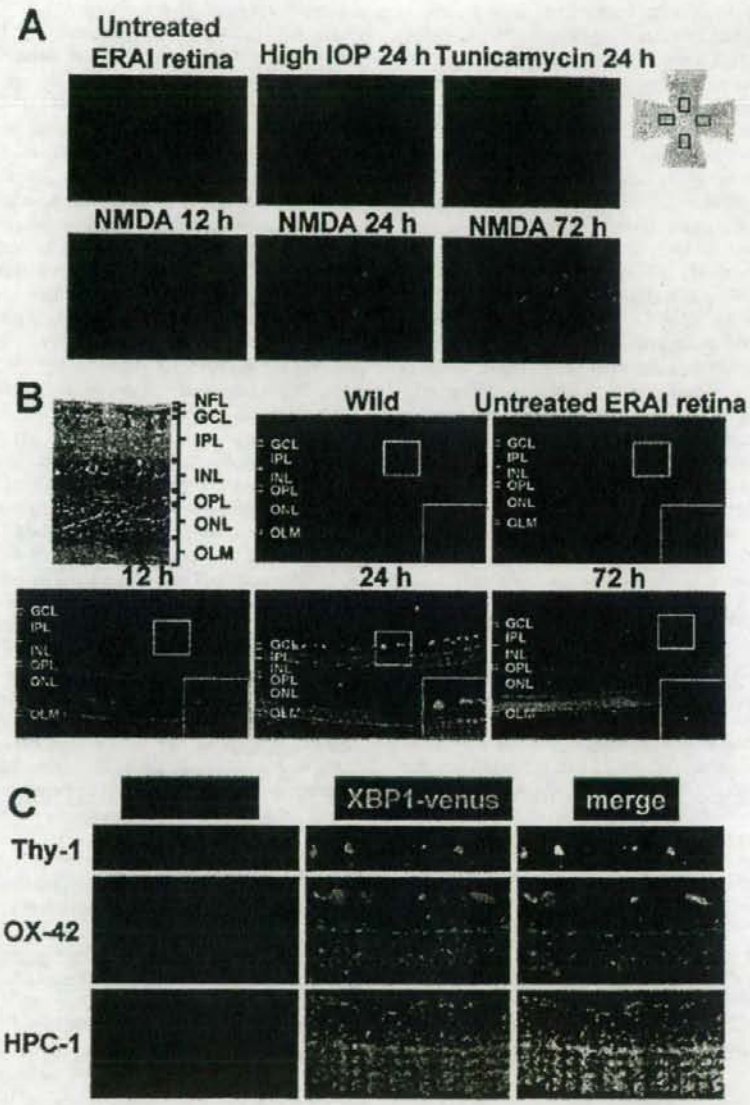

Figure 4. Expression and localization of XBP-1-venus fusion protein in ERAI mouse retinas after various types of retinal damage. **A**: Representative fluorescence photographs of increased XBP-1-venus fusion protein in ERAI mouse flatmounted retina after *N*-methyl-D-aspartate (NMDA), intraocular pressure (IOP) elevation, or tunicamycin insult. The fluorescence (green) arising from XBP-1-venus fusion protein was observed under an epifluorescence microscope. The scale bar represents 25 μm. **B**: Distribution of increased XBP-1-venus fusion protein in retinal cross-sections from ERAI mice after NMDA injection at 40 nmol/eye. The distribution of fluorescence (green) arising from XBP-1-venus fusion protein was observed under a laser confocal microscope. Each large box shows an enlargement of the area within the corresponding small box. **C**: Localization of XBP-1-venus fusion protein in ERAI mouse retina after NMDA injection. In the retinal nerve fiber layer (upper panels), Thy-1-positive cells (red) can be seen to merge with XBP-1-venus fusion protein (green). In the middle panels, OX-42 (a microglia marker)-positive cells (red) are partly merged with XBP-1-venus fusion protein (green). In the inner plexiform layer (lower panels), HPC-1 (an amacrine marker)-positive cells (red) are merged with XBP-1-venus fusion protein (green).

pressure (IOP) elevation, and tunicamycin all induced increases in fluorescence intensity at the time-points indicated in Figure 4A. In the NMDA-treated retinas of ERAI mice, the background fluorescence intensity was time-dependently increased in the period from 12 to 72 h, but little change was observed in the NMDA-treated retinas of wild-type mice.

These changes in background could reflect increases in the lower part of the ganglion cell layer, such as the inner plexiform layer and neurocpithelial layer, of the retina. In transverse sections, increases in fluorescence intensity were first observed in cells of the GCL and inner plexiform layer at 12 and 24 h, respectively, after NMDA injection, and the increases peaked in GCL cells at 24 h (Figure 4B). The increase in fluorescence had diminished at 72 h after the NMDA injection, but morphologically distinct cells (such as microglia cells) had appeared in GCL. On the other hand, the retinas of wild-type and non-treated ERAI mice showed a low fluorescence intensity (below background), while a slight fluorescence intensity was observed in the neuroepithelial layer of the retina (Figure 4B). These cells merged with Thy-1-positive cells (ganglion cells) and some OX-42-positive cells (microglia) in GCL, and with HPC-1-positive cells (amacrine cells) in IPL (Figure 4C). Together, these results suggest that XBP-1 splicing, representing activation of the ER-stress signal pathway, may be induced in retinal ganglion and amacrine and microglia cells during the early stages of retinal cell damage.

Increases in GRP78/BiP and CHOP in mouse retina after NMDA injection: To clarify whether ER stress-related proteins other than XBP-1 are induced in the mouse retina by NMDA stimulation, we examined the changes in BiP, a biomarker of ER stress, in the retina after intravitreal injection of NMDA. As shown in Figure 5B, cell loss in GCL and thinning of IPL were observed at 72 h after NMDA injection (versus non-treated control retinas; Figure 5A). Using immunoblots, as shown in Figure 5C, we found that BiP was significantly increased at 12 h after the NMDA injection, and that the increase persisted for the remainder of the 72 h observation period. Next, we investigated the distribution and time-course of changes in GRP78/BiP and CHOP, a proapoptosis protein, after NMDA injection. In the non-treated control retina, slight immunoreactivities for BiP and CHOP were observed in a number of cells in GCL and IPL (Figure 5D). Increases in these immunoreactivities were observed in retinal ganglion cells at 12 h after NMDA injection, and time-dependent increases were noted in the inner retina (Figure 5D).

DISCUSSION

In the present study, we could detect pathological changes and time-dependent changes related to ER stress in retinal flatmount and transverse sections and in the retinas of living mice after retinal damage. Moreover, we demonstrated that ER stress signals were activated in the retina in vivo after tunicamycin, elevating IOP, or NMDA treatment.

Agents or conditions that adversely affect ER protein folding lead to an accumulation of unfolded or misfolded proteins in the ER, a condition defined as ER stress. ER stress can be induced by agents or conditions that interfere with (a) protein glycosylation (e.g., glucose starvation, tunicamycin, glucosamine), (b) disulfide-bond formation (e.g., DTT, homocysteine), (c) Ca^{2+} balance (A23187, thapsigargin, EGTA), and/or (d) a general overloading of the ER with proteins (e.g., viral or non-viral oncogenesis) [1,13,14]. However, little is known about any involvement of ER stress in retinal damage. In the present study, we found that tunicamycin induced the ER stress-associated proteins BiP, p-eIF2α, and CHOP in cultured RGC-5 cells. These protein levels started to increase at 2 to 6 h after the start of tunicamycin treatment, and increased time-dependently until 24 h after the start of the treatment, while apoptotic cell death with condensation and fragmentation of nuclei was observed 24 h later. BiP acts as an ER resident molecular chaperon that is induced by ER stress, and this protein refolds the unfolded proteins, thereby tending to maintain homeostasis in the ER [15,16]. Since CHOP is a member of the CCAAT/enhancer-binding protein family that is induced by ER stress and participates in ER-mediated apoptosis, CHOP may be a key molecule in retinal cell death [17]. In the present study, the phosphorylation of eIF2α was increased concomitantly with the increases in the expression of BiP and CHOP proteins, even through p-eIF2α might be expected to suppress protein synthesis. Boyce et al.[18] reported that selective inhibition of eIF2α dephosphorylation increases both p-eIF2α and CHOP protein. These data suggest that during ER stress, p-eIF2α (inactive form) is still able to stimulate the translation of ATF4 mRNA, thereby increasing the transcription of BiP or CHOP mRNA, but that enough unphosphorylated-eIF2α (active form) may remain to translate BiP and CHOP mRNAs to proteins. On the other hand, we found that staurosporine, which mediates mitochondrial dysfunctions resulting in apoptotic cell death, did not induce any increases in BiP and CHOP proteins in RGC-5 [unpublished data]. Taken together, these findings suggest that persistent ER stress may induce apoptotic cell death through the eIF2α-CHOP signal pathway in RGC-5.

Next, we tried to determine whether tunicamycin could induce retinal damage in vivo. Intravitreal injection of low-dose tunicamycin induced a significant loss of cells in the retinal ganglion cell layer (GCL), but no thinning of the inner plexiform layer (IPL). These findings suggest that retinal ganglion cells are more sensitive to ER stress-induced cell death than other retinal cells. High-dose tunicamycin significantly decreased both the cell count in GCL and the thickness of IPL. The concentration of tunicamycin in the vitreous body after an intravitreal injection of low-dose tunicamycin was estimated to be 10 μg/ml. The tunicamycin concentration achieved within the retina will have been less than this. Interestingly, in the present in vitro study, tunicamycin at 1 to 4 μg/ml induced cell death with an increase in ER-stress signals, suggesting that the in vivo concentration of tunicamycin in the retina was roughly similar to that employed in vitro. Use of tunicamycin at a high dose also led to decreases in IPL, INL (inner nuclear layer), and ONL (outer nuclear layer) in the retina. In guinea pigs, a single subcutaneous injection of tunicamycin at 0.4 mg/kg has been reported to induce hepatotoxicity with dilation of the cisternae of the ER [19]. Fur-

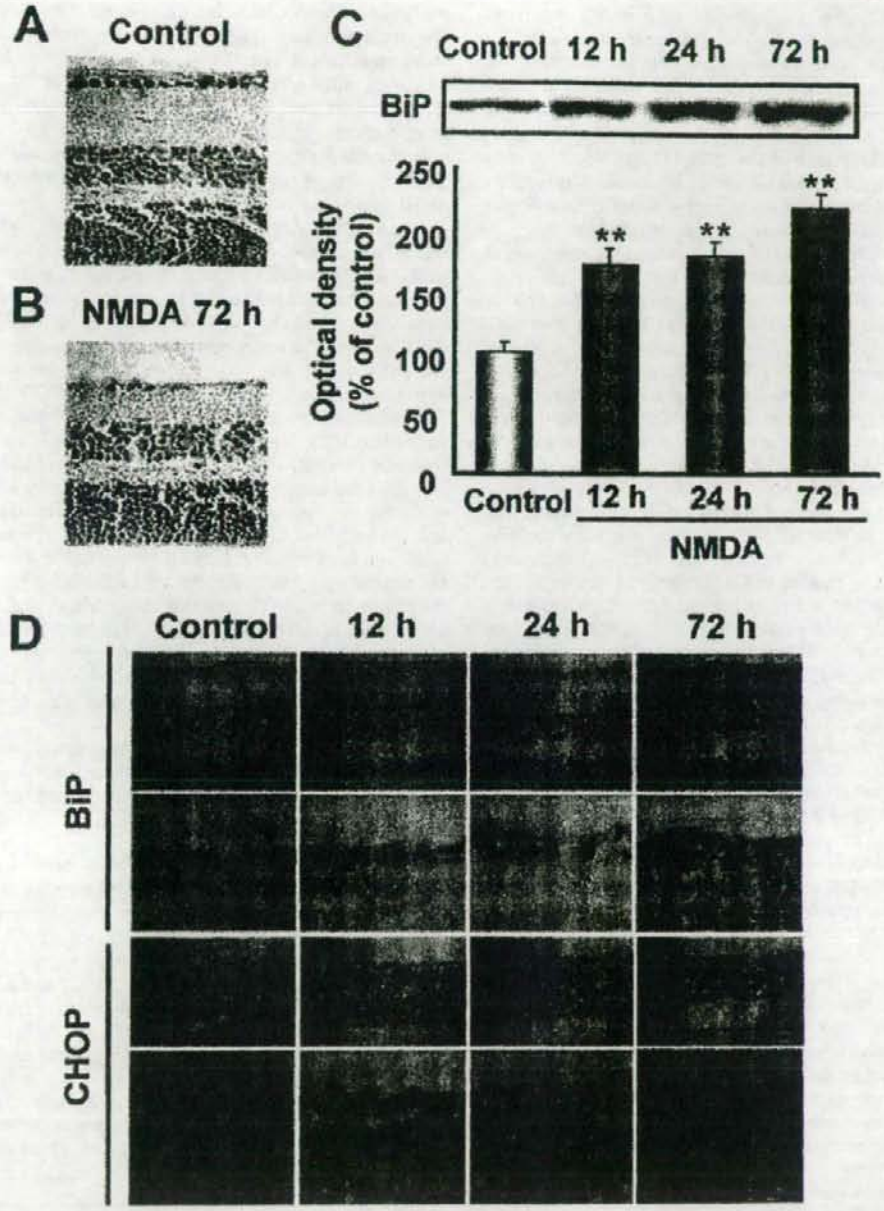

Figure 5. Increases in GRP78/BiP and CHOP in retinal extracts following stimulation by intravitreal injection of *N*-methyl-D-aspartate (NMDA) in mice. **A, B**: Representative photographs showing retinal cross-sections stained with hematoxylin and eosin after NMDA injection at 40 nmol/eye. **C**, upper panel: Representative immunoblots showing the time-course of changes in GRP78/BiP protein levels after intravitreal injection of NMDA. **C**, lower panel: Quantitative analysis of GRP78/BiP band densities. Data are expressed as mean±SEM (n=6) of values (in arbitrary units) obtained for single band density. Double asterisks represents $p<0.01$ versus vehicle-treated control group (Dunnett's test). **D**: Immunostainings for GRP78/BiP and CHOP in mouse retina after NMDA injection at 40 nmol. The scale bar represents 25 μm.

thermore, Zinszner et al.[20] noted that in mice, a single sublethal intraperitoneal injection of tunicamycin (1 mg/kg) induces CHOP expression and subsequent severe histological damage with an increase in TUNEL-positive cells, and a characteristic transient renal insufficiency. They also found that CHOP-deficient mice show an attenuated increase in TdT-mediated dUTP nick-end labeling (TUNEL)-positive cells during the renal damage induced by tunicamycin. These findings suggest that in vivo, tunicamycin-induced retinal cell death is due, at least in part, to an ER-stress mechanism.

NMDA receptors may participate in the processes of excitotoxicity and neuronal death in the retina [21,22]. Previous studies have found that TUNEL-positive cells can be observed in the GCL and INL of the mouse retina at an early stage (within 24 h) after an intravitreal injection of NMDA [23,24]. The hallmark of NMDA-induced neuronal death is a sustained increase in the intracellular Ca^{2+} concentration accompanied by overactivation of vital Ca^{2+}-dependent cellular enzymes [25]. Thus, the signal-transduction pathways for NMDA-mediated cell death in the retina are well studied, but not yet fully understood.

To illuminate the role and distribution of ER stress in vivo, we focused on the retina of ERAI mice. Information about the status of ER stress during the course of a given disease might be obtained by crossing an ERAI transgenic mouse (the indicator mouse for ER stress in living cells) with a mouse model of the human disease of interest. In flatmounted retinas, fluorescence was detected following various stimulations [tunicamycin, NMDA, and intraocular pressure (IOP) elevation]. To our knowledge, this is the first report demonstrating that NMDA and ischemic insult (elevating IOP), in addition to tunicamycin, can activate the ER stress signal (measured as the splicing of the XBP-1 and venus fusion gene in ERAI transgenic mice) in the retina in vivo. Interestingly, ER stress was also induced in the retina after a transient IOP elevation, defined as an ischemia-reperfusion model. It has been reported that this model exhibits retinal cell damage similar to that induced by NMDA, and that both of these examples of damage are protected against by MK-801, an NMDA receptor antagonist, and by NO synthetase-inhibitor treatment [8,26]. Although little is known about the precise mechanisms responsible for activation of ER stress after NMDA or IOP elevation (ischemia-reperfusion), both stimuli cause intracellular Ca^{2+} overload and increased NO production, resulting in apoptotic cell death. Several lines of study suggest that intracellular Ca^{2+} overload and excessive production of NO deplete Ca^{2+} in the ER, thereby resulting in ER stress [27,28]. Recently, Uehara et al.[10] reported that NO induces S-nitrosylation of protein-disulphide isomerase (PDI), an enzyme that assists in the maturation and transport of unfolded secretory proteins and thereby helps to prevent the neurotoxicity associated with ER stress. S-nitrosylated-PDI exhibits reduced enzymatic activity and induces cell death through the ER stress pathway. These mechanisms may contribute to the activation of ER stress in the retina after NMDA stimulation or IOP elevation. Accordingly, our findings may provide important new insights into the mechanisms underlying the retinal cell damage induced by NMDA and by ischemia-reperfusion. In transverse retinal sections, we observed an increase in fluorescence intensity within the cells of the ganglion cell layer (GCL) and inner plexiform layer (IPL) at 12 and 24 h, respectively, after NMDA injection. The cells displaying increased fluorescence were ganglion cells (at 12 h after the injection), amacrine cells in IPL (at 24 h), and microglia in GCL (at 72 h). These data indicate that ganglion cells may be more sensitive to ER stress than the other retinal cells examined.

To further clarify the participation of ER stress, we examined the changes in BiP and CHOP in the retina after NMDA-induced injury. We found (a) that NMDA induced BiP proteins in the retina at 12 h after its injection (on the basis of immunoblots), and (b) that, NMDA induced both BiP and CHOP in the retina (especially within retinal ganglion cells and INL) at 12 h after its injection (on the basis of our immunostaining results). The expression of the CHOP gene reportedly increases in the rat retina after intravitreal injection of NMDA [29]. Furthermore, Awai et al.[30] found that treatment with MK-801, an NMDA receptor antagonist, inhibited the increases in CHOP mRNA and protein in the mouse retina that are observed after intravitreal injection of NMDA, and moreover that CHOP-deficient mice were resistant to NMDA-induced retinal damage. However, CHOP-deficient mice partially suppressed the NMDA-induced cell death, and therefore other pathways, such as mitochondrial dysfunction, may be engaged in the retinal cell death. Collectively, the above results indicate that NMDA can cause ER stress in the retina, and that the neurotoxicity induced by NMDA is due in part to a mechanism dependent on CHOP protein induction through excessive ER stress.

In conclusion, we have identified a close association between ER stress and retinal damage, and our results suggest that the ER stress-signal pathway might be a good target in the treatment of retinal diseases.

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Involvement of Double-Stranded RNA-Dependent Protein Kinase in ER Stress-Induced Retinal Neuron Damage

Masamitsu Shimazawa, Yasushi Ito, Yuta Inokuchi, and Hideaki Hara

Purpose. To clarify whether the activation of double-stranded RNA-dependent protein kinase (PKR) participates in the cell death induced by endoplasmic reticulum (ER) stress, the authors used cultured retinal ganglion cells (RGC-5, a rat ganglion cell line transformed with the E1A virus) in vitro and the effect of a PKR inhibitor (an imidazolo-oxindole derivative) on *N*-methyl-D-aspartate (NMDA)–induced retinal damage in mice in vivo.

Methods. In RGC-5 culture, cell damage was induced by tunicamycin (an ER stress inducer), and cell viability was measured by Hoechst 33342, YO-PRO-1, or propidium iodide (PI) double staining or by the resazurin-reduction test. Levels of glucose-regulated protein (GRP) 78/BiP, activating transcription factor 4 (ATF4), C/EBP-homologous protein (CHOP) and the phosphorylated form of PKR were analyzed by immunoblot. The PKR inhibitor and two siRNAs that recognize nonoverlapping sequences of rat PKR were tested for their effects on tunicamycin-induced cell death. In vivo, retinal cell damage was induced by intravitreal injection of NMDA (20 nmol/eye) in mice. To examine its effect in vivo, the PKR inhibitor (1 nmol/eye) was intravitreally injected with NMDA, and ganglion cell layer cell loss and inner plexiform layer thinning were evaluated 7 days after NMDA injection.

Results. Treatment with tunicamycin at 1, 2, and 4 μg/mL for 24 hours increased the number of YO-PRO-1 and PI-positive (apoptosis or necrosis indicator) cells in a concentration-dependent manner. Immunoblotting analysis showed that tunicamycin at 2 μg/mL induced BiP, ATF4, and CHOP protein production and PKR phosphorylation. Both the PKR inhibitor (0.03–1 μM) and the PKR knockdown (using siRNA) inhibited tunicamycin-induced RGC-5 cell death. The same inhibitor also reduced NMDA-induced retinal damage in vivo. The PKR inhibitor reduced the tunicamycin-induced increase in CHOP but not that in BiP protein production.

Conclusions. These results indicate that inhibiting PKR activation is neuroprotective against ER stress-induced retinal damage, suggesting that PKR activation may be involved in the mechanisms underlying ER stress-induced cell death. (*Invest Ophthalmol Vis Sci.* 2007;48:3729–3736) DOI:10.1167/iovs.06-1122

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Endoplasmic reticulum (ER) stress has recently been linked to the pathogenesis of several diseases, including vascular and neurodegenerative diseases such as stroke, Alzheimer disease, and Parkinson disease.[1–3] Little is known about any role of ER stress in retinal damage.

Retinal ganglion cell (RGC) death is a common feature of many ophthalmic disorders, such as glaucoma, optic neuropathies, and of various retinovascular diseases (diabetic retinopathy, retinal vein occlusions). A variety of factors, including oxidative stress,[4] excitatory amino acids,[5] and nitric oxide,[6] have been reported to induce retinal cell death. These reports emphasize the importance of a better understanding of the precise mechanisms underlying retinal diseases.

Recently, it has been reported that one of the proapoptotic proteins involved in ER stress-mediated apoptosis (tunicamycin-induced apoptosis) is a double-stranded RNA-dependent protein kinase (PKR), as identified using a randomized ribozyme library.[7] PKR, an interferon-induced protein kinase identified initially in a study of responses to viral infection, is activated by the extensive secondary structure of viral RNA.[8] On binding to double-stranded RNA, PKR is autophosphorylated, and it then increases the cellular sensitivity to apoptotic stimuli through a number of putative pathways, including the phosphorylation of eukaryotic initiation factor 2α (p-eIF2α).[9,10] Thus, PKR is involved in the apoptosis induced not only by viral infection but also by ER stress. Hence, the purpose of the present study was to examine whether a PKR inhibitor (an imidazolo-oxindole derivative that acts as an ATP-binding site-directed inhibitor of PKR) or PKR silencing (by means of siRNA) might inhibit the retinal neuronal death induced by either ER stress (tunicamycin) or *N*-methyl-D-aspartate (NMDA).

Materials and Methods

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

Dulbecco modified Eagle medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, MO). The drugs used and their sources were as follows: double-stranded RNA-dependent protein kinase (PKR) inhibitor {8-[imidazol-4-ylmethylene]-6H-azolidino[5,4-g]benzothiazol-7-1}[11] and tunicamycin were obtained from Calbiochem (San Diego, CA) and Wako (Osaka, Japan), respectively; isoflurane was acquired from Nissan Kagaku (Tokyo, Japan); and fetal bovine serum (FBS) was obtained from Valeant (Costa Mesa, CA).

Retinal Ganglion Cell Line Culture

Cultures of RGC-5 were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin (Meiji Seika Kaisha, Ltd., Tokyo, Japan), and 100 μg/mL streptomycin (Meiji Seika Kaisha, Ltd.) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. RGC-5 cells were passaged by trypsinization every 3 days, as described in a previous report.[12]

Cell Viability Assay after Tunicamycin

RGC-5 cells were plated at a density of 1000 cells/well in 96-well culture plates (Falcon 3072; Becton Dickinson Labware, Oxnard, CA). Twenty-four hours later, cells were washed twice with DMEM and then immersed in DMEM supplemented with 1% FBS. One hour later, tunicamycin at 1 to 4 μg/mL was added to the media. Vehicle or PKR inhibitor at 0.03 to 1 μM was added to the media 1 hour before, concomitantly with, or 6 hours after tunicamycin treatment. Twenty-four hours after the addition of tunicamycin, cell viability was measured according to one of two methods. The first was a single-cell digital imaging-based method using fluorescent staining of nuclei. Briefly, cell death was assessed on the basis of combination staining with fluorescent dyes, namely, Hoechst 33342 (Molecular Probes, Eugene, OR) and YO-PRO-1 (YO; Molecular Probes) or propidium iodide (PI; Molecular Probes), observations made using an inverted epifluorescence microscope (IX70; Olympus, Tokyo, Japan). At the end of the culture period, Hoechst 33342 and YO-PRO-1 or PI dyes were added to the culture medium (8 μM, 0.1 μM, and 1.5 μM, respectively) for 30 minutes. Images were collected with a digital camera (Coolpix 4500; Nikon, Tokyo, Japan). At least 400 cells per condition were counted by a masked observer who used image-processing software (ImageJ, version 1.33f; National Institutes of Health, Bethesda, MD). Cell mortality was quantified by expressing the number of YO-PRO-1- or PI-positive cells as a percentage of the number of Hoechst 33342-positive cells.

As the second method for measuring cell viability, the effect on cell viability of tunicamycin treatment was quantitatively assessed by examining the fluorescence intensity changes induced by the cellular reduction of resazurin to resorufin. These experiments were performed in DMEM at 37°C. Cell viability was assessed by the use of 10% resazurin solution for 3 hours at 37°C, and then cells were examined for fluorescence at 560/590 nm. Fluorescence was expressed as a percentage of that in control cells in DMEM containing 1% FBS (after subtraction of background fluorescence).

RNA Interference

For rat PKR and negative control siRNAs, we used a duplex (Stealth PKR RNAi; Invitrogen, Carlsbad, CA) and a control duplex (Stealth RNAi Negative Control Duplex; no. 12935 to 200; Low GC Duplex; Invitrogen), respectively. Sense and antisense strands of rat PKR siRNA were: sequence 1, 5′-UACUUUGUGUAUCUGGGAGUAUUUG-3′ (sense) and 5′-CAAAUACUCCCAGAUACACAAAGUA-3′ (antisense); sequence 2, 5′-AAUUCCAUUUGGAUAAAGAGGCACC-3′ (sense) and 5′-GGUGCCUCUUUAUCCAAAUGGAAUU-3′ (antisense).

Transfection with siRNA In Vitro

RGC-5 cells were seeded at a density of 1000 cells/well into 96-well plates (for cell viability assay) or at a density of 5000 cells/well into 24-well plates (for assessment of the effects of PKR silencing) using the standard medium. Twenty-four hours later, cells were washed twice with medium (Opti-Mem I; Invitrogen) supplemented with 1% FBS without antibiotics and then were immersed in the same medium. Reagent (Lipofectamine 2000; Invitrogen) was used as the transfection agent. PKR and control siRNAs, each at a concentration of 10 nM, were transfected by incubation for 6 hours, according to the manufacturer's instructions (Invitrogen). Subsequently, the medium containing siRNA and complex (Lipofectamine 2000; Invitrogen) was replaced by DMEM supplemented with 1% FBS. Forty-eight hours after the infection, tunicamycin was added to each well, and incubation continued for another 24 hours.

In Vivo NMDA–Induced Retinal Damage

Male adult ddY mice weighing 36 to 43 g each (Japan SLC, Hamamatsu, Japan) were used for these experiments and were kept under controlled lighting conditions (12 hours light/12 hours dark). Anesthesia was induced with 3.0% isoflurane and maintained with1.5% isoflurane in 70% N_2O and 30% O_2, delivered through an animal general anesthesia machine (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). Body temperatures were maintained between 37.0°C and 37.5°C with the aid of a heating pad and a heating lamp. Retinal damage was induced by injection (2 μL/eye) of NMDA (Sigma-Aldrich) at 10 mM dissolved in 0.01 M phosphate-buffered saline (PBS) injected into the vitreous body of the left eye under the anesthesia described. One drop of levofloxacin ophthalmic solution (Santen Pharmaceuticals Co. Ltd., Osaka, Japan) was applied topically to the treated eye after the intravitreous injection. The PKR inhibitor (1 nmol/eye) or an identical volume of vehicle (0.5% DMSO in PBS) was coadministered with NMDA injection at 20 nmol/eye.

Immunoblotting

RGC-5 cells were lysed using a cell-lysis buffer (RIPA buffer [R0278; Sigma] with protease [P8340; Sigma] and phosphatase inhibitor cocktails [P2850 and P5726; Sigma]; and 1 mM EDTA). Cell lysates were solubilized in SDS-sample buffer, separated on 10% SDS-polyacrylamide gels, and transferred to PVDF membrane (Immobilon-P; Millipore, Bedford, MA). Transfers were blocked for 1 hour at room temperature (5% Blocking One-P; Nakarai Tesque, Inc., Kyoto, Japan) in 10 mM Tris-buffered saline with 0.05% Tween 20 (TBS-T) and were incubated overnight at 4°C with the primary antibody. Transfers were then rinsed with TBS-T and incubated for 1 hour at room temperature in horseradish peroxidase goat anti-rabbit or goat anti-mouse (Pierce, Rockford, IL) diluted 1:2000. Immunoblots were developed with chemiluminescence (Super Signal West Femto Maximum Sensitivity Substrate; Pierce) and visualized with the aid of a digital imaging system (FAS-1000; Toyobo Co., Ltd., Osaka, Japan). Primary antibodies used were as follows: mouse anti-BiP (BD Bioscience, San Jose, CA), rabbit anti-ATF4 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-PKR (Abcam, Cambridge, MA), rabbit anti-PKR (Cell Signaling, Beverly, MA), mouse anti-CHOP (Santa Cruz Biotechnology), and rabbit anti-actin (Santa Cruz Biotechnology).

Immunostaining

To clarify whether NMDA or tunicamycin induced PKR phosphorylation in the mouse retina in vivo and whether a PKR inhibitor would prevent PKR phosphorylation, immunocytochemistry was performed. At 12 or 24 hours after intravitreal injection of NMDA (20 mmol/eye) or tunicamycin (1 μg/eye), with or without PKR inhibitor (1 nmol/eye), eyes were enucleated, fixed in 4% paraformaldehyde overnight at 4°C, immersed in 20% sucrose for 48 hours at 4°C, and embedded in optimum cutting temperature (OCT) compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Transverse, 10-μm–thick cryostat sections were cut and placed onto slides (Mas Coat). Sections were subsequently processed for immunocytochemistry using antibodies against phospho-PKR (Abcam) diluted 1:500 in immunoreaction enhancer solution A (CanGet signal immunostain; Toyobo Co., Ltd.). Sections were incubated with biotin-conjugated secondary antibody for 1 hour at room temperature and were visualized with an immunodetection kit (Vector M.O.M.; Vector, Burlingame, CA).

Histologic Analysis of Mouse Retina

Seven days after the NMDA or tunicamycin injection, eyeballs were enucleated for histologic analysis. In mice under anesthesia produced by intraperitoneal injection of sodium pentobarbital (80 mg/kg), each eye was enucleated and kept immersed for at least 24 hours at 4°C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (3-μm thick) cut through the optic disc of each eye were prepared in a standard manner and were stained with hematoxylin and eosin. Retinal damage was evaluated as described previously, with three sections from each eye used for the morphometric analysis. Light microscope images were photographed, and the cell counts in the ganglion cell layer (GCL), at a distance between 350 and 650 μm from the optic disc, were measured on the photographs in a masked fashion by a single observer (Y.I.). Data from three sections (selected

randomly from the six sections) were averaged for each eye, and the values obtained were used to evaluate the cell count in the GCL.

Statistical Analysis

Data are presented as the mean ± SEM. Statistical comparisons were made, using a Student's *t*-test or a Dunnett test (by means of Stat View version 5.0; SAS Institute Inc., Cary, NC). $P < 0.05$ was considered statistically significant.

Results

Retinal Cell Death and Changes in Endoplasmic Reticulum Stress-Related Protein Induced by Tunicamycin

The time-course of the changes in cell morphology occurring after tunicamycin treatment at 2 μg/mL are shown in Figure 1A. An increase in nonadherent cells was observed 12 hours after tunicamycin treatment compared with that in nontreated control cells. Representative fluorescence stainings of nuclei—using Hoechst 33342, YO-PRO-1, and propidium iodide (PI) dyes—are shown in Figure 1B. Nontreated control cells displayed normal nuclear morphology and negative staining with YO-PRO-1 dye (which stains early apoptotic and later-stage cells) and PI dye (which stains late-stage apoptotic cells) (Fig. 1B, upper panels). Treatment with tunicamycin led to shrinkage and condensation of nuclei and to positive staining with each of these dyes (Fig. 1B, lower panels).

Changes in the protein levels of BiP, pATF-4, and CHOP 24 hours after tunicamycin treatment at 1–4 μg/mL are shown in Figure 1C. BiP, ATF-4, and CHOP were all concentration dependently increased, whereas actin levels remained unchanged.

Phosphorylation of a PKR in RGC-5 Cells Is Induced by Tunicamycin

Although no phosphorylated PKR was observed in vehicle-treated normal cells, marked and slight increases in phosphorylated PKR were noted at 6 and 24 hours, respectively, after tunicamycin treatment (Fig. 2A).

Effects of a PKR Inhibitor and PKR Silencing on the RGC-5 Cell Death Induced by Tunicamycin

Representative fluorescence stainings of nuclei with Hoechst 33342 and YO-PRO-1 dyes are shown in Figure 2B. Nontreated cells showed normal nuclear morphology and negative staining with YO-PRO-1 dye (which stains early-stage apoptotic and necrotic cells). Treatment with tunicamycin at 2 μg/mL for 24 hours led to shrinkage and condensation of nuclei and to positive staining with YO-PRO-1 dye. Treatment with a PKR inhibitor at 0.3 and 1 μM reduced the tunicamycin-induced morphologic changes in nuclei and the number of cells stained with YO-PRO-1. The number of cells exhibiting YO-PRO-1 fluorescence was counted, and the positive cells were expressed as the percentage of YO-PRO-1- to Hoechst 33342-

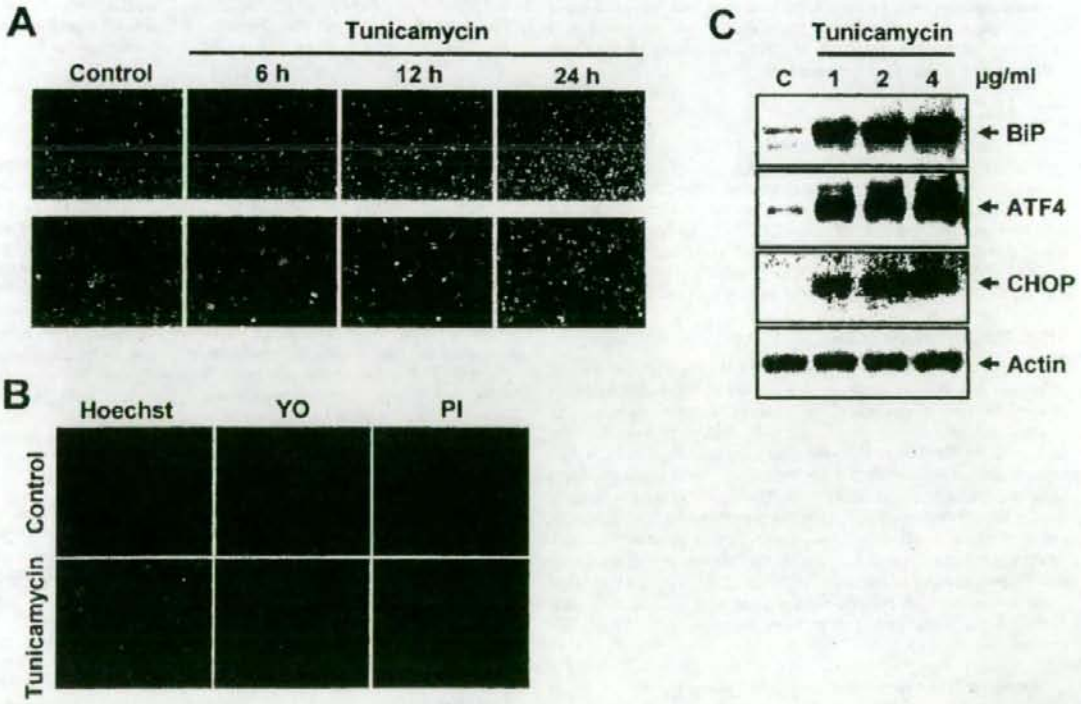

FIGURE 1. RGC-5 cell death and changes in ER-stress related proteins induced by tunicamycin. (A) Representative phase-contrast microscopy showing time-course of changes in RGC-5 cells after addition of tunicamycin at 2 μg/mL (*upper panels*, low power; *lower panels*, high power). Bar = 250 μm. (B) Representative fluorescence microscopy showing nuclear stainings for Hoechst 33342, YO-PRO-1, and PI 24 hours after tunicamycin treatment at 2 μg/mL. (C) Representative immunoblots showing protein levels (BiP, ATF4, CHOP, and actin) 24 hours after tunicamycin treatment at 1–4 μg/mL.

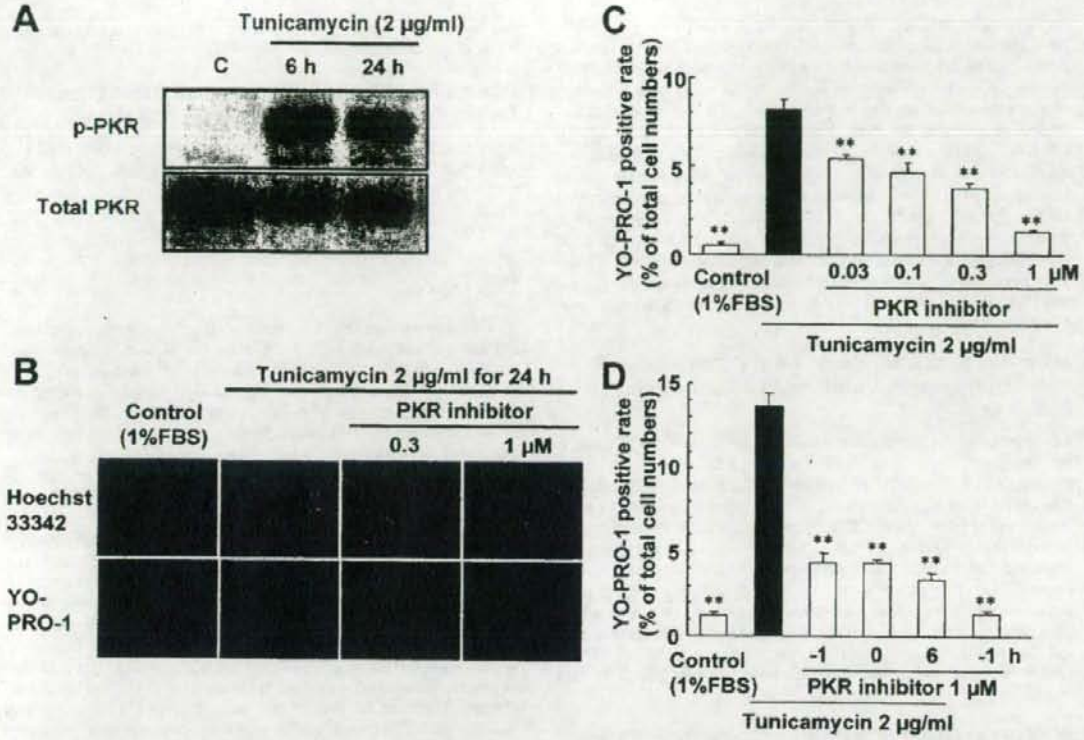

FIGURE 2. Phosphorylation of a PKR and its inhibitory effect on tunicamycin-induced cell damage in RGC-5 cells. (A) Phosphorylation of PKR in RGC-5 cells is induced by tunicamycin. Representative band images show immunoreactivity toward phosphorylated PKR and total PKR. (B–D) Effects of a PKR inhibitor on the RGC-5 cell death induced by tunicamycin. (B) Representative fluorescence microscopy showing nuclear stainings for Hoechst 33342 and YO-PRO-1 after 24-hour tunicamycin treatment. The PKR inhibitor was added 1 hour before the tunicamycin. (C) Number of cells exhibiting YO-PRO-1 fluorescence was counted, and positive cells were expressed as the percentage of YO-PRO-1 to Hoechst 33342. Each column represents the mean ± SEM ($n = 8$). $^{**}P < 0.01$ versus tunicamycin alone (Dunnett test). (D) The PKR inhibitor at 1 μM was added 1 hour before, concomitantly with, or 6 hours after tunicamycin at 2 μg/mL. Number of cells exhibiting YO-PRO-1 fluorescence was counted, and the positive cells were expressed as the percentage of YO-PRO-1 to Hoechst 33342. Each column represents the mean ± SEM ($n = 8$). $^{**}P < 0.01$ versus tunicamycin alone (Dunnett test).

positive cells (Fig. 2C). After treatment with tunicamycin for 24 hours, the percentage of YO-PRO-1–positive cells was 8.2% ± 0.5% ($n = 8$), whereas in the control group (supplemented with 1% FBS) it was 0.5% ± 0.1% ($n = 8$). Treatment with the PKR inhibitor at 0.03 to 1 μM significantly reduced the increase in YO-PRO-1–positive cells induced by tunicamycin (in a concentration-dependent manner). Treatment with the PKR inhibitor at 1 μM, either concomitantly with or 6 hours after tunicamycin, protected against RGC-5 cell death at 24 hours after tunicamycin (Fig. 2D). As shown in Figures 3A and 3B, each of the PKR siRNAs (#1 and #2) that recognize nonoverlapping sequences of rat PKR decreased the PKR protein ex-

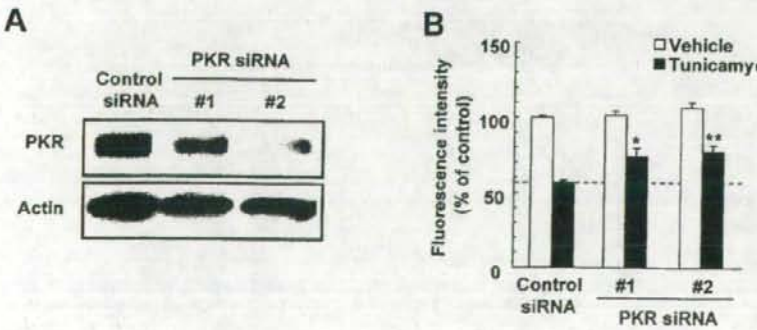

FIGURE 3. Effect of PKR siRNAs on tunicamycin-induced RGC-5 cell death. (A) Representative band images show immunoreactivities toward PKR and actin. PKR protein was decreased by 48-hour treatment with one of two PKR siRNAs. (B) RGC-5 cells were transiently transfected with control siRNA or one of two PKR siRNAs. After 48 hours, cells were treated with 2 μg/mL tunicamycin and maintained in this condition for another 24 hours. Each column represents the mean ± SEM ($n = 8$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control siRNA-treated group (Dunnett test).

pression and led to a significant level of resistance to the tunicamycin-induced decrease in cell viability. Some standard siRNAs have been reported to induce the activation of PKR/interferon pathways as nonspecific stress responses resulting in growth inhibition and cytotoxicity.[13] To avoid such undesirable responses, we used duplex and control duplex siRNA (Stealth; Invitrogen) with chemical modifications to enable the elimination of nonspecific stress response affecting the PKR/interferon pathways.[14] In the present study, negative control siRNA (Stealth; Invitrogen) at 10 nM did not increase PKR protein at 48 hours after treatment compared with the nontreated control (Shimazawa et al., unpublished data, 2006). This result indicates that under our experimental conditions, Stealth siRNA does not induce the activation of PKR/interferon pathways.

Effects of a PKR Inhibitor on the Tunicamycin-Induced Increases in BiP and CHOP Proteins in RGC-5 Cells

To determine the mechanism underlying the action of tunicamycin, we examined the effect of the PKR inhibitor on the increases in BiP and CHOP proteins induced in RGC-5 cells by treatment with tunicamycin at 2 μg/mL for 24 hours. Tunicamycin induced BiP and CHOP proteins in such cells (Fig. 4). Pretreatment with the PKR inhibitor at 1 μM significantly reduced the increase in CHOP protein but not that in BiP protein. On the other hand, treatment with the same PKR inhibitor alone (at 0.3 or 1 μM) had little effect on the production of these proteins (vs. vehicle-treated controls). Staurosporine (200 nM), an apoptosis inducer, did not increase BiP or CHOP protein, but it did induce apoptotic cell death in this condition.

Phosphorylation of a PKR in Mouse Retina after NMDA or Tunicamycin

No phosphorylated PKR was observed in the vehicle-treated normal retina, but phosphorylated PKR was observed in both the GCL and the inner plexiform layer (IPL) at 12 and 24 hours after intravitreal injection of NMDA (Fig. 5). Phosphorylated PKR was also observed in GCL and IPL at 12 and 24 hours after tunicamycin injection (Fig. 5). The PKR inhibitor (1 nmol), when coadministered with NMDA or tunicamycin, inhibited the increases in phosphorylated PKR in mouse retina induced by NMDA and tunicamycin (Fig. 5).

Effect of a PKR Inhibitor on the Retinal Damage Induced by Intravitreal Injection of NMDA

Intravitreal injection of NMDA at 20 nmol/eye decreased the cell count in the GCL (vs. nontreated normal retinas; Figs. 6A, 6B). Coadministration of the PKR inhibitor at 1 nmol/eye significantly reduced the NMDA-induced loss of GCL cells (Figs. 6A, 6B).

Discussion

In the present study, we demonstrated a close association between PKR and ER stress-induced retinal damage and showed that the inhibition of PKR reduced such damage.

Nutrient deprivation and agents that cause unfolded or misfolded proteins in the ER can activate the ER stress response, though the cell normally survives the insult.[1] However, excessive or prolonged ER stress can induce cell death, usually in the form of apoptosis. In the present study, we found that tunicamycin induced the ER stress-associated proteins BiP, ATF-4, and CHOP in cultured RGC-5 cells. In our previous study, the phosphorylation of eukaryotic translation initiation factor 2 kinase (eIF2α) was increased concomitantly with the increases in the expressions of BiP, ATF-4, and CHOP proteins from 6 hours onward.[15] BiP, ATF-4, and the phosphorylation of eIF2α increased time-dependently throughout the 24-hour tunicamycin treatment period, whereas actin levels remained unchanged. CHOP was first detected 6 hours after the addition of tunicamycin, and it persisted thereafter. On the other hand, a slight increase in cell death was observed 12 hours after tunicamycin treatment, and it was time-dependently increased thereafter.[15] BiP acts as an ER-resident molecular chaperone induced by ER stress, and this protein refolds the unfolded proteins, thereby tending to maintain homeostasis in the ER.[16,17] CHOP is a member of the CCAAT/enhancer-binding protein family that is induced by ER stress and participates in ER-mediated apoptosis; hence, CHOP may be a key molecule in retinal cell death.[18]

PKR is involved in the phosphorylation of eIF2α.[19,20] Overexpression of PKR leads to apoptosis.[21] In recent years, PKR

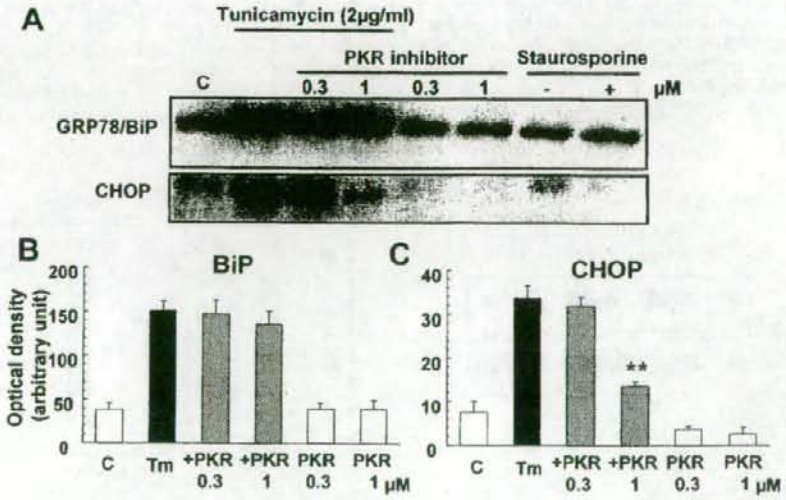

FIGURE 4. Effect of a PKR inhibitor on the tunicamycin-induced increases in BiP and CHOP proteins in RGC-5 cells. (A) Representative band images show immunoreactivities against BiP and CHOP. Quantitative analysis of band densities for BiP (B) and CHOP (C). Data are expressed as mean ± SEM (n = 4) of values (in arbitrary units) obtained from each band. **$P < 0.01$ versus tunicamycin alone (Dunnett test).

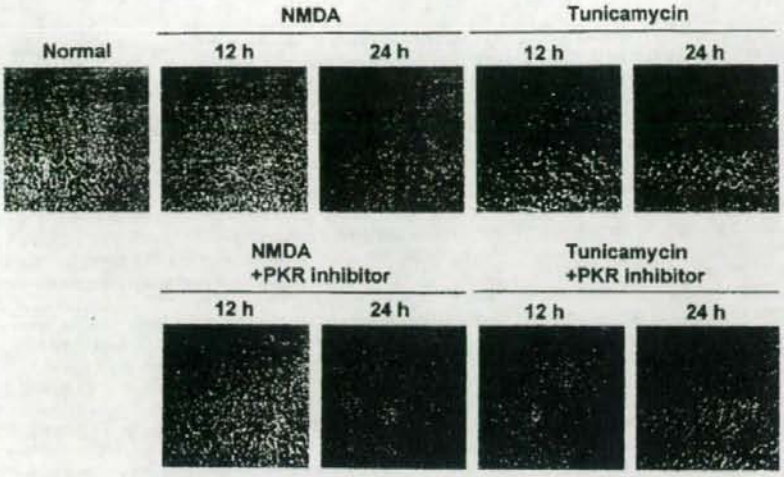

FIGURE 5. Phosphorylation of a PKR in mouse retina is induced by NMDA and by tunicamycin. Retinal cross-sections were labeled with antibody against phosphorylated PKR. Phospho-PKR-like immunoreactivity was increased in the inner retina 12 and 24 hours after intravitreal injection of NMDA (20 nmol/eye) or of tunicamycin (1 μg/eye). Coadministration of PKR inhibitor (1 nmol/eye) with the NMDA or tunicamycin inhibited the increase in phosphorylated PKR. Bar = 25 μm.

has been reported to be involved in Alzheimer disease,[7,22–24] Huntington disease,[25] Parkinson disease,[26] and amyotrophic lateral sclerosis,[27] indicating that PKR may be implicated in the neuronal damage induced by ER stress in these neurologic diseases. In the present study, PKR phosphorylation was observed in RGC-5 cells 6 and 24 hours after tunicamycin treatment. We therefore tested, both in vitro and in vivo, whether the activation of PKR might participate in the retinal neuron death induced by ER stress. In the in vitro study, both the PKR inhibitor and the knockdown of PKR (using siRNA) inhibited tunicamycin-induced RGC-5 cell death, and the inhibitor attenuated the tunicamycin-induced increase in CHOP protein but not the tunicamycin-induced BiP protein production. In our previous study, we reported that the same PKR inhibitor protects against the cell death induced by tunicamycin in a different cell line, SH-SY5Y cells.[28] Furthermore, treatment with the PKR inhibitor at 1 μM, either concomitantly with or 6 hours after tunicamycin, protected against RGC-5 cell death 24 hours after tunicamycin. This result suggests that PKR may operate in the late phase. In this study, *pretreatment* with the PKR inhibitor at 1 μM reduced the expression of CHOP protein 24 hours after tunicamycin. However, treatment with the PKR inhibitor (1 μM) *6 hours after* tunicamycin, when CHOP protein was being induced, also protected against RGC-5 cell death. This result suggests that CHOP production does not necessarily have to be decreased for cell survival to be enhanced. Indeed, pretreatment with the PKR inhibitor at 0.3 μM also inhibited tunicamycin-induced RGC-5 cell death, but it did not reduce the increase in CHOP protein. Although CHOP-deficient mice have been reported to show resistance to ER stress-induced cell death, a dimerization partner such as C/EBPβ is needed for the induction of such cell death.[29] In addition, CHOP protein

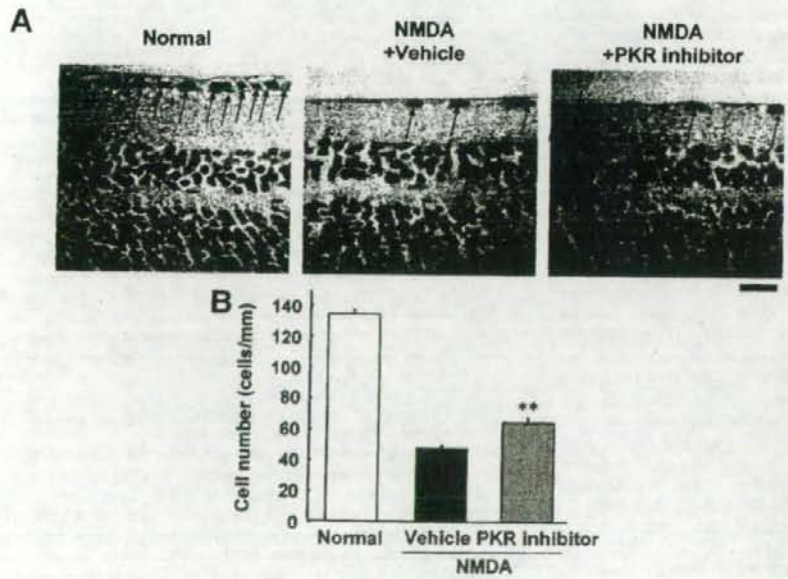

FIGURE 6. Effect of a PKR inhibitor on the mouse retinal damage induced by intravitreal injection of NMDA. (A) Representative photographs showing nontreated normal retina, NMDA-injected vehicle-treated retina, and NMDA-injected PKR inhibitor-treated retina. PKR inhibitor at 1 nmol/eye or vehicle was coadministered with the NMDA (20 nmol/eye). Quantitative analysis of cell number in the GCL (B). Each column represents the mean ± SEM ($n = 12$). $^{**}P < 0.01$ versus NMDA plus vehicle-treated group (Student's t-test). Bar = 25 μm.

undergoes stress-inducible phosphorylation by stress-inducible members of the p38 mitogen-activated protein kinase (MAPK) family, and such phosphorylation is associated with an enhancement of transcriptional activation by CHOP.[30] Takizawa et al.[31] reported that a dominant-negative mutant of PKR inhibited the apoptosis and the p38 MAPK activation induced by apoptosis signal-regulating kinase 1 (ASK1), a member of the MAPK kinase kinase (MAPKKK) family, which is activated by a variety of apoptosis-inducers. Both ASK1 and PKR are known to bind proteins associated with death receptors, such as tumor necrosis factor (TNF) receptor-associated protein 2 (TRAF2).[32,33] During ER-stress, ASK1 is recruited to oligomerized inositol-requiring enzyme-1 (IRE1) complexes containing TRAF2, thereby activating this kinase and causing downstream activation of c-Jun N-terminal kinase (JNK) and p38 MAPK.[34] Thus, PKR may activate the ASK1-p38 MAPK/-JNK signaling pathways to execute apoptosis. Furthermore, aggregated β-amyloid peptide has been reported to activate PKR through its phosphorylation or cleavage through calcium release from the ER, with activation of caspase-8 and caspase-3 as upstream signals.[23] In another possible mechanism, the eIF2α phosphorylation induced by activated PKR might result in an upregulation of CHOP, a proapoptotic transcription factor. In addition, excessive ER stress leads to activation of PKR-like ER kinase (PERK), just as it does to activation of PKR.[35,36] The luminal ER stress-sensing domains of PERK regulate its dimerization, and this leads to activation of its protein kinase activity under ER stress. Activation of PERK induces phosphorylation of eIF2α, contributing to a suppression of protein translation after the initiation of ER stress. Therefore, even when PKR activation is inhibited by a PKR inhibitor or by PKR downregulation (using siRNA), the phosphorylation of eIF2α may not be reduced. Accordingly, eIF2α may not be a target molecule through which activated PKR executes cell death, at least in ER stress. However, further studies will be needed to clarify the precise mechanisms. Regarding the specificity of the PKR inhibitor against PKR, we must consider the possibility of effects on other targets as a limitation.

The present results were obtained in cultured RGC-5 cells, and it could be agreed that the protective effect observed in vitro cannot be extrapolated directly to animal models in vivo. However, 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.[15] Moreover, expression of the CHOP gene is reportedly increased in the rat retina after intravitreal injection of NMDA.[37] Furthermore, Awai et al.[38] found that treatment with MK-801, an NMDA-receptor antagonist, inhibited the increases in CHOP mRNA and protein in the mouse retina observed after intravitreal injection of NMDA and that CHOP-deficient mice were resistant to NMDA-induced retinal damage. However, CHOP-deficient mice partially suppressed NMDA-induced retinal cell death; therefore, other pathways (e.g., leading to mitochondrial dysfunction) may be engaged in the induction of this cell death. These findings indicate that NMDA can cause ER stress in the retina and that the neurotoxicity induced by NMDA is caused in part by a mechanism dependent on the induction of CHOP protein through excessive ER stress. On the other hand, the neuroprotective effect of the PKR inhibitor on tunicamycin-induced RGC-5 cell death did not appear to depend on CHOP suppression. Although little is known about the precise mechanisms responsible for the NMDA-induced activation of ER stress, NMDA is known to cause intracellular Ca^{2+} overload and increased NO production, resulting in apoptotic cell death.[39,40] Several lines of study suggest that intracellular Ca^{2+} overload and excessive production of NO deplete Ca^{2+} in the ER, thereby resulting in ER stress.[41,42] Recently, Uehara et al.[43] 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. Furthermore, they found that NO induces S-nitrosylation of protein-disulfide isomerase (PDI), an enzyme that assists in the maturation and transport of unfolded secretory proteins and thereby helps to prevent the neurotoxicity associated with ER stress. S-nitrosylated PDI exhibits reduced enzymatic activity and induces cell death through the ER stress pathway. These mechanisms may contribute to the activation of ER stress in the retina after NMDA stimulation. Collectively, the results indicate that NMDA can cause ER stress in the retina and that the neurotoxicity induced by NMDA is caused in part by a mechanism dependent on ER stress.

In the present study, the phosphorylation of PKR was increased in the inner retina in mice in vivo after intravitreal injection of either NMDA or tunicamycin. The PKR inhibitor, when coadministered with NMDA or tunicamycin, inhibited the increase in phosphorylated PKR. This result indicates that PKR may be activated in the mouse retina in vivo during ER stress. The PKR inhibitor also reduced NMDA-induced retinal damage in mice in vivo, but this was a partial effect. Possibly, the observed difference in potency between the in vitro and in vivo situations may be attributed to the poor distribution or rapid excretion in ocular tissues of the PKR inhibitor after its intravitreal injection. Taken together, our results indicate that ER stress plays a role in retinal ganglion cell death and that PKR activation forms part of the underlying mechanisms.

In conclusion, we have identified a close association between PKR and ER stress-induced retinal damage, and our results suggest that PKR might be a good target in the search for better treatments for retinal diseases.

Acknowledgments

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〔特集：学会シンポジウム〕

分子シャペロン誘導剤の神経変性疾患治療への応用*

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要約：小胞体（ER）ストレスは，折りたたみ不整な蛋白が細胞内に蓄積することによって生じ，アルツハイマー病などの神経変性疾患の病理過程に関与しているとされる．ERストレスに対し，細胞は反応機構を元来備えており，ストレス状況からの離脱を図る．本研究はERストレス反応機構の1つである分子シャペロン誘導を人為的に行い，ストレスからの離脱を図ることで，アルツハイマー病（AD）などの神経変性疾患の治療に応用しようとするものである．分子シャペロンBiPのプロモーターを用いた解析から，我々はBiP誘導剤（BIX: BiP inducer X）を得た．細胞実験から，BIXはBiPのみ誘導し，他のERストレス反応分子を誘導させないことが示された．また，BIXで処理した細胞はERストレスに耐性を示し，ERのアポトーシス誘導分子の発現を抑えることが示された．さらに，マウスの脳室にBIXを前投与し，脳虚血を負荷すると梗塞巣の面積の減少をもたらし，神経症状の軽減が認められた．この BIX の効果は，梗塞周辺領域で ER のアポトーシス誘導分子の発現を抑えることによることが示された．以上のように，BIXはERストレスから生じるアポトーシスを抑制し，ADをはじめとする神経変性疾患の治療薬になることが示唆された．

キーワード：小胞体（ER）ストレス，分子シャペロン，アポトーシス，脳虚血，神経変性疾患

近年，アルツハイマー病（AD）の治療薬開発は，アミロイド前駆体蛋白の β/γ セクレターゼ阻害薬，非ステロイド消炎鎮痛剤，アミロイドワクチンなど様々な可能性が検討されているが，実用化に至ったものは未だない．したがって，現時点では多くの可能性を模索すべき時期であろうと思われる．また，パーキンソン病，レビー小体病，前頭側頭型認知症，ポリグルタミン病などの神経変性疾患は未だ病態過程が不明な点が多く，確立された根治療法がない．ただ，ADを含むこれらの疾患では，異常蛋白が神経細胞内に蓄積凝集するという点が共通であり，興味深い．

我々は，従来からアルツハイマー病の病態を小胞体（ER）ストレスに対する反応，すなわち unfolded protein response（UPR）の見地から検討してきた．このUPRは異常蛋白蓄積に直接リンクしていると考えられ，神経変性疾患研究の1つのトレンドとして多くの検討が行われている．近年我々は，ERストレスについて蓄積してきた知見を踏まえ，治療戦略への応用を検討し始めている．

Unfolded Protein Response（UPR）

細胞小器官の1つであるERは，分泌蛋白や膜構成蛋白などの折りたたみや翻訳後修飾を行う蛋白の「組み立て工場」のような役割を担う．「組み立て工場」であるが故に「不良品」すなわち折りたたみが不十分な，あるいは不正な蛋白（unfolded protein）の出現は宿命のようなものである．様々なストレスは，ERストレスとしてこの unfolded protein の ER 内への蓄積を生じ，カスペース12の活性化（Nakagawa et al, 2000）（ヒトではカスペース4（Hitomi et al, 2004））, JNK系路の活性化（Nishitoh et al, 2002），CHOPの誘導（Friedman, 1996）等のER発動のアポトーシスにつながる．その防御機構として，細胞には3つのUPRが備わっている．

1. 分子シャペロンの誘導

UPRの働きの1つは，小胞体から核へのシグナル伝達を活性化し，GRP78/BiPやGRP94などの分子シャペロンを発現誘導することである．これら分子シャペロンは，unfolded protein に作用し，折りたたみの促進や是正を行う（Sidrauski et al, 1998）．

2. 蛋白翻訳抑制

第2の戦略は，これ以上 unfolded protein を生じないように，蛋白の翻訳自体を抑制する方策であり，翻訳開始因子の eIF2α がリン酸化されることで発動される（Harding et al, 1999）．

3. ER-associated degradation（ERAD）

ERに蓄積した unfolded protein を処理しきれない場合，unfolded protein はERから細胞質にはき出され，ユビキチン化を受け，26Sプロテオソームで分解される（Bonifacino and Weissman, 1998）．

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図2　プレセニリン1変異体発現細胞のERストレス脆弱性とBiP誘導によるレスキュー．プレセニリン1変異体（A246E）発現細胞は野生型（PSW）細胞に比し，ERストレス（tunicamycin: Tm）に脆弱性を示す（矢印は神経細胞死）が，あらかじめウイルスベクターを用いてBiPを発現させておくと，この脆弱性はレスキューできる．

示唆する事実である．

また，遺伝性若年性パーキンソン病の原因遺伝子であるParkinはユビキチンリガーゼであることが示され，Parkinの変異体は基質であるPeal受容体のERADを発動することができず，unfoldedなPeal受容体を溜め，ER発動のアポトーシスを招くとされる（Imai et al, 2001）．ハンチントン病などのポリグルタミン病は，CAGのリピートが異常に伸張したために神経変性を呈する疾患であるが，伸張したポリグルタミンはプロテオソームの機能低下をきたし，ERストレスを惹起し，ASK1を活性化してJNKを介するアポトーシスが発動することが示されている．以上のように，UPRの異常は神経変性疾患の共通した病理因子である可能性が示唆されている．

分子シャペロン誘導剤開発

以上の知見から，UPRを人為的に活性化することは，アルツハイマー病をはじめとする神経変性疾患の治療につながる可能性が考えられる．我々の過去の検討で，BiPを発現するウイルスベクターを神経細胞にあらかじめ感染させておくと，プレセニリン1変異体によるERストレス脆弱性が改善した結果を得ている（図2）．そこで我々は，UPRのうち分子シャペロン誘導に着目し，分子シャペロンBiP誘導剤の検索を行った．BiPのプロモーター配列を利用してBiPレポーターシステムを構築し，コンパウンドライブラリーをハイスループットスクリーニングにて検索し，最もBiPのメッセージを誘導するコンパウンドBiP inducer X（BIX）を得た．このBIXを神経芽細胞腫SK-N-SHに投与すると，BIXの濃度依存的にBiPを誘導することが示されたが，古典的なERストレス誘導剤であるthapsigargin等が誘導するようなBiPの高レベルよりは低く観察された．また，BIXによるBiP誘導は，投与4時間後から起こり，6時間後をピークに減衰していくことが観察された．以上の観察されたメッセージレベルのBiP誘導には，BiP蛋白発現を伴っていることが，ウェスタンブロット法にて確認された．

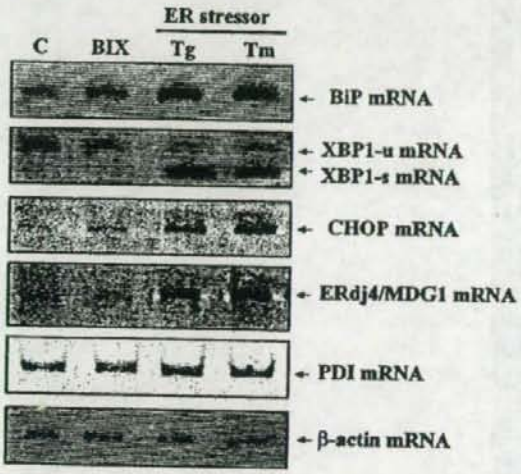

図3　BIXのBiP誘導効果とその他のERストレス．BIXはBiPのみを誘導し，thapsigargin（Tg）やtunicamycin（Tm）などのERストレッサーが誘導するようなその他のERストレス誘導分子は誘導しない．

このBIXが，もしBiP以外のERストレスによって発現される分子を活性化すれば，ERストレスによるアポトーシスにつながりかねず，治療法とはなり得ない．そこで，BiP以外のERストレス分子XBP1，CHOP，ERdj4/MDG1，PDIの活性化について，BIXの効果を検討した．BIXをSK-N-SH細胞に投与しても，BiPは誘導するが，XBP1のスプライシング（活性化），CHOPの誘導，ERdj4/MDG1の誘導，PDIの誘導は起きないことが確認された（図3）．また，BiP誘導と相対する蛋白翻訳抑制を起こすeIF2αのリン酸化についてもBIXの効果を検討したが，そのリン酸化は観察されなかった．以上の結果から，BIXはBiPのみを誘導し，他のERストレス反応分子を誘導しないことが示された．

BIXの神経細胞におけるERストレス保護作用

BIXのERストレス保護作用を検討する目的で，BIXをSK-N-SH細胞の培地に添加し，12時間後にtunicamycin

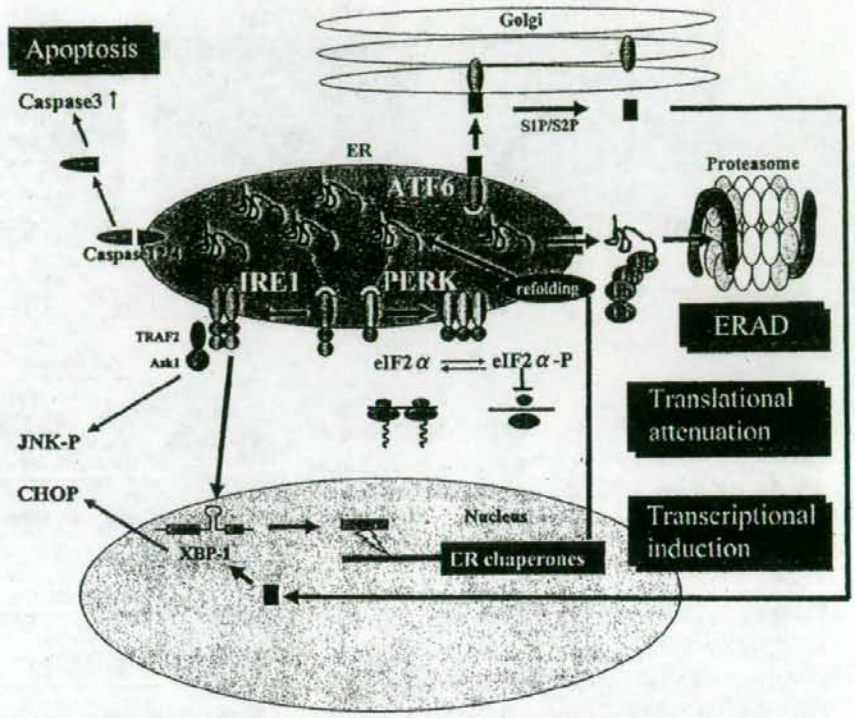

図1 小胞体（ER）ストレス反応（unfolded protein response: UPR）とアポトーシス．

ERストレストランスデューサー（図1）

UPRは，ER内のunfolded proteinの蓄積を感知することから始動する．現在まで，unfolded proteinのセンサーとしてER膜上のIRE1，PERK，ATF6が報告されている．IRE1は，二量体形成と自己リン酸化を経て，XBP-1のalternative splicingを起こし，成熟型のXBP-1を生じさせる（Yoshida et al, 2001）．この成熟型XBP-1は，GRP78/BiP等の分子シャペロンのプロモーターに働き，分子シャペロンを誘導する（Wang et al, 1998）．PERKは，多量体化と自己リン酸化を経て，eIF2αをリン酸化する（Harding et al, 1999）．このリン酸化されたeIF2αは43S initiation complexの形成を阻害し，翻訳開始を阻害する．ATF6は，ゴルジに運ばれ，S1PおよびS2Pによって細胞質側の膜貫通領域近傍で切断を受け，できたN末端領域がXBP1の転写を促進して分子シャペロンの誘導につながる（Haze et al, 1999）．これら3つのトランスデューサーは，非ストレス下ではGRP78/BiPが結合しており，unfolded proteinのER内での蓄積に際しその結合がはずれ，UPRが開始されるという共通の機序が想定されている．

ERストレスと神経変性疾患

我々は，家族性アルツハイマー病の主要な原因遺伝子であるプレセニリン1がERに局在することから，ERストレスとプレセニリン1変異体の関係について検討した．プレセニリン1変異体発現神経細胞は，ERストレスに脆弱であることが示され，ERストレス下における分子シャペロンBiPの誘導が抑制されることが見いだされた．プレセニリン1変異体神経細胞のUPRを検討すると，IRE1，PERK，ATF6全てのトランスデューサーの活性化が障害されており，UPR抑制のためにERストレス脆弱性をきたしていることが示唆された（Katayama et al, 1999, 2001; Yasuda et al, 2002）．また，プレセニリン1変異体神経細胞にUPRの1つであるBiPをウイルスベクターにより発現させておくと，ERストレス脆弱性をレスキューできることも示された（図2）．したがって，プレセニリン1変異による家族性アルツハイマー病の神経変性には，UPR障害が関与すると考えられる．実際，家族性アルツハイマー病患者脳ではBiPの発現が低いことが観察されており，おもしろいことに孤発性AD患者脳でもBiP発現が低下していることが観察された．これは，ADの神経変性に共通してBiP誘導障害，すなわちUPR障害が関与すること

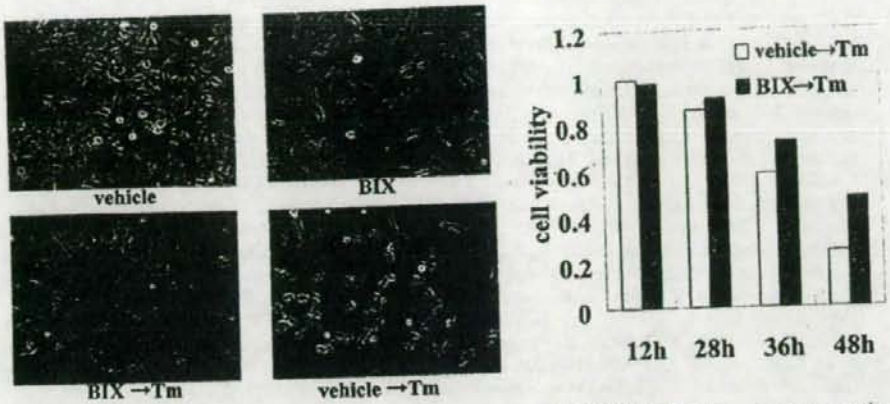

図4 BIX の抗 ER ストレス効果．BIX を 12 時間前に前投与しておくと，vehicle 投与神経細胞に比し，ER ストレス（tunicamycin: Tm）による神経細胞死を抑制できる．

を投与して ER ストレスをかけた．BIX を投与していない細胞では，tunicamycin 投与後 36 時間後より細胞死が観察されたが，BIX 投与細胞では有意な細胞死の減少が観察された（図 4）．ER ストレスから誘導されるアポトーシスで活性化されるカスペース 4 についてウェスタンブロット法で検討したところ，BIX 投与細胞ではその活性化が抑制されていることが確認され，BIX は ER ストレスによるアポトーシスを抑制し，神経細胞を保護することが示された．

BIX の脳虚血モデルにおける効果

脳虚血は ER ストレスを惹起するので，BIX の効果を検証する *in vivo* の実験系として，マウスの中大脳動脈閉塞（MCAO）モデルを採用した．BIX のマウス脳室内投与は，6 時間後で脳実質内の BiP を誘導することが確認された．今回は，脳虚血 30 分前に BIX を脳室内投与し，MCAO 24 時間後に薬剤の効果を検討した．通常 MCAO 後 24 時間では，歩行不可，反対側への旋回運動，反対側前足の伸展障害，あるいは神経症状なしのいずれかの神経障害が認められ，多くのマウスは旋回運動程度の神経障害を示すが，BIX 投与マウスでは神経障害は軽度となり，前足の伸展障害程度を示していた．MCAO 24 時間後の脳切片を作製し，TTC 染色により脳梗塞部位の計測を行ったところ，BIX 投与マウスでは脳梗塞部位面積の有意な減少が認められ，特に梗塞周辺領域（penumbra）の減少が顕著であった．また，MCAO 24 時間後の脳浮腫についても検討したところ，BIX 投与マウスでは有意に脳浮腫が軽減されていた．これらの結果は，BIX の脳室内前投与が MCAO による脳梗塞の侵襲を軽減して神経症状発現を抑えうることを示している．

BIX の効果は，penumbra の減少に顕著に認められたが，その部位での ER ストレスによるアポトーシスについて検討した．アポトーシス発現細胞を見るために脳切片を TUNEL 染色したところ，BIX 投与マウスの penumbra における TUNEL 陽性細胞は有意に減少していることが観察された．さらに，ER ストレスによるアポトーシス誘導分子である CHOP の誘導について *in situ* hybridization にて検討したところ，CHOP の誘導も BIX 投与マウスの penumbra において有意に抑制されていることが観察された．これらのことから，BIX 投与は penumbra の ER ストレスを軽減し，それによるアポトーシスを抑制することで梗塞巣の増大を抑え，また *in vivo* においても，BIX は ER ストレスによるアポトーシスに効果があることを示している．

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Abstract: Takashi KUDO[*1], Kazunori IMAIZUMI[*2] and Hideaki HARA[*3] ([*1]Department of Psychiatry, Osaka University Graduate School of Medicine, D3, 2–2 Yamadaoka, Suita, 565–0871 Japan; [*2]Department of Anatomy, Faculty of Medicine, University of Miyazaki; [*3]Department of Biofunctional Molecules, Gifu Pharmaceutical University) *A molecular chaperone inducer as potential therapeutic agent for neurodegenerative diseases*. Jpn. J. Neuropsychopharmacol., 27: 63–67 (2007).

Recent reports have shown that ER stress is involved in the pathology of some neurodegenerative diseases. In a screen for compounds that induce the ER-mediated chaperone BiP/GRP 78 (BiP), we identified BiP inducer X (BIX). BIX induced BiP only, in a dose-dependent manner, without induction of other molecules involved in the ER stress response. Pretreatment of neuroblastoma cells with BIX reduced cell death induced by ER stress. Intracerebroventricular pretreatment with BIX reduced the area of infarction due to focal cerebral ischemia in mice. In the penumbra of BIX-treated mice, ER stress-induced apoptosis was suppressed, leading to a reduction in the number of apoptotic cells. Taken together, BIX induces BiP to prevent neuronal death by ER stress, suggesting that it may be a potential therapeutic agent for cerebral diseases caused by ER stress.

Key words: ER stress, Molecular chaperone, Apoptosis, Cerebral ischemia, Neurodegenerative diseases

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A molecular chaperone inducer protects neurons from ER stress

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The endoplasmic reticulum (ER) stress response is a defense system for dealing with the accumulation of unfolded proteins in the ER lumen. Recent reports have shown that ER stress is involved in the pathology of some neurodegenerative diseases and cerebral ischemia. In a screen for compounds that induce the ER-mediated chaperone BiP (immunoglobulin heavy-chain binding protein)/GRP78 (78 kDa glucose-regulated protein), we identified BiP inducer X (BIX). BIX preferentially induced BiP with slight inductions of GRP94 (94 kDa glucose-regulated protein), calreticulin, and C/EBP homologous protein. The induction of BiP mRNA by BIX was mediated by activation of ER stress response elements upstream of the BiP gene, through the ATF6 (activating transcription factor 6) pathway. Pretreatment of neuroblastoma cells with BIX reduced cell death induced by ER stress. Intracerebroventricular pretreatment with BIX reduced the area of infarction due to focal cerebral ischemia in mice. In the penumbra of BIX-treated mice, ER stress-induced apoptosis was suppressed, leading to a reduction in the number of apoptotic cells. Considering these results together, it appears that BIX induces BiP to prevent neuronal death by ER stress, suggesting that it may be a potential therapeutic agent for cerebral diseases caused by ER stress.
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The endoplasmic reticulum (ER) is an 'assembly plant' for the manufacture of secretory and membrane proteins. However, from time to time 'inferior goods', that is, unfolded/misfolded proteins in the ER are inevitable. Under normal physiological conditions, unfolded proteins are degraded; under conditions of ER stress, however, unfolded proteins can accumulate in the ER lumen. Eukaryotes utilize the unfolded protein response (UPR) to overcome the critical status induced by ER stress.[1] The UPR consists of the following three pathways: (1) suppression of protein translation to prevent the generation of more unfolded proteins; (2) facilitation of refolding of unfolded proteins by the induction of ER chaperones; and (3) activation of ER-associated degradation (ERAD) to degrade the unfolded proteins accumulated in the ER, by the ubiquitin–proteasome pathway. If these strategies are unsuccessful, cells go into ER stress-induced apoptosis.

Recent reports show that dysregulation of the UPR is implicated in much important pathology, including some neurodegenerative diseases and cerebral ischemia. Previously, the ER transducers inositol-requiring kinase 1 (IRE1), PKR(protein kinase regulated by RNA)-like ER-associated kinase (PERK), and activating transcription factor 6 (ATF6) were reported to be downregulated in presenilin-1 mutant neurons, causing the vulnerability to ER stress seen in cases of familial Alzheimer disease (AD).[2–4] Caspase 4, the human homologue of murine caspase 12, was also reported to play a critical role in ER stress-induced neuronal cell death in AD.[5] One inherited form of Parkinson's disease is associated with the accumulation of the protein Parkin-associated endothelin receptor-like receptor in the ER of dopaminergic neurons as a result of defective ERAD by mutant Parkin, a ubiquitin protein ligase (E3).[6,7] Analysis of the polyglutamine tract associated with the spinocerebrocellular atrophy protein (spinocerebellar ataxia type 3) in Machado–Joseph disease suggests that cytoplasmic accumulation of this protein can inhibit proteasome function, thereby interfering with ERAD and eliciting ER stress-induced apoptosis.[8,9] It was also reported that cerebral ischemia activates the UPR.[10,11] These findings show that many cerebral disorders are related to an impaired UPR and ER stress-induced apoptosis.

These accumulated data concerning the involvement of ER stress in cerebral disorders encouraged us to investigate this

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Keywords: endoplasmic reticulum stress; chaperone; apoptosis; cerebral ischemia; neurodegeneration
Abbreviations: AD, Alzheimer disease; ATF6, activating transcription factor 6; BiP, immunoglobulin heavy-chain binding protein; CHOP, C/EBP homologous protein; EDEM, ER degradation-enhancing α-mannosidase-like protein; eIF2α, eukaryotic translation initiation factor 2 subunit α; ERAD, ER-associated degradation; ERdj4/MDG1, ER-localized DnaJ 4/microvascular differentiation gene 1; ERSE, ER stress response element; GRP78, 78 kDa glucose-regulated protein; GRP94, 94 kDa glucose-regulated protein; HSP70, 70 kDa heat-shock protein; IRE1, inositol-requiring kinase 1; MCA, middle cerebral artery; MEF, mouse embryonic fibroblast; PERK, PKR(protein kinase regulated by RNA)-like ER-associated kinase; $p58^{IPK}$, protein kinase inhibitor of 58 kDa; Tg, thapsigargin; Tm, tunicamycin; TTC, 2,3,5-triphenyltetrazolium chloride; UPR, unfolded protein response; XBP1, X-box binding protein 1
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field in an effort to identify therapeutic targets for the treatment of these disorders. We speculate that a therapeutic strategy that induces the UPR might prevent neuronal death induced by ER stress. According to the UPR pathway, we could try to (1) induce the expression of ER molecular chaperones; (2) block translation of proteins; or (3) artificially stimulate the degradation of misfolded proteins by the proteasome, as therapeutic approaches. Indeed, some chemical compounds that induce particular UPR pathways have been developed. For example, Boyce *et al.*[12] identified salubrinal, a selective inhibitor of cellular complexes that dephosphorylates eukaryotic translation initiation factor 2 subunit α (eIF2α), and thereby blocks translation. They concluded that salubrinal might be useful in the treatment of diseases involving ER stress.[12] Kim *et al.*[13] reported that valproate, a widely prescribed drug for epilepsy and bipolar disorder, increases the expression levels of the ER chaperones immunoglobulin heavy-chain binding protein (BiP), GRP94 (94 kDa glucose-regulated protein), protein disulfide isomerase, and calreticulin as well as the cytosolic chaperone HSP70 (70 kDa heat-shock protein). They also showed that valproate induces these chaperones without evoking the UPR, and speculated that inhibition of glycogen synthase kinase-3 by valproate might lead to the induction of chaperones.[13]

Previous reports have shown that induction of BiP, an ER molecular chaperone, prevents neuronal death induced by ER stress.[2,14–16] By contrast, inhibition of GRP78 (78 kDa glucose-regulated protein) mRNA induction increases cell death in response to calcium release from the ER, oxidative stress, hypoxia, and T-cell-mediated cytotoxicity.[17–19] Therefore, in this paper, we searched for a chemical compound that induces BiP for possible use as a neuroprotective agent against ER stress. We have identified such a chemical compound, BiP inducer X (BIX), and shown its protective effect against ER stress-induced apoptosis *in vivo* and *in vitro*, suggesting that this compound might be useful in the treatment of cerebral disorders associated with ER stress, such as cerebral ischemia.

Results

BIX preferentially induces BiP. To identify chemical compounds that induce BiP mRNA, we utilized high-throughput screening (HTS) with a BiP reporter assay system. Of the screened compounds, 1-(3,4-dihydroxy-phenyl)-2-thiocyanate-ethanone (Figure 1a) showed the highest level of induction of BiP mRNA; thus, we named this compound BiP inducer X (BIX).

First, we examined whether BIX does indeed induce the expression of BiP mRNA. Northern blot analysis and real-time PCR of SK-N-SH neuroblastoma cells treated with BIX showed that BIX induces BiP mRNA in a dose-dependent manner; however, the level of BiP mRNA induced by BIX is less than that induced by thapsigargin (Tg) (Figure 1b). Additionally, treatment of cells with 50 μM BIX caused the highest induction of BiP and little toxicity to cells. Because BIX generated cytotoxicity at higher dosages, we did not use it at concentrations greater than 50 μM in further analyses. Semiquantitative RT-PCR and real-time PCR showed that the BiP signal peaked at 4 h after the addition of 5 μM BIX and remained at that level until 6 h after treatment, with a subsequent reduction in level after this point (Figure 1c). To determine whether the induction of BiP mRNA by BIX results in an increase in the level of BiP protein, we carried out immunoblot analysis. As shown in Figure 1d, the level of BiP protein was increased by 5 μM BIX in a time-dependent manner, consistent with the changes in mRNA levels.

Next, we performed semiquantitative RT-PCR analysis to investigate whether BIX affects the expression of any other ER stress response-related genes, such as GRP94, calreticulin, X-box binding protein 1 (XBP1), ER-localized DnaJ 4/microvascular differentiation gene 1 (ERdj4/MDG1), ER degradation-enhancing α-mannosidase-like protein (EDEM), protein kinase inhibitor of 58 kDa ($p58^{IPK}$), C/EBP homologous protein (CHOP), and asparagine synthetase (ASNS) (Figure 2a). According to the time-course study of BiP induction by BIX (Figure 1c), a 6 h treatment of cells with BIX was adopted into this study. XBP1 mRNA, which is spliced under ER stress induced by 1 μM Tg or 1 μg/ml tunicamycin (Tm), was not processed in cells treated with 5 μM BIX. Compared with a control sample, ERdj4/MDG1, EDEM, $p58^{IPK}$, and ASNS were scarcely induced by BIX. On the other hand, GRP94, calreticulin, and CHOP were induced by BIX, but their inductions were not as prominent as that of BiP. We also performed time-course analyses on the expressions of ER stress response-related genes by real-time PCR in SK-N-SH cells treated with 50 μM BIX as well as 5 μM BIX. A 5 μM portion of BIX induced GRP94, calreticulin, and CHOP mRNA as well as BiP. However, BiP was definitely induced from 2 to 6 h (Figure 2b). The time courses for EDEM, $p58^{IPK}$, and ASNS were not changed by treatment with 5 μM BIX (Figure 2b). A 50 μM portion of BIX also induced BiP from 4 to 6 h and transiently induced calreticulin and CHOP. Even 50 μM BIX did not induce EDEM, $p58^{IPK}$, or ASNS (Figure 2b).

Moreover, we performed immunoblot analysis of GRP94 and phosphorylated eIF2α to examine whether BIX affects other signaling pathways that participate in the ER stress response. Treatment of SK-N-SH cells with BIX caused very slight induction of GRP94 protein compared with its induction by 1 μM Tg (Figure 2c). This result was consistent with that of semiquantitative RT-PCR analysis of the GRP94 mRNA level. Treatment of cells with 1 μM Tg increased the level of phosphorylated eIF2α, but BIX did not cause its phosphorylation (Figure 2c). These results indicate that BIX invokes little ER stress, but almost preferentially induces BiP.

The induction of BiP by BIX is mediated by activation of ERSEs through the ATF6 pathway. To investigate the mechanism by which BiP is induced by BIX, we performed reporter assays using 132 bp BiP-pGL3 reporter plasmids as described in the Materials and Methods section. A BiP (132)-pGL3 plasmid (Figure 3a) was transfected into SK-N-SH cells and the cells were treated with 5 μM BIX, 300 nM Tg, or 0.5 μg/ml Tm for either 6 or 16 h. The reporter activities in transfectants were elevated in response to stimulation with Tg or Tm, and maintained at a long-lasting high level (Figure 3b). By contrast, reporter activities in cells treated with BIX were transiently induced at 6 h after stimulation and then downregulated to basal levels by 16 h (Figure 3b). This