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Expression of Vascular Endothelial Growth Factor by Retinal Pigment Epithelial Cells Induced by Amyloid-β Is Depressed by an Endoplasmic Reticulum Stress Inhibitor

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Key Words
4-phenylbutyl phosphonylacetate · Vascular endothelial growth factor · Amyloid-β · Endoplasmic reticulum stress · Age-related macular degeneration

Abstract
Purpose: Amyloid-β (Aβ) is a 36- to 43-amino-acid peptide that is a constituent of drusen, and it has been demonstrated to upregulate vascular endothelial growth factor (VEGF) expression by retinal pigment epithelial (RPE) cells. This study aimed to determine whether 4-phenylbutyl phosphonylacetate (PBA), a known endoplasmic reticulum (ER) stress inhibitor, can reduce Aβ-induced expression of VEGF in RPE cells. ***Methods:*** Aβ was added to the medium of regularly cultured or polarized ARPE-19 cells, a human RPE cell line, with or without PBA. The levels of VEGF and ER stress markers, namely GRP78/Bip, cleaved caspases 4 and 12 and GADD153/C-EBP homologous protein, were determined by enzyme-linked immunoassay, immunocytochemistry and Western blotting. ***Results:*** Exposure of ARPE-19 cells to Aβ induced GRP78/Bip expression and activated caspases 4 and 12; however, their expression was decreased by simultaneous exposure to PBA. Aβ increased the expression of VEGF both in regularly cultured and polarized ARPE-19 cells, but it was suppressed by PBA. PBA did not cause RPE cell apoptosis. ***Conclusion:*** Aβ has been suggested to be involved in the development of age-related macular degeneration; therefore, our findings suggest that drugs that target ER stress should be considered for the treatment of age-related macular degeneration.

Introduction

Drusen are deposits that accumulate beneath the retinal pigment epithelial (RPE) cells and are early signs of age-related macular degeneration (AMD) [1]. Amyloid-β (Aβ) is a constituent of drusen [2–4], and it has been demonstrated to upregulate the expression of vascular endothelial growth factor (VEGF) in RPE cells in vitro [5].

When proteins with abnormal configurations are present in a cell, they are removed by the endoplasmic reticulum (ER). When the number of abnormally configured proteins is large, the cell is considered to be under ER stress, and the unfolded protein response (UPR) is activated to maintain homeostasis of the cell [6]. Relevant to our study, the UPR has also been reported to enhance the expression of VEGF in RPE cells [7, 8].

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Markers for ER stress have been identified. GRP78, a heat-shock protein, is a molecular chaperone that is upregulated in response to ER stress, and it can increase the protein folding capacity of cells as protection from ER stress [9–11]. The expression of caspases 4 and 12 is increased by ER stress; however, these proteins are also degraded in response to ER stress, resulting in activation of the caspases. These activated caspases can then participate in cellular apoptosis and inflammation [10, 12–16]. The expression of C-EBP homologous protein (CHOP) is also known to be increased by ER stress, and it enhances apoptosis [11, 17].

The low-molecular-weight fatty acid 4-phenylbutyl phosphonylacetate (PBA) functions as a molecular chaperone, and it is used to treat sickle cell disease, β-thalassaemia, urea cycle disorders and cystic fibrosis [18–24]. Although PBA is known to reduce ER stress, it has not been determined whether PBA can decrease the expression of VEGF in RPE cells induced by Aβ.

The purpose of this study was to determine whether PBA, a blocker of ER stress, can decrease the Aβ-induced expression of VEGF in RPE cells. To accomplish this, we exposed cultured human RPE cells to Aβ alone or in combination with PBA and measured VEGF expression levels.

Methods

Cell Cultures

ARPE-19 cells, a human RPE cell line, were grown on 24-well plates at 37°C under 5% CO_2 until confluence was reached. The culture medium consisted of DMEM mixed with HAM-12 (Sigma-Aldrich, St. Louis, Mo., USA) containing 10% FBS, 1% penicillin and 1% streptomycin. After reaching confluence, the medium was switched to a serum-free medium containing 1, 10 or 25 μM Aβ ($A\beta_{1-40}$ oligomer; Peptide Institute, Osaka, Japan) for 24 h. To examine the protective role of PBA against Aβ-induced changes in ARPE-19 cells, some of the wells were pretreated with 2.5 mM PBA (Sigma-Aldrich) for 14 h, followed by the change to the medium containing 2.5 mM PBA and 25 μM Aβ for 24 h. All the experiments were repeated at least twice to confirm reproducibility, with values calculated for each individual well (n = number of wells).

Polarized Cell Culture

The polarized culture was performed as previously described with minor modifications [25–27]. In brief, ARPE-19 cells (approx. 1.65×10^5 cells/well) were plated on precoated Matrigel (BD Biosciences, San Jose, Calif., USA) on Transwell filters (0.4 μm pore size; Corning, N.Y., USA). The cells were maintained for >8 weeks in a mixed medium of MEM-α, N1 supplement, nonessential amino acids, L-glutamine-penicillin-streptomycin, taurine, hydrocortisone, triiodothyronine and 1% FBS. Aβ (25 μM) and/or PBA (2.5 mM) were added to the medium at the top (apical) side of polarized culture of ARPE-19 cells for 24 h, with and without pretreatment with PBA (2.5 mM) for 14 h, before the measurement of VEGF levels in the bottom (basal) medium.

Enzyme-Linked Immunosorbent Assay

Supernatants of the regularly cultured ARPE-19 cells and basal medium of the polarized ARPE-19 cells were collected after the treatments, and the concentration of VEGF was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine; R&D Systems, Minneapolis, Minn., USA) following the manufacturer's protocol [28, 29].

Immunocytochemistry

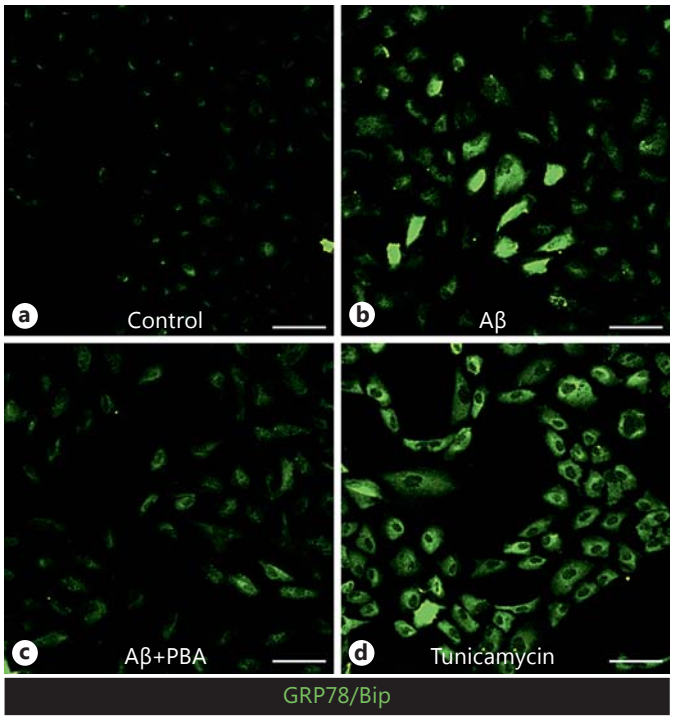
ARPE-19 cells were cultured on chambered cell culture slides. Cells were washed twice with PBS, fixed with 10% formalin for 20 min at room temperature (RT) and permeabilized with 100% methanol for 20 min at –20°C. After rinsing in 0.1% TBS-T, the cells were blocked with 3% donkey serum/PBS for 30 min at RT, incubated with anti-GRP78 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) for 90 min at RT, washed with TBS-T three times and incubated with FITC-conjugated anti-goat IgG (1:3,000, Santa Cruz Biotechnology) for 45 min at RT. After washing with TBS-T three times, the cells were examined by confocal microscopy. Photographs were taken of three wells (n = 3) from each group and analyzed using SCION imaging software to measure relative signal intensities. Experiments were independently conducted six times and the average of the signal intensities in each experiment was used as one index for the experiment. The average signal intensity was calculated (n = number of experiments). Tunicamycin, an ER stress inducer, was used as a positive control to confirm GRP78/Bip upregulation.

TUNEL Staining

TUNEL-positive cells were detected as previously described [29]. In brief, cells were pretreated with either 2.5 mM PBA or standard media for 14 h before incubation with control medium, 2.5 mM PBA, 25 μM Aβ, or 25 μM Aβ and 2.5 mM PBA for 24 h. The cells were then fixed with 2% PFA for 20 min at RT on the chambered cell culture slides. Furthermore, the cells were stained with the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) and 0.3 mg/ml 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, Calif., USA) for 1 h. The entire procedure was performed on 8-well chamber slides (SCS-008; Matsunami, Osaka, Japan). The stained cells were observed using a BioImaging Navigator fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The number of TUNEL-positive cells was calculated from images obtained with a 20× lens (537 × 710 μm). The average number of TUNEL-positive cells observed in three independent areas was calculated per well (n = number of wells).

Western Blotting

Collected cells were lysed in Laemmli sample buffer (Bio-Rad, Hercules, Calif., USA) containing 2% SDS, 25% glycerol and 5% mercaptoethanol, boiled at 100°C for 2 min and centrifuged at 12,000 rpm for 5 min. 20 μl of the supernatant were applied to each lane, and the proteins were separated by SDS-PAGE (Ready-Gel J; Bio-Rad). The proteins were transferred to PVDF membranes using a dry-blotting system (iBlot system; Invitrogen). The membranes were blocked in 5% skim milk/0.1% TBS-T and incubated at 4°C overnight with anti-caspase 12 (1:1,000; Sigma-Aldrich),



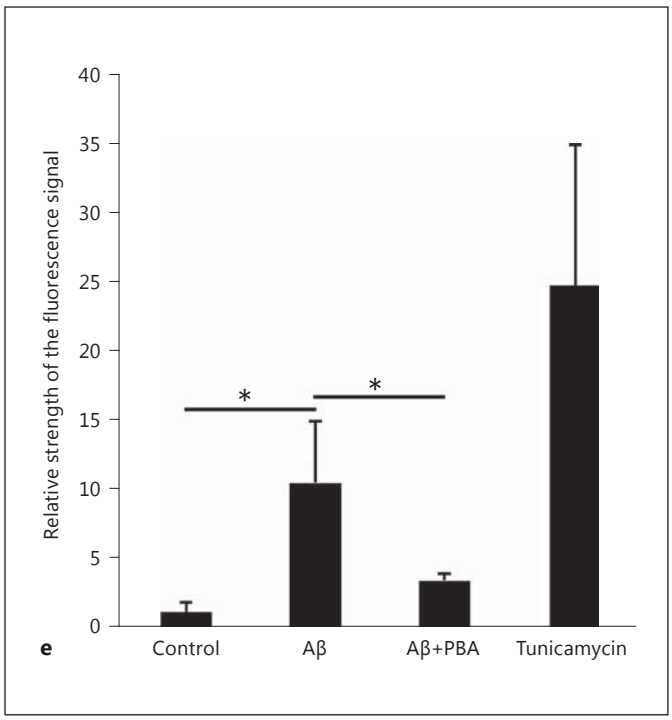


Fig. 1. a–d Immunostaining of the ARPE-19 cells with anti-GRP78/Bip antibody. Scale bars = 50 μm. **e** The results with tunicamycin are shown as a positive control (24.7 ± 10.2; n = 6). GRP78/Bip signal intensities in ARPE-19 cells treated with Aβ (10.4 ± 4.39; n = 6) and was significantly higher than those in cells treated with control media (1.00 ± 0.74; n = 6) and those with Aβ + PBA (3.35 ± 0.46; n = 6). GRP78/Bip signal intensity was increased in response to Aβ and decreased in response to PBA. Groups were compared using ANOVA with Scheffé's test for multiple comparisons. * $p < 0.05$.

anti-caspase 4 antibodies (1:1,000; MBL International, Carlsbad, Calif., USA) or anti-GADD153/CHOP antibody (1:1,000; Abcam, Cambridge, UK). For controls, the membranes were exposed to anti-GAPDH (1:3,000; Abcam) antibody. Furthermore, the membranes were incubated with HRP-conjugated anti-mouse IgG (1:3,000; Santa Cruz Biotechnology) for 1 h at RT. The signals were detected by an ECL detection system (GE Healthcare UK Ltd., UK) and captured by ImageQuant LAS-4000 (GE Healthcare). The experiments were repeated three times to confirm reproducibility.

Confluent cells were used for Western blotting and ELISA, whereas subconfluent cells were used for immunocytochemistry and TUNEL staining [30].

Results

Immunocytochemistry for GRP78/Bip

To confirm that the cells were under ER stress when Aβ was added to the medium, the cells were immunostained for GRP78/Bip, a marker for ER stress. To confirm the reliability of GRP78/Bip signal intensity as a measure of ER stress, 1 μM tunicamycin, a known ER stress inducer [14, 15, 31], was added as a positive control to confirm increased signal intensity (n = 6). The relative fluorescence intensity was increased 10.4-fold (10.4 ± 4.39, n = 6) in the cells treated with 25 μM Aβ compared with control media (1.00 ± 0.74, n = 6, $p < 0.05$). However, the fluorescence intensity decreased by 68% in cells treated with 25 μM Aβ + 2.5 mM PBA (3.35 ± 0.46, n = 6) compared with 25 μM Aβ ($p < 0.05$; fig. 1).

Western Blotting for Caspase-4, Caspase-12 and GADD153/CHOP

Increased levels of cleaved caspase-12 and cleaved caspase-4, the activated forms, were observed following exposure to 10 or 25 μM Aβ. The addition of 2.5 mM PBA to the medium containing 25 μM Aβ decreased the levels of cleaved caspase-12 and cleaved caspase-4. The levels of GADD153/CHOP were also increased by exposure to Aβ and decreased by the addition of PBA (fig. 2).

VEGF Expression in Cultured RPE Cells

VEGF is the main driver of neovascular AMD, and Aβ upregulates VEGF secretion by RPE cells [5]. Therefore, we examined whether VEGF upregulation by Aβ is

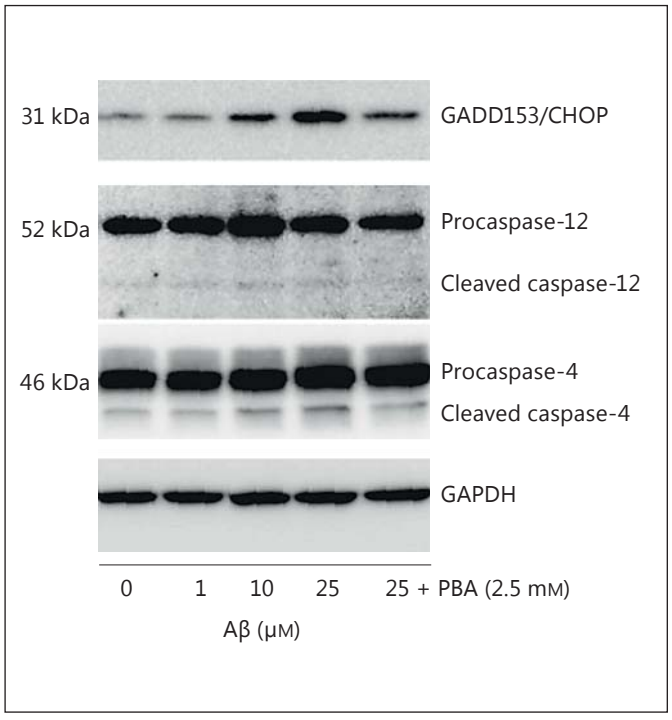


Fig. 2. Western blotting using anti-GADD153/ CHOP, anticaspase 12 and anticaspase 4 antibodies. The levels of CHOP, cleaved caspase-12 and cleaved caspase-4 were increased when the cells were exposed to Aβ, and they were decreased when PBA was added.

blocked by an ER stress inhibitor. First, we prepared regularly cultured ARPE-19 cells and exposed them to Aβ. The VEGF concentration in the culture medium was 1,257 pg/ml in the absence of Aβ, and the concentration was increased to 1,340, 1,755 and 2,345 pg/ml when the cells were exposed to 1, 10 and 25 μM Aβ, respectively. When the cells were exposed to 2.5 mM PBA before exposure to 25 μM Aβ, the concentration of VEGF was 741 pg/ml, which was not significantly different from that in cells cultured without Aβ but significantly lower than that in cells cultured with Aβ without PBA ($p < 0.01$; ANOVA with Scheffé's test for multiple comparisons; fig. 3). Currently, a polarized RPE cell culture system is more appropriate to simulate the physiological condition of human eyes, including VEGF measurements [25]. To confirm our findings that an ER stress inhibitor blocks VEGF under Aβ exposure, we prepared polarized ARPE-19 cells and examined VEGF concentrations in the basal medium after Aβ treatment with or without PBA. The VEGF concentration in the basal medium was 612 pg/ml in the absence of Aβ. Compared with the VEGF concentration (906 pg/ml) when the cells were exposed to 25 μM Aβ, VEGF levels were significantly decreased to 559 pg/ml in cells exposed to both 2.5 mM PBA and 25 μM Aβ ($p < 0.01$; ANOVA with Scheffé's test for multiple comparisons; fig. 3).

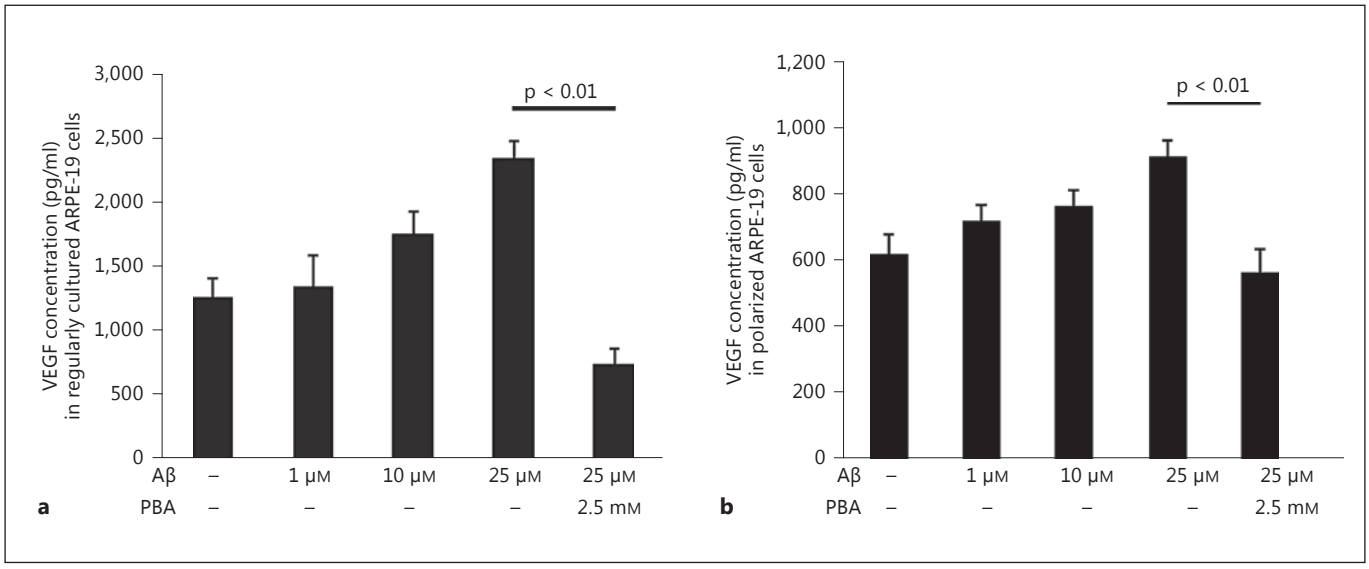


Fig. 3. a Concentration of VEGF in the medium of regularly cultured ARPE-19 cells. The VEGF concentration increased after the cells were exposed to Aβ. However, when PBA was also added to the medium, the expression of VEGF was significantly lower than that in the absence of PBA. **b** Concentration of VEGF in the basal medium of polarized ARPE-19 cells. The VEGF concentration changed after the cells were exposed to Aβ and PBA, and the same manner was observed as in regularly cultured ARPE-19. Compared with the VEGF levels in the absence of Aβ and PBA, its levels increased after Aβ administration but decreased when PBA was also added to the medium to a level significantly lower than that in the absence of PBA ($p < 0.01$). ANOVA with Scheffé's test for multiple comparisons.

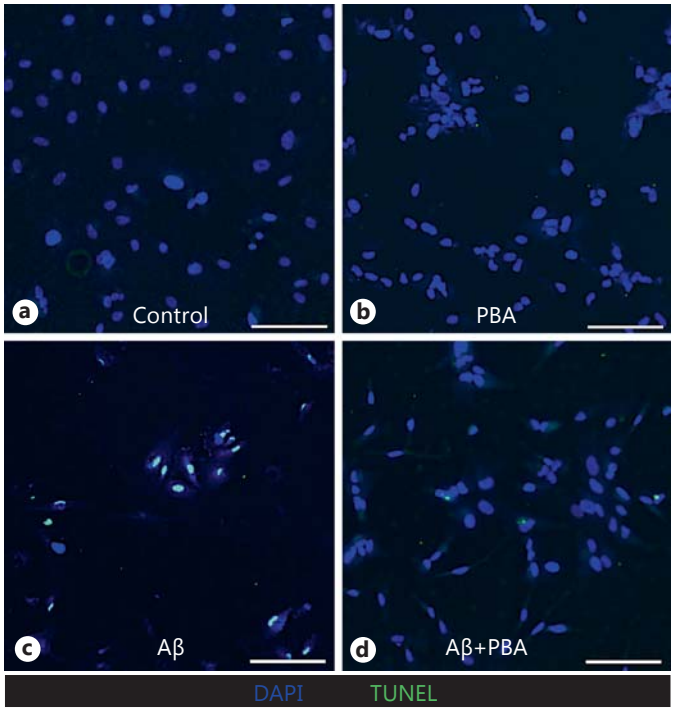
TUNEL Staining

Furthermore, we evaluated whether Aβ and PBA induce RPE toxicity. The number of TUNEL-positive (apoptotic) cells was counted among ARPE-19 cells cultured with 25 μM Aβ with or without 2.5 mM PBA. Compared with the numbers of TUNEL-positive ARPE-19 cells in the absence of Aβ and PBA (control) and the cells with 2.5 mM PBA only, the cells with 25 μM Aβ were increased 3.86- and 3.37-fold, respectively ($p < 0.01$; ANOVA with Scheffé's test for multiple comparisons; fig. 4). Although there was no significant difference ($p = 0.089$), the number of TUNEL-positive cells in the ARPE-19 cells with 25 μM Aβ and 2.5 mM PBA was relatively decreased (by 44%) compared to those with 25 μM Aβ only. These findings indicated that PBA blocked cell apoptosis induced by Aβ and that PBA itself did not induce strong RPE toxicity. Corroborating our results, these data suggest that the decrease of VEGF expression was not due to PBA-induced cell apoptosis (fig. 4).

Discussion

Previously, only a few studies have examined the role of ER stress in the development of ocular diseases. ER stress induces retinal ganglion cell apoptosis in a rodent glaucoma model [32], and ER stress was activated in the retina in animal models of diabetes and oxygen-induced retinopathy [33]. It is also reported that ER stress possibly prevents photoreceptor degeneration in rat retinitis pigmentosa [34].

Previous studies have indicated the importance of ER stress and Aβ in the pathogenesis of AMD [2, 35]. Our results illustrated that the expression of VEGF in ARPE-19 cells was increased after exposure to Aβ and was lowered by the simultaneous addition of PBA. In addition, we demonstrated increased secretion of VEGF in response to Aβ exposure and decreased secretion of VEGF in response to PBA not only in the cells cultured under normal conditions but also in the polarized cells that supposedly have a more similar condition as that in the human eye.



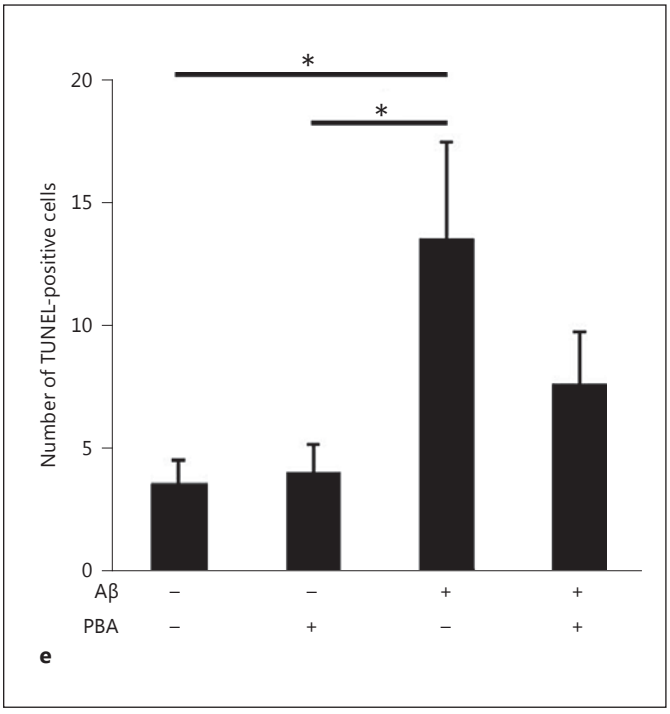


Fig. 4. The number of TUNEL-positive cells were confirmed by incubating cells with medium only (**a**) and 2.5 mM PBA (**b**), 25 μM Aβ (**c**), and 2.5 mM PBA and 25 μM Aβ (**d**). Scale bars = 100 μm. **e** Apoptotic cell numbers were increased when the cells were incubated with Aβ alone, and they were decreased by the addition of PBA. There was a significant difference in the number of TUNEL-positive cells between the negative control (medium only) and treatment with Aβ alone (3.86-fold), and between PBA alone and treatment with Aβ alone (3.37-fold). * $p < 0.01$; ANOVA with Scheffé's test for multiple comparisons.

The number of apoptotic RPE cells was not increased by the addition of PBA alone; therefore, the decrease of VEGF expression was not due to the toxicity of PBA. Signals for UPR markers, including GRP78, also became significantly stronger after Aβ exposure and became weaker following the addition of PBA. From these results, we concluded that ER stress caused by Aβ increased the expression of VEGF in RPE cells.

It has been reported that ER stress activates PERK, an eIF2α kinase, and phosphorylation of eIF2α leads to an increase in the expression of ATF4 [6, 11]. ATF4 induces the expression of numerous genes including those encoding GRP78 and VEGF [8, 12]. Our results suggest that PBA prevents the expression of VEGF caused by Aβ by inhibiting ER stress in the RPE cells.

RPE damage in AMD is multifactorial [36]. Although we have demonstrated that Aβ causes ER stress in cultured RPE cells similar to that in other neurons, why extracellular deposits of Aβ cause ER stress in RPE cells remains unknown. In addition, a limitation of the present study was lack of Aβ aggregate measurements in cultured ARPE-19 cells or evaluation of the correlation between Aβ aggregates and RPE pathology. However, studies on neuronal cells have suggested that Aβ is taken up by the cells, leading to proteasomic dysfunction and disturbances of the calcium homeostasis, which in turn activate the UPR [8, 37, 38]. Previous studies have demonstrated elevated levels of intracellular calcium in ARPE-19 cells treated with thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic Ca^{2+}-ATPase, but not following treatment with tunicamycin [3]. Further studies are required to validate these findings.

Recently, anti-VEGF therapy improved vision in patients with AMD to some degree [39, 40]. However, in most patients, visual acuity does not recover to normal levels. Therefore, preventing the onset of AMD would be more important, although directly suppressing VEGF is difficult to justify for patients with only drusen because of the need for intravitreal injections. Additional treatments including oral medicines are fully desired, particularly for elderly patients [41]. It should be determined whether PBA reaches the targeted RPE layer following oral administration.

PBA is a low-molecular-weight fatty acid that functions as a molecular chaperone and reduces ER stress [20, 22–24]. Because this molecule is currently being used systemically without severe side effects for some diseases [18, 19, 21], it should be considered for patients with drusen to prevent the development of AMD.

Levels of CHOP, cleaved caspase-12 and cleaved caspase-4, which are known to be involved in ER stress-induced apoptosis and inflammation, were increased in response to Aβ and blocked by the addition of PBA. In contrast, no significant increase in the number of apoptotic ARPE-19 cells was detected following treatment with PBA. Caspase-12 plays an important role in ER stress-induced apoptosis in mice [15]; however, the function of caspase-12 differs between humans and mice, and caspase-4 is the mediator of ER stress-induced apoptosis in humans [14]. Although no significant difference in the number of TUNEL-positive ARPE-19 cells was observed following treatment with the combination of 25 μM Aβ and 2.5 mM PBA versus 25 μM Aβ alone, our results indicate that PBA blocked Aβ-induced apoptosis in ARPE-19 cells without inducing apoptosis itself. Because the appearance of drusen is a precursor for both wet- and dry-type AMD, characterized by the death of photoreceptors and RPE cells [1], continuous exposure of RPE cells and photoreceptors to Aβ via drusen may result in the activation of caspase-4 and cause apoptosis, resulting in the development of dry-type AMD.

In human eyes, there are other cells (i.e. macrophages) around drusen, and other factors (i.e. complement factors) exist in drusen [42]. These factors and cells may also participate in the development of AMD. This is a limitation of our in vitro study using cultured RPE cells and Aβ. In addition, the contribution of Aβ toxicity to the pathogenesis of AMD in human eyes remains unclear. However, the results of the present study, in corroboration with previous studies, indicate that the ER stress inhibitor PBA may inhibit the development of AMD by reducing ER stress-induced upregulation of VEGF by RPE.

In conclusion, exposure of cultured ARPE-19 cells to PBA suppressed the expression of VEGF caused by Aβ. These findings suggest that drugs targeting ER stress should be considered for the prevention of AMD.

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Disclosure Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This work was supported by a Grant-in-Aid for Young Scientists (H.K.) and a Grant-in-Aid for Challenging Exploratory Research from the Japan Society for the Promotion of Science (H.K.). The study was also supported by the Japan Intractable Diseases Research Foundation (H.K.), Takeda Science Foundation (H.K.), Takeda Medical Research Foundation (H.K.), Yokoyama Foundation for Clinical Pharmacology (YRY1411, H.K.) and The Uehara Memorial Foundation (H.K.).

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