the group exposed to OGD alone; $^{\&}$P<0.05, $^{\&\&}$P<0.01 as compared to the group exposed to OGD and treated with 3-Br-7-NI. nNOS, neuronal nitric oxide synthase; OGD, oxygen-glucose deprivation; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein.

effects of 3-Br-7-NI on the viability of the OGD-exposed PC12 cells, indicating that OGD induced cytotoxicity via NOS-activated AMPK. In addition, we found that pre-treatment with AICAR markedly abolished the inhibitory effects of 3-Br-7-NI on OGD-induced ER stress, as evidenced by the upregulated expression of GRP-78 (Fig. 3B), CHOP (Fig. 3C) and cleaved caspase-12 (Fig. 3D). These results indicated that the OGD-induced increase in nNOS/NO/AMPK signaling contributed to OGD-induced ER stress.

Blockage of the CBS/H_2S system contributes to OGD-induced ER stress in PC12 cells. We then investigated the role of the CBS/H_2S system in OGD-treated PC12 cells. As shown in Fig. 4, we found that exposure of the PC12 cells to OGD for 12 h markedly reduced the level of H_2S in the culture supernatant (Fig. 4A). In addition, the activity of CBS was also attenuated by exposure to OGD (Fig. 4B). These results suggest that exposure to OGD results in the downregulation of the CBS/H_2S system. In order to further confirm the role of the CBS/H_2S system in OGD-induced neuronal injury, NaHS (a donor of H_2S) and SAM (a CBS agonist) were used. We found that pre-treatment with NaHS (200 μM) for 30 min and SAM (100 μM) for 1 h markedly attenuated the OGD-induced decrease in cell viability, while NaHS or SAM treatment alone had no effect on the viability of PC12 cells (Fig. 4C). At the same time, both NaHS and SAM abolished the OGD-induced increase in the expression of ER stress-related marker proteins, including GRP78 (Fig. 4D), CHOP (Fig. 4E) and cleaved caspase-12 (Fig. 4F) in PC12 cells. These results indicate that OGD causes cytotoxicity and ER stress via the inhibition of the CBS/H_2S system.

Enhancement of CBS/H_2S by NaHS and SAM mitigates the OGD-induced activation of nNOS/NO and AMPK in PC12 cells. Finally, we further investigated the association of CBS/H_2S, nNOS/NO and AMPK in the PC12 cells exposed to OGD. We found that the increased levels of H_2S induced by NaHS (200 μM) and the enhanced activity of CBS induced by SAM (100 μM) distinctly reversed the OGD-induced NO overproduction, as evidenced by a decrease in the levels of NO (Fig. 5A) and in the activity of nNOS (Fig. 5B), indicating that the inhibition of the CBS/H_2S system mediates the OGD-induced upregulation of the nNOS/NO system. Simultaneously, NaHS or SAM treatment also reduced the ratio of p-AMPK (Thr-172)/AMPK in the PC12 cells exposed to OGD (Fig. 5C), suggesting that OGD induced AMPK activation by suppressing the CBS/H_2S system.

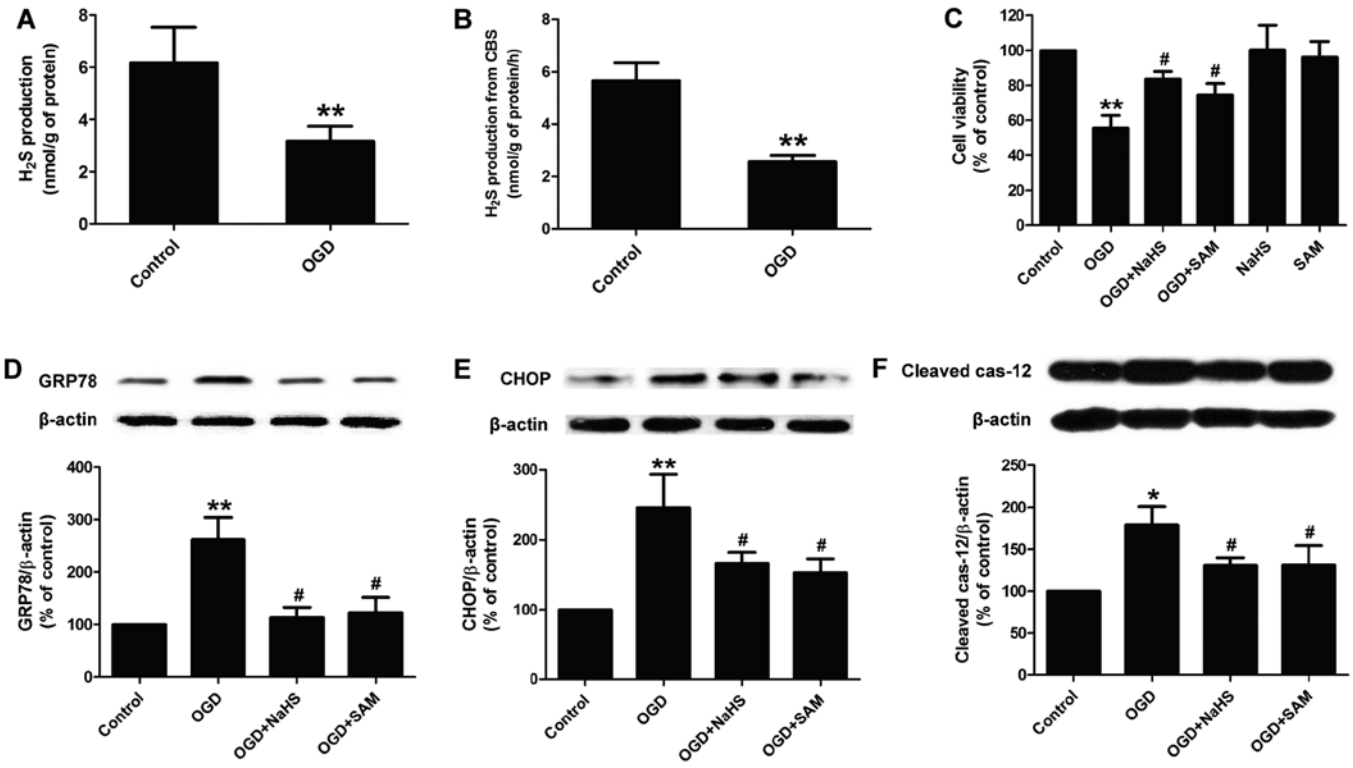

Figure 4. Effect of the CBS/H_2S system on OGD-induced ER stress in PC12 cells. (A) The level of H_2S in thye culture supernatant was detected using the methylene blue spectrophotometric method. (B) The activity of CBS was determined by the *N,N*-dimethyl-*p*-phenylenediamine sulphate method. (C) The viability of PC12 cells was measured by CCK-8 assay. The expression levels of (D) GRP78, (E) CHOP, (F) cleaved caspase-12 proteins were measured by western blot analysis and β-actin was used as a loading control. Data are presented as the means ± SEM from independent experiments performed in triplicate. $^{**}P<0.01$ as compared to the untreated control group; $^{\#}P<0.05$ as compared to the group exposed to OGD alone. CBS, cystathionine β-synthase; H_2S, hydrogen sulfide; OGD, oxygen-glucose deprivation; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein.

Discussion

In the present study, we explored the roles of nNOS/NO/AMPK signaling and the CBS/H_2S system in OGD-induced ER stress in PC12 cells. In this study, to the best of our knowledge, we demonstrate for the first time that OGD causes ER stress through the activation of the nNOS/NO/AMPK pathway resulting from the inhibition of the CBS/H_2S system.

According to the World Health Organization, approximately 15 million individuals each year suffer from cerebral ischemic injury, such as stroke (30). Despite this, the understanding of the mechanisms underlying cerebral ischemic injury remains extremely limited. Accumulating evidence has indicated that ER stress plays an important role in the progression of brain neuronal injury resulting from I/R (31-33). Certain stimuli, such as ischemia and hypoxia may trigger the accumulation of unfolded proteins in the ER, leading to excessive or aberrant ER stress and accelerated nerve damage (34). These findings suggest that the elucidation of mechamisms responsible for ER stress signaling may provide a novel target for effective therapeutic approaches for cerebral ischemia.

NO is a physiological mediator generated from L-arginine and oxygen by various forms of NOS, including eNOS, nNOS and iNOS in the brain (35). Increasing evidence reveals a wide range of roles for NOS/NO signaling involved in the occurrence and development of ischemic brain or brain ischemic injury (36). However, NO acts in a protective or deleterious manner depended upon the NOS isoform (37). It has been reported that hypoxic-ischemic injury is attenuated in mice deficient in nNOS, but is exacerbated in eNOS-deficient mice (38,39), indicating that NO overproduction released from nNOS contributes to brain damage. Other studies have further demonstrated that NO is a mediator of neuronal damage following cerebral ischemia (40,41) and the inhibition of NOS decreases BBB disruption, leading to neuroprotective effects under conditions of I/R injury during acute hypertension in rats (42). Furthermore, Nadjafi *et al* found that NO production was increased during OGD/reperfusion in OLN-93 oligodendrocytes (43). Consistent with these studies, our study demonstrated that exposure to OGD markedly increased the level of NO and the activity of nNOS in PC12 cells. Notably, an experimental study detected that 3-Br-7-NI, a potent and selective nNOS inhibitor, attenuated brain ischemic injury in diabetic stroke via the inhibition of the ER stress pathway (44), implying the mediation of NOS/NO signaling in ER stress under conditions of cerebral ischemia. Similar to this, the current study demonstrated that 3-Br-7-NI pre-treatment also mitigated OGD-induced ER stress, as evidenced by the down-regulated expression of ER-related proteins, such as GRP78, CHOP and cleaved caspase-12 in PC12 cells. Thus, these results suggest that OGD induces ER stress through the enhancement of nNOS/NO signaling.

AMPK has been reported to be present in most mammalian tissues, including the brain (45). It is increasingly becoming recognized that alternations in AMPK activation are not only related to metabolic needs, but are also related to sensing and

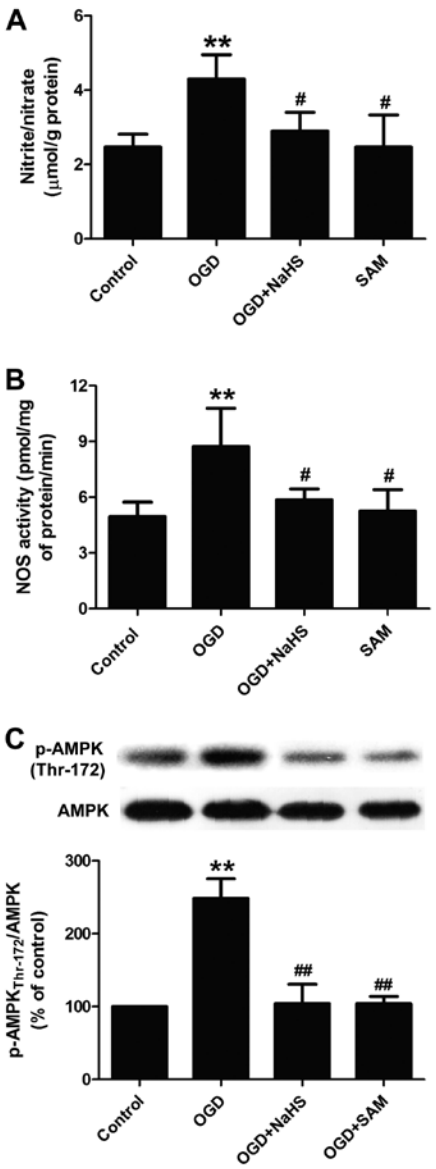

Figure 5. Effect of NaHS and SAM on the OGD-induced NO overproduction and AMPK activation in PC12 cells. PC12 cells were pre-treated with NaHS (200 μM) for 30 min and SAM (100 μM) for 1 h followed by exposure to OGD for 12 h. (A) The levels of NO were measured using the Nitric Oxide Assay kit as described in the Materials and methods. (B) The activity of NOS was detected using the Total Nitric Oxide Synthase Assay kit. (C) The expression levels of p-AMPK (Thr-172) and AMPK were measured by western blot analysis and β-actin was used as a loading control. Data are presented as the means ± SEM from independent experiments performed in triplicate. **P<0.01 as compared to the untreated control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 as compared to the group exposed to OGD alone. OGD, oxygen-glucose deprivation; NO, nitric oxide; NOS, nitric oxide synthase; p-AMPK, phosphorylated AMP-activated protein kinase.

responding to 'various cell stress', such as ischemia, hypoxia and energy depletion (46). However, little is known regarding the physiological and pathological functions of AMPK and the mechanisms through which AMPK activation occurs in the brain during ischemia. Of note, some studies have shown that cerebral ischemia increases the activation of AMPK in the brain in an NO-dependent manner, which is muted in mice lacking nNOS (17,18), indicating the important role of NOS/NO signaling in AMPK activation. Nevertheless, the interactions between AMPK, NO and NOS warrant further investigation, particularly under the condition of cerebral ischemic injury. Our finds indicated that exposure to OGD increased the phosphorylation of AMPK in PC12 cells, while this effect was abolished by pre-treatment with 3-Br-7-NI, indicating that the OGD-induced AMPK activation is dependent on nNOS/NO signaling. In addition, we found that the AMPK activator, AICAR, attenuated the protective effects of 3-Br-7-NI against OGD-induced cytotoxicity and ER stress in PC12 cells. These results suggest that OGD causes ER stress by increasing the activity of AMPK, as a result of nNOS/NO signaling promotion.

Emerging evidence indicates that H_2S plays a broad range of roles in cerebral ischemic injury (20,47). In *in vivo* model of cerebral I/R injury, H_2S pre-conditioning improved neurological function and decreased the infarct size, implying that H_2S plays a therapeutic role in cerebral ischemic stroke (21). In addition, a number of studies have confirmed that H_2S attenuates ER stress induced by multiple stresses and neurotoxins, including chronic unpredictable mild stress, homocysteine and 6-OHDA (23,24,48), exerting neuroprotective effects. However, the association of H_2S and ER stress in cerebral ischemic injury is not yet understood. Thus, we hypothesized that the disruption of H_2S may be also involved in ER stress in PC12 cells exposed to OGD. In the current study, we found that exposure to OGD markedly reduced the level of H_2S in the culture supernatant, as well as the activity and expression of CBS, indicating that the CBS/H_2S system was inhibited by OGD. These findings are consistent with those of the study by Shen *et al*, who demonstrated that in acute ischemic conditions, CBS is upregulated and activated followed by causing an increased production of H_2S (49). In addition, we found that NaHS abolished the OGD-induced decrease in the viability of the PC12 cells and the increase in ER stress, as evidenced by the increased expression of GRP78, CHOP and cleaved caspase-12 in the PC12 cells following treatment with NaHS. These results indicated that blocking the CBS/H_2S system contributes to OGD-induced ER stress. .

An increasing number of studies have revealed the complex association between H_2S and NO, which has an indispensable effect in multiple diseases (50,51). In another study, Grossi proved that H_2S is regarded as a co-factor responsible for the generation of NO (52). In addition, another finding is that H_2S directly inhibits eNOS, as well as nNOS, contributing to the dual modulation of vascular tension (53). In the present study, we further investigated the association between the CBS/H_2S system and nNOS/NO signaling in OGD-exposed PC12 cells. We found that pre-treatment with NaHS markedly attenuated OGD-induced NO overproduction by the excessive activation of nNOS and AMPK activation in PC12 cells. These results indicated that OGD induced the activation of nNOS/NO/AMPK signaling activation through the inhibition of CBS/H_2S, leading to ER stress.

In conclusion, our findings demonstrate that exposure to OGD induces ER stress, and the activation of the nNOS/NO/AMPK pathway results from the attenuation of the CBS/H_2S system. Our findings provide a better understanding of the signal transduction mechanisms involved in the pathophysiological process of cerebral ischemia-induced ER stress, which provides novel targets and guidance for the development of neuroprotective agents.

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