Sigma-1 receptors regulate Bcl-2 expression by ROS-dependent transcriptional regulation of NF-κB

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Abstract

The expression of Bcl-2, the major anti-apoptotic member of the Bcl-2 family, is under complex controls of several factors including reactive oxygen species (ROS). The sigma-1 receptor (Sig-1R), which was recently identified as a novel molecular chaperone at the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM), has been shown to exert robust cellular protective actions. However, mechanisms underlying the antiapoptotic action of the Sig-1R remain to be clarified. Here, we found that the Sig-1R promotes cellular survival by regulating the Bcl-2 expression in CHO cells. Though both Sig-1Rs and Bcl-2 are highly enriched at the MAM, Sig-1Rs neither associate physically with Bcl-2 nor regulate stability of Bcl-2 proteins. However, Sig-1Rs tonically regulate the expression of Bcl-2 proteins. Knockdown of Sig-1Rs downregulates, whereas overexpression of Sig-1Rs upregulates bcl-2 mRNA, indicating that the Sig-1R transcriptionally regulates the expression of Bcl-2. Importantly, the effect of Sig-1R siRNA downregulating Bcl-2 was blocked by ROS scavengers as well as by the inhibitor of the ROS-inducible transcription factor NF-κB. Knockdown of Sig-1Rs upregulates p105, the precursor of NF-κB, while concomitantly decreases IκBα. Sig-1R knockdown also accelerates the conversion of p105 to the active form p50. Lastly, we demonstrated that knockdown of Sig-1Rs potentiates H2O2-induced apoptosis; the action is blocked by either the NF-κB inhibitor oridonin or overexpression of Bcl-2. These findings thus suggest that Sig-1Rs promote cell survival, at least in part, by transcriptionally regulating Bcl-2 expression via the ROS-NF-κB pathway.
Introduction

Bcl-2 is a key molecule regulating “apoptosis,” a deliberate life-relinquishment of the cell. Bcl-2 has been implicated in pathophysiology of several human diseases such as Alzheimer’s disease and cancer, thus providing a clue that Bcl-2 may serve as a potential target for treating human diseases (Danial and Korsmeyer, 2004; Youle and Strasser, 2008). Bcl-2 prevents apoptosis caused by a variety of cellular stress such as oxidative stress, heat shock, and cytokine deprivation (Reed, 1994; Danial and Korsmeyer, 2004; Youle and Strasser, 2008). Bcl-2 localizes at mitochondrial outer membranes, as well as at the endoplasmic reticulum (ER) (Krajewski et al., 1993). One of the primary actions of Bcl-2 is to block homodimerization of pro-apoptotic Bax at mitochondria (Oltvai et al., 1993). Bcl-2 also regulates the Ca\textsuperscript{2+} mobilization at the ER (Pinton and Rizzuto, 2006). However, the precise localization and function of Bcl-2 at the ER remain to be clarified.

Regulating Bcl-2 expression is an important element in promoting cellular survival. The bcl-2 gene possesses 3 exons and two major promoters (i.e., P1 and P2) (Seto et al., 1988). The P1 site is the major transcriptional promoter containing the Sp1-binding site and cyclic AMP response element (CRE), which are under controls of transcription factors such as c-Jun and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Heckman et al., 2002). Recent evidence indicates that ROS are the potent regulators of the Bcl-2 expression (Hildeman et al., 2003; Li et al., 2004). ROS decrease the expression of bcl-2 mRNA by promoting CRE-binding protein or NF-κB to CRE and κB sequences on the bcl-2 promoter (Sohur et al., 1999; Pugazhenthi et al., 2003).
The Sig-1R is an ER protein that shares no homology with any mammalian proteins (Hanner et al., 1996). The Sig-1R was recently identified as a novel molecular chaperone targeting the ER subdomain associating with mitochondria (i.e., the mitochondrion-associated ER membrane: MAM) (Hayashi and Su, 2007). Sig-1Rs stabilize the conformation of the proteins at the MAM such as inositol 1,4,5-trisphosphate receptor type III (IP3R3) (Hayashi and Su, 2007). The Sig-1R has been implicated in several human diseases, including neurodegenerative diseases, drug abuse, and cancer (Snyder and Largent, 1989; Maurice and Lockhart, 1997; Matsumoto et al., 2007; Palmer et al., 2007; Fontanilla et al., 2009). Of the most prominent actions of Sig-1Rs or those ligands is their robust cellular protective effect (Maurice and Lockhart, 1997; Bowen, 2000). Sig-1R agonists have been shown to promote cellular survival by preventing oxidative stress caused by ischemia (Schetz et al., 2007), diabetes (Smith et al., 2008), inflammation (Wang et al., 2008), and β-amyloid toxicity (Meunier et al., 2006).

A few studies have recently suggested a possible relationship between Sig-1Rs and the Bcl-2 family in neuronal survival. Sig-1R agonists prevent upregulation of Bax in cortical neurons exposed to β-amyloid 25-35 (Marrazzo et al., 2005). The Sig-1R agonist 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) was shown to prevent downregulation of bcl-2 mRNA caused by ischemic conditions (Yang et al., 2007). The action of PPBP is fully abolished by the Sig-1R antagonist rimcazole (Yang et al., 2007). In contrast, dehydroepiandrosterone-sulfate, a neurosteroid activating Sig-1Rs, promotes cellular survival in PC-12 cells by inducing Bcl-2, but the action is insensitive to both Sig-1R agonist SKF-10047 and antagonist haloperidol (Charalampopoulos et al., 2004). Therefore, although recent findings support the notion that regulating the Bcl-2 family
may be of potential elements in the cellular protective action of Sig-1Rs, discrepancies (possibly due to variance with selectivity and potency of Sig-1R ligands) remain to be solved. Here we sought to provide direct evidence to prove the involvement of the ROS-Bcl-2 pathway in the cellular protection induced by Sig-1Rs. Instead of using Sig-1R ligands, we employed molecular biological approaches (e.g., gene transfer or silencing) to control Sig-1R’s activity. Although the physical interaction of Sig-1Rs with Bcl-2 proteins was originally expected in lights of nature of the Sig-1R as a molecular chaperone, the data provided evidence that Sig-1Rs transcriptionally control expression of Bcl-2 by regulating the ROS-NF-κB pathway.
Methods

Materials.

Chinese hamster ovary (CHO) cells were from ACTT (Manassas, VA). MEM-alpha medium + Glutamax, heat-inactivated fetal bovine serum (FBS), lipofectamine-2000, Hoechst 33342, and Alexa-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA). Cycloheximide, H$_2$O$_2$, N-acetyl cysteine (NAC), nitro-L-arginine (NLA), and Tempol were from Sigma-Aldrich (St Louis, MO). Oridonin was from EMD Biosciences (Gibbstown, NJ). Specific antibodies were purchased as follows: anti-FLAG from Sigma-Aldrich; monoclonal Bcl-2, NF-κB p105/50 subunit, extracellular signal-regulated kinase (ERK), and calnexin from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal Bcl-2, monoclonal Bcl-2 (SPM117), ERp57, and IκBα from Abcam Inc (Cambridge, MA); cytochrome c oxidoreductase (OxyR) from Invitrogen; cytochrome C, monoclonal IP3R3, Nucleoporin p63, and BiP from BD Biosciences (San Jose, California); polyclonal IP3R3 from Millipore (Billerica, MA). Anti-Sig-1Rs were raised as described previously (Hayashi and Su, 2003).

Cell culture and vector transfection

CHO cells were cultured in MEM-alpha containing 10% heat-inactivated FBS and 2 mM Glutamax at 37°C with 5% CO$_2$. Cells were treated with compounds or transfected in 6-well plates. The cDNA encoding siRNA was constructed in the pSIREN vector (Clontech, Mountain View, CA) as previously described (Hayashi and Su, 2004). The expression vector for FLAG-tagged Sig-1Rs was constructed by ligating the PCR-amplified rat Sig-1R cDNA (Hayashi and Su, 2001) in the pFLAG vector (Sigma). The
Bcl-2 pClneo vector was donated by Drs. Xingming Deng and W. Stratford May, Jr. (University of Florida Shands Cancer Center). Cells were transfected one day prior to experiments by applying the DNA/lipofectamine-2000 mixture (1 μg to 2 μl) to the culture medium. Concentrations of vectors in medium are 1μg/ml for the FLAG-tagged Sig-1R, 0.20-0.50 μg/ml for Bcl-2, 0.33 μg/ml for the wild-type Sig-1R, 1μg/ml for ER-DsRed, 0.3 μg/ml for Mito-DsRed, and 1μg/ml for Sig-1R siRNA / control siRNA. After 6 h, transfected cells were harvested and spread on a new plate with or without ROS inhibitors, H2O2, or oridonin. The membrane fractionation and immunocytochemistry verified that Bcl-2 expressed by the optimized transfection condition shows the identical subcellular distribution to that of endogenous Bcl-2 in CHO cells.

**Immunocytochemistry and nuclear staining for apoptosis**

CHO cells were grown on a round coverslip coated with poly-D-lysine. After a fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (10 min at room temperature), cells were permeabilized with 0.2% Triton X-100 for 5 min followed by blocking with 10% non-fat dry milk. Fixed cells were incubated with primary antibodies in PBS containing 4% bovine serum albumin, 0.1% Triton X-100, and 5% FBS. Primary antibodies used were: monoclonal Bcl-2 (1:50), polyclonal Bcl-2 (1:100), monoclonal IP3R3 (1:50), polyclonal IP3R3 (1:100), monoclonal ERp57 (1:100), monoclonal OxyR (1:100). After incubation with Alexa-conjugated secondary antibodies, images were captured by using the UltraView confocal system (PerkinElmer). The monoclonal anti-Bcl-2 recognizes the amino acids 1-205, whereas the polyclonal Bcl-2 recognizes the amino acids 1-18 of human Bcl-2 with a cross reactivity to endogenous Bcl-2 in CHO cells. Since the
expression level of endogenous Bcl-2 is considerably low in CHO cells, immunocytochemistry was performed basically in CHO cells transfected with Bcl-2 cDNA under the optimized overexpression condition (see above). For nuclear staining, fixed cells were incubated with Hoechst-33342 (10 μg/ml) for 15 min followed by a brief washing with PBS. Nuclear images were captured by fluorescence microscopy. Five fields were captured randomly from single wells of a 12-well plate. Six wells were analyzed in each group.

**MAM preparation**

The MAM fraction was prepared as described previously (Rusinol et al., 1994). Briefly, CHO cells grown on two 15-cm dishes were homogenized by a glass Dounce homogenizer in the homogenization buffer (0.25 M sucrose, 10 mM HEPES/KOH, pH 7.4). The homogenate was centrifuged at 500 x g to yield the P1 nuclear fraction. The supernatant was centrifuged at 10,300 x g for 20 min to yield the crude mitochondrial fraction. The supernatant was centrifuged at 100,000 x g for 1 hr to obtain P3 microsomal and cytosolic fractions. The crude mitochondrial fraction in 0.5 ml of isolation medium (250 mM mannitol, 5 mM HEPES/KOH at pH 7.4, 0.5 mM EGTA/KOH at pH 8.0) was layered on a Percoll solution [225 mM mannitol, 25 mM HEPES/KOH at pH 7.4, 1 mM EGTA/KOH at pH 8.0, 30% (v/v) Percoll] followed by a centrifugation at 95,000 x g for 30 min. The purified mitochondrial fraction and the MAM fraction were collected followed by washings with the isolation medium.

**Immunoprecipitation**
Cells were suspended in PBS followed by crosslinking with dithiobis succinimidyld propionate (Thermo Scientific; Rockford, IL) at 150 μg/ml (15 min at 4 °C). The reaction was stopped by adding Tris-HCl (pH 8.8, 30 mM). Cell lysates were prepared by suspending the crosslinked cells in the buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.2% SDS, 0.5% sodium deoxychorate, 150 mM NaCl, and the protease inhibitor cocktail (Sigma). After a centrifugation at 12,000 x g, the supernatant was incubated overnight with primary anti-FLAG or anti-Bcl-2 antibodies. The cell lysate was incubated with Sepharose protein A (GE Healthcare; Piscataway, NJ) or with protein A/G Plus-agarose beads (Santa Cruz) for 90 min. After washing with lysis buffer, immunoprecipitants were boiled in 2x sample buffer and applied to Western blotting.

Nuclear preparation

Cells were incubated with Nuclei EZ lysis buffer (Sigma-Aldrich) for the purification of nuclei. Cell lysates were centrifuged at 500 x g for 5 min. The supernatant was kept as a post-nuclear cell lysate. The pellet was washed twice with the same lysis buffer, and kept at -80 °C until used in Western blotting.

Total RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA in CHO cells were extracted with Total RNA Isolation NucloeSpin® RNA II (Macherey-Nagel Inc). The RT-PCR for bcl-2 mRNA was performed by using Titanium® one-step RT-PCR kit (Clontech) with 0.5 μg of total RNA and 24.5 μL of the reaction mixture under the following thermal cycle: 50°C for 60 min, 94°C for 5 min, 40 cycles of 94°C (30s)-57°C (30s)-68°C (45s), followed by 72°C for 2 min and 4°C (GeneAmp®
PCR System 9700, PerkinElmer Applied Biosystems; Foster City, CA). Sig-1R mRNA was amplified by the same RT-PCR condition, but with 25 cycles. Following primers were used: the bcl-2 antisense primer 5’-CTACTGCTTTTAGTGAACC-3’, the bcl-2 sense primer 5’-GGAAGGATGGCGCAAGCCGGGAG-3’, the Sig-1R sense primer 5’-CCAGGCTGCCCCGCT-3’, the Sig-1R antisense primer 5’-TGAGTCCCAGCGAGTAGAAATGG-3’. PCR products were analyzed by 2%-agarose electrophoresis followed by imaging with Image Station 440CF under UV light (Kodak; New Heaven, CT).

Western-Blotting

Cells were briefly washed and harvested in ice-cold PBS. Cell pellets after a centrifuge at 3000 x g for 10 min were suspended into 2x sample buffer (0.13M Tris-HCl, pH 6.8, 4.2% SDS, 20% glycerol). After a brief sonication, samples were centrifuged at 16,000 x g (10 min) and the supernatants kept at -20 °C. Protein assays were performed by using the Micro BCA assay (Thermo Scientific). After SDS-polyacrylamide electrophoresis, proteins were transferred onto a polyvinylidene fluoride membrane (BioRad; Hercules, CA) by Trans-Blot Electrophoretic Transfer Cell (BioRad). The membrane was blocked with 10% non-fat dry milk in TBST (20 mM Tris-base, 500 mM NaCl, 0.005% Tween-20, pH 7.5) followed by incubation overnight with primary antibodies. After washing with TBST, the membrane was incubated for 1h with secondary antibodies conjugated with horseradish peroxidase (Thermo Scientific). Protein bands were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and Image...
Station 440CF (Kodak). Data were analyzed by Prism 3.0cx (GraphPad Software; San Diego, CA).

**Statistical analysis**

All quantifications for Western blotting and RT-PCR were performed by Kodak 1D Image Analysis Software (Kodak). Data were submitted to statistical analyses for the Prism 3.0cx software. Data were presented as percentage of control with SEM. The level of statistical significance is $p < 0.05$. For comparison of two groups, Student-$t$ test was employed. For data with multiple groups, two-way ANOVA followed by Bonferroni post-hoc test was employed.
Results

Sig-1Rs regulate the Bcl-2 protein expression

Sig-1R ligands have been demonstrated to block the alterations of Bax or Bcl-2 expression induced by pathological conditions (Marrazzo et al., 2005; Yang et al., 2007). To clarify whether regulating the expression of Bcl-2 family is the bona fide action of Sig-1Rs, we first examined if knockdown or overexpression of Sig-1Rs per se may affect the expression of two Bcl-2 family proteins in CHO cells. Western blotting using whole CHO cell lysates found that siRNA against Sig-1Rs significantly decrease the protein level of Bcl-2, but not that of Bax (Fig 1). Conversely, overexpression of Sig-1Rs significantly upregulated Bcl-2 (Fig 1).

MAM localization of Bcl-2 and Sig-1R

Since both Sig-1Rs and Bcl-2 are known to regulate Ca\(^{2+}\) transmission from ER to mitochondria (Pinton and Rizzuto, 2006; Hayashi et al., 2009), we speculated that these proteins may localize at the same ER locus (i.e., MAM), thus may physically interact each other. The association might thus regulate the protein stability/degradation of Bcl-2 via the chaperone activity of Sig-1Rs. To address the possibility, we first examined the cellular localization of Bcl-2 in CHO cells. The differential centrifugation combined with a Percoll gradient centrifugation demonstrated that Bcl-2 is present in P1 nuclear and mitochondrial fractions with a marginal level in the P3 fraction. However, Bcl-2 was also present in the MAM fraction (Fig 2A). Under our optimized condition for transfection of Bcl-2 (see Methods), transiently transfected Bcl-2 also showed the same cellular
distribution to that of endogenous Bcl-2 (Fig. 2A). On the other hand, Bax was present mostly in P1, mitochondrial, and cytosolic fractions, but much lower in the MAM and P3 fractions (Fig. 2A). Sig-1R and IP3R3, the well-characterized MAM proteins (Hayashi and Su, 2007), were most enriched in the MAM fraction (Fig. 2A). OxyR and cytochrome c were present exclusively in the mitochondrial fraction (Fig. 2A), indicating few contaminations of mitochondrial membranes in the MAM fraction.

We examined whether downregulation of Bcl-2 caused by Sig-1R siRNA in the total lysate (Fig. 1) is attributed to organelle-specific downregulation of Bcl-2, particularly that at the MAM. As shown in Fig. 2B, knockdown of Sig-1Rs decreased Bcl-2 proteins similarly in any fractions containing Bcl-2 (Fig. 2B). Sig-1R knockdown thus decreases the total Bcl-2 proteins without affecting the subcellular distribution.

To further confirm the MAM localization of Bcl-2, immunocytochemistry using the monoclonal anti-Bcl-2 antibody was performed. Fluorescence confocal microscopy demonstrated that a portion of Bcl-2 indeed colocalizes with Mito-DsRed expressed in mitochondria (yellow in Fig 3A and B). However, the higher magnification revealed that a significant amount of Bcl-2 is also present in close vicinity to mitochondria (green in Fig 3A, lower panels). The polyclonal antibody recognizing the different domain of Bcl-2 also showed the exactly same pattern: presence of Bcl-2 in both mitochondria and their vicinity (Fig 3B). The same distribution of Bcl-2 was also observed when endogenous mitochondrial protein OxyR was immunocytochemically visualized (Fig 3C).

Next, the localization of Bcl-2 was compared to that of ER-specific proteins. ER-DsRed, similar to ERp57, predominantly expressed at reticular structures of the ER (Fig. 3D), whereas MAM-specific IP3R3 localized dominantly at punctate structures inside the
cell (Fig 3D). Confocal microscopy demonstrated that a number of IP3R3-containing punctates apposes mitochondria that are visualized by expressing Mito-DsRed (Fig 3E, upper panels). Importantly, Bcl-2 showed its partial colocalization with IP3R3 (Fig. F), supporting the notion that a portion of Bcl-2 is present at the MAM.

**Bcl-2 is not a substrate of Sig-1R chaperones**

The Sig-1R is a molecular chaperone that regulates stability of proteins at the MAM via direct protein-protein interactions (Hayashi and Su, 2007). To examine if Sig-1Rs directly associate with Bcl-2, thus stabilizing the latter, we performed immunoprecipitation in CHO cells overexpressing Sig-1R-FLAG and Bcl-2. The same immunoprecipitation protocol successfully detected the physical association of Sig-1Rs with BiP in CHO cells (Hayashi and Su, 2007). Although FLAG antibodies efficiently immunoprecipitated Sig-1R-FLAG, the antibodies failed to co-immunoprecipitate Bcl-2 (Fig. 4A). Conversely, Bcl-2 antibodies, though efficiently immunoprecipitated Bcl-2, also failed to co-immunoprecipitate Sig-1R-FLAG (Fig 4B), suggesting no potential interaction between the two proteins.

We next examined whether knockdown of Sig-1Rs may regulate stability of Bcl-2. Degradation of Bcl-2 was monitored in CHO cells treated with cycloheximide, a protein synthesis inhibitor. Although Sig-1R siRNA cause downregulation of Bcl-2 (Fig. 1), the degree of the Bcl-2 degradation remained the same between control siRNA- and Sig-1R siRNA-transfected CHO cells (Fig. 4B).

**Sig-1Rs regulate the mRNA level of bcl-2**
We next examined whether knockdown of Sig-1Rs might promote downregulation of Bcl-2 at the mRNA level. The RT-PCR found that bcl-2 mRNA were significantly decreased by siRNA against Sig-1Rs (Fig. 5A-B). Conversely, overexpression of Sig-1Rs increased the mRNA level of bcl-2 (Fig. 5C), suggesting that Sig-1Rs tonically regulate the transcription of bcl-2 mRNA or stability of bcl-2 mRNA. Because Sig-1R siRNA failed to alter the expression level of transiently transfected Bcl-2 (data not shown), where the transcription is driven by the exogenous promoter on the expression vector, Sig-1Rs likely regulate the transcription of bcl-2 processed at the endogenous promoter of the bcl-2 gene.

Sig-1Rs regulate transcription of bcl-2 by inhibiting the NF-κB pathway.

As previously reported (Sohur et al., 1999; Pugazhenthi et al., 2003), our sequence analyses using TFsearch (Heinemeyer et al., 1998) found multiple consensus κB sequences in the bcl-2 promoter (base pairs at -14 to -23, -1788 to -1797, -1792 to -2001, or -2088 to – 2097 of the human bcl-2 DNA; Accession # NG-009361). Since Sig-1Rs have been implicated in neuroprotection against oxidative stress (Meunier et al., 2006), we hypothesized that Sig-1Rs transcriptionally regulate the Bcl-2 expression via the pathway involving the ROS-responsive transcription factor NF-κB.

First, we examined whether free radical scavengers could prevent Sig-1R siRNA-induced downregulation of Bcl-2. Downregulation of Bcl-2 caused by Sig-1R knockdown was completely abolished by Tempol (the superoxide anion scavenger) or NAC (the scavenger of both superoxide and H₂O₂), but not by NLA (the nitric oxide synthase inhibitor: Fig. 6A), indicating that ROS, particularly O₂⁻ and/or H₂O₂, but not
nitric oxide, are involved in downregulation of Bcl-2 caused by Sig-1R siRNA. Next, we examined whether knockdown of Sig-1Rs may alter the activation and/or expression of NF-κB. Knockdown of Sig-1Rs promoted the moderate increase of p105, the precursor of the NF-κB complex, as well as the large increase of p50, the active component in the NF-κB complex (Fig. 6B). KCl-induced upregulation of p105 as well as the induction of p50 were accelerated by knocking down Sig-1Rs (Fig. 6C). The increase of p50 caused by Sig-1R knockdown was abolished by Tempol or NAC, but not significantly by NLA (Fig. 6D). It is known that the NF-κB complex containing p50 translocates to the nucleus upon activation of the complex (Karin, 2006). Sig-1R siRNA in fact increased p50 in the nuclear fraction (Fig. 6E). Sig-1R siRNA also caused the decrease of IκBα, the key inhibitor of NF-κB (Fig. 6F). On the other hand, overexpression of Sig-1Rs promoted suppression of p105 accompanied by nearly complete abolishment of p50 (Fig. 6G). Overexpression of Sig-1Rs, however, did not significantly affect the level of IκBα (Fig. 6G).

The NF-κB pathway is involved in Sig-1R siRNA-induced Bcl-2 downregulation and apoptosis

Oridonin is a selective inhibitor of NF-κB that disrupts the interaction between the active form of the NF-κB complex and DNA (Ikezoe et al., 2005). To confirm whether NF-κB is involved in downregulation of Bcl-2 caused by Sig-1R knockdown, CHO cells transfected with either control or Sig-1R siRNA were treated with oridonin at different concentrations. As shown in Fig. 7A, oridonin itself promoted slight upregulation of Bcl-2 in control cells. Oridonin blocked downregulation of Bcl-2 caused
by Sig-1R siRNA in a dose-dependent manner (Fig. 7A). The effect of Sig-1R overexpression to upregulate Bcl-2 was also abolished by oridonin (Fig. 7B).

It has been shown that knockdown of Sig-1Rs increases vulnerability of cells to proapoptotic stimuli (Hayashi and Su, 2007). To examine whether the identified pathway plays a role in the cellular protective action of Sig-1Rs, we tested oridonin and Bcl-2 overexpression in apoptosis promoted by Sig-1R siRNA. Apoptosis was initiated in CHO cells by challenging 50 μM of H₂O₂ for up to 48 h. Control cells showed an increase in apoptosis 8 h after the H₂O₂ exposure, and the proportion of apoptotic cells continuously increased up to 48 h (Fig. 7C). Sig-1R siRNA accelerated H₂O₂-induced apoptosis as shown in increased apoptotic cells particularly 8 h after the H₂O₂ exposure (Fig. 7C). Cells overexpressing Bcl-2 showed slightly higher resistance to H₂O₂ when compared to control cells, but the difference was not statistically significant, suggesting that endogenously expressed Bcl-2 may provide the nearly maximum anti-apoptotic action in control cells. On the other hand, overexpression of Bcl-2 markedly inhibited apoptosis potentiated by Sig-1R siRNA (Fig. 7C).

We also tested whether the inhibition of NF-κB by oridonin may prevent the action of Sig-1R siRNA potentiating H₂O₂-induced apoptosis. Oridonin, although showed a marginal effect on H₂O₂-induced apoptosis in control cells, it inhibited the action of Sig-1R siRNA to potentiate H₂O₂-induced apoptosis (Fig. 7D).
Discussion

In the present study we demonstrated that 1) Bcl-2 localizes at the MAM, but does not serve as a substrate protein for Sig-1R chaperones; 2) Sig-1Rs transcriptionally and tonically regulate the expression of Bcl-2 via the ROS-NF-κB pathway; 3) blocking NF-κB or upregulating Bcl-2 abolishes the potentiation of H2O2-induced apoptosis caused by Sig-1R knockdown. These findings imply the significant importance of the ROS-NF-κB pathway in the cellular protective action promoted by Sig-1Rs.

Regulating the expression of Bcl-2, thus maintaining the Bcl-2:Bax ratio at the high level, is a vast importance for cellular survival (Reed, 1994; Danial and Korsmeyer, 2004; Youle and Strasser, 2008). The cell possessing multifunctional regulatory systems can control the expression of Bcl-2 at various steps including transcription, translation, and protein degradation. ROS as a potent regulator of the Bcl-2 regulate the expression of Bcl-2 via both transcription and protein degradation (Hildeman et al., 2003; Li et al., 2004). ROS-activated transcription factors such as NF-κB often negatively regulate transcription of the bcl-2 gene (Sohur et al., 1999; Pugazhenthi et al., 2003). Under certain conditions ROS decrease a half-life of Bcl-2 protein by facilitating protein degradation (Li et al., 2004). In squamous cell carcinoma OSC-4, knocking down Mn-superoxide dismutase activates ubiquitination and proteasomal degradation of Bcl-2, but suppresses degradation of Bax without activating NF-κB (Li et al., 2004). Interestingly, in our system knocking down Sig-1Rs, downregulation of Bcl-2 is caused mainly by activation of NF-κB. Knockdown of Sig-1Rs affected neither the degradation of Bcl-2 proteins nor the Bax protein level. This discrepancy might be in part due to the difference
in the cell types used in these studies. However it would be worth noting that the Sig-1R agonist PPBP is also shown to selectively prevent downregulation of the bcl-2 mRNA, but not the bax mRNA, in primary cortical neurons under hypoxia and glucose deprivation (Yang et al., 2007). Thus, the action of Sig-1Rs regulating the bcl-2 mRNA level could be seen in a wide variety of cell types. Sig-1R might be regulating the generation of specific type(s) of ROS (e.g., H₂O₂), and/or controlling sensitivity of NF-κB to ROS, thus might be more specifically involved in the process activating the ROS-NF-κB pathway. It should be also important to point out that the up-/down-regulation of Bcl-2 caused by manipulations of the Sig-1R expression in our study was induced without any stressors; the mode of action different from that in previous reports where Sig-1R ligands exert the action on the bcl-2 mRNA level only in the presence of proapoptotic stimuli (Yang et al., 2007). Thus, our study demonstrate the novel action of Sig-1Rs that tonically and intrinsically regulate the expression of Bcl-2 proteins, but not merely doing so by ameliorating stress caused by pathological insults.

The tight link between Sig-1Rs and NF-κB is observed particularly in results shown in Figures 6 and 7. We showed that knockdown of Sig-1Rs increases the expression of p105 precursors, but also the formation of the active form p50 and its nuclear localization; all leading to activation of the NF-κB complex (Karin, 2006). Further, we found that the NF-κB inhibitor oridonin completely inhibits both downregulation of Bcl-2 caused by Sig-1R siRNA and upregulation of Bcl-2 caused by overexpression of Sig-1Rs. Although oridonin showed only a marginal effect on apoptosis induced by H₂O₂ in our system, indicating the presence of NF-κB-independent cell death pathways activated by H₂O₂, it selectively abolished the effect of Sig-1R
siRNA potentiating H$_2$O$_2$-induced apoptosis. Our findings thus clearly indicate that the ROS-NF-kB-Bcl-2 pathway is a crucial element in constituting the cellular protective action of the Sig-1R against oxidative stress.

The regulation of the Ca$^{2+}$ transmission between ER and mitochondria play important roles in controlling apoptosis and cellular survival (Pinton and Rizzuto, 2006). Previous studies showed that Bcl-2 interacts with the regulatory domain of IP3R to inhibit the channel opening of IP3R, thus Ca$^{2+}$ overloading in mitochondria as well (Rong et al., 2008). On the other hand, some studies demonstrated that Bcl-2 rather activates the release of Ca$^{2+}$ from ER pools, leading to the decrease of the Ca$^{2+}$ content in ER Ca$^{2+}$ pools (Lam et al., 1994). The lowered Ca$^{2+}$ content is postulated to prevent overloading of mitochondrial Ca$^{2+}$ under cellular stress (Pinton and Rizzuto, 2006). In lights of the functional similarity, we originally speculated that Sig-1R chaperones might associate with Bcl-2, thus stabilizing Bcl-2 at the MAM to regulate Ca$^{2+}$ signaling. However, immunoprecipitation failed to demonstrate the physical interaction of Bcl-2 with Sig-1Rs. Although results from immunoprecipitation may not be sufficient to totally negate the potential physical interaction, the data showing that Sig-1R knockdown failed to alter stability of Bcl-2 strongly indicate that Bcl-2 does not serve as a substrate protein for Sig-1R chaperones, thus the physical interaction is unlikely present. It is demonstrated that the almost entire sequence of the Bcl-2 polypeptide is either anchored to the cytoplasmic surface of mitochondria or embedded in the outer mitochondrial membrane (Youle and Strasser, 2008). On the other hand, the chaperone domain of the Sig-1R is shown to reside in the lumen of the ER (Hayashi and Su, 2007). Therefore it is likely that Bcl-2 may take the same membrane topology at the ER (i.e., cytoplasmic localization) as shown
previously (Kim et al., 2004), thus the physical association of these two proteins may not be achieved \textit{in vivo}.

Although a number of studies postulated that Bcl-2 at ER membranes may localize in close vicinity to mitochondria based on the function of Bcl-2 regulating the Ca\textsuperscript{2+} transport from ER to mitochondria (Pinton and Rizzuto, 2006), it is worth noting that few studies have examined the MAM localization of Bcl-2. Thus, our findings provide direct evidence to support the notion that Bcl-2 is highly concentrated at the MAM.

We here presented a molecular mechanism by which Sig-1Rs promote cellular survival under oxidative stress. Data indicate that Sig-1Rs transcriptionally regulate the expression of Bcl-2 via the ROS-NF-\kappa B pathway, which may partly explain the robust neuroprotective action of Sig-1Rs seen \textit{in vitro} systems as well as in animal models of neurodegeneration (Meunier et al., 2006; Yang et al., 2007). Emerging questions that should be answered in the future may include: 1) how does the Sig-1Rs’ innate chaperone activity at the MAM promote the suppression of ROS generation, thus prevent activation of NF-\kappa B; 2) are there any molecules/signaling pathways that are involved in linking Sig-1Rs at the MAM to ROS generation? Sig-1Rs are shown to inhibit cell death induced by thapsigargin (Hayashi and Su, 2007), a typical ER stress inducer promoting accumulation of protein aggregates in the ER. However, Sig-1Rs also prevent cell death caused by a variety of stressors including glucose deprivation (Hayashi and Su, 2007) and H\textsubscript{2}O\textsubscript{2} (Fig. 7) that may promote protein aggregation and activation of cell death signals more predominantly in the cytoplasm or in mitochondria. Since Sig-1Rs regulate the Ca\textsuperscript{2+} influx from the MAM to mitochondria that leads to activation of mitochondrial
metabolisms as well as generation of ROS (Pinton and Rizzuto, 2006; Hayashi and Su, 2007; Hayashi et al., 2009), dysregulation of the mitochondrial Ca^{2+} signaling might be involved in underlying mechanisms by which Sig-1Rs regulate ROS accumulation/scavenging. Alternatively, since Sig-1Rs are implicated in regulation of lipid transport/metabolisms (Hayashi and Su, 2003), and since the MAM plays the crucial role in regulating metabolisms of lipids and glucose, the major determinant of cellular Redox (Rusinol et al., 1994; Voelker, 2005), Sig-1Rs might regulate the Redox state of the cell by regulating lipid/glucose metabolisms.

In conclusion, we demonstrated that the ROS-NF-κB pathway plays a pivotal role in the Sig-1R’s regulation of the Bcl-2 expression. The findings suggest that Sig-1R ligands that activate or upregulate Sig-1Rs may serve as a new class of cellular protective agents that promote upregulation of Bcl-2.
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Footnotes:

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Figure 1. Sig-1Rs tonically regulate the expression of Bcl-2 in CHO cells

Sig-1Rs were knocked down or overexpressed (OE) by transfecting vectors in CHO cells as described in Methods. Total cell lysates (30 μg /lane) were analyzed by Western blotting. As controls, the pSIREN vector carrying an inactive siRNA (control siRNA) or the empty pCR3.0 vector was transfected. ERK is for the loading controls. The graphs represent mean±SEM from six separate samples. *p<0.05, **p<0.01 by Student-t test.

Figure 2. Subcellular distribution of Sig-1Rs and Bcl-2

A. Enrichment of Sig-1Rs and Bcl-2 in the MAM fraction. The differential centrifugation combined with a Percoll gradient centrifugation was performed to obtain five different fractions (see Methods). Ten μg of proteins from each fraction except were analyzed by Western blotting. Thirty μg/lane was used for Bax immunoblotting. The second panel from the top represents the distribution of transfected Bcl-2 (OE) with the similar distribution to that of endogenous Bcl-2 (top panel). Transfected Bcl-2 was selectively detected by the monoclonal Bcl-2 antibody clone SPM117. Marker proteins of mitochondria or ER/MAM were also measured by Western blotting. The graph represents fraction distributions of proteins where the sum of 5 fractions was taken as 100% for each protein. B. No effect of Sig-1R knockdown on the subcellular distribution of Bcl-2. Membrane fractions were prepared from CHO cells transfected with Sig-1R siRNA or control siRNA as described in A. The images represent the result from three independent experiments. The graphs show fraction distributions of Sig-1Rs, Bcl-2, and Bax, where
the sum of 4 fractions was taken as 100% for each protein. Note that Sig-1R siRNA
decrease Bcl-2 in all fractions without affecting the subcellular distribution. OxyR,
cytochrome c oxidoreductase; IP3R3, type-3 IP3 receptors.

Figure 3. Confocal microscopy for the intracellular distribution of Bcl-2

A. Cellular localization of Bcl-2 and mitochondria in CHO cells. Mitochondria were
visualized by expressing Mito-DsRed fluorescent proteins (red). Bcl-2 (green) was
labeled with monoclonal Bcl-2 (1:50) primary antibodies and Alexa-480 secondary
antibodies (bar=10 μm). The second row is at a higher magnification (bar=2 μm). The
arrow indicates the mitochondrial localization of Bcl-2, whereas the arrowhead indicates
Bcl-2 in close vicinity to mitochondria. B. Spatial distribution of Mito-DsRed and Bcl-2
(in green) labeled with polyclonal Bcl-2 (1:100) primary antibodies (bar=10 μm). The
second row is at a higher magnification (bar=2 μm). Arrow: mitochondrial localization of
Bcl-2; arrowhead: Bcl-2 in close vicinity to mitochondria. C. Spatial distribution of Bcl-2
and cytochrome c oxidoreductase (OxyR). Polyclonal Bcl-2 (1:100) and monoclonal
OxyR (1:100) antibodies were used (bar=10 μm). Note that a portion of Bcl-2 (in green)
is not colocalized with the mitochondrial protein OxyR. D. Cellular localization of ER
proteins in CHO cells. Note: ERp57 and ER-DsRed distributing on reticular network
structures of the ER, whereas IP3R3 specifically targeting to punctate structures (bar=10
μm). E. Type-3 IP3 receptors (IP3R3) localize at the MAM. Monoclonal IP3R3 (1:50)
primary antibodies and Alexa-480 secondary antibodies were used. IP3R3-containing
MAMs appose mitochondria expressing Mito-DsRed (arrows and inset; bar=10 μm). F.
Partial co-localization between IP$_3$R3 (in green) and Bcl-2 (in red). Monoclonal Bcl-2 (1:50) and polyclonal IP$_3$R3 (1:100) were primary antibodies used. Bar=10 μm.

**Fig 4. Bcl-2 is not a substrate of Sig-1R chaperones**

A. No interaction between Sig-1Rs and Bcl-2 detected in immunoprecipitation. Protein lysates from CHO cells overexpressing Bcl-2 and/or Sig-1R-FLAG were prepared as described in Methods. For immunoprecipitation, either monoclonal Bcl-2 antibodies or FLAG antibodies were used. Immunoprecipitants as well as total cell lysates (INPUT) were analyzed by Western blotting. Upper two panels represent the successful immunoprecipitation of Sig-1R-FLAG with FLAG antibodies (IB: FLAG), but without co-immunoprecipitation of Bcl-2 (IB: Bcl-2). IgG: the immunoglobulin light chain of monoclonal FLAG antibodies used for immunoprecipitation. Lower two panels represent efficient immunoprecipitation of Bcl-2 with monoclonal Bcl-2 antibodies (IB: Bcl-2), but without co-immunoprecipitation of Sig-1R-FLAG (IB: FLAG). Bcl-2 bands seen in samples without the Bcl-2 transfection (the bottom panel) represent endogenous Bcl-2. B. Kinetics of Bcl-2 degradation in CHO cells transfected with control (closed circle) or Sig-1R siRNA (open circle with dotted lines). Twenty-four hours after transfection, CHO cells were treated with cycloheximide (50 μg/ml) for different time of periods to prevent the protein synthesis. Levels of Bcl-2 were measured by Western blotting. The protein level at each time point is shown as % of time 0 h of respective groups. At the 0 h-time point, the absolute level of Bcl-2 in CHO cells transfected with Sig-1R siRNA is 54.6% of the level in cells transfected with control siRNA (data not shown).
Fig 5. Sig-1Rs regulate the expression of the bcl-2 mRNA

A. Amplifications of Sig-1R mRNA and bcl-2 mRNA by RT-PCR. Total mRNA from CHO cells (0.5 µg) were amplified with specific primer sets for Sig-1R and bcl-2 (see Methods) at different cycles. Amplified cDNA were measured by agarose electrophoresis (N=2). B. Effect of Sig-1R knockdown on the mRNA level of bcl-2. The level of the bcl-2 or Sig-1R mRNA was measured by RT-PCR by using total RNA extracts (0.5 µg for each) from CHO cells transfected with either control siRNA or Sig-1R siRNA. Amplified cDNA as well as total RNA were visualized in the agarose gel. PCR cycles are 40 cycles for bcl-2 and 25 cycles for Sig-1Rs. See Methods for details of RT-PCR. The graphs represent mean±SEM (N=5). *** p < 0.001 by Student-t test. C. Effect of Sig-1R overexpression on the mRNA level of bcl-2. Total RNA were extracted from CHO cells transfected with either control vectors or Sig-1R overexpression (OE) vectors. The mRNA level of bcl-2 or Sig-1Rs was measured by RT-PCR. The graphs represent mean±SEM (n=4). *** p < 0.001 by Student-t test.

Fig. 6. Sig-1R knockdown promotes activation NF-κB

A. Sig-1R siRNA promote downregulation of Bcl-2 in a ROS-dependent manner. Free radical scavenger Tempol (TPL, 0.5 mM), NAC (5 mM), or NLA (0.5 mM) were applied to culture medium 6 h after the transfection of CHO cells with control or Sig-1R siRNA. After further incubation for 18 h, cell lysates were analyzed by Western blotting (30 µg/lane). Bcl-2 levels were normalized to the internal controls (total ERK) and presented as % of control. Two-way ANOVA of effect of siRNA transfection or scavenger treatments on the Bcl-2 level is: F(2;29; transfection) = 16.23, p=0.0006; F(4;29;
treatment) = 9.578, p=0.0003. *p<0.05 compared to control siRNA without scavenger, ##
p<0.01, # p<0.05 compared to Sig-1R siRNA without scavenger (Bonferroni post-hoc

test; n=4). B. Sig-1R siRNA increase levels of p50 and p105 subunits of the NF-κB 

complex. Levels of p50 or p105 analyzed by Western blotting were normalized to ERK, 
and shown as % of control in the graph (n=10). *p<0.05, ** p<0.001 compared to control 
siRNA by Student-t test. C. Increased KCl-induced induction of p50 and p105 in CHO 
cells knocking down Sig-1Rs. KCl at 50 mM was applied to culture medium for indicated 
periods of time. Protein levels of p50 and p105 were measured by Western blotting 
followed by normalization to ERK. Two-way ANOVA of effect of time or KCl treatment 
on p105 level is: F(4;40; time) = 102.1, p<0.0001; F(4;40; treatment) = 52.92, p<0.0001; 
F(4;40; interaction) = 7.754, p = 0.0014. * p<0.05, ** p<0.01 compared to control siRNA 
by Bonferroni post-hoc tests. Two-way ANOVA of effect of time or KCl treatment on 
p50 level is: F(4;40; time) = 84.54, p<0.0001; F(4;40; treatment) = 34.81, p<0.0001. ** 
p<0.01 compared to control siRNA by Bonferroni post-hoc test (n=4). D. Effect of free 
radical scavengers on Sig-1R siRNA/H2O2 -induced induction of p50. After transfection 
with either control siRNA or Sig-1R siRNA, CHO cells were treated with free radical 
scavengers for 18 h at the same concentrations used in A or with H2O2 (50 μM) for 4 h. 
Twenty-four h after transfection, cells were harvested. Levels of p50 in total cell lysates 
were analyzed by Western blotting followed by normalization to ERK. Two-way 
ANOVA of effect of siRNA transfection or scavenger treatments on the Bcl-2 level is: 
F(3;72; transfection) = 130.0, p<0.0001; F(4;72; treatment) = 52.99, p<0.0001. * p<0.05,
 ** p<0.01 compared to control siRNA; # p<0.05, ## p<0.01 compared to Control (no 
scavenger) with the same siRNA transfection or H2O2 treatment. (Bonferroni post-hoc
test, n=4). **E.** Nuclear p50 is increased by Sig-1R siRNA. Nuclei and post-nuclear lysate were prepared from CHO cells transfected with control or Sig-1R siRNA as described in Methods. In Western blotting, 30 μg/lane of lysates were loaded. Nucl, nucleoporin p63. In the graph, nuclear p50 was normalized to nucleoporin p63 (n=6). ** p<0.01 compared to control siRNA by Student- t test. **F.** Decreased IκBα in CHO cells transfected with Sig-1R siRNA. IκBα levels were measured by Western blotting (20 μg/lane) followed by normalization to ERK. * p<0.01 compared to control siRNA by Student- t test (n=6). **G.** Effect of Sig-1R overexpression on levels of p105, p50 and IκBα. Twenty-four hours after transfection of wild-type Sig-1Rs, the levels of each protein were measured by Western blotting (20 μg/lane) followed by normalization to ERK. **p<0.05 compared to control vector by Student- t test. N=5.

**Fig 7. Involvement of the NF-κB-Bcl-2 pathway in cell protection of Sig-1Rs**

**A.** Dose-dependent inhibition by oridonin of Bcl-2 downregulation caused by Sig-1R siRNA. Six hours after transfection with control siRNA or Sig-1R siRNA, cells were cultured with oridonin for following 18 h. Bcl-2 protein levels were measured by Western blotting with normalization to internal controls (total ERK). The graph represents mean±SEM (n=6). Two-way ANOVA of effect of siRNA or concentrations of oridonin is: F(4;60; siRNA) = 11.87, p=0.0012; F (4;60; [oridonin])=32.61, p<0.0001. ** p<0.01 compared to control siRNA at the same dose of oridonin (Bonferroni post-hoc test). **B.** Dose-dependent inhibition by oridonin of Bcl-2 upregulation caused by Sig-1R overexpression (OE). Six hours after transfection with empty vectors or Sig-1R overexpression vectors, cells were cultured with oridonin for following 18 h. Bcl-2 levels
were measured as described in A. Two-way ANOVA of effect of vector transfection or concentrations of oridonin is: $F(4;60; \text{OE}) = 4.324, p=0.0427$; $F(4;60; \text{oridonin}) = 12.85, p<0.0001$. *p<0.05 compared to control vector at the same dose of oridonin (Bonferroni post-hoc test; n=6). C. Effect of Sig-1R siRNA and/or Bcl-2 overexpression on H$_2$O$_2$-induced apoptosis in CHO cells. Twenty-four hours after transfection with siRNAs and/or Bcl-2 expression vectors, CHO cells were treated with H$_2$O$_2$ (50 μM) for indicated periods of time. Apoptotic cells were counted as described in Methods. Two-way ANOVA of effect of transfection or exposure time of H$_2$O$_2$ is: $F(4;80; \text{transfection}) = 5.586, p = 0.0016$; $F(4; 80; \text{exposure time}) = 46.02, p<0.0001$. **p<0.01 compared to control siRNA at 8 h (Bonferroni post-hoc test; n=6). D. Effect of oridonin on H$_2$O$_2$-induced apoptosis. CHO cells were transfected with control siRNA or Sig-1R siRNA. Six h after transfection, oridonin (2 μg/ml) was applied to culture medium for following 18 h. Twenty-four hours after transfection, H$_2$O$_2$ (50 μM) was challenged for 8 h with or without oridonin. Apoptotic cells were counted as described in Methods. Two-way ANOVA of effect of drug treatment or transfection is: $F(4;48; \text{treatment}) = 32.61, p <0.0001$; $F(2; 48; \text{transfection}) = 1.155, p=0.2889$. *p<0.05, ** p<0.001 compared to Control (no drug treatment) with the same siRNA transfection. ## p<0.01 compared to H$_2$O$_2$ alone transfected with Sig-1R siRNA. +p<0.05 compared to H$_2$O$_2$ alone transfected with control siRNA (Bonferroni post-hoc test; n=6).
Figure 3
Figure 6

A. Bcl-2 Level (% of control)

- TPL | NAC | NLA
Control siRNA | 100 | 100 | 100
Sig-1R siRNA | 100 | 100 | 100

B. NF-κB Level (% of control)

- TPL | NAC | NLA
Control siRNA | 100 | 100 | 100
Sig-1R siRNA | 100 | 100 | 100

C. p105 level (% of control)

- TPL | NAC | NLA
Control siRNA | 100 | 100 | 100
Sig-1R siRNA | 100 | 100 | 100

D. p50 level (% of control)

- TPL | NAC | NLA
Control siRNA | 100 | 100 | 100
Sig-1R siRNA | 100 | 100 | 100

E. Western Blot

- Nuclei | Post-nuclear lysate
Control siRNA | 100 | 100
Sig-1R siRNA | 100 | 100

F. IkBα level (% of control)

- TPL | NAC | NLA
Control siRNA | 100 | 100 | 100
Sig-1R siRNA | 100 | 100 | 100

G. Protein level (% of control)

- TPL | NAC | NLA
Control siRNA | 100 | 100 | 100
Sig-1R siRNA | 100 | 100 | 100

** and * indicate significant differences.