

Involvement of Bid and Caspase-2 in Endoplasmic Reticulum Stress- and Oxidative Stress-Induced Retinal Ganglion Cell Death

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Endoplasmic reticulum (ER) stress and oxidative stress are involved in many diseases, including retinal disorders, causing toxicity in various tissues and cells; however, intracellular signaling of ER stress and cross-talk between ER stress and oxidative stress are unknown in retinal ganglion cells (RGC), whose degeneration is associated with glaucoma. The aim of the study was to clarify the mechanisms of ER stress- and oxidative stress-induced RGC death, using cultured retinal ganglion cells (RGC-5) in vitro and N-methyl-D-aspartate (NMDA)- or ER stress-induced retinal damage in mice in vivo. We focused on both BH3-interacting domain death agonist (Bid) and caspase-2, which work as apoptosis promotion factors. In an in vitro study, both Bid and caspase-2 inhibitors protected against RGC-5 death from ER stress or oxidative stress. A caspase-2 inhibitor did not inhibit Bid cleavage, although a Bid inhibitor reduced the increase of caspase-2 activity in ER stress-induced RGC-5 death. A Bid inhibitor also reduced the increase of caspase-2 activity in oxidative stress-induced RGC-5 death. Moreover, both Bid and caspase-2 inhibitors reduced the increase of caspase-3 activity. In an in vivo study, a Bid inhibitor inhibited NMDA- or ER stress-induced retinal damage. These findings indicate that a common mechanism through Bid and caspase-2 exists in both ER stress- and oxidative stress-induced RGC death and that they are activated in the order of Bid, caspase-2, and caspase-3. © 2011 Wiley-Liss, Inc.

Key words: glaucoma; ER stress; caspase-2; Bid; RGC

Retinal ganglion cell (RGC) death is a common feature of many ophthalmic disorders, such as glaucoma, optic neuropathies, and retinovascular diseases (diabetic retinopathy and retinal vein occlusions). RGC death has been reported to occur via a variety of mechanisms involving oxidative stress (Bonne et al., 1998), excitatory amino acids (Dreyer, 1998), nitric oxide (NO; Neufeld, 1999), and endoplasmic reticulum (ER) stress (Shimazawa et al., 2007a). Among these mechanisms, ER stress is caused by the accumulation of unfolded proteins in the

ER lumen and is associated with various neurodegenerative diseases, such as Alzheimer's, Huntington's, and Parkinson's diseases (Katayama et al., 2001; Oyadomari et al., 2002b; Ryu et al., 2002). Recent reports have shown that ER stress is also involved in a variety of experimental retinal neurodegenerative models, such as diabetic retinopathy (Roybal et al., 2004), retinitis pigmentosa, and glaucoma (Joe et al., 2003; Gould et al., 2007).

Oxidative stress may occur because of an imbalance between the production and the removal of reactive oxygen species (ROS) and is also considered a critical mediator in RGC injury of various etiologies and RGC death (Maher and Hanneken, 2005b). Thus, oxidative stress is associated with various retinal diseases, such as diabetic retinopathy (Abu el-Asrar et al., 1995), glaucoma (Chalasan et al., 2007), age-related macular degeneration (Kindzelskii et al., 2004), and retinitis pigmentosa (Doonan et al., 2005).

Caspases, a highly conserved family of cysteine proteases, are key apoptotic effectors and play a critical role in apoptosis through a cascade of cleavage events (Schmitz et al., 2000; Shi, 2002). Among caspase family members, caspase-2 is unique because it has features of both the long prodomain of upstream caspases and the optimal recognition motif of downstream caspases. The

Additional Supporting Information may be found in the online version of this article.

R. Uchibayashi and K. Tsuruma contributed equally to this work.

Contract grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan; Contract grant number: 20592082; Contract grant sponsor: Takeda Science Foundation.

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Received 24 August 2010; Revised 12 April 2011; Accepted 14 April 2011

Published online 29 July 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.22691

prodomain is essential for the dimerization and autoprocessing of precursor caspase-2, which allows caspase-2 to be directly activated and further initiate the caspase cascade in response to various apoptotic stimuli (Harvey et al., 1997; Droin et al., 2001). Caspase-2 is activated by ER stress and oxidative stress and activates caspases-3, -8, and -9 and BH3-interacting domain death agonist (Bid; Cheung et al., 2006; Prasad et al., 2006; Gu et al., 2008; Tamm et al., 2008; Upton et al., 2008; Huang et al., 2009). Activation of caspase-2 occurs prior to the activation of caspase-3 and the cleavage of Bid (Cheung et al., 2006; Prasad et al., 2006; Tamm et al., 2008; Upton et al., 2008).

Bid is a member of the BH-3-only family of proapoptotic proteins that initiates apoptotic cell death (Puthalakath and Strasser, 2002). It is constitutively expressed in many cells, and its main mechanism of activation involves a series of posttranslational modifications, including proteolytic cleavage, phosphorylation, and translocation to mitochondria (Li et al., 1998; Luo et al., 1998; Desagher et al., 2001). Bid is also activated by ER stress and oxidative stress, inducing the release of cytochrome c from mitochondria to cytosol, activity of caspases-3 and -9, and nuclear translocation of apoptosis-inducing factor (AIF; Abdelrahim et al., 2006; Rabi et al., 2007; Landshamer et al., 2008; Tamm et al., 2008; Lee et al., 2009).

In a previous study, we showed that ER stress and oxidative stress played pivotal roles in RGC death in vitro and in vivo (Shimazawa et al., 2005; Suemori et al., 2006; Nakajima et al., 2008; Inokuchi et al., 2009a,b); however, the mechanisms, i.e., key proteins, have not been elucidated sufficiently. In the present study, we sought to clarify the mechanism of ER stress- and oxidative stress-induced RGC death, especially Bid and caspase-2.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagles's medium (DMEM), Bid inhibitor (BI-6C9), and N-methyl-D-aspartate (NMDA) were purchased from Sigma-Aldrich (St. Louis, MO). Caspase-2/Ich1 inhibitor (Z-VDVAD-fmk) was purchased from BioVision (Mountain View, CA). L-buthionine-(S,R)-sulfoximine (BSO) and tunicamycin were purchased from Wako (Osaka, Japan). L-glutamic acid monosodium salt (glutamate) was purchased from Nacalai Tesque (Kyoto, Japan). Isoflurane was purchased from Nissan Kagaku (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Valeant (Costa Mesa, CA).

RGC Line (RGC-5) Culture

The RGC-5 line is a transformed cell line for RGCs, which was generously provided by Dr. Neeraj Agarwal (UNT Health Science Center, Fort Worth, TX). Cultures of RGC-5 were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin (Meiji Seika Kaisha, Tokyo, Japan), and 100 µg/ml streptomycin (Meiji Seika Kaisha) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The RGC-5

were passaged by trypsinization every 3 days, as in a previous report (Tsuruma et al., 2010).

Cell Death Assay

RGC-5 were plated at a density of 1,000 cells/well in 96-well culture plates (3072; Falcon, Becton Dickinson and Company, Franklin Lakes, NJ). Twenty-four hours later, cells were washed twice with DMEM and then immersed in DMEM supplemented with 0.1% or 1% FBS. After 1 hr of pretreatment with BI-6C9 (2 and 10 µM) or Z-VDVAD-fmk (2 and 10 µM), tunicamycin (2 µg/ml), or BSO (500 µM) plus glutamate (10 mM) was added to these cultures for 24 hr. Cell mortality was measured using a single-cell digital imaging-based method employing fluorescent staining of nuclei. Briefly, cell death was assessed on combination staining with fluorescent dyes [namely, Hoechst 33342 (Molecular Probes, Eugene, OR) and propidium iodide (PI; Molecular Probes)]. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan), as in a previous report (Tsuruma et al., 2010).

Immunoblotting

RGC-5 were lysed using a cell-lysis buffer (RIPA buffer; Sigma-Aldrich) with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Cell lysates were solubilized in SDS-sample buffer, separated on 5–20% SDS-polyacrylamide gels, and transferred to PVDF membrane (Immobilon-P; Millipore, Bedford, MA). Transfers were blocked for 30 min at room temperature with blocking with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan), then incubated overnight at 4°C with the goat anti-Bid (R&D Systems, Minneapolis, MN) or anticaspase-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-β-actin (Sigma-Aldrich) primary antibody. The transfers were then rinsed with TBS-T and incubated for 1 hr at room temperature in horseradish peroxidase rabbit anti-goat (Pierce, Rockford, IL) diluted 1:200,000, goat anti-rabbit or goat anti-mouse (Pierce) diluted 1:2,000. The immunoblots were developed using chemiluminescence (Super Signal West Femto Maximum Sensitivity Substrate; Pierce), and visualized with the aid of a digital imaging system (LAS-4000UVmini; Fujifilm, Tokyo, Japan). Four immunoreactive bands were detected using anti-Bid antibody. The highest molecular weight band is nontruncated Bid. Yi et al. (2003) reported that the second band is a Bid antibody-reactive band that was occasionally detected in Bcl-2-transfected cell lysates, and the third and lowest molecular weight bands are tBid p15 and p13, respectively. tBid p13 exists only in membrane fraction, although p15 was observed in both membrane and cytosol (Yi et al., 2003). Because tBid acts in mitochondrial membrane, we considered p13 as the activated Bid by BSO plus glutamate or ER stress.

Caspase-2 Activity Assay

Caspase-2 activity was measured using the Caspase-2 Fluorometric Assay Kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. Briefly, cells were harvested and resuspended in cell lysis buffer. After lysis, cell lysates were mixed with 50 µl assay buffer and 5 µl of the

1 mM VDVAD-afc substrate. After incubation at 37°C for 1 hr, fluorescence of reactions was measured using a microplate reader (Skanlt RE for Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA) with 400-nm excitation filter and 505-nm emission filter. Fluorescence of each sample was determined by subtraction of the mean fluorescence of the blank from that of the sample.

Caspase-3 Activity Assay

Caspase-3 activity was measured using the CaspACE Fluorometric Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, cells were harvested and resuspended in cell lysis buffer. After lysis, cell lysates were mixed with 32 μ l assay buffer, 2 μ l DMSO, 10 μ l of 100 mM DTT, and 2 μ l of 2.5 mM Ac-DEVD-amc substrate. After incubating at 37°C for 1 hr, fluorescence of reactions was measured using a microplate reader with 360-nm excitation filter and 460-nm emission filter. Fluorescence of each sample was determined by subtraction of the mean fluorescence of the blank from that of the sample.

Animals

Male adult ddY mice (Japan SLC, Hamamatsu, Japan) weighing 36–43 g were kept under controlled lighting conditions (12 hr:12 hr light/dark). All experimental procedures were performed in accordance with the NIH *Guide for the care and use of laboratory animals* and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

NMDA- or Tunicamycin-Induced Retinal Damage

Male mice were anesthetized with 3.0% isoflurane and maintained using 1.5% isoflurane in 70% N₂O and 30% O₂, delivered via an animal general anesthesia machine (Soft Lander; Sin-ei Industry, Saitama, Japan). The body temperature was maintained at 37.0°C to 37.5°C with the aid of a heating pad and heating lamp. Retinal damage was induced by injection (2 μ l/eye) of NMDA at 5 nmol/eye or tunicamycin at 1 μ g/eye. This was injected into the vitreous body of the left eye with the animal under the anesthesia described above. One drop of 0.01% levofloxacin ophthalmic solution (Santen Pharmaceuticals, Osaka, Japan) was applied topically to the treated eye immediately after the intravitreal injection. Seven days after the injection, eyeballs were enucleated for histological analysis. For comparative purposes, nontreated retinas from each mouse strain were also investigated. BI-6C9 (1 or 4 nmol/eye) or vehicle (5% DMSO in saline) was coadministered with the tunicamycin or vehicle in each mouse.

Histological Analysis

Seven days after the tunicamycin injection, eyeballs were enucleated for histological analysis. In mice under anesthesia, produced by an intraperitoneal injection of sodium pentobarbital (80 mg/kg), each eye was enucleated and then kept immersed for at least 24 hr at 4°C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness 3 μ m) cut through the optic disc of each eye were prepared in a standard manner and stained with

hematoxylin and eosin. Retinal damage was evaluated as described previously, and three sections from each eye were used for the morphometric analysis (Inokuchi et al., 2009b).

TUNEL Staining

TUNEL staining was performed according to the manufacturer's protocol (In Situ Cell Death Detection Kit; Roche Biochemicals, Mannheim, Germany) to detect the retinal cell death induced by NMDA. Mice were anesthetized with pentobarbital sodium at 80 mg/kg, i.p., 24 hr after intravitreal injection (NMDA 5 nmol/eye). The eyes were enucleated, fixed overnight in 4% paraformaldehyde, and immersed in 25% sucrose in PBS for 48 hr at 4°C. The eyes were then embedded in a supporting medium for frozen tissue specimens (OCT compound; Tissue-Tek, IL). Retinal sections 10 μ m thick were cut at –25°C using a cryostat and stored at –80°C until staining. After twice washing with PBS, sections were incubated with fluorescein-labeled terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C for 1 hr. The sections were washed three times in PBS for 1 min each at room temperature and then developed. Fluorescent microscope images were photographed, and the labeled cells in the GCL at a distance between 375 and 625 μ m from the optic disc were counted in two areas of the retina in a masked fashion by a single observer (Y.I.). The number of TUNEL-positive cells was averaged for these two areas and plotted as the number of TUNEL-positive cells.

Statistical Analysis

Data are presented as means $\pm$ SEM. Statistical comparisons were made using a one-way ANOVA followed by either a Student's *t*-test or a Dunnett's or Tukey's multiple-comparison test. A value of *P* < 0.05 was considered to indicate statistical significance.

RESULTS

Bid Inhibitor and Caspase-2 Inhibitor Suppressed ER Stress- and Oxidative Stress-Induced RGC-5 Death

In the present study, tunicamycin was used as an ER stress inducer, and BSO plus glutamate was used as an oxidative stress inducer. Tunicamycin, a naturally occurring antibiotic, blocks the first step in the biosynthesis of N-linked oligosaccharides in cells (Mahoney and Duksin, 1979). BSO depletes intracellular glutathione (GSH), and glutamate inhibits cystine uptake. We first examined which apoptotic pathways were involved in ER stress-induced RGC death using RGC-5. ER stress is known to trigger cell death via caspase-12, JNK pathway, AIF, and CHOP induction; therefore, we investigated the participation of these proteins using specific inhibitors or siRNAs. However, these proteins were not associated with tunicamycin-induced RGC death (Supp. Info. Fig. 1). Consequently, we examined whether Bid and caspase-2, which were also reported as proteins existing downstream of ER stress, might be involved in tunicamycin-induced RGC death. To clarify this issue, we examined cell mortality using a Bid

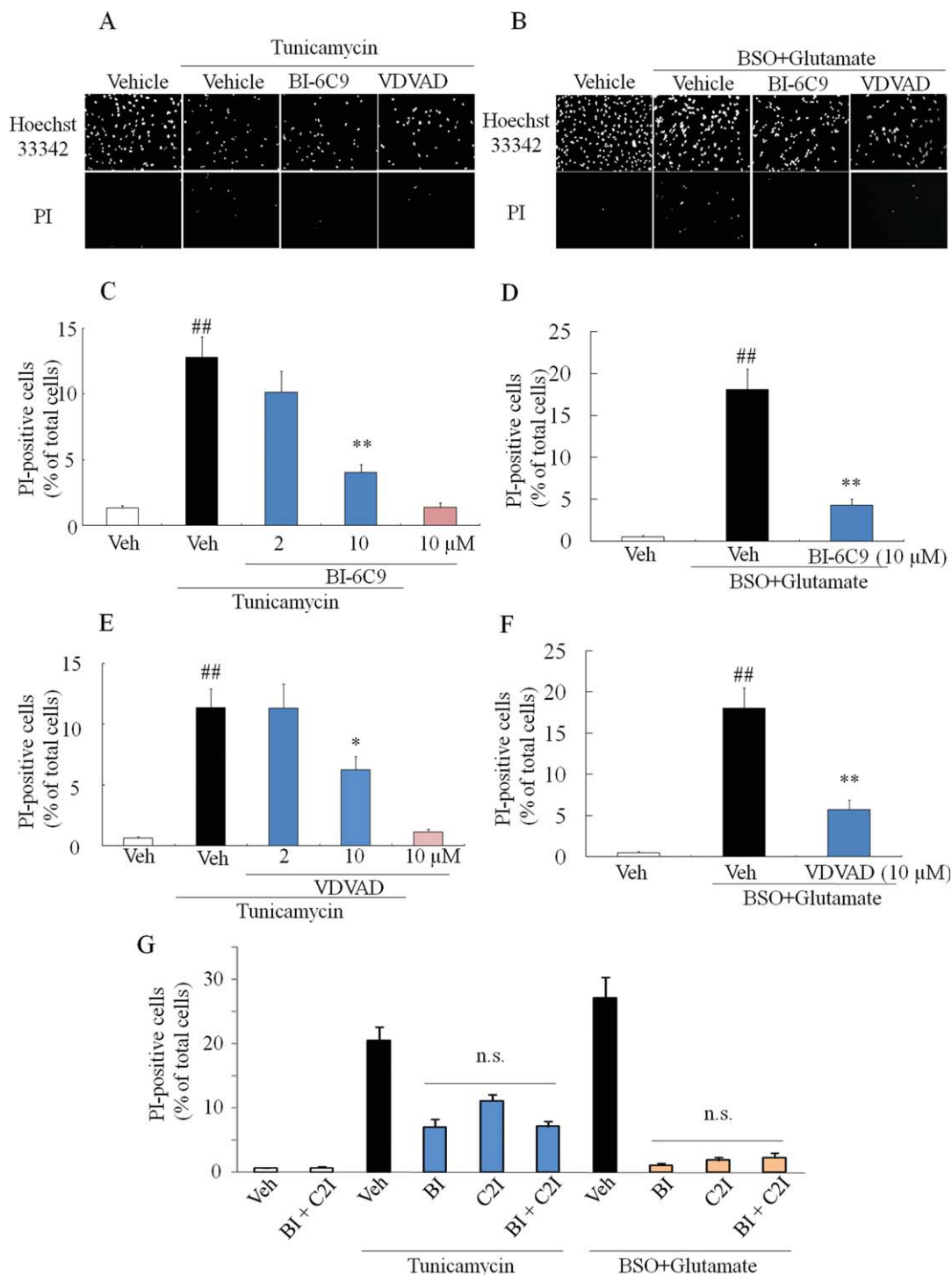


Fig. 1. Bid inhibitor and caspase-2 inhibitor suppressed ER stress- and oxidative stress-induced RGC-5 death. **A,B**: Typical image of nuclear staining. **A**: After 1 hr pretreatment with BI-6C9 (10 μ M) or Z-VDVAD-fmk (10 μ M), tunicamycin (2 μ g/ml) was added for 24 hr. Vehicle is 1% DMSO. **B**: After 1 hr pretreatment with BI-6C9 (10 μ M) or Z-VDVAD-fmk (10 μ M), BSO (500 μ M) and glutamate (10 mM) were added for 24 hr. **C–G**: Quantification of cell death was calculated as described in Materials and Methods. **C**: BI-6C9 with tunicamycin. **D**:

BI-6C9 with BSO plus glutamate. **E**: Z-VDVAD-fmk with tunicamycin. **F**: Z-VDVAD-fmk with BSO plus glutamate. **G**: Effect of combination treatment with BI-6C9 (10 μ M) and Z-VDVAD-fmk (10 μ M) on tunicamycin or BSO plus glutamate-induced cell death. Data are expressed as the mean $\pm$ SEM ($n = 6–8$). $^{##}P < 0.01$ vs. vehicle alone; $^{**}P < 0.01$ vs. vehicle. n.s., Not significant (Tukey's test); Veh, vehicle; BI, BI-6C9; C2I, Z-VDVAD-fmk (caspase-2 inhibitor). [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

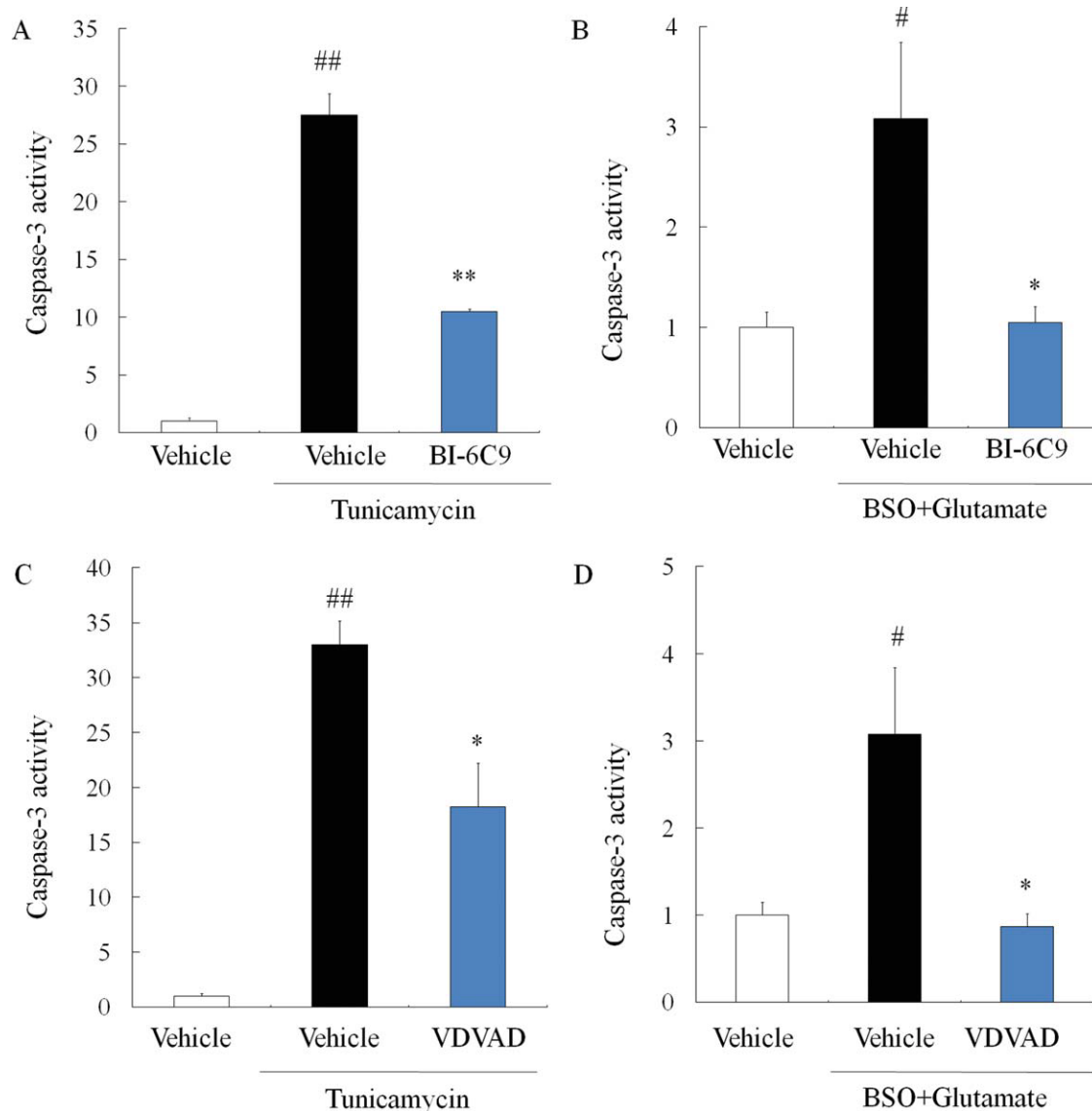


Fig. 2. Bid inhibitor and caspase-2 inhibitor suppressed ER stress- and oxidative stress-induced increases of caspase-3 activity. Caspase-3 activity measured after 24 hr tunicamycin (2 μ g/ml) or BSO (500 μ M) plus glutamate (10 mM) treatment. BI-6C9 (10 μ M) or Z-VDVAD-fmk (10 μ M) was added at 1 hr before tunicamycin or BSO plus glutamate. Data are expressed as the mean $\pm$ SEM ($n = 3$ or 4). # $P < 0.05$, ## $P < 0.01$ vs. control; * $P < 0.05$, ** $P < 0.01$ vs. vehicle. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

inhibitor (BI-6C9) and a caspase-2 inhibitor (Z-VDVAD-fmk). In comparison with the vehicle-treated group, tunicamycin significantly increased the number of dead cells (stained with PI; Fig. 1A,C,E). Pretreatment with BI-6C9 (10 μ M) and Z-VDVAD-fmk (10 μ M) protected against tunicamycin-induced RGC-5 death (Fig. 1C,E). These data indicate that Bid and caspase-2 are involved in tunicamycin-induced RGC-5 death. These effects were also observed in human neuroblastoma SH-SY5Y cells (Supp. Info. Fig. 2A–D). We also examined other ER stress inducer, thapsigargin (inhibitor of the ER Ca^{2+} pump),

and Bid and caspase-2 inhibitor reduced thapsigargin-induced RGC-5 death (Supp. Info. Fig. 2E–H). Moreover, BI-6C9 (10 μ M) and Z-VDVAD-fmk (10 μ M) also protected against BSO plus glutamate-induced RGC-5 death (Fig. 1D,F). These data indicate that Bid and caspase-2 are involved not only in ER stress- but also BSO plus glutamate-induced RGC-5 death. On the other hand, combination treatment with BI-6C9 and Z-VDVAD-fmk did not alter the reduction of cell death induced by tunicamycin nor BSO plus glutamate compared with single treatment (Fig. 1G).

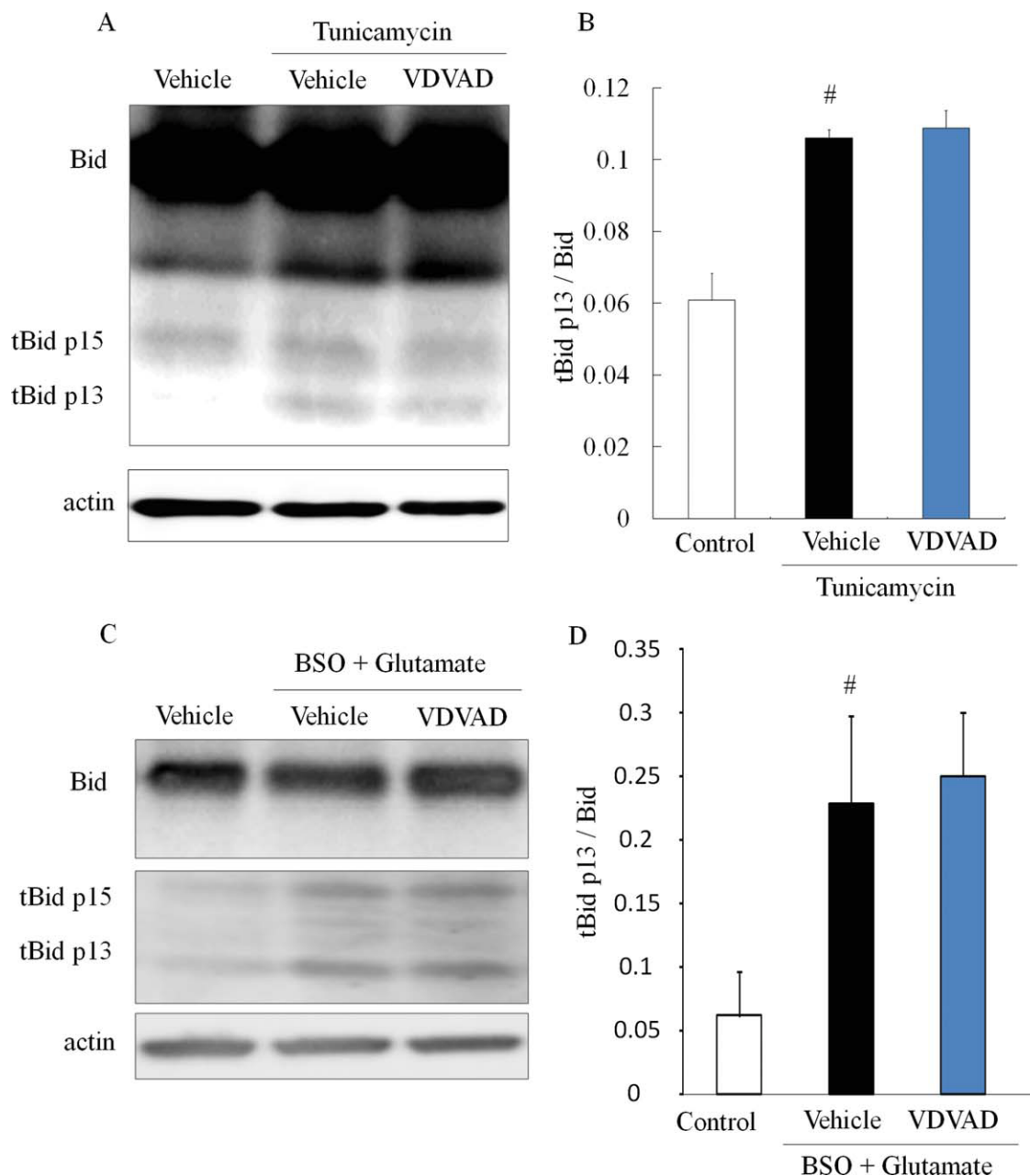


Fig. 3. Caspase-2 inhibitor did not inhibit Bid cleavage in RGC-5. **A,C:** Representative immunoblots showing Bid, tBid, and actin protein levels at 24 hr after tunicamycin (2 μ g/ml; **A**) or BSO (500 μ M) plus glutamate (10 mM; **C**) treatment. Z-VDVAD-fmk (10 μ M) was added 1 hr before stress-inducer treatment. **B,D:** Quantitative analysis of band densities for tBid/Bid. Relative intensity was described by the

intensity of tBid p13 (active form of tBid) divided by Bid (nontruncated Bid). The details of each band are described in Materials and Methods. Data are expressed as the mean $\pm$ SEM ($n = 3$) of values (in arbitrary units) obtained from each band. [#] $P < 0.05$ vs. vehicle alone. VDVAD, Z-VDVAD-fmk. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.wileyonlinelibrary.com).]

Bid Inhibitor and Caspase-2 Inhibitor Inhibited ER Stress- and Oxidative Stress-Induced Increases of Caspase-3 Activity

We next examined whether caspase-3 was activated downstream of Bid and caspase-2 in tunicamycin- and BSO plus glutamate-induced RGC-5 death. Both tunica-

mycin and BSO plus glutamate-treated groups increased caspase-3 activity in comparison with the control, and both BI-6C9 and Z-VDVAD-fmk reduced the increase of caspase-3 activity (Fig. 2). These data indicate that caspase-2 and Bid exist upstream of caspase-3 in both tunicamycin- and BSO plus glutamate-induced RGC-5 death.

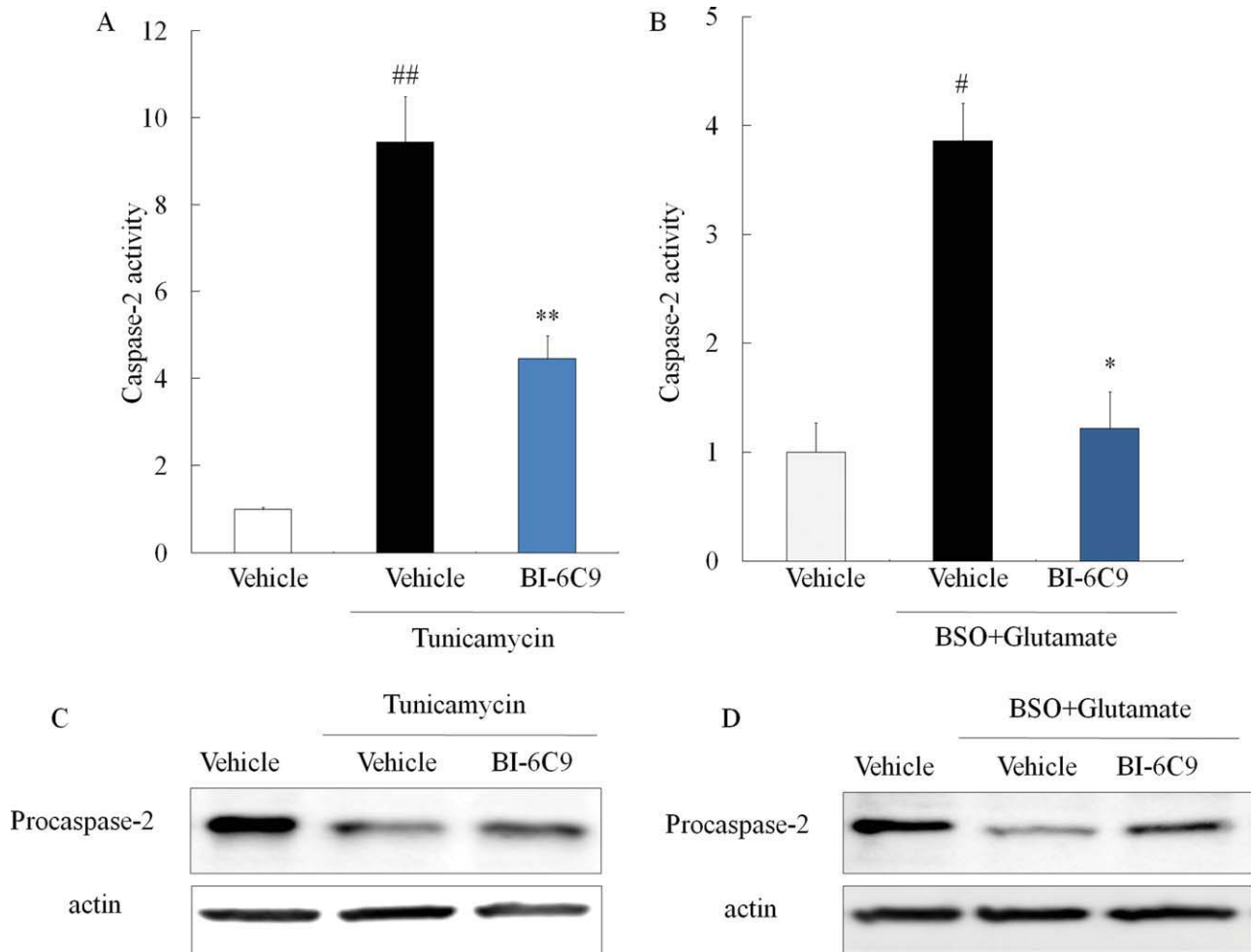


Fig. 4. Bid inhibitor suppressed ER stress- and oxidative stress-induced increase of caspase-2 activity in RGC-5. **A:** Cells were exposed to tunicamycin (2 μ g/ml) for 24 hr. BI-6C9 (10 μ M) was added 1 hr before tunicamycin. **B:** Cells were exposed to BSO (500 μ M) plus glutamate (10 mM) for 18 hr. BI-6C9 (10 μ M) was added 1 hr before the BSO plus glutamate. These cells were also subjected to

immunoblot analysis. Reduction of caspase-2 activation (degradation of procaspase-2) by BI-6C9 was observed in tunicamycin (**C**)- or BSO plus glutamate (**D**)-treated cells. Data are expressed as the mean $\pm$ SEM ($n = 3$ or 4). [#] $P < 0.05$, ^{##} $P < 0.01$ vs. vehicle alone; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs. vehicle. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Caspase-2 Inhibitor Did Not Inhibit Bid Cleavage

The above results showed that Bid and caspase-2 were involved in tunicamycin-induced RGC-5 death (Figs. 1, 2). Truncated Bid (tBid), an active form of Bid, is generated by various proteases. Previous studies have indicated that caspase-2 cleaves Bid by ER stress in mouse embryonic fibroblasts (MEFs; Upton et al., 2008). To clarify this issue, we examined by using immunoblotting whether Z-VDVAD-fmk might inhibit Bid cleavage. In tunicamycin-treated groups, cleaved Bid and Bid activity (i.e., rate of Bid cleavage; p13) was significantly increased, but, unexpectedly, Z-VDVAD-fmk did not inhibit Bid cleavage (Fig. 3A,B). Moreover, Z-VDVAD-fmk did not inhibit Bid cleavage induced by BSO plus glutamate (Fig. 3C,D). These data indicate that Bid exists upstream of caspase-2 in tunicamycin- and BSO plus glutamate-induced RGC-5 death.

Bid Inhibitor Inhibited ER Stress- and Oxidative Stress-Induced Increase of Caspase-2 Activity

To clarify whether BI-6C9 might inhibit the increase of caspase-2 activity, we measured caspase-2 activity using caspase-2 specific fluorescence substrate. Both tunicamycin- and BSO plus glutamate-treated groups increased caspase-2 activity compared with the control group, and BI-6C9 reduced the increases of caspase-2 activity in both tunicamycin- and BSO plus glutamate-induced RGC-5 death (Fig. 4A,B). We also investigated caspase-2 activation using immunoblot analysis. Procaspase-2 was degraded (i.e., activated) in tunicamycin- or BSO plus glutamate-treated cells, and BI-6C9 suppressed this degradation (Fig. 4C,D). These data indicate that Bid regulates caspase-2 activation in both tunicamycin- and BSO plus glutamate-induced RGC-5 death.

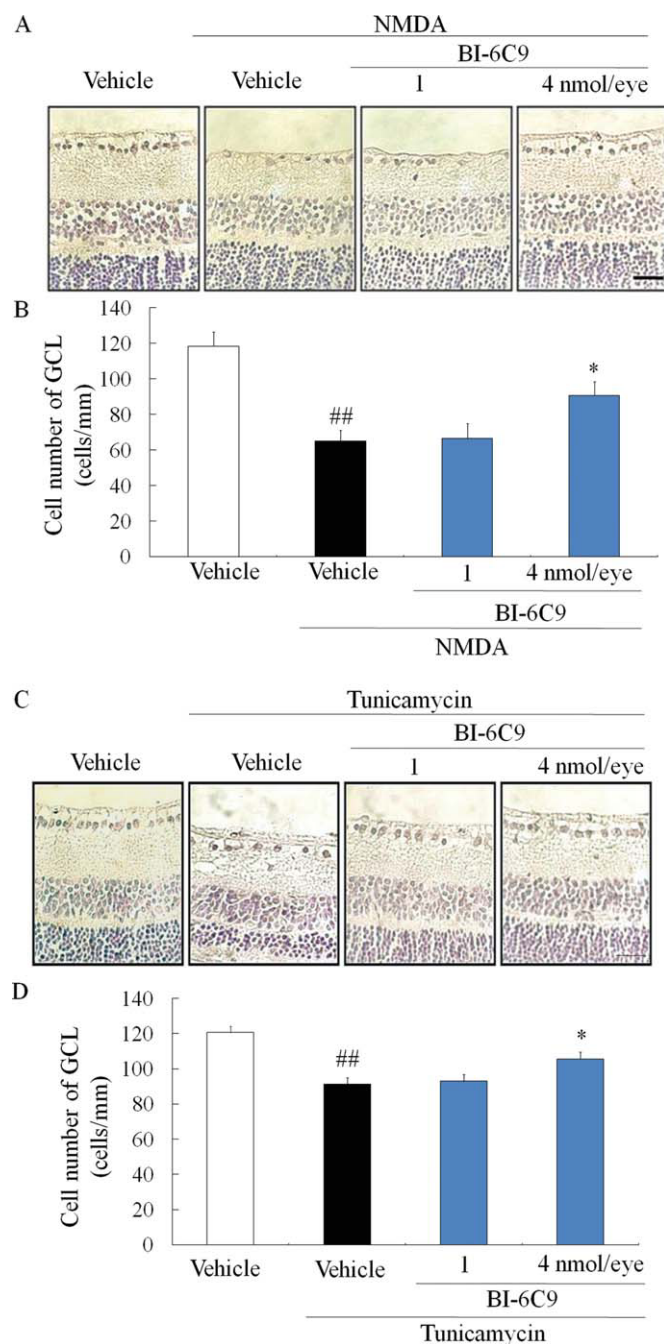


Fig. 5. Bid inhibitor reduced NMDA- and tunicamycin-induced RGC death in vivo. **A,C**: Representative photographs showing nontreated normal retina, NMDA (5 nmol/eye)- or tunicamycin (1 μ g/eye)-treated retina, and NMDA or tunicamycin and BI-6C9 (1 and 4 nmol/eye)-treated retinas at 7 days after intravitreal injection. Samples were stained with hematoxylin and eosin as described in Materials and Methods. **B,D**: Retinal damage was evaluated by counting cell numbers in GCL at 7 days after intravitreal injection. Data are expressed as the mean $\pm$ SEM ($n = 6$ or 9). ^{##} $P < 0.01$ vs. vehicle alone; ^{*} $P < 0.05$ vs. vehicle. Scale bar = 25 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

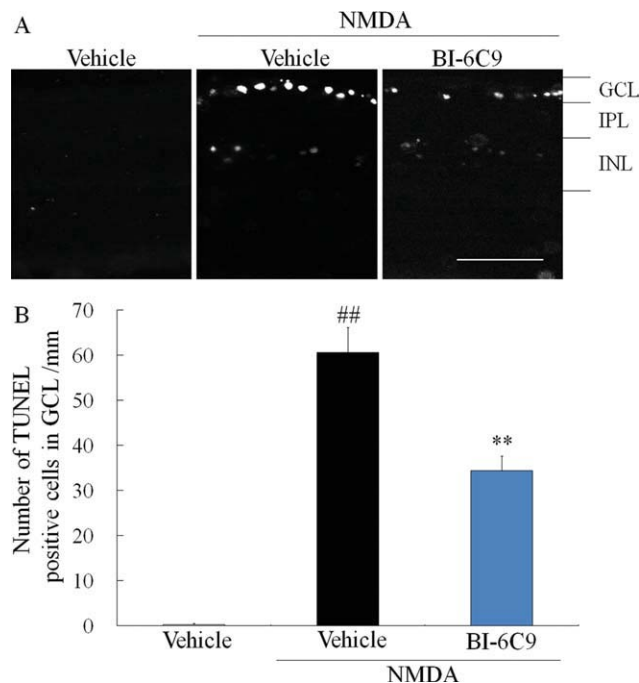


Fig. 6. Bid inhibitor reduced TUNEL-positive cells induced by intravitreal injection of NMDA in mice. **A**: Representative photographs showing nontreated normal retina, NMDA (5 nmol/eye)-treated retina, and NMDA and BI-6C9 (4 nmol/eye)-treated retinas at 24 hr after intravitreal injection. **B**: Number of TUNEL-positive cells in GCL at 24 hr after intravitreal injection of NMDA. Data are expressed as the mean $\pm$ SEM ($n = 6-8$). ^{##} $P < 0.01$ vs. vehicle alone; ^{**} $P < 0.01$ vs. vehicle. IPL, inner plexiform layer; INL, inner nuclear layer. Scale bar = 50 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Bid Inhibitor Inhibited NMDA- and Tunicamycin-Induced RGC Death In Vivo

The NMDA receptor is one of the excitatory glutamate receptors whose activation leads to neuronal death. NMDA-induced cell death is believed to play a role in retinal diseases, such as diabetic retinopathy and glaucoma (Kalloniatis, 1995). NMDA could cause ER stress and oxidative stress in a mouse retina (Shimazawa et al., 2007a,b). To clarify whether Bid might be involved in RGC death in the mouse model, we examined histological changes in the retina at 7 days after intravitreal injection of NMDA and tunicamycin. Intravitreal injection of NMDA at 5 nmol/eye or tunicamycin at 1 μ g/eye decreased the number of cells counted in the GCL in the mouse retina, compared with that in the nontreated normal retina (Fig. 5). Treatment with BI-6C9 (4 nmol/eye) significantly suppressed the decrease (induced by injection of NMDA or tunicamycin) cells of the GCL (Fig. 5), indicating that Bid is involved in both NMDA- and tunicamycin-induced RGC death in vivo. In addition, intravitreal injection of NMDA increased the number of TUNEL-positive cells in GCL at 24 hr (Fig. 6). BI-6C9 (4 nmol/eye), when

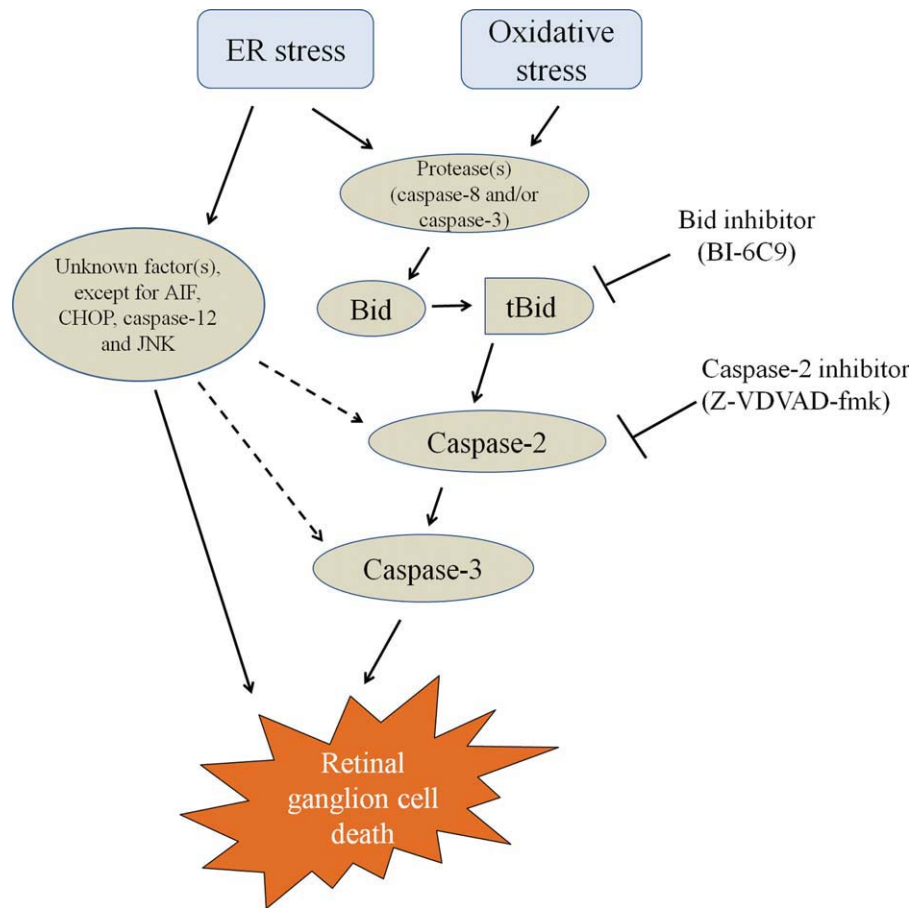


Fig. 7. Putative mechanism of ER stress- and oxidative stress-induced retinal ganglion cell death. ER stress and oxidative stress activate some proteases that may be caspase-8 and caspase-3 and lead to the activation of Bid-caspase-2 cascade. On the other hand, ER stress also activates unknown factors, except for AIF, CHOP, caspase-12 and JNK, and these factors lead to activated caspases and retinal ganglion cell death. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

coadministered with NMDA, significantly reduced the number of TUNEL-positive cells (Fig. 6).

DISCUSSION

In the present study, we demonstrated that Bid and caspase-2 are involved in ER stress- and oxidative stress-induced RGC-5 death in vitro. Bid was cleaved by ER stress, and caspase-2 and caspase-3 were activated by both ER stress and oxidative stress in RGC-5. These stresses were activated in the order Bid, caspase-2, and caspase-3. Moreover, Bid is involved in both NMDA- and ER stress-induced RGC death in vivo (Fig. 7).

Recently, the origin of the RGC-5 line has been recharacterized (Van Bergen et al., 2009). The RGC-5 line, designated as a transformed rat RGC line, is actually of mouse origin (Van Bergen et al., 2009). However, when differentiated by treating with staurosporine, the RGC-5 cells express some proteins characteristic to RGCs, including Thy1 and NMDA receptor, which indicates that RGC-5 is a precursor cell line of RGCs (Frassetto et al., 2006). Because the purpose of this study was to determine

what kind of molecules participate in retinal damage by ER stress and oxidative stress, the origin of the RGC-5 cell line was less relevant to our results.

In in vitro study, we used glutamate in combination with BSO to induce oxidative stress, and it did not induce any ER stress in RGC-5. RGC-5 cells do not express any glutamate receptors. Therefore, elevated levels of extracellular glutamate inhibit cystine uptake, leading to a marked decrease in intracellular glutathione (GSH) levels and resulting in the induction of oxidative stress (Murphy et al., 1989). Furthermore, combining glutamate with BSO, which depletes intracellular GSH, potentiates the oxidative stress (Maher and Hanneken, 2005a). On the other hand, we have already confirmed that intravitreal injection of NMDA leads to increases in X box binding protein (XBP-1) mRNA splicing and in BiP and CHOP proteins in the mouse retina, representing activation of the unfolded protein response (UPR) signaling pathway (Shimazawa et al., 2007b). NMDA receptor activation is known to cause intracellular Ca^{2+} overload and increased NO production, resulting in apoptotic cell death

(Bonfoco et al., 1996; Leist et al., 1997). Several lines of study suggest that intracellular Ca^{2+} overload and excessive production of NO deplete Ca^{2+} in the ER, resulting in ER stress (Li et al., 1993; Oyadomari et al., 2002a). Recently, Uehara et al. (2006) reported that, in primary cortical culture, even mild exposure to NMDA induces apoptotic cell death through an accumulation of polyubiquitinated proteins and increases in XBP-1 mRNA splicing and CHOP mRNA, representing activation of the UPR signaling pathway.

RGC death has been reported to occur via a variety of mechanisms. Among these mechanisms, the present study focused on ER stress and oxidative stress. In our previous study, antioxidants (trolox, edaravone, and minomycin) did not inhibit ER stress-induced RGC-5 death (data not shown); therefore, ER stress did not induce oxidative stress in RGC-5. On the other hand, BSO plus glutamate did not induce ER chaperones such as 78-kDa glucose regulated protein, which is a well-known induced by ER stress (data not shown). Identifying common proteins involved in RGC death generated by different mechanisms might lead to retina disease treatments with a marked effect.

In the present study, we found common proteins, Bid and caspase-2, involved in ER stress- and oxidative stress-induced RGC death. Especially in BSO plus glutamate stress, single treatment with BI-6C9 or Z-VAD-fmk completely reduced cell death (Fig. 1G). This result suggested that Bid and caspase-2 are probably activated in the same pathway and play critical roles in BSO plus glutamate stress. On the other hand, those inhibitors partially attenuated cell death induced by tunicamycin, and an additive effect was not observed (Fig. 1G). These results seemed to be reflected in caspase-3 activity (Fig. 2) and caspase-2 activation (Fig. 4). Judged from these results, the Bid-caspase-2-dependent pathway may not be a unique signal cascade under the ER stress condition in RGC-5, although we demonstrated that well-defined ER stress-related proteins such as caspase-12, JNK, and CHOP were not involved in ER stress-induced cell death in RGC-5. Both Bid and caspase-2 inhibitors suppressed ER stress-induced SH-SY5Y cell death (Supp. Info. Fig. 2A–D). Moreover, Bid inhibitor and caspase-2 inhibitor also suppressed thapsigargin (disrupting Ca^{2+} homeostasis)-induced RGC-5 death (Supp. Info. Fig. 2E–H). From these results, as for Bid and caspase-2, the possibility of involvement in cell death of various cells and various ER stresses was suggested.

Caspase-2 cleaved Bid in ER stress-induced mouse embryonic fibroblast cell death (Upton et al., 2008); therefore, we expected that Bid exists downstream of caspase-2. Contrary to our expectations, caspase-2 existed downstream of Bid in both ER stress- and oxidative stress-induced RGC-5 death (Figs. 3, 4). Caspase-2 is unique because it has features of both the long prodomain of upstream caspases and the optimal recognition motif of downstream caspases. Many reports have indicated that caspase-2 acts as an upstream caspase (Bonne et al., 1998; Wagner et al., 2004; Upton et al., 2008;

Lee et al., 2009). On the other hand, caspase-2 acts as a downstream caspase (O'Reilly et al., 2002; Paroni et al., 2002; He et al., 2004; Bonzon et al., 2006). In the present study, caspase-2 might have acted as a downstream caspase, inducing cell death. From these results, in both ER stress- and oxidative stress-induced RGC-5 death, caspase-2 existed downstream of Bid and caspase-3 was activated downstream of caspase-2.

Caspase-8 (Abdelrahim et al., 2006), caspase-2 (Upton et al., 2008), and calpain (Lee et al., 2009) are thought to be proteins related to the cleavage of Bid. We have demonstrated that caspase-2 existed downstream of Bid (Figs. 3, 4); however, calpain was not involved in ER stress-induced RGC-5 death (data not shown). Caspase-8 generates tBid p15 and p13 and also activates caspase-3 that induces tBid p13 generation (Bossy-Wetzel and Green, 1999; Degli Esposti et al., 2003). Considering these reports and our results, caspase-8 may be activated and involved in the cleavage of Bid though the caspase-3 activation in ER stress and oxidative stress-induced RGC death. Although the mechanism of Bid activation remains unclear, we consider that endogenous caspase-2 inhibitor(s) such as ARC (Koseki et al., 1998) and GMEB1 (Tsuruma et al., 2004) may have important roles. In the early period after tunicamycin or BSO/Glu stimulation, these proteins interacted with caspase-2 and inhibited caspase-2 activation. In the middle or late state, these proteins may be reduced by some mechanisms, such as degeneration by protease or proteasome or translational inhibition, which is triggered by tBid. Actually, GMEB1 is reduced by hypoxia, associated with oxidative stress and ER stress, and knockdown of GMEB1 increases caspase-2 activation and cell death (Tsuruma et al., 2004, 2006).

In an *in vivo* study, Bid was involved in NMDA-induced (related to oxidative stress) and ER stress-induced retinal damage (Figs. 5, 6). This is the first study to use a Bid inhibitor. The involvement of Bid in RGC death was shown *in vivo*, and the further utility of a Bid inhibitor was shown in our study.

In conclusion, we have identified a common mechanism through Bid and caspase-2 in ER stress- and oxidative stress-induced RGC death, activated in the order Bid, caspase-2, and caspase-3 *in vitro*. Furthermore, Bid was involved in NMDA- and ER stress-induced retinal damage *in vivo*. Our results may lead to clarification of the mechanism of various retinal diseases.

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