Down-modulation of Bis sensitizes cell death in C6 glioma cells induced by the oxygen-glucose deprivation through inhibiting NF-kappa B activity

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Directed by Professor Young Soo Ahn

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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Bcl-2 interacting cell death suppressor (Bis) has been implicated in anti-stress and anti-apoptotic pathways. Previous study showed a significant induction of Bis in the reactive astrocytes of rat hippocampus after transient forebrain ischemia. To investigate the significance of its induction in the ischemic injury, the expression of Bis was reduced by si-RNA strategy in C6 glioma cells, which were then exposed to oxygen-glucose deprivation (OGD). *Bis* knock-down resulted in a decrease in viability after OGD accompanied by ROS accumulation. Among the cellular antioxidants, superoxide dismutase (SOD) activity was significantly perturbed in *bis* si-RNA treated cells (*bis*-kd C6). A Subsequent analysis of in-gel reduction ability and quantification of mRNA revealed that induction of SOD1 in response to OGD was impaired at transcriptional levels as a result of *bis* knock-down. As a candidate for the transcription factor for *sod1* gene, the activity of NF-κB was determined using a DNA binding assay and the nuclear translocation of p65 subunit, showing that activation of NF-κB was attenuated in *bis* si-RNA transfected C6 cells. The treatment of SOD mimetic resulted in the recovery of the viability of C6 cells upon OGD, which is more prominent in *bis* si-RNA treated C6 cells. Furthermore, the inhibition of NF-κB suppressed the induction of SOD1 transcription and aggravated the cell death, which was potentiated with

reduction of Bis, after OGD. The constitutive levels of p65 were decreased by Bis reduction while Bis overexpression increased p65 protein levels, but not mRNA levels. These results suggest that one of the physiological function of Bis is the regulation of p65 protein stability and thereby NF-κB activity which affects the induction of SOD1 after oxidative stress and subsequent cell viability. The critical mechanisms by which Bis control the p65 stability remain to be determined.

Key words: Bis, oxygen-glucose deprivation, NF-κB, superoxide dismutase, C6

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I. INTRODUCTION

The Bcl-2 interacting death suppressor (Bis), also referred to as Bag3 and CAIR-1, has been shown to be a Bcl-2 binding protein as evidenced by protein interaction techniques [1-4]. *In vitro* DNA transfer experiments show that Bis significantly enhances anti-apoptotic activity of Bcl-2, indicating that Bis is a modulator of apoptotic processes [1]. In support of this, Bis expression is increased in several tumors including pancreatic cancer, thyroid tumors and certain forms of leukemia, while its down-modulation sensitizes B-CLL to apoptotic cell deaths induced by several anti-cancer drugs [5-8]. In addition to pro-survival ability of Bis, its ability in regulating adhesion and migration may also contribute to tumorigenesis and metastasis of malignant tumor *in vivo* [9, 10]. Bis is also thought to play a role in cellular reaction to stress, as evidenced by the observation that Bis is induced in response to several stressful conditions, including exposure to high temperature, heavy metals, low intensity pulsed ultrasound and proteasome inhibitors [4, 11]. Although the precise function of Bis in the cells that are exposed to stressful environment is not clear, the modulating activity of Bis on chaperon machinery via interaction with ATPase domain of HSP70 suggest that Bis is involved in the

regulating protein stability. Moreover, when Bis is overexpressed, the degradation of Htt43Q, an aggregation-prone protein is accelerated, via stimulating autophagy in concert with HSPB8 [12, 13]. Thus, these results suggest that Bis is implicated in the protein quality control under non-physiological conditions.

It is also noteworthy that, in addition to the stressful conditions given for cellular levels, the expression of Bis is significantly increased in animal disease models for stroke, seizure and retinal degeneration [14-16]. Specifically, Bis is up-regulated in reactive astrocytes in the rat hippocampus after transient forebrain ischemia or kainic acid treatment [14, 15]. Moreover, Bis is strongly expressed in reactive astrocytes in area of gliosis in the brain of human immunodeficiency virus (HIV) encephalopathy patients, suggesting that Bis is involved in adaptive response of glial cells [17]. However, the physiological significance of Bis induction in the reactive astrocytes is currently unclear.

Oxidative stress has been implicated as a major aspect of the pathophysiology of ischemic brain injury, in which the reactive oxygen species (ROS), generated during reperfusion, induce various cellular damages [18]. Light damage, which induces Bis expression in mouse retina, has been also shown to involve oxidative stress, as evidenced by the fact that several antioxidant genes are up-regulated following photo injury [16, 19]. Furthermore, down-modulation of Bis potentates diethylmaleate-induced apoptosis [20]. The latter is a glutathione-deprivation agent in normal and neoplastic leukocytes. Collectively, these previous findings suggest that Bis could participate in regulation of cellular response to oxidative stress *in vivo* as well *in vitro*.

The family of nuclear factor-kappaB (NF-κB) is well known ubiquitous transcription factors that mediate diverse biological processes, ranging from inflammation to cell death [21]. In mammals, NF-κB family consists of the five subunits p50, p52, RelA (p65), c-Rel and RelB. Generally, NF-κB is sequestered in the cytoplasm, bound to an inhibitory molecule, inhibitor kappa B (IκB). Exposure to a variety kind of signals results in the phosphorylation and subsequent degradation of IκB by the 26S proteasomes. Then the hetero- or homo-dimer of NF-κB translocate into the nucleus and bind to κB-sites of

target genes and initiate gene expression. There is plenty of *in vivo* and *in vitro* evidence for activation of NF-κB in astrocytes during oxidative stress. For examples, hydrogen peroxide, oxygen-glucose deprivation, ionizing radiation and HIV-1 Tat have been shown to activate NF-κB and to modulate cell death [22-27]. However, the consequence of activation of NF-κB by oxidative stress is thought to be cell- and stimulus-type dependent. In non-neuronal cells, NF-κB is usually considered as a cell survival factor [28]. However, in neuronal system, it remains the question of whether NF-κB is a cell survival factor or a cell death factor [29]. Therefore, the anti-cell death or pro-cell death role of NF-κB against oxidative stress remains unclear to the present.

Superoxide dismutases (SODs) are the primary line of antioxidant enzymes against oxidative stress, which is considered as pivotal factor in several neurodegenerative diseases [30]. They constitute a family of enzymes that catalyzes the conversion of superoxide anions to molecular oxygen and hydrogen peroxide. They include the copper-zinc-containing SOD (SOD1) in the cytoplasm with a small fraction in the mitochondria intermembrane space and the manganese-containing SOD (SOD2) in the mitochondria and the Ec-SOD (SOD3) in the extracellular spaces. An ample of literatures demonstrates that the SOD overexpression might protect while, the SOD depletion might increase vulnerability against oxidative stress conditions [31].

Lim at al. recently reported that GMF, when transfected in rat glioma cells activates NF-κB, leading to an induction of SOD transcript and enzyme activity [32]. Additionally, study from other laboratory has shown that NF-κB is activated during OGD in mouse primary astrocytes and has a biphasic role on cell survival depending SOD expression [25]. Furthermore, other researcher has demonstrated that activation of NF-κB induces SOD and protects hippocampal neurons against reactive oxygen species-induced apoptosis [33]. Overall, these data indicate that oxidative stress could activate NF-κB and may induce SOD as a down stream target gene.

In the present study, therefore, the effect of repression of Bis expression on the cellular response to oxidative stress was investigated using an *in vitro*

hypoxia model based on oxygen-glucose deprivation (OGD) in C6 rat glioma cells [34]. The findings herein indicate that a decrease in Bis expression in C6 cells increases cell death induced by OGD, accompanied by impairment in the induction of superoxide dismutase (SOD), which is linked to inhibition of NF-κB.

II. MATERIALS AND METHODS

1. Cell culture and reagents

C6 cells, originating from a rat brain glioma, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained with DMEM (Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37℃ in a humidified incubator under 5% CO_2 and 95% humidified atmosphere.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless specified otherwise. TEMPOL was purchased from Calbiochem (La Jolla, CA, USA). DCF-DA was from Molecular Probe (Eugene, OR, USA).

2. Electroporation of small interfering RNA

Suppression of Bis expression was performed by transfection of specific si-RNA targeted for *bis* (5'-AAGGUUCAGACCAUCUUGGAA-3') using a microporator (MP-10; iNCYTO, Suwon, Korea) The *bis* si-RNA duplex targeted against nucleotides 1496-1518 of the *bis* messenger RNA sequence and the nonfunctional negative control si-RNA did not target any gene (Bioneer, Daejeon, Korea) [17]. Transfection of C6 cells were performed with si-RNA for *bis* and negative control si-RNA using electorporation according to the manufacturer's instruction. Cells, adjusted to a density of 2.5 x 10^5 cells in phosphate buffered saline (PBS), were transfected with si-RNA oligos using a microporator at 1,700 V and pulse width of 20 msec. The efficacy of si-RNA was confirmed by Western blotting.

3. OGD treatment and assessment of cell viability

After 48 hours of transfection, C6 cells were washed twice with degassed DMEM without glucose and serum, and then placed in an anaerobic chamber (Thermo Forma, Marietta, Ohio, USA) containing 85% (v/v) N_2, 10% (v/v) H_2, 5% (v/v) CO_2 at 37℃ for 6 hours. The viability was determined by reducing ability of 2-(4, 5-dimethyltriazol-2- yl)-2,5-diphenyl tetrazolium bromide (MTT; Duchefa, Haarlem, the Netherlands), an indication of metabolic activity [35]. The viability of C6 cells placed in DMEM without glucose and serum for the same time was used as control.

4. Measurement of ROS production

The levels of ROS in cells following OGD-treatment were determined using the fluorescent probe DCF-DA. At the end of treatment, cells were washed three times with PBS, incubated for 30 min with 10 μM DCF-DA, and then washed twice with PBS. Fluorescent intensities were observed at 4 hours after OGD using a fluorescence inverted microscope (IX71; Olympus, Tokyo, Japan) and photographed.

5. Determination of superoxide dismutase level

SOD activity was determined by zymography according to the method of Beauchamp and Fridovich with slightly modification [36]. Briefly, the cells were lysed and sonicated for 15 sec in ice-cold 50 mM potassium phosphate buffer pH 7.8, containing 1 mM EDTA. An equal amount of each sample (20 μg of protein) was electrophoresed in 12% (w/v) non-denaturing polyacrylamide gel. The gel was stained in 2 mg/ml NBT (nitroblue tetrazolium), 0.028 mM riboflavin, 30 mM TEMED, 50 mM potassium phosphate buffer pH 7.8, 1 mM EDTA for 15 min in the dark. The gel was rinsed in water and illuminated until clear zones of SOD activity were evident. SOD activity was

also determined by Superoxide Dismutase Activity Kit (Stressgen Bioreagents, Victoria, Canada).

6. Measurement of catalase activity and glutathione (GSH)

Catalase activity was determined as the ability of the cell extract to decompose a given concentration of H_2O_2. Hydrogen peroxide was measured fluorometrically using the Amplex Red catalase assay kit (Molecular Proves, Eugene, OR, USA). The method is based on the ability of the reagent, Amplex Red. Briefly, a solution of H_2O_2 was added to cell extracts in a total volume of 50 μl of a solution containing Amplex Red and HRP was added to each reaction and incubated for 30 min at 37℃ in the dark. The fluorescence was measured in a microplate reader (Victor3; Perkin Elmer, Turku, Finland) using excitation at 530 nm and emission detection at 595 nm.

Total amounts of GSH were determined using a commercially available kit (OxisResearch, Foster city, CA, USA) according to the manufacturer's instructions. For total GSH, OGD treated cells were harvested with 60 μl of ice-cold cell lysis buffer. Cell lysates were centrifuged and supernatants were mixed with 60 μl of 5% metaphosphoric acid and recentrifuged, and the supernatants obtained were mixed with assay buffer at a 10-fold dilution. Changes in absorbance at 412 nm were monitored for 3 min.

7. Quantitative real-time reverse transcription polymerase chain reaction (RT–PCR) and semi-quantitative RT-PCR

Total RNA isolated from cells using RNA-Bee (Tel-Test, Friendswood, TX, USA) and quantified by spectrophotometer (ND-1000; NanoDrop Technologies Inc., USA). 2 μg RNA was used for reverse transcription by using an AccuPower CycleScript (dN6) (Bioneer, Daejeon, Korea). To validate the expression level of the SOD1 and SOD2, a quantitative real time PCR was performed using the cDNA as templates and the specific primer sets [37, 38] (Table. 1). The amplification was carried out using Mx3000p cycler

(Stratagene, La Jolla, CA, USA) in a 25 μl reaction mixture containing 0.5~ 2 μl of diluted cDNA templates, 5 pmol of each primer and 12.5 μl of 2x SYBR® premix Ex Taq™ (TaKaRa Biotechnology Co., Dalian, China) including ROX dye. The reaction of each sample was performed in duplicate. 18S rRNA was used to normalize the expression levels of each sample [39].

Semi-quantitative RT-PCR was performed using a Top-Taq™ DNA polymerase kit (CoreBio Lifescience & Biotech, Seoul, Korea) following a protocol provided by the manufacturer. Briefly, 2 μl cDNA was added to a 18 μl reaction premix containing 1x buffer, 0.2 mM dNTP mix, 10 pmole forward and reverse primers (Table. 1) and 1.2 units DNA polymerase. The PCR reactions were performed with a master cycler gradient (Eppendorf, Hamburg, Germany). The reaction condition was an initial step of denaturation (5 min at 95°C), 35 cycles of amplification (95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min), and an extension (72°C for 5 min). Then, PCR products were electrophoretically analyzed on 1.5% agarose gels containing ethidium bromide.

Table 1. Primers used for RT-PCR

Gene		Sequences	GeneBank#
Bis	F	AAGCCAACAG CTAGAAGCCA AAGC	DQ631552
	R	AAGTTACTGC ATACCAGGCG GCTA	
SOD1	F	GGATTAACTGAAGGCGAGCA	NM_017050
	R	CAATCACACCACAAGCCAAG	
SOD2	F	CACGACCCACTGCAAGGAA	NM_017051
	R	GCGTGCTCCCACACATCA	
NF-κB p65	F	CATCAAGATCAATGGCTACA	NM_199267.2
	R	CACAAGTTCATGTGGATGAG	
β-actin	F	TCATGAAGTGTGACGTTGACATCCGTAAAG	NM_031144
	R	CCTAGAAGCATTTGCGGTGCACGATGGAGG	
18S rRNA	F	GTAACCCGTTGAACCCCATT	NM_213557
	R	CCATCCAATCGGTAGTAGCG	

8. Western blot analysis

Cells were lysed with radioimmunoprecipitation assay buffer (RIPA) on ice for 30 min. After centrifugation, the supernatant was used for Western blotting. The concentration of protein was measured by a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA) with BSA as a standard. Equal proteins were separated on 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated for 1 hour with 5% dry skim milk in TBST buffer to block nonspecific binding and then incubated with antibodies against Bis (1:10,000), HIF-1α (1: 500; Novus biologicals, Littleton, CO, USA), beta (β)-actin (1:10,000; Sigma-Aldrich, St. Louis, MO, USA), NF-κB p65, and LaminB (1:1,000; Santa Cruz Biotechnology, Santa Cruz, USA) [14, 15]. After washing with TBST (Tris, NaCl, 0.1% Tween 20), the membranes were then incubated with peroxidase-conjugated secondary antibodies (1:2000; Promega, Madison, WI, USA) for 1 hour. Immunoreactive bands were visualized with the enhanced chemiluminescence detection system (ECL; Amersham Pharmacia Biotech, Bucks, UK) according to manufacturer's protocol.

9. Electrophoretic mobility-shift assay (EMSA)

Cytoplasmic and nuclear extracts of OGD-treated cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Kit (PIERCE, Rockford, IL, USA). An EMSA was performed according to the method of Byun et al [40]. The oligonucleotide containing consensus recognition sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3') for NF-κB was obtained from Santa Cruz Biotechonology, and end-labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP. Binding reaction was performed with 10 μg of nuclear extract and the reaction products were analyzed on 6% PAGE in 0.5 x TBE buffer [22.5 mM Tris-HCl, pH 8.5, 22.5 mM borate, 0.5 mM EDTA]. The dried gel was exposed to film on intensifying screen for overnight at -70℃.

10. Statistics

Values are expressed as mean ± SEM of at least three experiments. Results were analyzed with Student's *t* test or analysis of variance (ANOVA), with $P < 0.05$ as the significant level.

III. RESULTS

1. Induction of Bis after OGD

In an earlier study, a significant increase of Bis expression has observed in reactive astrocytes after transient forebrain ischemia [14]. To determine if Bis is also induced by *in vitro* hypoxia, the expression levels of Bis were determined after OGD in C6 rat glioma cells. As shown in Fig. 1A, the levels of Bis in C6 cells that were exposed to OGD increased in a time dependent manner. The induction of Bis during reperfusion period was greater than that during hypoxic period. The significant increase in HIF-1α levels indicates the effective induction of hypoxic states in this system. The increase in Bis protein levels was due to an increase in *bis* mRNA levels, as evidenced by the semi-quantitative RT-PCR (Fig. 1B).

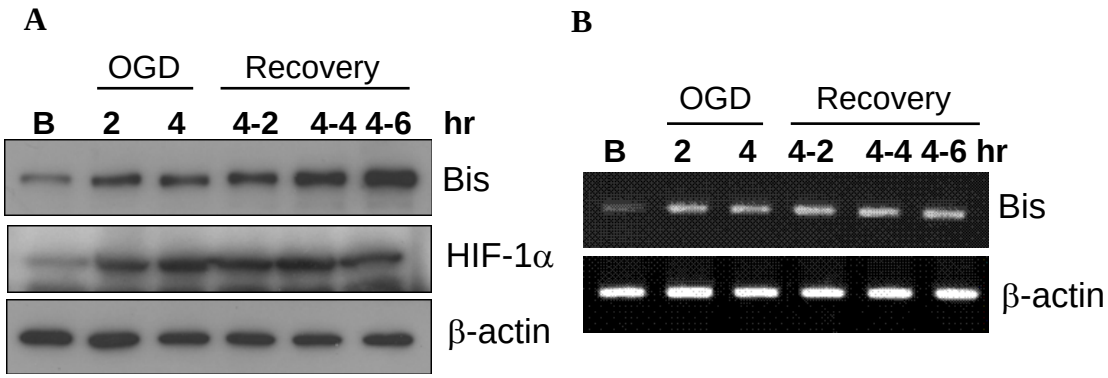

Figure 1. Induction of Bis after OGD in C6 cells.
C6 cells were exposed to OGD for 4 hours and then allowed to recover in normal medium for additional 6 hours. Bis expression at the indicated times was determined by Western blotting assay with the whole cell lysates (A) and by semi-quantitative RT-PCR (B). Beta (β)-actin expression was shown as internal control.

2. *Bis* knock-down increases cell death and ROS production following OGD

To define the physiological significance of Bis induction in response to OGD, Bis expression was down-regulated by *bis* specific siRNA oligonucleotides prior to exposure to OGD. Western blot shows that *bis* knock-down C6 (*bis*-kd C6) cells show a decrease in Bis protein levels to 30% of C6 cells transfected control siRNA (control C6) by densitometry analysis (Fig. 2A). Next, cell viability experiment was done to examine the effect of reduced Bis expression on cell death induced by OGD-insults. As determined by MTT assay, the viability of control C6 cells after 6 hours of OGD was 69 % as compared to the C6 cells that had not been exposed to OGD, whereas the viability of *bis*-kd C6 cells was 26%, indicating that reduced Bis expression increases the sensitivity of C6 cells to OGD insults (Fig. 2B).

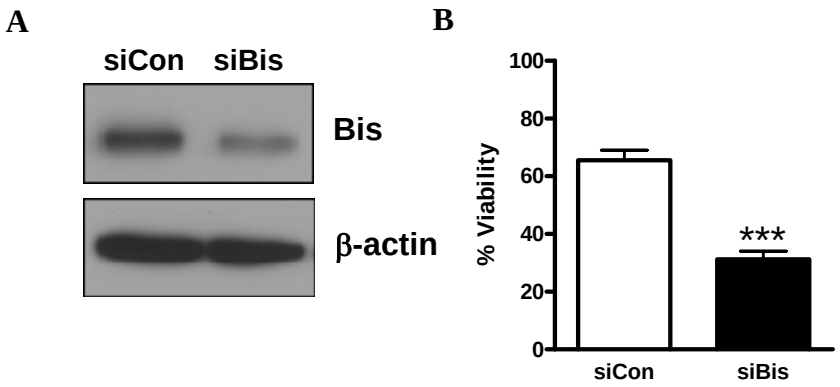

Figure 2. *Bis* knock-down increased cell death in C6 cells after OGD.

(A) Western blotting for Bis expression after transfection of control siRNA or specific siRNA targeted for *bis* mRNA into C6 cells for 48 hours.

(B) After transfection with control or *bis*-siRNA, C6 cells were exposed OGD for 4 hours and the viability was determined by MTT assay. The relative viability was represented as percent (%) of the viability of control or *bis*-kd C6 cells exposed to OGD to the viability of the corresponding cells exposed to glucose-deprived medium for 4 hours. *** $P < 0.001$ compared with control.

Since the down-regulation of Bis increased cell death as a result of OGD insults, ROS levels in the cells were measured to determine whether the reduced expression of Bis may be correlated with accumulation of reactive oxygen species, which are implicated in hypoxic damage to cells [18]. For this purpose, intracellular ROS accumulation was estimated by DCF-DA staining. As showed in Fig. 3, DCF fluorescence was elevated significantly in *bis*-kd C6 cells after 4 hours of OGD exposure.

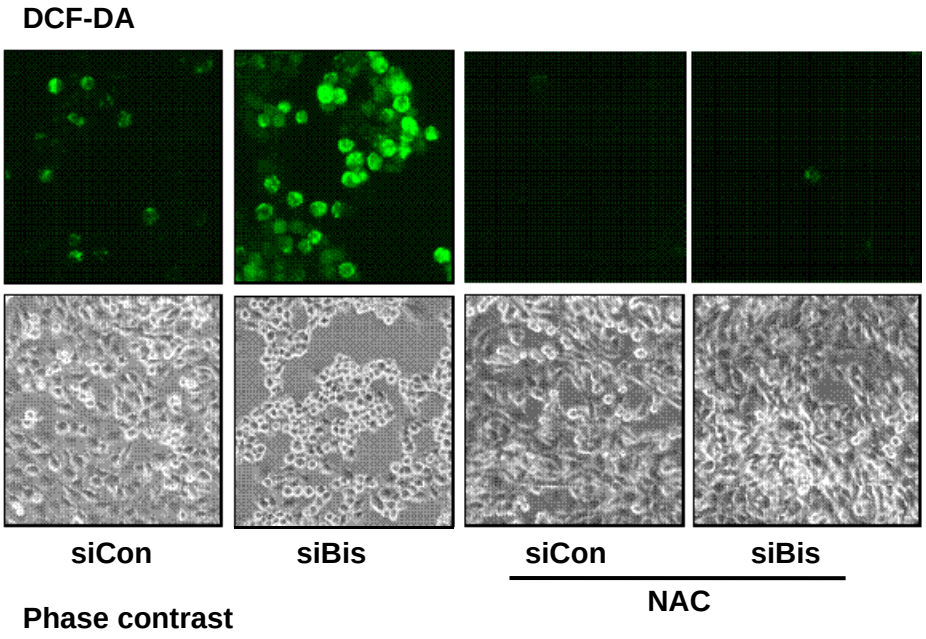

Figure 3. *Bis* knock-down increased accumulation of intracellular ROS in C6 cells after OGD.

ROS accumulation following OGD was examined under fluorescence microscopy after incubation with the DCF-DA in the presence or absence of NAC as described in materials and methods (upper panels). The cell morphology was observed by phase contrast microscopy (lower panels)

Furthermore, a pretreatment of 1 mM N-acetyl-L-cysteine (NAC), the representative ROS scavenger [41], nearly eliminated the DCF signals both in control and *bis*-kd C6 cells. These findings strongly suggest that Bis may well play a role in ROS generation or ROS elimination in the oxidative stress signaling pathway, which is associated with survival of cells.

3. Induction of superoxide dismutase upon OGD is impaired by *bis* knock-down

In order to explain the relation of Bis reduction and the accumulation of ROS upon OGD, the activities or amounts of several cellular antioxidants were measured after OGD. In control C6 cells, the activity of catalase was increased to 170% by OGD. In *bis*-kd C6, the basal activity of catalase was higher than that in control C6 cells which was further increased by OGD, from 160% to 200%, compared to the catalase activity of control C6 cells that had not been exposed OGD. Total GSH levels were not affected by OGD in either control C6 cells or *bis*-kd C6 cells (Fig. 4). In contrast, SOD activity was decreased by OGD in *bis*-kd C6 cells while it was increased in control C6 cells. It is known that total cellular SOD activity is determined by the sum of activities cytosolic SOD (SOD1) and mitochondrial SOD (SOD2) [42, 43]. Then, SOD zymography was performed to determine which SOD activity is affected by Bis expression levels [36]. Fig. 5A shows that a gradual increase in the intensity of SOD1 in control C6 cells occurs, which is decreased in *bis*-kd C6 cells during OGD. The quantitative analysis of mRNA levels for SOD1 indicated that SOD1 mRNA levels were significantly increased by OGD in control C6 cells but it was rather decreased in *bis*-kd cells (Fig. 5B). The induction of SOD2 mRNA levels was not as significant as SOD1 in control C6 cells but in *bis*-kd C6 cells, the transcript levels for SOD2 were decreased only slightly after 4 h of OGD. To confirm if the effect of the down-modulation of Bis in decreasing cell survival is related with insufficient levels of SOD, a SOD mimetic, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL) was added to the media before OGD and determined the degree of

recovery in cell death. As shown in Fig. 6, the pretreatment with TEMPOL increased the viability of control cells from 60% to 69% to OGD, while pretreatment with TEMPOL led to a more marked recovery in cell viability in *bis*-kd C6 cells, from 38% to 54%. These findings suggest that the decrease in Bis expression resulted in an impairment of SOD1 induction to OGD and the subsequent accumulation of ROS, which sensitize C6 cells to oxidative stress.

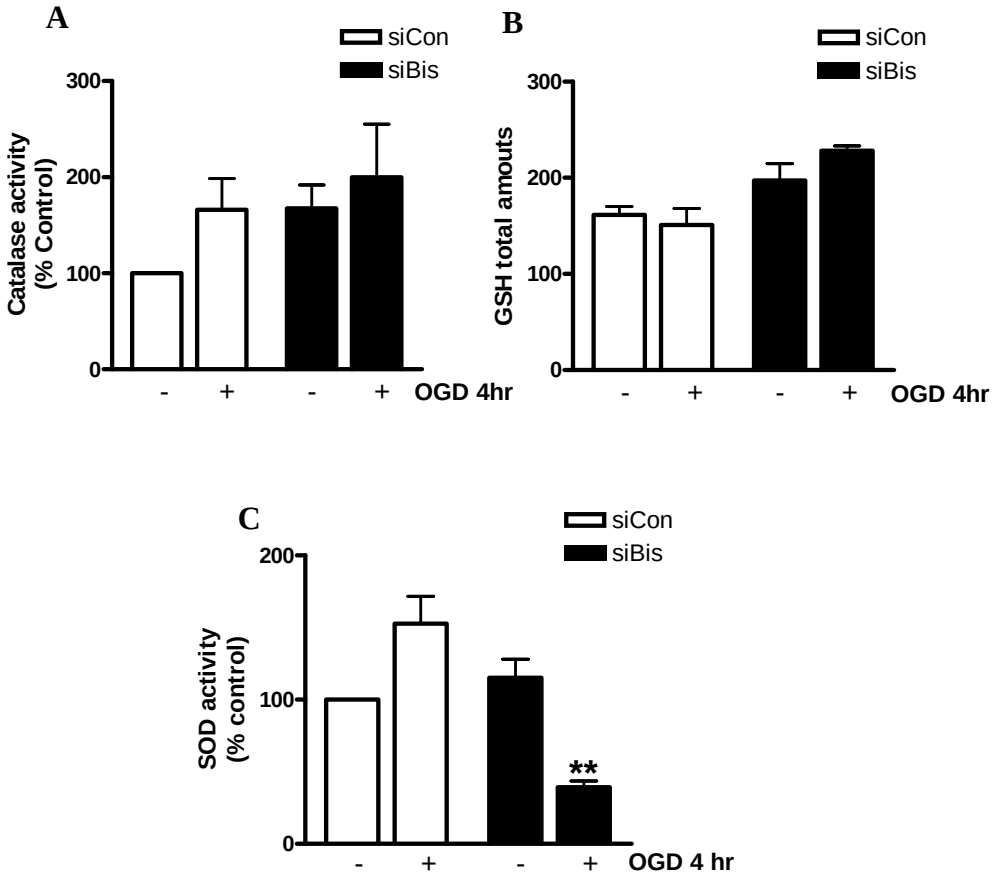

Figure 4. Bis depletion was correlated with repression of SOD activity to OGD in C6 cells.

After 4 hours of OGD, the activity of catalase (A) and the total amounts of GSH (B), and SOD activity (C) were determined from control C6 and *bis*-kd C6 cells using commercial kits as described in materials and methods. The relative activity for catalase or SOD was represented as percent (%) of the activity at the indicated times of OGD to the activity of C6 cells before exposure to OGD. ** $P < 0.01$ compared with control.

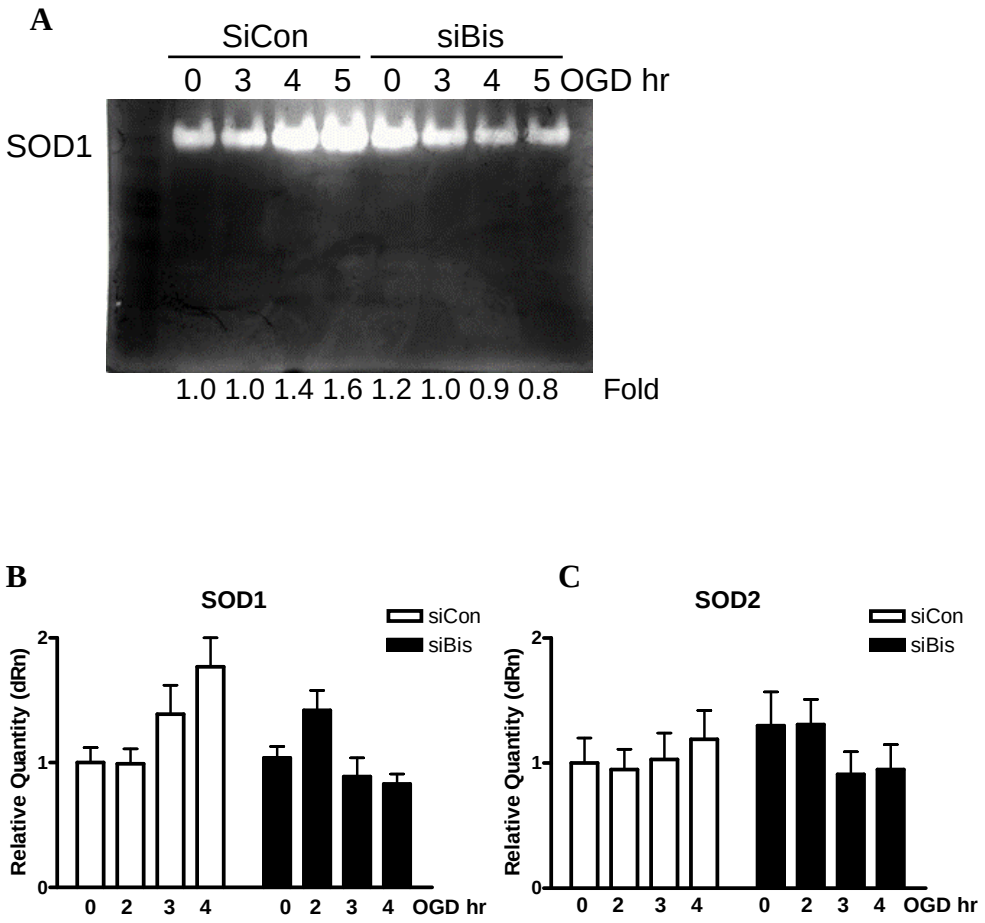

Figure 5. Induction of SOD by OGD was impaired by *bis* knock-down in C6 cells.

(A) SOD activity was evaluated by zymography with the cell extracts from the indicated times during OGD in control and *bis*-kd C6 cells. The fold inductions at the indicated times were shown compared to that of control C 6 cells unexposed to OGD.

(B) The induction of SOD1 or SOD2 mRNA following OGD was examined by quantitative RT-PCR in control and *bis*-kd C6 cells. The transcripts level of SOD1 or SOD2 of control C6 cells before exposure to OGD was arbitrarily designated as 1.

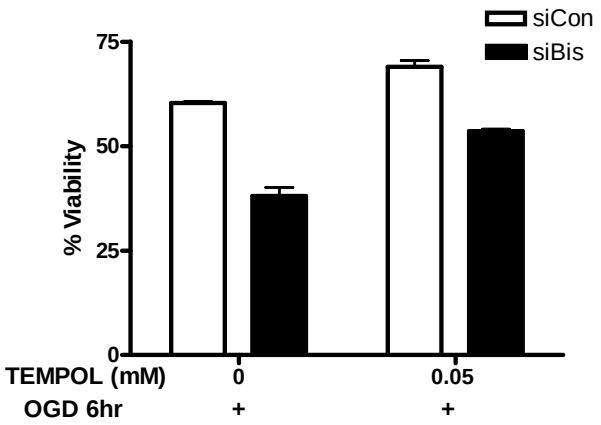

Figure 6. Effect of TEMPOL on the viability following OGD.
Effect of SOD mimetic on the survival of control or *bis*-kd C6 cells after OGD. Percent (%) viability was determined by MTT assay as in Fig.2.

4. Bis is involved in the activation of NF-κB

Since SOD1 induction in response to OGD is impaired at mRNA levels in *bis*-kd C6 cells, the activation of NF-κB, a redox-regulated transcription factor was investigated, after OGD. Using a consensus probe for NF-κB, EMSA was performed with nuclear extracts from C6 cells after OGD to determine DNA binding activity of NF-κB. As shown in Fig. 7A, DNA binding activity of NF-κB increased in a time dependent manner in control C6 cells, but was less prominent in *bis*-kd C6 cells. The activation of NF-κB was also examined by nuclear translocation of p65 of NF-κB after OGD. A Western blotting analysis shows that p65 protein levels in nuclear extracts were obviously increased by 4 hours of OGD insults in control C6 cells. On the contrary, p65 levels at the basal as well as after OGD in nucleus compartment in *bis*-kd C6 cells was significantly lower in *bis*-kd C6 cells. Fig. 7B also shows that the levels of p65 in cytosol fraction was also gradually increased in control C6 cells during

OGD insults, which is not obvious in *bis*-kd C6 cells. Next, it was determined whether inhibition of NF-κB affects OGD-induced SOD1 induction. C6 cells were treated with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which has been shown to inhibit NF-κB activation by blocking degradation of IκB, prior to exposure to OGD, and SOD1 mRNA levels were determined by semi-quantitative RT-PCR. After 4 hours of OGD, mRNA levels for SOD1 were increased in control cells, which were dose dependently down-regulated by TPCK. In addition, SOD1 transcripts levels in *bis*-kd C6 cells were also decreased as a result of TPCK with a similar inhibitory potency (Fig. 8A). Then, MTT assay was performed to see whether the inhibitory effect of TPCK on the induction of SOD mRNA resulted in an increase of cell death after OGD. As shown in Fig. 8B, TPCK pretreatment increased cell death both control and *bis*-kd C6 cells following OGD but the susceptibility was greater in *bis*-kd C6 cells.

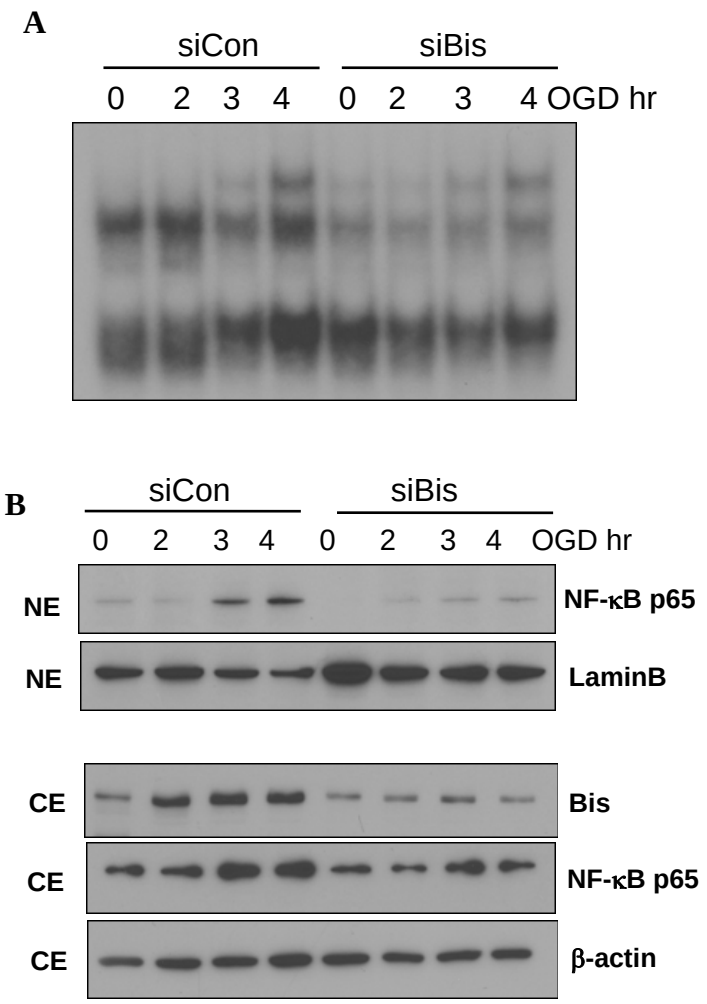

Figure 7. Inhibition of OGD-induced NF-κB activation by *bis* knock-down in C6 cells.

(A) DNA-binding activity of NF-κB was examined using a radiolabeled consensus probe and nuclear fractions extracted from control or *bis*-kd C6 cells at the indicated times of exposure to OGD.

(B) Nuclear translocation of p65 subunit of NF-κB was shown by immunoblotting for p65 with nuclear extracts (NE) or cytopalsmic extracts (CE) after OGD for the indicated times. Bis expression in cytoplasmic fraction was also examined. Lamin and β-actin levels were shown as loading controls.

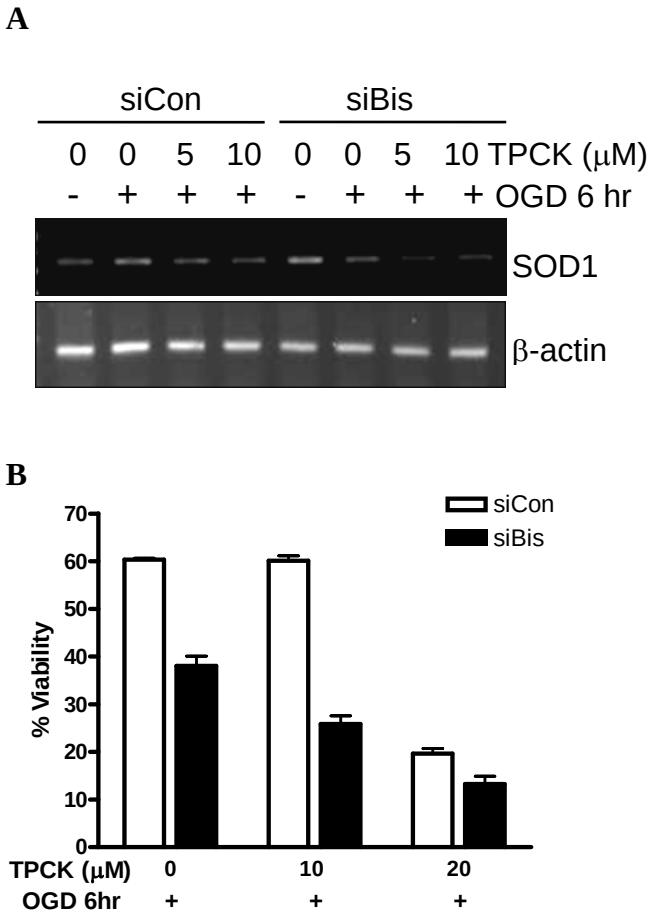

Figure 8. Effect of TPCK on the induction of SOD1 and cell death following OGD.

TPCK was pretreated 1 hour before OGD and exposed to OGD for 4 hours. The induction of SOD1 mRNA (A) and viability (B) in control C6 and *bis*-kd C6 cells were determined by semi-quantitative RT-PCR and MTT assay as in Figure 4 and Figure 2, respectively.

5. Bis regulates constitutive expression of p65/RelA

Since *bis* knock-down caused the decrease in p65 levels both in cytosol and nuclear fractions, it was examined the possibility that *bis* knock-down decreases the constitutive levels of p65. Western blotting of total protein extracts with p65 antibody shows a decrease of p65 protein levels in *bis*-kd cells compared to control C6 cells (Fig. 9A). On the other hands, the transient overexpression of Bis up-regulates p65 protein levels (Fig. 9B). The levels of p65 mRNA were not significantly affected by Bis expression as shown in RT-PCR experiments. Thus, Bis appears to modulate constitutive p65 levels via its ability to regulate protein stability.

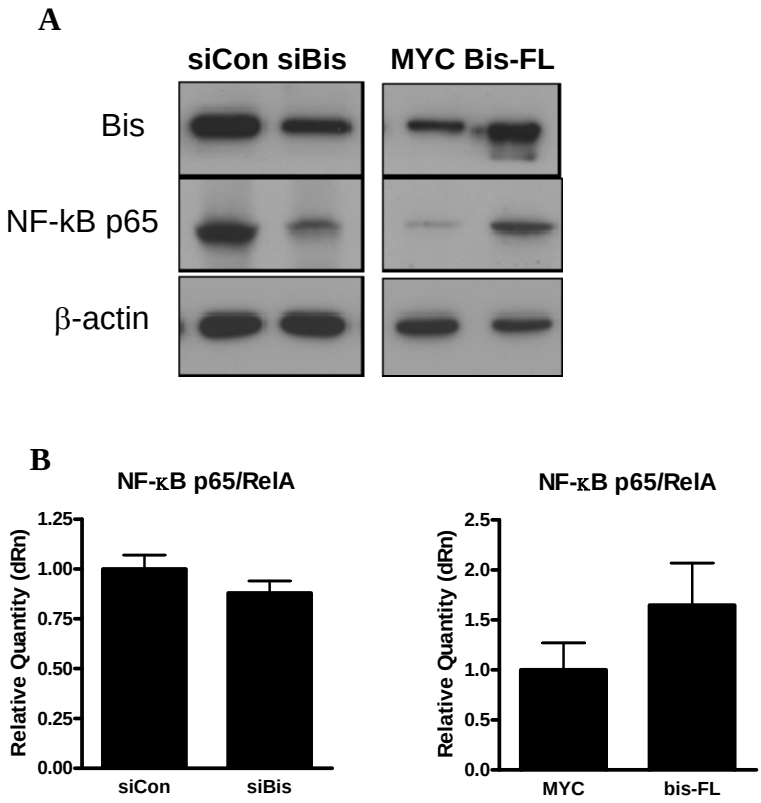

Figure 9. Bis regulates constitutive expression of p65 subunit of NF-κB.

(A) Bis expression was down-modulated by transient transfection of *bis* siRNAs or up-regulated by transfection of *bis*/ myc-3.1 vector for 48 hours. The Bis and p65 expressions were evaluated by immunoblotting.

(B) The mRNA levels of p65 expressions were analyzed by quantitative RT-PCR.

IV. DISCUSSION

Previous experiment showed that Bis is up-regulated in the reactive astrocytes of hippocampus of rats after transient global ischemia [14]. The induction of Bis *in vivo* upon oxidative stresses has been also reported by others, i.e., in case of focal ischemia and retinal damage by light [16, 44]. In this report, it was demonstrated that Bis expression was also induced in C6 glioma cells using an *in vitro* hypoxia model, OGD, which was regulated at transcriptional level, indicating that Bis is an important reactive protein in oxidative stresses (Fig. 1). To understand the physiological significance of induction of Bis following OGD better, si-RNA system was used in this experiment to reduce Bis expression in C6 cells in which the basal level of Bis is quite high.

The down-modulation of Bis expression led to C6 cells more susceptible to OGD-induced death (Fig. 2A, B). Thus, it can be postulated that the induction of Bis *in vitro* as well as in the reactive astrocytes *in vivo* might be related with protective mechanism against oxidative stress.

It has been generally accepted that the production of ROS in reperfusion period following ischemic period has been implicated in pathophysiology of brain damage after cerebral ischemia [18]. During reperfusion, ROS accumulate as a result of a perturbation in the balance between the production of oxygen radicals and the antioxidant mechanisms by which the cells counteract the deleterious effect of ROS, as exerted by several antioxidants such GSH, catalase and SOD. Although the recovery time was not separately given in this OGD system, current study also revealed that ROS accumulation occurred in C6 cells following OGD, which was significantly increased by Bis reduction. Since the cells in the media were immediately exposed to atmosphere as soon as they were removed from hypoxic chamber, this could mimic the reperfusion condition. On the other hands, it has been previously shown that oxygen radicals are generated during brief OGD, probably as a result of mitochondrial dysfunction, which activates cellular antioxidant systems,

resulting in protective effects against the subsequent ischemia in neurons [45, 46]. In primary astrocytes culture, even glucose deprivation led to an increase in ROS accumulation [47, 48]. Thus, Bis might be involved in controlling the generation of ROS during OGD or during the short recovery time. However, based on the findings presented herein, it is not clear when the generation of ROS occurs.

Present results suggest that SOD1 can be considered to be a candidate protein which is responsible for the accumulation of ROS in *bis*-kd C6 cells as follows. While total glutathione levels as well as catalase activity was not decreased in *bis*-kd C6, the SOD activity measured by direct reduction of superoxide anion was decreased *bis*-kd C6 cells compared to significant increase in control C6 cells (Fig. 4). In addition, an indirect reduction of NBT in-gel assay and quantitative measurement for mRNA shown that induction of SOD1 activity failed in *bis*-kd C6 cells (Fig. 5A, B). And the clear bands of SOD2, which should appear in a lower section of the native gel than SOD1, were not detected in this system. This is not likely due to an error in experimental procedure. This observation is supported by previous reports showing that SOD2 was not detected by zymography in C6 cells and several cancer cells [49, 50]. Furthermore, treatment of SOD mimetic led to the recovery in viability following OGD, which is more pronounced in *bis*-kd C6 cells (Fig. 6). In addition, the induction of mRNA levels for NADPH oxidase 2, which is mainly involved in ROS generation to oxidative stress, was not influenced by Bis reduction (data not shown), excluding the possibility that Bis modulates ROS production. Since Bis is mainly localized in cytosol, it is not likely that Bis directly regulates the transcription of SOD1 gene [1]. Thus, it is probable that Bis regulates some transcription factor, which is activated by oxidative stresses.

A number of transcription factors such as activator protein-1, NF-κB, specificity protein-1 and hypoxia-inducible factors are known to modulate gene expressions in response to cellular oxidative stress [51]. Among the redox-sensitive transcription factors, NF-κB plays an important role in cellular response to a variety of stress signals including environmental hypoxia as well

as traumatic brain injury [25]. It has been known that 5'-flanking region of *sod1* gene has putative binding sites for NF-κB in addition to HSE, c/EBP and ARE [52]. Furthermore, PI3K/Akt pathway protects cells against oxidative stress, involving the up-regulation of SOD1 expression via NF-κB [25]. These findings indicate that SOD1 is one of the target genes of NF-κB, of which activation could be modulated by Bis. This presumption is supported by the current results as follows. NF-κB activation after OGD was suppressed in *bis*-kd C6 cells as evidenced by DNA binding activity and nuclear translocation of p65 (Fig. 7A, B). The inhibition of NF-κB also decreased the induction of SOD1 mRNA level, which was more profound in *bis*-kd C6 cells, after OGD (Fig. 8A). The decrease in the viability upon OGD by NF-κB inhibition was also exacerbated in *bis*-kd C6 cells (Fig. 8B). Therefore, taken together, one of the physiological significances of the increase of Bis following hypoxia appear to be the activation of SOD1 to protect cells from oxidative damage, probably via activation of NF-κB.

The mechanism by which Bis regulates activation of NF-κB is not certain at the present. It has been reported that Bis physically interacts with p65 subunit of NF-κB in the cells derived from human astrocytoma [17]. However, their interaction appears to prevent the nuclear entry of p65, suppressing the DNA-binding activity of p65 on LTR of HIV, which is contrary to the postulation provided in current results, although the resulting effect is to protect host cells from viral replication. In this study, in addition to nuclear level of p65, the cytoplasmic levels of p65 were also increased during OGD in a time dependent manner, which was markedly attenuated by *bis* knock-down (Fig. 7B). The overexpression of Bis was also found to increase p65 protein levels, but not mRNA levels (Fig. 9). Thus, it is feasible that an association of NF-κB with Bis may stabilize the p65 protein, permitting to accumulate in the cytosol, which promotes the nuclear entry upon dissociation with IκB. However, further studies are required to define the precise mechanism by which Bis stabilizes p65 protein. It should be also noted that the basal levels of catalase as well as SOD1 were higher in *bis*-kd C6 cells than control C6 cells (Fig. 4), indicating that the antioxidant system may be already triggered

by *bis* knock-down even before OGD. In this respect, the direct effect of Bis on the antioxidant systems should be examined in more detail to clarify its role in subsequent OGD insults.

In summary, this study demonstrates that Bis reduction sensitizes C6 cells to OGD-induced death, which is probably related with the impairment of SOD1 induction due to the decrease of constitutive levels of p65 subunit of NF-κB.

V. CONCLUSION

To investigate the significance of Bis induction in the reactive astrocytes of rat hippocampus after transient forebrain ischemia, si-RNA system was employed to reduce Bis expression in C6 glioma cells. And then, oxygen-glucose deprivation (OGD), as known by *in vitro* hypoxia model, was used for studying both the effect and the underlying mechanisms of Bis induction following oxidative stress.

1. Bis was markedly induced after OGD in C6 rat glioma cells.

2. The repression of Bis expression by si-RNA aggravated cell death with accumulation of reactive oxygen species in C6 rat glioma cells subjected to OGD exposure.

3. Down-modulation of Bis expression resulted in the impairment of induction of superoxide dismutase1 expression after OGD injury, which was due to the attenuation of transcription of *sod1* gene.

4. Bis reduction attenuated NF-κB activation induced by OGD insults and the constitutive levels of p65/RelA was down- or up-regulated by reduction or overexpression of Bis, respectively.

Therefore, Bis might have a protective role in oxidative stress in rat C6 glioma cells, probably via modulating the stability of p65/RelA and subsequent NF-κB activity.

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ABSTRACT(IN KOREAN)

C6 신경교종 세포에서 산소/포도당 결핍으로 유도되는 세포사에 미치는 Bis 단백질 발현 억제 효과

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Bis (Bcl-2 interacting cell death suppressor)는 항 세포사멸 단백질인 Bcl-2와 결합한다고 알려진 단백질로서 항 스트레스와 항 세포사멸 과정에 관여함이 보고되었다. 이전의 연구결과에서 Sprague-Dawley 쥐에 일시적인 전뇌 허혈을 유도한 뒤 해마의 별아교 세포에서 Bis 단백질의 발현을 관찰한 결과 현저하게 증가한 것을 확인하였다. 이러한 허혈 손상에 의한 유의적인 증가의 기전을 조사하기 위해 C6 신경교종 세포에서 Bis 발현을 si-RNA 방법으로 감소시킨 뒤 산소/포도당 결핍 (oxygen-glucose deprivation; OGD)에 노출시켰다. 그 결과 생존도가 크게 감소하였고 활성 산소 종 (reactive oxygen species; ROS) 의 축적이 관찰되었다. 세포 내 항산화제 중 *bis* si-RNA 처리 세포 (*bis*-kd C6) 에서 항 산화 효소인 초과산화물 불균등화 효소 (superoxide dismutase; SOD)의 유도가 저해되었다. 더욱이 in-gel 분석 결과와 전사 수준을 조사한 결과 산소/포도당 결핍 유발에 의한 SOD1의 유도가 *bis* knock-down 세포에서 전사 수준에서부터 손상되어있었다. *Sod1* 유전자의 전사조절인자의 후보로서 NF-κB의 활성을 DNA 결합 분석방법인 겔 지연방법과 p65 subunit 단백질의 핵 내로의 이동을 조사한 결과 *bis* si-RNA를 처리하여 Bis 발현을 억제시킨 C6 신경교종 세포에서 NF-κB의 활성이 감소되어있었다. 또한, 산소/포도당 결핍 유발 손상에서 SOD mimetic을 처리한 신경교종 세포의 생존도가 특히 *bis* si-RNA를 처리한 세포에서 더 뚜렷하게 회복되었다. 더욱이 NF-κB 활성 저해 시 SOD1 전사의 유발이 감소되었고

세포사가 심화되었는데 이는 Bis 단백질이 감소된 세포에서 확연한 차이를 보였다. 또한 constitutive 수준의 p65 단백질의 양은 *bis* knock-down에서 감소되어있는 반면 Bis 과발현 시 p65/RelA가 mRNA의 변화는 미미하나, 단백질 수준이 증가되어있었다. 이러한 결과들로 미루어보아 Bis 단백질의 생리학적 기능 중 하나는 p65 단백질의 안정성을 조절하는 것으로 보이며 그럼으로써 NF-κB의 활성을 조절하여 산화 스트레스에 의한 SOD1의 유발과 세포의 생존도에 영향을 주는 것으로 보여진다. 그리고 Bis에 의한 p65의 안정성 조절의 정확한 기전은 추후에 조사해야 될 것으로 생각된다.

핵심되는 말: Bis, 산소/포도당 결핍, NF-κB, 초과산화물 불균등화 효소, C6 신경교종 세포