投与した。NMDA 投与 7 日後に眼球を摘出し、網膜薄切標本による組織評価を行った。

〔結果〕*In vitro*: BIX は RGC-5 において BiP を濃度及び時間依存的に誘導した（図 4）。また BIX は tunicamycin による細胞死に対して濃度依存的な保護効果を示した（図 5）。ツニカマイシン誘発 CHOP タンパク発現に対して BIX は抑制作用を示した（図 6）。しかし、スタウロスポリン誘発細胞死に対しては BIX は明らかな作用を示さなかった（図 7）。マウス硝子体内に BIX を投与すると BiP の発現が経時的に認められた（図 8）。*In vivo*: NMDA 投与マウス網膜障害モデルにおいても BIX は有意な細胞保護作用を示した（ツニカマイシンモデル; 図 9、NMDA モデル; 図 11）。ERAI マウスを用いた検討で、ツニカマイシンは網膜で XBP-1-Venus の発現を誘導したが、BIX はその誘導を抑制した（図 10）。ツニカマイシンまたは NMDA 誘発による CHOP の誘導を BIX は抑制した（図 12）。

〔考察〕BiP を選択的に誘導する薬剤は、小胞体ストレスと関連する網膜障害に対して有効である可能性が示唆された。

図 2　BIX の化学構造式

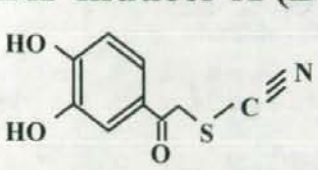
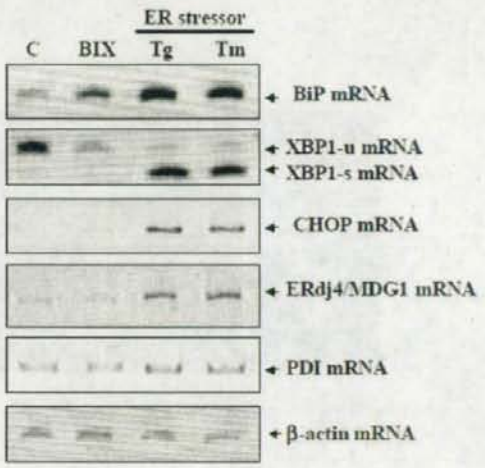



図 3　BIX の BiP 誘導効果とその他の ER ストレス




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。

図 4 ラット網膜神経節細胞（RGC-5）における BiP mRNA 発現に及ぼす BIX の影響

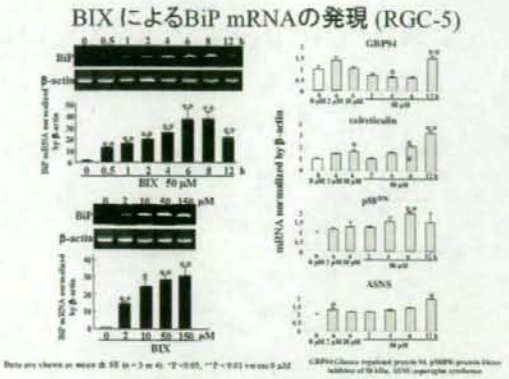



図５RGC-5におけるツニカマイシン誘発細胞死に対するBIXの作用

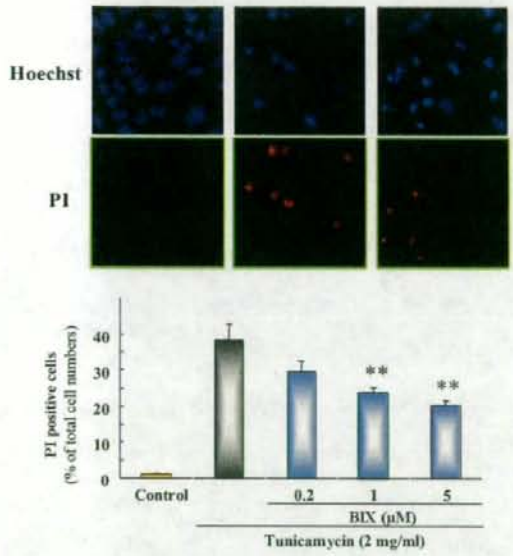



図６RGC-5 におけるツニカマイシン誘発CHOPタンパク発現に対するBIXの作用

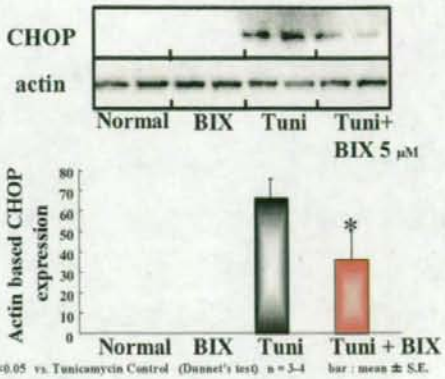
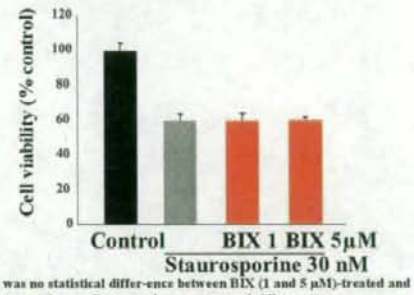
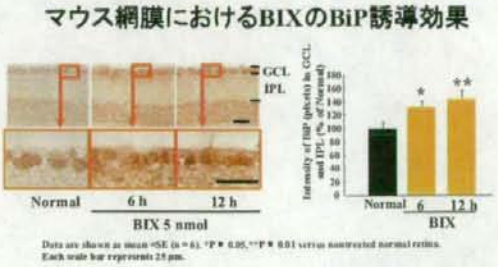



図７RGC-5におけるスタウロスポリン誘発細胞死に対するBIXの作用



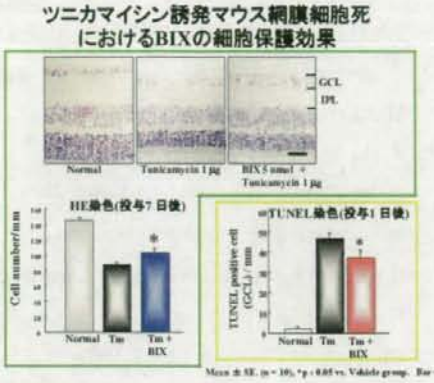

BIXはスタウロスポリン細胞死に対して作用を示さなかった。

図８マウス網膜におけるBIXのBiP誘導効果



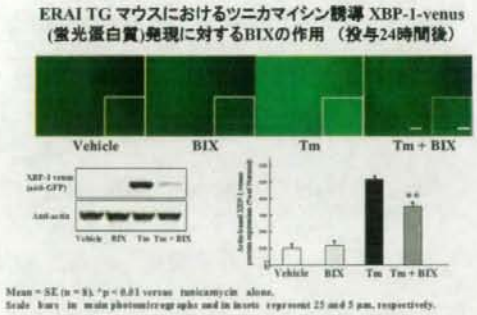

BIX(5 nmol)はマウス硝子体内に投与され、6または12時間後に眼を摘出された。

図９　ツニカマイシン誘発マウス網膜細胞死に対するBIXの細胞保護効果



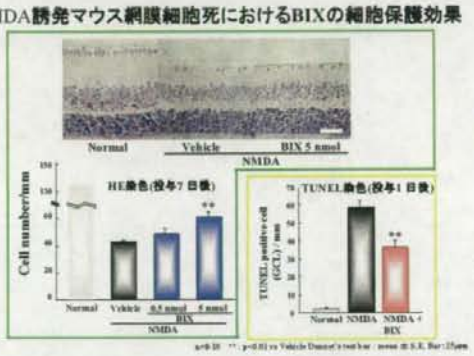

BIX（5 nmol）の硝子体内投与はツニカマイシン誘発網膜神経節細胞死に対して１日後のアポトーシスおよび７日後の細胞死を抑制した。

図１０　ERAI マウスにおけるツニカマイシン誘発XBP-1ピーナス発現に対するBIXの作用




小胞体ストレス条件下においてスプライシングを受ける X-box binding protein-1 (XBP-1)遺伝子の下流に蛍光蛋白質 VENUS 遺伝子を結合させた遺伝子を組み込んだ遺伝子改変マウス（ERAI マウス）を用いた。ツニカマイシン投与 24 時間後に XBP-1-venus の発現による蛍光が網膜において観察され，とくに網膜神経節細胞層及び内網状層において強い蛍光が認められた。BIX はその発現を抑制した。

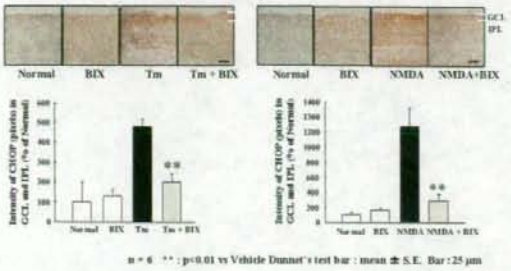
図１１　NMDA 誘発マウス網膜神経細胞死に対するBIXの細胞保護効果




BIX（5 nmol）の硝子体内投与は、NMDA 誘発網膜神経節細胞死に対して１日後のアポトーシスおよび７日後の細胞死を抑制した。

図１２　ツニカマイシンまたは NMDA 誘発 CHOP 発現に対するBIXの効果

ツニカマイシンまたはNMDA誘導CHOP発現
に対するBIXの効果（投与3 日後）




n = 6 ** ; p<0.01 vs Vehicle Dunnet's test bar : mean ± S.E. Bar : 25 μm

D．考察

1. 分子シャペロンBiPのプロモーターを用いた解析から、BiP選択的誘導剤（BIX）を見出した。
2. BIXは各種網膜障害モデルにおいて保護作用を示した。

以上、BiP の誘導は網膜細胞障害に対して有効であることが示唆された。

E．結論

以上のことから、小胞体ストレスを制御しうる薬物は、網膜疾患治療薬の新規な創薬ターゲットになる可能性が示唆された。

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3） Inokuchi Y., Nakajima Y., Shimazawa M., Kurita T., Kubo M., Saito A., Sajiki H., Kudo T., Aihara M., Imaizumi K., Araie M. and Hara H. Effect of an inducer of BiP, a molecular chaperone,

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第31回日本神経科学大会（東京、2008、7、9-11）

2） 井口勇太、嶋澤雅光、中島佳美、佐治木弘尚、工藤喬、今泉和則、原英彰
小胞体ストレスによる網膜障害におけるBiP誘導剤（BIX）の保護効果
第82回日本薬理学会年会（横浜、2009、3、16-18）

H．知的財産権の出願・登録状況（予定を含む）

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

厚生労働科学研究費補助金(感覚器障害研究事業)分担研究報告書

# 緑内障性網膜神経障害に対する新規治療薬の開発

分担研究者　金本尚志　広島大学講師

## 研究要旨

免疫機能を司るT細胞受容体(T cell receptor)ファミリーやインテグリン・ファミリーの一部が、緑内障性網膜視神経障害に関与することが判明した。

## A.研究目的

緑内障性網膜神経障害に関与するタンパクについてプロテオミクス解析により候補因子を確定しているが、本研究ではそれらの機能解析を行い新規神経保護薬を開発すること。

## B.研究方法

まず、緑内障性網膜神経障害の関連タンパクの可能性のあるT細胞受容体(T cell receptor: TCR)を解析した。まずは、In vivoの実験として、網膜内におけるTCRの発現部位を同定するために、マウス網膜サンプルを用いて、TCRのサブタイプである TCR-α、TCR-β、TCR-γ、TCR-δの免疫染色を行った。また、緑内障モデルマウスであるDBA2Jの網膜内での生後７ヶ月から１１ヶ月における経時的なTCRの発現変化を、ウェスタン・ブロット法にて分析した。次に、In vitroの実験として、TCRの網膜神経保護効果を検討するために、、継代培養系の網膜神経細胞であるＲＧＣ５細胞に対して、TCRのsiRNAを行った後、酸化ストレスなどによる神経細胞死を誘導し、その生存率をＭＴＳアッセイ等を用いて検討した。

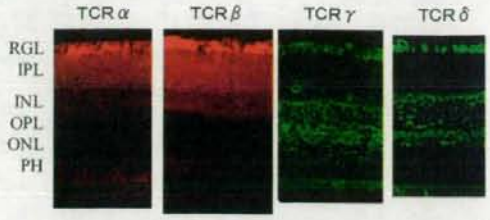
上記と同じく免疫系に関与するインテグリン・ファミリーのうち、緑内障性網膜神経障害の関連タンパクの可能性のあるインテグリン・β７（Integrin-beta7: Intb7）を主として解析した。免疫染色を行って、マウス網膜内における Intb7 の発現部位を同定した。次に、In vitro の実験として、Intb7 を介した の網膜神経保護効果を検討するために、ウィルスベクターを用いたsiRNA法を導入し、ＲＧＣ５細胞に対してIntb7のsiRNAを行った後、グルタミン酸神経毒性による神経細胞死を誘導し、その生存率を検討した。

（倫理面への配慮）

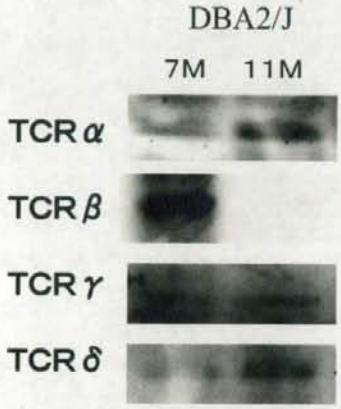
文部科学省告示の「研究機関等における動物実験等の実施に関する基本指針」および，広島大学動物実験指針，広島大学動物実験実施規則に則った。

## C.研究結果

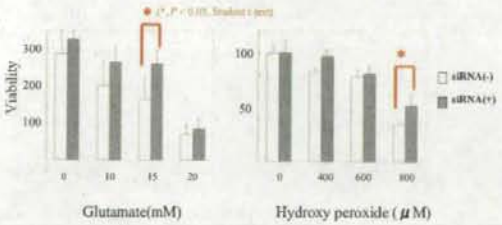
1. TCRのサブタイプ全てが、少なくとも、マウス網膜の網膜神経節細胞層(RGC)に発現していた（下図)。



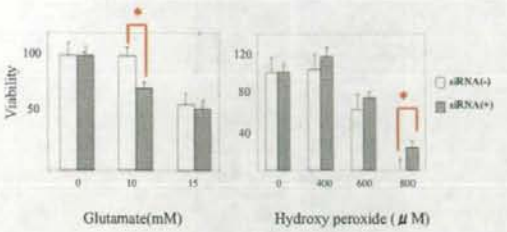

2. βサブユニットでは発現が減少していたが、その他のサブタイプでは発現が減少していた(下図)。



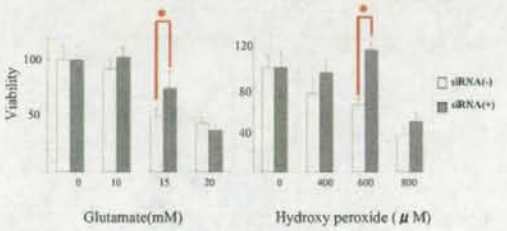

3. TCRα発現の抑制によって、グルタミン酸神経毒性と酸化ストレスによって誘導される細胞死は、いずれにおいても、抑制された(下図)。



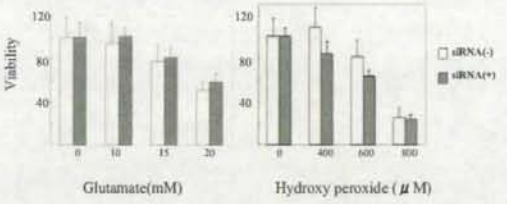

4. グルタミン酸神経毒性によって誘導される細胞死は、TCRβ発現の抑制によって促進された。逆に、酸化ストレスによって誘導される細胞死は、抑制された(下図)。



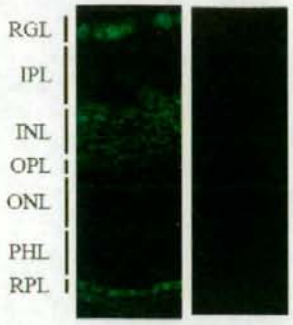

5. グルタミン酸神経毒性と酸化ストレスによって誘導される細胞死は、いずれにおいても、TCRγ発現の抑制によって抑制された(下図)。



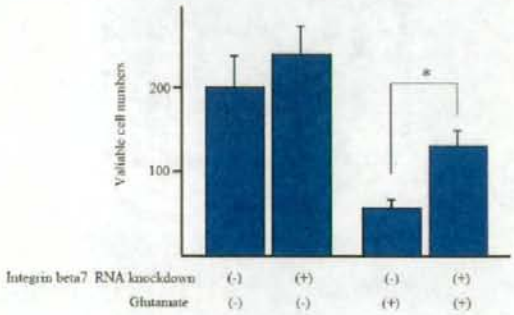

6. グルタミン酸神経毒性と酸化ストレスによって誘導される細胞死は、いずれにおいても、TCRδ発現の抑制に影響されなかった(下図)。




7. 免疫染色の結果から、Intb7は網膜神経節細胞層に豊富に存在することが明らかになった(下図)。




8.Intb7のsiRNAによって、グルタミン酸神経毒性で誘導される網膜神経節細胞死は抑制された（下図）。




D.考察

今回の In vitro assay の結果を基盤として、今後は動物モデルを用いた免疫系による、神経保護効果について、検討していく必要があると考えられた。

E.結論

1．TCR の網膜神経節細胞層の局在性と、網膜神経節細胞死への関与が明らかになった(下図)

| | TCRα | TCRβ | TCRγ | TCRδ |
|---|---|---|---|---|
| マウスRGCにおける発現 | (+) | (+) | (+) | (+) |
| マウスRGC減少に伴う発現変化 | 増加 | 大きく減少 | やや増加 | 増加 |
| siRNAによるグルタミン酸誘導性細胞死 (=グルタミン酸誘導性細胞死への作用) | 抑制 (=促進) | 強く促進 (=抑制) | 抑制 (=促進) | 変化なし |
| siRNAによる酸化ストレス誘導性細胞死 (=酸化ストレス誘導性細胞死への作用) | 抑制 (=促進) | 抑制 (=促進) | 強く抑制 (=促進) | 変化なし |

2．網膜神経節細胞に局在する Intb7 は、その細胞死を促進している可能性がある。

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G. 知的財産権の出願・登録状況(予定を含む。)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧表

書籍

| 著者氏名 | 論文タイトル名 | 書籍全体の編集者名 | 書　籍　名 | 出版社名 | 出版地 | 出版年 | ページ |
|---|---|---|---|---|---|---|---|
| なし | | | | | | | |

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# Inhibitor of double stranded RNA-dependent protein kinase protects against cell damage induced by ER stress

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**Abstract**

Endoplasmic reticulum (ER)-stress is known to induce neuronal cell death and to play roles in neurodegenerative diseases. Phosphorylation of double stranded RNA-dependent protein kinase (PKR) has been demonstrated in brain tissues in patients with Alzheimer's, Parkinson's, and Huntington's diseases. Here, we examined the effect of a PKR inhibitor (an imidazolo–oxindole derivative that acts as an ATP-binding site-directed inhibitor of PKR) on the neuronal cell death induced by ER-stress in cultured human neuroblastoma cells (SH-SY5Y). Cell damage was induced by tunicamycin (an ER-stress inducer), and cell viability was measured by Hoechst 33342 and YO-PRO-1 double staining and by the resazurin-reduction test (to evaluate metabolic activity). Treatment with tunicamycin at 2 μg/ml for 24 h induced apoptotic cell death accompanied by nuclear condensation and/or fragmentation, and these cells were positive for YO-PRO-1 (early-phase apoptosis and necrosis indicator). Treatment with the PKR inhibitor at 0.1 or 0.3 μM led to a decrease in the number of apoptotic cells induced by tunicamycin. In the resazurin-reduction test, the PKR inhibitor (at 0.1 and 0.3 μM) concentration-dependently inhibited the tunicamycin-induced decrease in metabolic activity. On the other hand, treatment with the PKR inhibitor alone (at 0.3 μM) had no effect on cell morphology or viability (versus in normal control cells). These results indicate that inhibition of PKR activation may be neuroprotective against ER stress-induced cell damage.


*Keywords:* Endoplasmic reticulum (ER) stress; Double stranded RNA-dependent protein kinase (PKR); Tunicamycin; Neuroblastoma; SH-SY5Y cells

In chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington' s disease, and amyotrophic lateral sclerosis (ALS), abnormally unfolded proteins are known to aggregate and accumulate in neurons, and they are thought to be closely related to the initiation and development of these neurodegenerative diseases [1,5,8]. Recently, endoplasmic reticulum (ER)-stress has been reported to induce neuronal cell death, and moreover to play roles in neurodegenerative diseases [5]. Furthermore, 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 [12]. PKR, an interferon-induced protein kinase that was initially identified in a study of responses to viral infection, is activated by the extensive secondary structure of viral RNA [3]. Upon 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) [14,17]. Interestingly, phosphorylation of PKR has been demonstrated in brain tissues in patients with Alzheimer's disease, Parkinson's disease, Huntington's disease, and ALS [2,6,12,13]. Consequently, it has been suggested that PKR may be involved not only in the apoptosis induced by viral infection, but also in that induced by ER-stress. Recently, Jammi et al. discovered a potent, small-molecule PKR inhibitor, an imidazolo–oxindole derivative, by screening a library of 26 different ATP-binding site-directed inhibitors with a variety of structure [7]. However, there has been no report of any effect of a PKR inhibitor on ER-stress-induced cell death. Hence, our purpose in the present study was to examine whether the above PKR inhibitor might reduce the neuronal death induced by ER-stress (tunicamycin).

Cultures of human neuroblastoma (SH-SY5Y) cells were maintained in Dulbecco's modified Eagles's medium (D-MEM, St. Louis, MO) containing 10% FBS (Valeant, Costa Mesa, CA), 100 U/ml penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100 μg/ml streptomycin (Meiji Seika Kaisha Ltd.)

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in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C. The cells were passaged by trypsinization every 5–7 days, as described in a previous report [9]. To examine the effect of the above PKR inhibitor, {8-(imidazol-4-ylmethylene)-6*H*-azolidino[5,4-g]benzothiazol-7-one} (Calbiochem, San Diego, CA), on tunicamycin-induced cell death, cells were seeded at a density of $1 \times 10^4$ cells per well into 96-well plates, and then incubated in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C for 2 days. Tunicamycin (Wako, Osaka, Japan) was added to the cells at a final concentration of 2 μg/ml. The PKR inhibitor was added 1 h before the tunicamycin treatment, at which time the cell-culture medium was replaced with D-MEM containing 1% FBS to reduce the cell growth as long as possible. Assessment of cell viability was performed using two methods, each at 24 h after the addition of tunicamycin. The first method was a single-cell digital imaging-based method employing fluorescent staining of nuclei. Cell death was assessed on the basis of combination staining with fluorescent dyes (namely, Hoechst 33342 and YO-PRO-1 (Molecular Probes, Eugene, OR)), observations being made using an OLYMPUS IX70 inverted epifluorescence microscope (OLYMPUS, Tokyo, Japan). Hoechst 33342 ($\lambda_{ex}$ 360 nm, $\lambda_{em}$ > 490 nm) freely enters living cells and, therefore, stains the nuclei of viable cells, as well as those that have suffered apoptosis or necrosis. Apoptotic cells can be distinguished from viable and necrotic cells on the basis of nuclear condensation and fragmentation. YO-PRO-1 ($\lambda_{ex}$ 491 nm, $\lambda_{em}$ > 509 nm) is a membrane-impermeant dye that is generally excluded from viable cells, whereas early-stage apoptotic and necrotic cells are YO-PRO-1-positive. At the end of the culture period, Hoechst 33342 and YO-PRO-1 dyes were added to the culture medium (at 8 and 0.1 μM, respectively) for 30 min. Images were collected using a digital camera (COOLPIX 4500, Nikon, Tokyo, Japan). In a blind manner, a total of at least 400 cells per condition were counted using image-processing software (Image-J ver. 1.33f; National Institutes of Health, USA). As the second method for measuring cell viability, cell metabolic activity was quantitatively assessed by the fluorescence-intensity changes induced by the cellular reduction of resazurin to resorufin. All experiments were performed in D-MEM medium at 37 °C. Cell viability was assessed by treatment with 10% resazurin solution for 2 h at 37 °C following by examination of fluorescence at 560/590 nm. Fluorescence was expressed as a percentage of that shown by control cells in D-MEM containing 1% FBS, after subtraction of background fluorescence.

Representative fluorescence stainings of nuclei (using Hoechst 33342 and YO-PRO-1 dyes) are shown in Fig. 1A. Non-treated control cells displayed normal nuclear morphology and little staining with YO-PRO-1 dye (which stains early-stage apoptotic and necrotic cells). Treatment with tunicamycin led to condensation and fragmentation of nuclei, and to positive staining with YO-PRO-1 dye. Treatment with the PKR inhibitor at 0.1 and 0.3 μM reduced both of the tunicamycin-induced effects (morphological 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 positive cells were expressed as a percentage of YO-PRO-1- to Hoechst 33342-positive cells (Fig. 1B). After treatment with tunicamycin at 2 μg/ml for 24 h, the percentage of YO-PRO-1-positive cells was 44.1 ± 1.9% ($n$ = 8), while in the non-treated control group (supplemented with 1% FBS) it was 8.6 ± 1.2% ($n$ = 8). Treatment with the PKR inhibitor at either 0.1 or 0.3 μM significantly reduced the tunicamycin-induced increase in YO-PRO-1-positive cells. In the resazurin-reduction test, tunicamycin decreased cell viability to approximately 70% of control (Fig. 1C). The PKR inhibitor (at 0.1 or 0.3 μM) reduced this decrease, the effect being significant at each concentration (Fig. 1C). On the other hand, treatment with the PKR inhibitor alone (at 0.3 μM) led to no change in cell morphology or viability (versus normal control cells) (Fig. 1B and C).

In the present study, we used the small-molecule inhibitor of PKR reported by Jammi et al. [7] to be an effective inhibitor of RNA-induced PKR autophosphorylation ($IC_{50}$ = 0.21 μM) and to rescue PKR-dependent translation block ($IC_{50}$ = 0.1 μM) in human PKR. The above potencies are close to the concentrations (0.1 and 0.3 μM) at which this inhibitor displayed a protective effect against ER-stress-induced cell death in the present study. There has been no previous report of any effects of a PKR inhibitor in living cells. Hence, this is the first report demonstrating a protective effect of a PKR inhibitor on ER-stress-induced neuronal cell death, although regarding specificity against PKR we must consider the possibility of effects on other targets as a limitation. Furthermore, our results were obtained in cultured neuroblastoma cells in the present study. Therefore, the protective effect *in vitro* may be difficult to extrapolate directly to animal models *in vivo*.

Activation of PKR by an ER-stress inducer (tunicamycin) has been well described by Onuki et al. [12]. The phosphorylated form of PKR translocates into nuclei and forms an aggregate, while overexpression of the dominant-negative form of the PKR phosphorylation site attenuates the apoptosis induced by tunicamycin in human neuroblastoma cells (SK-N-SH). Furthermore, aggregated β-amyloid peptide has been reported to activate PKR via its phosphorylation and/or cleavage through calcium release from the ER, with activation of caspase-8 and caspase-3 as upstream signals [15]. However, little is known about the downstream signals or about the precise mechanisms by which PKR induces cell death. The following possible mechanism may be proposed: eIF2α phosphorylation induced by activated PKR results in an upregulation of CCAAT/enhancer-binding protein (C/EBP)-homologous protein/growth arrest and DNA damage-inducible protein 153 (CHOP/GADD153), a proapoptotic transcription factor. On the other hand, Takizawa et al. [16] reported that a dominant-negative mutant of PKR inhibited both the apoptosis and the p38 mitogen-activated protein kinase (MAPK) activation induced by apoptosis signal-regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase 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) [4,11]. 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

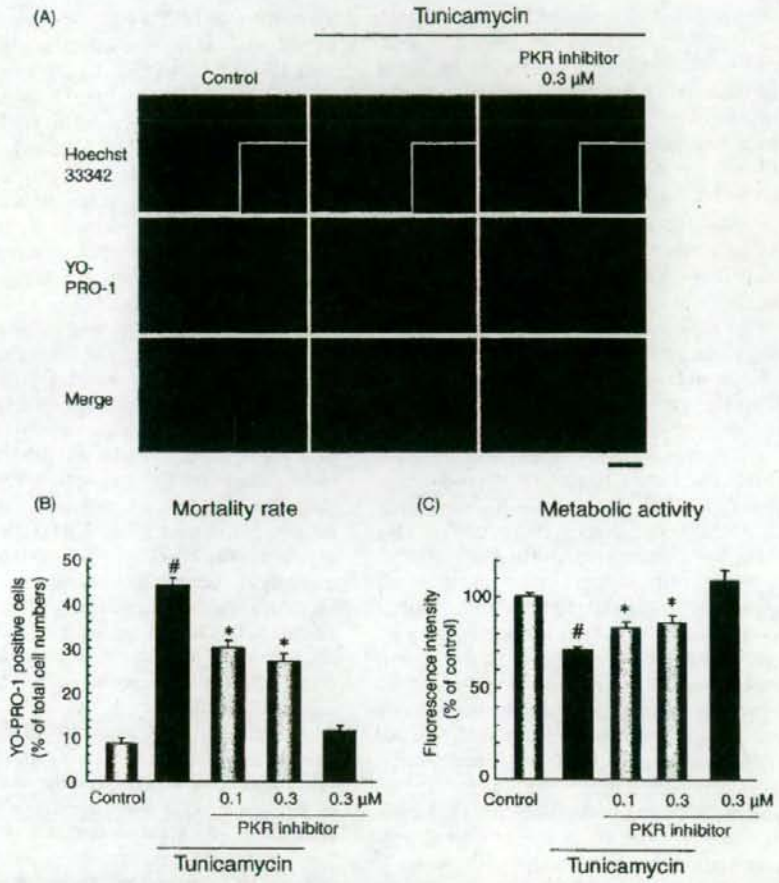


Fig. 1. Effects of the double stranded RNA-dependent protein kinase (PKR) inhibitor on the SH-SY5Y cell death induced by tunicamycin. (A) Representative fluorescence microscopy showing nuclear staining for Hoechst 33342 (blue) and YO-PRO-1 (green) following 24 h tunicamycin (2 μg/ml) treatment. Magnified images for Hoechst 33342 staining are shown in white frames. Control, 1% FBS; bar = 50 μm. (B) Number of cells exhibiting YO-PRO-1 fluorescence was counted, and positive cells were expressed as the percentage of YO-PRO-1-positive to Hoechst 33342-positive cells. Each column represents the mean ± S.E.M. ($n = 8$). $^{*}p < 0.01$ versus tunicamycin-treated group; $^{\#}p < 0.01$ versus control (1% FBS) group (Dunnett's test). (C) Cell metabolic activity was measured using a resazurin-reduction test. Each column represents the mean ± S.E.M. ($n = 8$). $^{*}p < 0.01$ versus tunicamycin-treated group; $^{\#}p < 0.01$ versus control (1% FBS) group (Dunnett's test).

kinase (JNK) and p38 MAPK [10]. Thus, PKR may activate the ASK1-p38 MAPK/-JNK signaling pathways that execute apoptosis. However, their activations alone would not be sufficient to explain the effects that occur following the translocation to nuclei and aggregation of PKR. Therefore, further studies will be needed to clarify the precise mechanisms.

In conclusion, our results indicate that PKR activation may play a pivotal role in the SH-SY5Y cell death induced by ER-stress.

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# Involvement of ER stress in retinal cell death

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**Purpose:** To clarify whether endoplasmic reticulum (ER) stress is involved in retinal cell death, using cultured retinal ganglion cells (RGC-5, a rat ganglion cell line transformed with E1A virus), and transgenic mice ER stress-activated indicator (ERAI) mice carrying a human XBP1 and venus a variant of green fluorescent protein (GFP) fusion gene.

**Methods:** RGC-5 damage was induced by tunicamycin, and cell viability was measured by double nuclear staining (Hoechst 33342 and either YO-PRO-1 or propidium iodide). The expressions of glucose-regulated protein 78(GRP78)/BiP, the phosphorylated form of eukaryotic initiation factor 2α (p-eIF2α), and C/EBP-homologous (CHOP) protein after tunicamycin (in vitro or in vivo) or *N*-methyl-D-aspartate (NMDA; in vivo) treatment were measured using immunoblot or immunostaining. ERAI mice carrying the F-XBP1-DBD-venus expression gene were used to monitor ER-stress in vivo. Twenty-four hours after intravitreal injection of tunicamycin or NMDA, or after raising intraocular pressure (IOP), the retinal fluorescence intensity was visualized in anesthetized animals using an ophthalmoscope and in retinal flatmount or cross-section specimens using laser confocal microscopy.

**Results:** Treatment with tunicamycin induced apoptotic cell death in RGC-5 and also induced production of ER stress-related proteins (BiP, the phosphorylated form of eIF2α, and CHOP protein). In vivo, tunicamycin induced retinal ganglion cell (RGC) loss and thinning of the inner plexiform layer, 7 days after intravitreal injection. In flatmounted retinas of ERAI mice, the fluorescence intensity arising from the XBP-1-venus fusion protein, indicating ER-stress activation, was increased at 24 h after tunicamycin, NMDA, or IOP elevation. In transverse cross-sections from ERAI mice, the fluorescence intensity was first increased in cells of the ganglion cell and inner plexiform layers at 12 and 24 h, respectively, after NMDA injection, and it was localized to ganglion and amacrine cells at 12 and 24 h, respectively, and to microglial cells at 72 h. BiP and CHOP were increased at 12 h after NMDA injection, and the increases persisted for the remainder of the 72 h observation period.

**Conclusions:** These data indicate that ER-stress may play a pivotal role in RGC death, whether induced by NMDA or IOP elevation.

Endoplasmic reticulum (ER) stress is caused by a number of biochemical and physiological stimuli that result in the accumulation of unfolded proteins in the ER lumen, and it is closely associated with the neuronal cell injury caused by vascular and neurodegenerative diseases such as stroke, Alzheimer disease, and Parkinson disease [1-3]. However, little is known about the role, if any, 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 retinovascular diseases, such as diabetic retinopathy and retinal vein occlusions. RGC death has been reported to occur via a variety of mechanisms involving, for example, oxidative stress [4], excitatory amino acids [5], nitric oxide (NO) [6], and apoptosis [7]. Glutamate, one of the excitatory amino acids, is the main neurotransmitter in the retinal signaling pathway. Excessive glutamate increases both intracellular $Ca^{2+}$ and NO production through activation of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor, resulting in retinal cell death [8,9]. Recently, Uehara et al. [10] reported that in primary cortical culture, even mild exposure of NMDA induces apoptotic cell death. They demonstrated to be caused by an accumulation of polyubiquitinated proteins and increases in X box binding protein (XBP-1) mRNA splicing and C/EBP-homologous (CHOP) mRNA, representing activation of the unfolded-protein response (UPR) signaling pathway. They also found that protein-disulphide isomerase (PDI), which assists in the maturation and transport of unfolded secretory proteins, prevented the neurotoxicity associated with ER stress. They suggest that neurodegenerative disorders might be mediated by *S*-nitrosylation of PDI, which would reduce its enzymatic activity. Their results strongly suggest that activation of ER stress may participate in the retinal cell death occurring after NMDA receptor activation and/or ischemic insult. Hence, the purpose of the present study is to examine how ER stress might induce retinal damage both in vitro using cultured retinal ganglion cells (RGC-5, a rat ganglion cell line transformed using E1A virus) and in vivo (using ER stress-activated indicator (ERAI) transgenic mice, in which effective identification of cells under ER-stress conditions is possible in vivo, as described in our previous report) [11]. Use of ERAI mice should provide valuable information regarding the dynamics of ER stress-induced retinal damage.

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## METHODS

*Materials:* Dulbeco's modified Eagles's medium (D-MEM) was purchased from Sigma-Aldrich (St. Louis, MO). The drugs used and their sources were as follows. Tunicamycin was obtained from Calbiochem (San Diego, CA) and Wako (Osaka, Japan). Isoflurane was acquired from Nissan Kagaku (Tokyo, Japan), and fetal bovine serum (FBS) was obtained from Valeant (Costa Mesa, CA).

*Retinal ganglion cell line (retinal ganglion cell-5) culture:* Cultures of RGC-5 were maintained in D-MEM 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. The RGC-5 cells were passaged by trypsinization every 3 days, as 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 (number 3072, Falcon®, Becton Dickinson and Company, Franklin Lakes, NJ). Twenty-four h later, cells were washed twice with D-MEM and then immersed in D-MEM supplemented with 1% FBS plus tunicamycin at 1 to 4 µg/ml. Twenty-four or forty-eight hours after the addition of tunicamycin, cell viability was measured using a single-cell digital imaging-based method employing 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 either YO-PRO-1 (Molecular probes) or propidium iodide (PI; Molecular probes)]. Observations were made using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan). At the end of the above culture period, Hoechst 33342 and YO-PRO-1 or PI dyes were added to the culture medium at 8 µM, 0.1 µM, and 1.5 µM, respectively, for 30 min. Images were collected using a digital camera (Coolpix 4500, Nikon Corp., Tokyo, Japan). In a blind manner, a total of at least 400 cells per condition were counted using image-processing software (Image-J ver. 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.

*Animals:* ER-stress-activated indicator (ERAI)-transgenic mice carrying the F-XBP1DDBD-venus expression gene [11] and their background wild-type mice (C57BL/6) aged 8-11 weeks or male adult ddY mice (Japan SLC, Hamamatsu, Japan) weighing 36-43 g for experiments other than the comparison with ERAI-transgenic mice were used, and were kept under controlled lighting conditions (12 h:12 h light/dark). 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.

*Retinal damage induced by N-methyl-D-aspartate (NMDA)-, tunicamycin-, or intraocular pressure (IOP) elevation:* Male mice were anesthetized with 3.0% isoflurane and maintained using 1.5% isoflurane in 70% $N_2O$ and 30% $O_2$, delivered via an animal general anesthesia machine (Soft Lander, Sin-ei industry Co. Ltd., Saitama, Japan). The body temperature was maintained at 37.0 - 37.5 °C with the aid of a heating pad and heating lamp. Retinal damage was induced by injection (2 µl/eye) either of NMDA (Sigma-Aldrich) at 20 mM dissolved in 0.01 M phosphate-buffered saline (PBS) or of tunicamycin at 50 and 500 µg/ml, or (b) by acutely increasing the intraocular pressure (IOP). For NMDA- or tunicamycin-induced injury, the relevant agent was injected into the vitreous body of the left eye under the above anesthesia. In the IOP elevation model, the pupils were dilated with topical 2.5% phenylephrine hydrochloride and 1% tropicamide (Santen Pharmaceuticals Co. Ltd., Osaka, Japan). After topical instillation of 0.4% oxybuprocaine hydrochloride (Santen Pharmaceuticals Co. Ltd.), the anterior chamber was cannulated with a 32-gauge needle connected to a reservoir containing 0.9% NaCl. IOP was elevated by raising the height of the reservoir, maintaining a pressure of 100 mm Hg for 45 min. Retinal ischemia was confirmed by the blanching of the iris and retinal circulation. At the end of the elevated IOP period, the needle was removed, and reperfusion of the retinal vasculature was confirmed by ophthalmoscopic examination (KOM 300; Konan Inc., Nishinomiya, Japan). One drop of levofloxacin ophthalmic solution (Santen Pharmaceuticals Co. Ltd.) was applied topically to the treated eye after each procedure (intravitreal injection or ischemia-reperfusion).

*Monitoring endoplasmic reticulum (ER) stress using ERAI-transgenic mice:* In anesthetized ERAI-transgenic or wild-type mice, retinal damage was induced by injection (2 µl/eye) of either NMDA at 20 mM or tunicamycin at 50 µg/ml into the vitreous body, or by elevating IOP to 100 mmHg for 45 min (see above). Twenty-four hours later, the fluorescence intensity arising from the XBP-1-venus fusion protein, which is translated from the F-XBP1DDBD-venus gene, was visualized in the retina of anesthetized animals using an ophthalmoscope (TRC-50; TOPCON, Tokyo, Japan) fitted with a fluorescence filter. In separate experiments, the distribution and time-course of changes in fluorescence intensity in the retina were measured in retinal flatmount and cross-section specimens using either laser confocal microscopy (Bio-Lad Laboratories, Inc, Hercules, CA) or epifluorescence microscopy (Power BX50; Olympus, Tokyo, Japan). At various times after the intravitreal injections (12, 24, and 72 h), eyes were enucleated, then fixed in 4% paraformaldehyde for 1 h or overnight at 4 °C as preparation for retinal flatmount and retinal cross-section, respectively. For the preparation of retinal flatmounts, detached retinas were flatmounted on slides (MAS COAT; MATSUNAMI GLASS IND., LTD., Osaka, Japan) by making radial incisions. They were then mounted under a coverslip and observed using the epifluorescence microscope. For the preparation of retinal cross-sections, fixed eyes were immersed in 20% sucrose for 48 h 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) under a coverslip, and observed using the laser confocal microscope.

*Immunoblotting:* RGC-5 cells or mouse retinas were lysed using a cell-lysis buffer (RIPA buffer (R0278; Sigma) with protease (P8340; Sigma Aldrich) 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 h at room temperature with 5% Blocking One-P (Nakarai Tesque, Inc., Kyoto, Japan) in 10 mM Tris-buffered saline with 0.05% Tween 20 (TBS-T), then incubated overnight at 4 °C with the primary antibody. The transfers were then rinsed with TBS-T and incubated for 1 h at room temperature in horseradish peroxidase goat anti-rabbit or goat anti-mouse (Pierce, Rockford, IL) diluted 1:2000. The immunoblots were developed using 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). The primary antibodies used were as follows: mouse anti-BiP (BD Bioscience, San Jose, CA), rabbit anti-phospho-eIF2α (Ser51; Cell Signaling, Beverly, MA), rabbit anti-eIF2α (Cell Signaling), mouse anti-CHOP (Santa Cruz, Santa Cruz, CA), and rabbit anti-actin (Santa Cruz).

*Immunostaining:* To clarify the distribution and localization of the XBP1-venus fusion protein in the retina of ERAI mice (as seen in the retinal flatmounts and cross sections), double-staining immunocytochemistry was performed. At various times after intravitreal injections (12, 24, and 72 h), eyes were enucleated, fixed in 4% paraformaldehyde overnight at 4 °C, immersed in 20% sucrose for 48 h 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 (MATSUNAMI GLASS IND., LTD.). Sections were subsequently processed for immunocytochemical localization using antibodies against CHOP (1:100 dilution in PBS; Santa Cruz), glucose-regulated protein 78(GRP78)/BiP (1:100 dilution in PBS), thymus cell antigen 1 (Thy-1; 1:100 dilution in PBS; Serotec Ltd, Oxford UK), microglia (OX-42, 1:100 dilution in PBS; Serotec Ltd), and amacrine cells (HPC-1/Syntaxin, 1:100 dilution in PBS; Santa Cruz). The sections were incubated either (a) with Alexa Fluor-568-conjugated secondary antibody (1:200 dilution in PBS; Molecular Probes, Eugene, OR) for 1 h at room temperature, mounted with a coverslip, and observed under a laser confocal microscope (Bio-Lad Laboratories, Inc), or (b) with biotin-conjugated secondary antibody for 1 h at room temperature, and visualized using a VECTOR M.O.M. Immunodetection kit (Vector, Burlingame, CA). Each image was taken using a digital camera (Coolpix 4500; Nikon, Tokyo, Japan) attached with epifluorescence microscope (Power BX50; Olympus).

*Histological analysis of mouse retina:* Seven days after the NMDA or 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, then kept immersed for at least 24 h at 4 °C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness, 3 µm) 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. Light-microscope photographs were taken using a digital camera (Coolpix 4500, Nikon) and the cell counts in the ganglion cell layer (GCL) and the thickness of the inner plexiform layer (IPL) at a distance between 350 and 650 µm from the optic disc were measured on the images 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 GCL cell count and the IPL thickness.

*Statistical analysis:* Data are presented as the means±SEM. Statistical comparisons were made using a Student's *t*-test or Dunnett's test, by means of STAT VIEW version 5.0 (SAS Institute Inc., Cary, NC). $P<0.05$ was considered to be statistically significance.

## RESULTS

*Retinal cell death and time-course of changes in endoplasmic reticulum (ER) stress-related protein induced by tunicamycin:* We examined whether tunicamycin treatment could induce cell death through ER stress in retinal ganglion cell using RGC-5. Representative fluorescence stainings of nuclei [using Hoechst 33342, YO-PRO-1, and propidium iodide (PI) dyes] are shown in Figure 1A. Vehicle-treated control cells displayed normal nuclear morphology and negative staining with both YO-PRO-1 dye (which stains early apoptotic and later-stage cells) and PI dye (which stains late-stage apoptotic cells; upper panels in Figure 1A). Treatment with tunicamycin led to shrinkage and condensation of nuclei, and to positive staining with each of these dyes (lower panels in Figure 1A). The number of cells exhibiting PI fluorescence was counted, and positive cells were expressed as the percentage of PI- to Hoechst 33342-positive cells (Figure 1B). After treatment with tunicamycin at 1, 2, or 4 µg/ml for 24 h, the percentages of PI-positive cells were 8.3±1.2% (n=6), 13.1±0.9% (n=6), and 11.3±0.6% (n=6), respectively, while in the non-treated control group the percentage was 0.5±0.2% (n=6). After treatment with tunicamycin at 1, 2, or 4 µg/ml for a longer time period (48 h), the corresponding values were 41.5±3.5% (n=6), 43.7±2.1% (n=6), and 50.7±2.6% (n=6), respectively (1.2±0.4% (n=6) for the non-treated control group). Time-course data for the changes in the protein levels of glucose-regulated protein (GRP)78/BiP, the phosphorylated form of eukaryotic initiation factor 2α (eIF2α), total eIF2α, and C/EBP-homologous protein (CHOP) occurring after tunicamycin treatment at 2 µg/ml are shown in Figure 1C. BiP, a biomarker of ER-stress, increased time-dependently throughout the 24 h tunicamycin treatment period, while actin levels remained unchanged. Treatment with tunicamycin time-dependently induced eIF2α phosphorylation, while total eIF2α levels were not changed during the 24 h observation period. CHOP was first detected at 6 h after addition of tunicamycin and persisted thereafter. These data indicate that treatment with tunicamycin can induce expres-

sions of ER stress-related proteins and subsequent apoptotic cell death in RGC-5 culture in vitro.

*Intravitreal injection of tunicamycin induces retinal cell death in mice:* To clarify whether tunicamycin would induce retinal cell death in vivo, we examined the histological changes in the retina at 7 days after intravitreal injection of tunicamycin. As shown in Figure 2, intravitreal injection of tunicamycin at 0.1 μg/eye (a low dose) induced a significant loss of cells in the retinal ganglion cell layer (GCL), but no thinning of the inner plexiform layer (IPL; versus vehicle-treated retinas). At a high dose of 1μg/eye, tunicamycin significantly decreased both the cell count in GCL and the IPL thickness (versus the non-treated normal retina; Figure 2). On the other hand, no retinal damage was induced by intravitreal injection of an identical volume of vehicle (versus the non-treated retina). Together, these findings suggest that tunicamycin at 0.1 μg/eye (giving an estimated concentration in the vitreous body of approximately 10 μg/ml) induces retinal ganglion cell death at a concentration similar to that inducing exhibiting the apoptotic cell death in RGC-5 in vitro.

*Increase in XBP-1-venus fusion protein in the retina in ER stress-activated indicator (ERAI)-transgenic mice:* To investigate whether ER stress is induced in the mouse retina during the early stages of retinal damage in vivo, we used ERAI-transgenic mice carrying the F-XBP1DDBD-venus expression gene, which allows effective identification of cells under ER stress in vivo, as previously described by Iwawaki et al. [11]. Twenty-four h after intravitreal injection of tunicamycin at 0.1 μg or of *N*-methyl-D-aspartate (NMDA) at 40 nmol, the fluorescence intensity arising from the XBP-1-venus fusion protein was visualized in the retina of anesthetized animals (using an ophthalmoscope) as shown in Figure 3. Both tunicamycin and NMDA increased the fluorescence intensity of this protein, while little change in fluorescence intensity was observed in the control fellow eyes. For further elucidation of this phenomenon, the distribution and time-course of changes in the fluorescence intensity derived from the XBP-1-venus fusion protein were measured in retinal flatmount and transverse sections, as shown in Figure 4A,C. In the flatmounts, such stimulations as NMDA, an intraocular

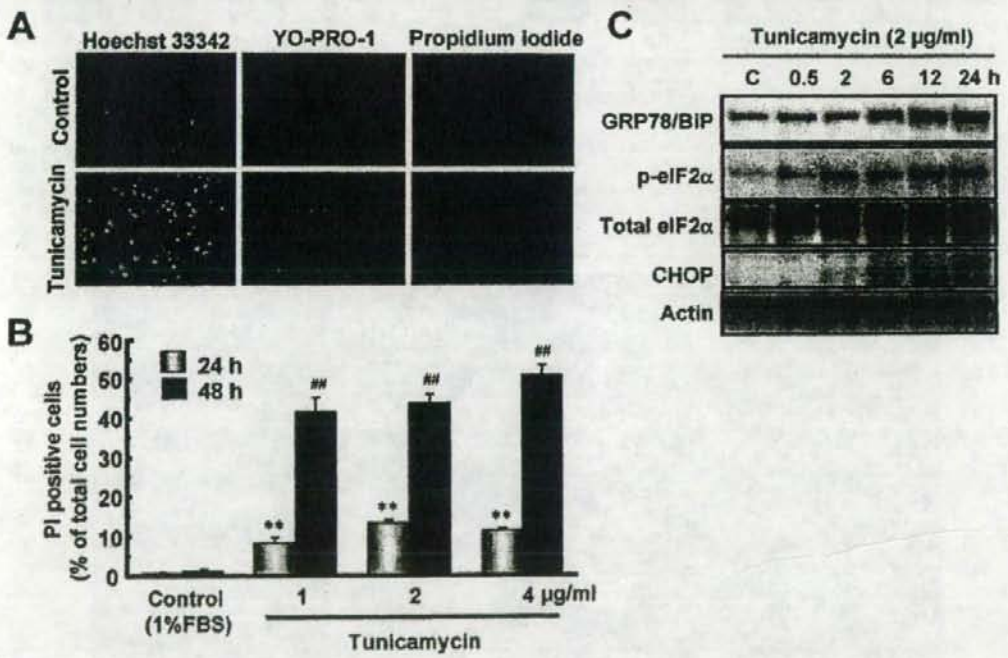


Figure 1. Retinal cell death and time-course of changes in endoplasmic reticulum (ER)-stress related proteins induced by tunicamycin. **A**: Representative fluorescence microscopy showing nuclear stainings for Hoechst 33342 (blue), YO-PRO-1 (green), and propidium iodide (PI, red) at 48 h after addition of tunicamycin at 1 μg/ml. **B**: The number of cells displaying PI fluorescence was counted at two time-points, and positive cells were expressed as the percentage of PI to Hoechst 33342. Each column represents the mean±SEM (n=6). Double asterisks and double hash marks; $p<0.01$ versus corresponding control group (Dunnett's test). **C**: Representative immunoblots showing the time-course of changes in protein levels (GRP78/BiP, phosphorylated-eIF2α, total eIF2α, and CHOP) after tunicamycin treatment at 2 μg/ml.

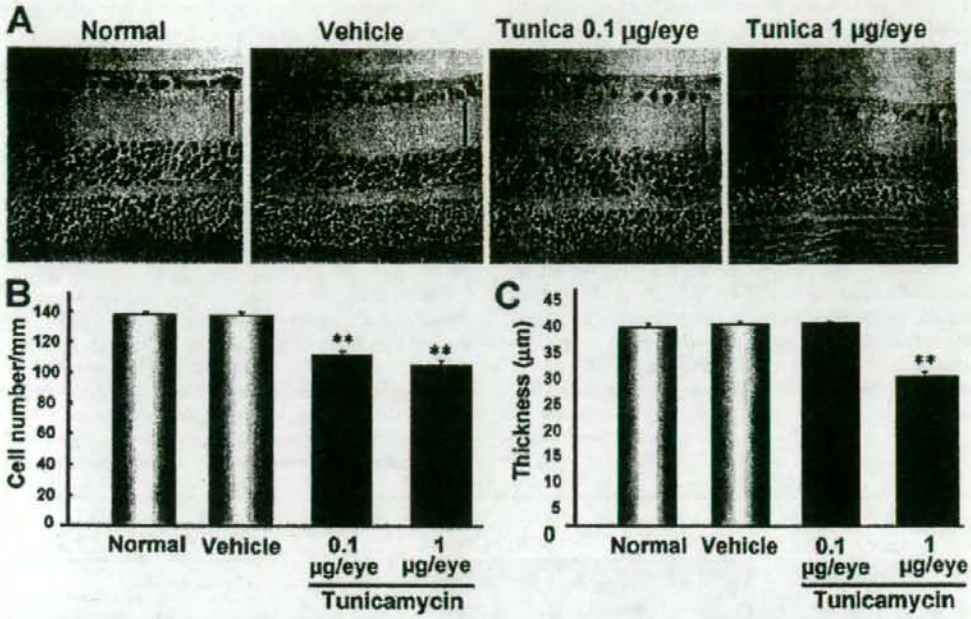


Figure 2. Intravitreal injection of tunicamycin induces retinal cell death in mice. A: Representative photographs showing non-treated normal retina, vehicle-treated retina, and low-dose (0.1 µg/eye) and high-dose (1 µg/eye) tunicamycin-treated retinas 7 days after intravitreal injection. Quantitative analysis of cell number in ganglion cell layer (B) and thickness of inner plexiform layer (IPL) C: Each column represents the mean±SEM (n=10). Double asterisks $p<0.01$ versus vehicle-treated control group (Dunnett's test). The horizontal scale bar represents 25 µm and the vertical bar indicates each thickness of IPL.

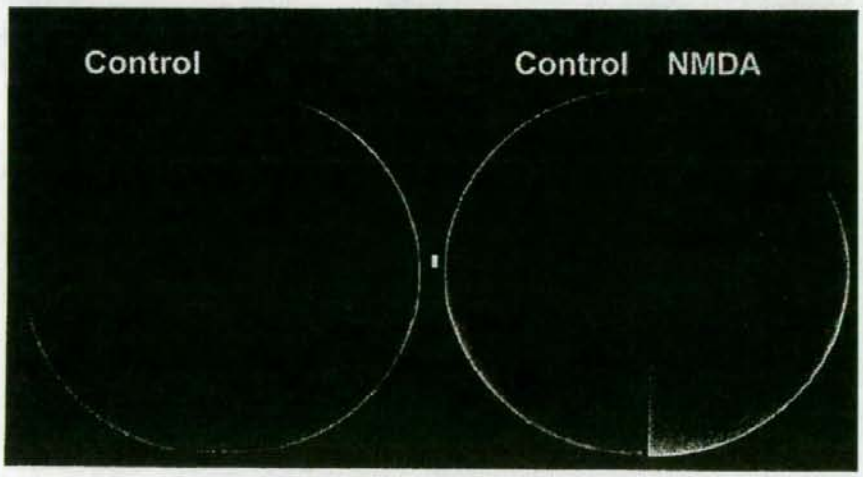


Figure 3. Non-invasive imaging of XBP-1-venus fusion protein in ERAI mouse retina in vivo. Twenty-four hours after intravitreal injection of either tunicamycin at 0.1 µg/eye or *N*-methyl-D-aspartate (NMDA) at 40 nmol/eye, the fluorescence intensity arising from XBP-1-venus fusion protein was visualized in the retinas of anesthetized animals using an ophthalmoscope fitted with a fluorescence filter.