Bis is Induced by Oxidative Stress via Activation of HSF1

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The Bis protein is known to be involved in a variety of cellular processes including apoptosis, migration, autophagy as well as protein quality control. Bis expression is induced in response to a number of types of stress, such as heat shock or a proteasome inhibitor via the activation of heat shock factor (HSF1). We report herein that Bis expression is increased at the transcriptional level in HK-2 kidney tubular cells and A172 glioma cells by exposure to oxidative stress such as H2O2 treatment and oxygen-glucose deprivation, respectively. The pretreatment of HK-2 cells with N-acetyl cysteine, suppressed Bis induction. Furthermore, HSF1 silencing attenuated Bis expression that was induced by H2O2, accompanied by increase in reactive oxygen species (ROS) accumulation. Using a series of deletion constructs of the bis gene promoter, two putative heat shock elements located in the proximal region of the bis gene promoter were found to be essential for the constitutive expression as well as the inducible expression of Bis. Taken together, our results indicate that oxidative stress induces Bis expression at the transcriptional levels via activation of HSF1, which might confer an expansion of antioxidant capacity against pro-oxidant milieu. However, the possible role of the other cis-element in the induction of Bis remains to be determined.

Key Words: Bis, HSF1, Oxidative stress, ROS

INTRODUCTION

Bis (Bcl-2 interacting cell death suppressor), also known as BAG3 and CAIR-1, was originally identified as a Bcl-2-binding protein that enhances the antiapoptotic activity of Bcl-2 and as a HSP70-binding protein which inhibits Hsc70/Hsp70 chaperone activity [1-3]. Subsequent studies have provided a growing body of evidence to indicate that Bis exerts diverse functions through specific interactions with various partners. While the pro-survival activity of Bis is assigned to its binding to Bcl-2 or Hsp70 through the BAG domain [1,4], cellular motility and invasion activity appear to be modulated by Bis via interactions with a variety of proteins including FAK, MMP-2, PDZGEF and CCT [5-8]. In addition, recent studies strongly suggest that, as the result of complex formation with HspB8 or HspB6, Bis also participates in promoting autophagy and the subsequent degradation of misfolded and aggregated proteins [9-11]. Bis is also reported to be involved in the different process of promyelocytes, glia cells as well as cardiac myoblasts, although the molecular mechanism for this is unclear [12-14].

Considering these diverse functions of Bis, the maintenance of Bis expression in an appropriate level is prerequisite for appropriate responses various changes that occur in cells. Bis is normally expressed in most tissues at different levels with the highest levels in cardiac and skeletal muscles [1]. However, Bis expression is constitutively up-regulated in human tumor tissues from various origins compared to corresponding normal tissues [15]. On the other hand, Bis expression is efficiently induced by a variety of stressors, including heat shock, heavy metal, ultrasound, UV, proteasome inhibitors, small thiol compounds, lipid oxidation products and HIV infection [16-21]. Among those, several stimuli such as heat shock, proteasome inhibitors and pyrrolidine dithiocarbamate (PDTC) are known to induce Bis expression via activation of the heat shock factor (HSF1) and subsequent interaction with the heat shock element (HSE) in the Bis gene promoter [20-23]. Activation of the bis gene promoter was also implicated in the upregulation of Bis expression through stimulation of the transcription factor Egr-1 and c-jun, which were directed by the fibroblast growth factor (FGF-2) and serum enrichment, respectively [24,25]. Moreover, in some cell types of glial ori-
gin, Bis protein enhances the transcriptional activity of its own promoter, probably through the BAG domain [26]. Thus, Bis expression is mainly regulated by transcriptional levels in response to diverse types of cellular stress, involving distinct transcription factors and cis-elements.

In a previous study, we reported that Bis expression was increased by ischemia-reperfusion in reactive astrocytes in vivo [27,28]. An in vitro study using C6 glioma cells demonstrated that oxygen-glucose deprivation significantly enhanced Bis protein expression, and Bis knockdown resulted in an increase in ROS levels and cell death upon oxygen-glucose deprivation (OGD), concomitant with an impairment in the induction of superoxide dismutase (SOD) activity [29]. Even though some oxidants such as 4-hydroxy-2-nonenal (HNE), a diffusible lipid species, have been reported to induce Bis expression via activating HSF1 [20], the issue of whether oxidative stress directly activates the Bis gene promoter remains unclear. Therefore, the primary purpose of this study is to investigate whether ROS derived from oxidative stresses such as H₂O₂ or OGD directly induce Bis expression at transcriptional levels. We also intended to examine the involvement of HSF1 in the activation of Bis and subsequent effect on cellular anti-oxidant capacity, by SHF1 silencing. Finally, we tried to define the cis-element for HSF1-binding on Bis gene promoter to prove the specific interaction between HSF1 and Bis gene promoter upon oxidative stresses.

METHODS

Cell culture and treatment

HK-2 human kidney-2 cells and A172 human glioma cells were purchased from American Type Culture Collection (ATCC) and maintained in DMEM (Hyclone) and then maintained for 48 h at 37°C in an incubator in a 5% CO² atmosphere. The cells were transfected with siRNA into 48-well plates using different distal primers and proximal primers of human bis genomic DNA with various length was amplified by PCR from HEK 293 genomic DNA using different distal primers and proximal primers of human bis genomic DNA.

MTT assay

The cells were transfected with siRNA into 48-well plates and then maintained for 48 h at 37°C in an incubator in a 5% CO² atmosphere. Cells were exposed to H₂O₂ in glucose/serum free DMEM for 3 h and then further incubated in the normal medium supplemented with glucose and serum. To observe the protective effect of ROS scavenger on the induction of Bis, 1 mM of N-acetyl-L-cysteine (NAC, Sigma-Aldrich) was treated to HK-2 cells before exposure to H₂O₂. For OGD, A172 cells were washed twice with degassed DMEM without glucose and serum (WelGene), and exposed to an anaerobic chamber containing 85% (v/v) N₂, 10% (v/v) H₂, 5% (v/v) CO² (Thermo Forma) at 37°C for 5 h. Then A172 cells were recovered in the normal medium in the indicated times.

Luciferase assay

Suppression of Bis or HSF1 expression was performed by transfection of specific siRNA targeted for Bis (5'-AAGGUUCAAGCACUAUUGGA-3') or HSF1 (5'-AAGGUCUUGAGAACCCUAUGGA-3') with G-fectin (Genolusion Pharmaceuticals) according to manufacturer’s instruction. AccuTarget™ Control siRNA (Bioneer, 5'-CCUAGCCACC-AUUUUUG-3) was used for negative control.

Protein analysis

Cells were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodiumdodecyl sulphate, 50 mM Tris-HCl pH 8.0) with protease inhibitor (Roche Diagnostics) on ice for 30 min. Equal amounts of protein were separated on polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated for 1 h with 5% dry skim milk in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween 20) buffer and then incubated with antibodies against Bis [1], HSF1 (Cell Signaling), or beta-actin (Sigma-Aldrich). After incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology), the immunoreactive bands were visualized by an enhanced chemiluminescence substrate (Thermo Fisher Scientific). Quantification for the intensities of each band was carried out on Multi Gauge 2.2 software (Fuji Photo Film Co.).
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Fig. 1. Induction of Bis expression by oxidative stress in HK-2 and A172 cells. (A) Western blot assay for Bis expression in HK-2 (left column) and A172 (right column) cells after exposure to H2O2 and OGD, respectively, and then incubated in the normal medium for indicated times. Beta-actin expression was used as a loading control. (B) The relative levels of Bis mRNA from HK-2 (left column) or A172 (right column) cells at the indicated conditions were determined by quantitative real time RT-PCR analysis after normalizing with beta-actin mRNA level in the same sample. The mean values from three independent experiments are shown with SD. The value from the control cells before exposure to oxidative stress is designated as 1.0. *p < 0.05 and **p < 0.01 vs. the value from control cells. (C) HK-2 (left column) or A172 (right column) cells were transfected with control or Bis specific siRNA with indicated doses for 48 h and then treated with 100 μM of H2O2 for 3 h followed by additional 6 h in normal medium. The relative cell viability was determined using MTT assay as described in METHODS section. Values from triplicate experiments were provided as mean±SD. *p < 0.05 and **p < 0.01 vs. the value from control cells treated only with H2O2. (D) ROS accumulation was determined in HK-2 cells after treatment of H2O2 by measuring DCF-DA fluorescence intensity using flow cytometric analysis. 1 mM of NAC was pretreated before exposure to H2O2. Fold changes in the mean from three experiments are provided as mean±SE. *p < 0.05 and **p < 0.01 vs. the value from control cells, ***p < 0.001 vs. the value in the absence of NAC. (E) Effect of NAC on the induction of Bis mRNA was determined by pretreatment of NAC prior to H2O2 treatment and then Bis mRNA level was determined as in (B). The mean values from four independent experiments are present with SD. ***p < 0.001 vs. the value from control cells, ##p < 0.01 vs. the value in the absence of NAC.

RESULTS

Bis expression is up-regulated by oxidative stress at transcriptional level

We previously reported that Bis expression was induced by oxygen-glucose deprivation in C6 glioma cells, as evidenced by a western blot assay [29]. To examine whether oxidative stress-induced Bis expression was restricted only to glia-derived cells, we determined Bis expression levels after a H2O2 treatment in HK-2 cells, human kidney proximal cells. As shown in Fig. 1A, treatment of the HK-2 cells with 50 μM of H2O2 for 3 h resulted in an increase in Bis expression at protein level by about 3.4-fold, which was further increased after additional incubation in normal media in a time dependent manner, showing an increase of about 4.6-fold after 6 h. Quantitative real time PCR assays revealed that Bis transcripts levels were also increased by exposure to H2O2 by about 3.3-fold, and then further increased to 4.8-fold after incubation for 6 h in normal media, with a similar pattern of protein levels (Fig. 1B). The increase in Bis expression in recovery or reperfusion period was verified in A172 glioma cells which were exposed to

tivity, the luciferase activities are presented as fold change relative to the normalized firefly luciferase activity in cells transfected with pBis-1(−1080/+289) without oxidative stress, which was taken as 1.0.

ROS determination

ROS accumulation in HK-2 cells following H2O2 treatment was determined using the oxidative sensitive fluorescent probe 2'7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes). This assay is based on the principle that the nonpolar, nonionic H2-DCFDA crosses cell membranes and is enzymatically hydrolyzed into nonfluorescent H2-DCF by intracellular esterases. In the presence of ROS, H2-DCF is rapidly oxidized to become highly fluorescent DCF. At the indicated time points, cells were washed three times with phosphate buffered saline, incubated for 10 min with 10 μM of DCF-DA, and then washed twice with PBS. Quantification of ROS levels were performed by flow cytometry analysis (BD FACS-Calibur; BD Bioscience) with excitation at 488 nm and emission at 525 nm. Mean fluorescence intensities were obtained by histogram statistics using the BD FACS-Diva software (BD Bioscience).

Statistics

Student’s t-tests were employed in order to compare the differences between two different groups. p value of < 0.05 was considered statistically significant.
OGD and subsequently allowed to recover in normal media supplemented with 5% O2 (Fig. 1A and B). It thus appears that the signals for Bis induction are persistently activated even after oxidative stress source is eliminated. To define the physiological significance of Bis induction by oxidative stress, we evaluated the susceptibility to cell death induced by H2O2 following Bis silencing. Bis depletion resulted in the significant decrease of the relative viability both in HK-2 and A172 cells; 84% and 78% were decreased to 61% and 55% by Bis depletion with 100 nM of Bis siRNA in HK-2 and A172 cells, respectively (Fig. 1C). Thus, the induction of Bis upon oxidative stress might exhibit pro-survival activity to protect cells from entering apoptosis program. As determined by DCF-DA staining and subsequent FACS analysis, cellular ROS levels were higher in the recovery period than in H2O2 exposure period (Fig. 1D). Furthermore, the pretreatment with NAC, an antioxidant, significantly repressed ROS levels as well as Bis mRNA levels (Fig. 1D and E), indicating that ROS accumulation directly stimulates Bis expression at the transcriptional level.

Silencing HSF1 suppressed Bis expression and ROS accumulation upon oxidative stress

Several types of stress such as heat shock and proteasome inhibitor have been reported to stimulate Bis expression through activation of HSF1 and subsequent its interaction with HSEs in the promoter of Bis gene [22,23]. To investigate if oxidative stress-induced Bis induction is also mediated by the activation of HSF1, we investigated the effect of HSF1 siRNA on Bis induction upon a H2O2 treatment. As shown in Fig. 2A, the transfection of HSF siRNA effectively suppressed HSF1 protein expression. Silencing HSF1 had no effect on the basal expression of Bis at the transcriptional level as well as the protein level. However, H2O2-induced Bis expression was decreased in HSF1-silenced cells. The increase in Bis expression by H2O2 in HSF1-silenced cells was 2.3-fold and 3.5-fold at protein and the mRNA levels, respectively, which correspond to 63% and 48% of the control cells. To define the physiological significance of Bis induction mediated by ROS and HSF1, we measured ROS levels after a H2O2 treatment in control or HSF1 siRNA-treated HK-2 cells. A shown in Fig. 2C, the transfection of HSF1 siRNA had no effect on basal ROS levels. Treatment with H2O2 resulted in an increase in ROS accumulation by about 2.7-fold, which was further increased the 3.4-fold by HSF1 silencing. Thus, the HSF1 deficiency appeared to be associated with anti-oxidant defense, probably via the regulation of Bis expression.

**HSF1 is involved in Bis expression upon oxidative stress**

It has been reported that three putative HSEs are present in the promoter of the Bis gene [22,31]. To define which region of the Bis gene promoter is responsible for oxidative stress-mediated Bis induction, we prepared a series of deletion constructs of the Bis gene promoter for use in a reporter assay (Fig. 3A). pBis-1, a luciferase plasmid containing all the three putative HSEs, showed similar time course profiles for the induction of luciferase activity with an increase in the endogenous Bis transcripts, showing a peak activity of about a 5-fold increase at 6 h after recovering from the H2O2 treatment. Thus, oxidative stress induced Bis expression can primarily be attributed to the activation of promoter activity, resulting in an increase in the transcripts level. The basal luciferase activity of pBis-2, which contain two HSEs (H1 and H2), was 1.5-fold higher compared to pBis-1. In addition, the transcriptional induction of pBis-2 by oxidative stress was also greater than pBis-1, by about 7-fold and 5-fold, respectively. Further deletion of HSE2 (pBis-3) or HSE1 (pBis-4) resulted in a marked reduction in basal activity of 9 and 15% of pBis-1, while partially restored oxidative stress-responsive ability by about 2-fold leading to 16 and 33% of pBis-1, respectively. Finally, as observed in endogenous Bis induction, HSF1 silencing clearly suppressed the transcriptional activation of pBis-2 upon H2O2 exposure, from 2.6-fold to 0.8-fold (Fig. 3D). Taken together, these results suggest that both HSE1 and HSE2 are required for basal expression as well as oxidative stress-inducible expression of Bis.

**DISCUSSION**

The findings of the present study demonstrate that Bis expression is increased at the transcriptional level in response to oxidative stress such as H2O2 and OGD. Various types of stress, such as heat shock or proteasome inhibitors have been reported to increase Bis expression through HSF1 activation and subsequent interaction with HSEs that are located in the bis gene promoter [22,23]. Our result

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Fig. 2. HSF1 suppressed induction of Bis mRNA upon oxidative stress. HK-2 cells were transfected with control or HSF1-specific siRNA and incubated with H2O2 for 3 h followed by additional 6 h in normal medium. HSF1 and Bis expressions were evaluated by Western assay (A) and by real-time RT-PCR (B) as in Fig. 1. (C) Effect of downregulation of HSF1 on ROS accumulation upon H2O2 treatment was examined by DCF-DA staining and FACS analysis. Data are presented with fold changes in the mean intensities from three independent experiments with SE. *p<0.05 vs. the value from control cells, †p<0.05 vs. the value from the control siRNA-treated cells.
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Fig. 3. Identification of oxidative stress-responsive region in the Bis promoter. (A) Schematic diagram of the deletion mutants for proximal region of Bis promoter, which were cloned into pGL3 basic vector. The relative position to the transcription site (+1) and the locations of putative HSE (H) are shown. (B) Transcriptional activation of pBis-1 was determined at the indicated time points following treatment of H$_2$O$_2$ after normalization with renilla activity. Luciferase activity before exposure to H$_2$O$_2$ is designated at 1.0. The mean value from triplicate experiments are present with SD. **p < 0.01 and ***p < 0.001 vs. the value from control cells. (C) Fold activation of various deletion mutants of Bis promoter in response to H$_2$O$_2$ treatment was shown. The luciferase activity of pBis-1 before exposure to H$_2$O$_2$ is designated at 1.0. The values for pBis-3 and pBis-4 were provided as a magnified graph (inlet). The mean values from triplicate experiments are present with SD. *p < 0.05 and **p < 0.01 vs. the value from H$_2$O$_2$-untreated cells in each construct. (D) Effect of HSF1 knockdown on the expression of pBis-2 was shown. Expression of HSF1 was suppressed by transfection of HSF1 specific siRNA for 24 h and then HK-2 cells were transfected with pBis-2 construct as described in Materials and Methods section. Fold change in luciferase activity compared with that of control HK-2 cells are presented as mean value with SD. *p < 0.05 vs. the value from H$_2$O$_2$-untreated control cells, #p < 0.05 vs. the value from the control siRNA-treated cells.

also show that the silencing of HSF1 significantly suppressed the induction of endogenous Bis mRNA as well as luciferase activity derived pBis-2 which include two HSEs upon H$_2$O$_2$ treatment, suggesting that HSF1 is involved in the activation of the bis gene promoter upon exposure to oxidative stress (Figs. 1 and 3). It should be noted that the H$_2$O$_2$-mediated increase of endogenous Bis mRNA was inhibited in 48% of control cells upon HSF1 siRNA while the induction of luciferase activity derived from pBis-2 was completely inhibited by HSF1 siRNA. As a possible explanation for this discrepancy, in addition to HSEs, another cis-element located outside the sequences that was used in the reporter assay, appears to participate in the activation of Bis gene transcription in response to oxidative stress. It is noteworthy that, the effect of regulatory elements in the coding region, if present, on the promoter activity should be excluded in the reporter assay. Furthermore, the Bis protein is reported to auto-regulate its own promoter in glia cells in a positive feedback manner [26]. Thus, it is likely that the activity of the Bis gene promoter in cells represents the net effects contributed from the interactions of individual cis-elements and the corresponding trans-acting factors, such as HSF1, Bis or other unknown factors.

We, and other groups, have reported that Bis expression is increased by oxidative stress or pro-oxidants, both in vivo and in vitro [20,27,28]. The physiological significance of Bis induction upon PDTC or HNE was verified by the knockdown of HSF1 [20,21], showing that HSF1-mediated Bis expression confers cellular protection from pro-apoptotic and inflammatory stress. In the present study, we observed that the knockdown of HSF1 increased ROS accumulation upon H$_2$O$_2$ treatment (Fig. 2C), suggesting that HSF1 is involved in cellular homeostasis for oxidative status via the modulation of Bis expression. In glioma cells, we also observed that the knockdown of Bis increases susceptibility to cell death upon OGD [29]. In addition, we recently demonstrated that diabetic nephropathy was aggravated in bis heterozygote (bis$^{+/-}$) mice by increased ROS accumulation and apoptosis in kidney tubular and mesangial cells, which was accompanied by an impaired induction in SOD activity [32]. Thus, a HSF1 deficiency or inactivation may result in a significant impairment in the antioxidant system, through the modulation of Bis expression and subsequent SOD. However, the survival of neuronal cells of the hippocampus following neonatal hypoxia-reperfusion was higher in bis-deleted (bis$^{-/-}$) mice than in the wild type (bis$^{+/-}$) mice, possibly because of the modulation of galectin 3 levels [28]. Taken together, these results suggest that the modulation of Bis expression resulted in diverse effects related to cell survival depending on the cellular contexts.

HSF1 has been known to orchestrate the transcriptional activation of heat shock proteins to protect cells from severe stresses by control of protein structures for proper folding for essential target proteins [33,34]. Bis was previously shown to be induced by heat shock, heavy metal as well as proteasome inhibitor, in addition to oxidative stress [16,
23,35]. Furthermore, Bis was shown to play an important role in protein quality control as a co-chaperone and as an activator for macroautophagy by aggresomal targeting of mis-folded proteins [2,9-11,36]. Thus, the ability of HSF1 in the activation of Bis implicates that, under various stresses including aggregation-prone stressor, HSF1-mediated regulation of Bis levels might determine the cellular fates by affecting the proper folding or solubility of specific proteins which are essential for cell viability.

In conclusion, Bis expression was found to be induced by oxidative stress at the transcriptional level. Although HSF1 is an important regulator of Bis induction in oxidative stress as in other types of stress, further studies will be required to define the role of other cis elements in the regulation of constitutive and inducible Bis expression.

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