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Elevated amyloid β production in senescent retinal pigment epithelium, a possible mechanism of subretinal deposition of amyloid β in age-related macular degeneration

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ABSTRACT

Age-related macular degeneration (AMD) is the most common cause of legal blindness in the elderly individuals in developed countries. Subretinally-deposited amyloid β (Aβ) is a main contributor of developing AMD. However, the mechanism causing Aβ deposition in AMD eyes is unknown. Aging is the most significant risk of AMD, thus, we examined the effect of aging on subretinal Aβ deposition. mRNAs and cell lysates were isolated from retinal pigment epithelial (RPE) cells derived from 24-month-old (24M RPE) and 2-month-old (2M RPE) C57BL/6 mice. Aβ concentration in culture supernatants was measured by ELISA. Activity and expression of proteins that regulate Aβ level were examined by activity assay and real time PCR. Effect of β-secretase (BACE) on Aβ production was examined by siRNA silencing. Aβ amounts in supernatants of 24M RPE were significantly higher than 2M RPE. Activity and mRNA levels of neprilysin, an Aβ degrading enzyme, were significantly decreased in 24M RPE compared to 2M RPE. PCR analysis found that BACE2 was significantly more abundantly expressed than BACE1 in RPE cells, however, inactivation of BACE2 gene did not affect Aβ production. BACE1 protein amounts did not differ between 24M and 2M RPE, however, BACE1 activity was significantly higher in 24M RPE compared to 2M RPE. There were no significant changes in the activities of α- or γ-secretase between 2M and 24M RPE. In conclusion, RPE cells produce more amounts of Aβ when they are senescent, and this is probably caused by a decrease in Aβ degradation due to a reduction in the expression and activity of neprilysin and an increase in Aβ synthesis due to increased activity of BACE1.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of legal blindness and central vision decrease in the elderly individuals in developed countries. Advanced AMD can be classified into geographic atrophy and exudative AMD [1,2]. Geographic atrophy is characterized by the retinal degeneration involving the photoreceptors and retinal pigment epithelial (RPE) cells. Exudative AMD is characterized by the growth of new blood vessels from the choroid into the subRPE and subretinal spaces, eventually resulting in the formation of disciform scars with loss of photoreceptors. Drusen, grayish-yellow deposits beneath RPE cells, have been identified as a significant risk for developing AMD [3]. Amyloid β (Aβ) has been found in drusen [4,5] and is a major component of senile plaques in the brains of patients with Alzheimer's disease (AD) [6]. The results of our earlier study on senescent neprilysin (NEP) gene-disrupted mice, which lack the Aβ-degrading enzyme, demonstrated that there was a significant increase in the deposition of Aβ in the subretinal space, and those mice developed several features of human eyes with AMD [7]. These results suggest that Aβ deposition in drusen may be a key contributor to the development of AMD. However, the mechanism that causes subretinal deposition of Aβ in eyes with AMD has yet been clarified.

Aβ peptides vary in length from 39 to 43 amino residues and are produced by the sequential proteolytic processing of amyloid precursor protein (APP). The steady state levels of Aβ peptides are maintained by metabolic balance between synthesis and degradation [8–11]. Activation of the amyloidogenic pathway leads to Aβ synthesis by sequential cleavage of APP by β-secretase (BACE) and γ-secretase [12,13]. Two isoforms of BACE exist; BACE1 and BACE2. BACE1 is the main isoform in the brain and BACE2 is dominant in peripheral tissues like colon, kidney and pancreas [14]. A

Abbreviations: Aβ, amyloid β; APP, amyloid precursor protein; RPE, retinal pigment epithelium; AMD, age-related macular degeneration; AD, Alzheimer's disease; NEP, neprilysin; BACE, beta-site amyloid precursor protein cleaving enzyme.

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cleavage of APP by α-secretase leads to a non-amyloidogenic pathway, thus precludes Aβ synthesis [15]. Synthesized Aβ can be degraded by various proteases such as insulin degrading enzyme [16], NEP [10] and cathepsin B [17].

RPE is a monolayer which lies beneath the neural retina and plays critical role in maintaining the homeostasis of the photoreceptors [18,19]. RPE cells constitutively expressed APP [7], α-secretase [20], BACE [7], γ-secretase [21] and NEP [7]. Activity and expression alterations of Aβ synthesis and degradation enzymes have been reported in the brain of both normal aging individuals and AD patients and contribute to Aβ deposition [22–27]. Recent studies suggested that Aβ deposition is not exclusive to AMD and AD but also occurs during normal aging, and most humans and mice accumulate Aβ in their brains and retinas as they age [28]. These findings suggest the possibility that the alterations in the activity and the expression of Aβ synthesis and degradation enzymes in senescent RPE cells may trigger subretinal deposition of Aβ in AMD. Thus, the purpose of the present study was to examine the effect of aging on Aβ production, expression and activity of Aβ synthesis and degradation enzymes in RPE cells isolated from senescent and young mice.

2. Materials and methods

Details for Materials, animals, RPE primary culture and cell lysates isolation, mRNA extraction and PCR analysis are available in Supplemental materials.

2.1. ELISA measurements of $A\beta_{1-40}$ and $A\beta_{1-42}$

The concentrations of $A\beta_{1-40}$ and $A\beta_{1-42}$ in the supernatants of cultured RPE cells isolated from 24-month-old (24M RPE) and 2-month-old (2M RPE) mice were determined with a commercial ELISA kit (Wako, Osaka, Japan). The absorbance was measured at 450 nm in a Bio-Rad Model 450 microplate reader (Bio-Rad Laboratories, Hercules, CA). All experiments were performed at least six times.

2.2. Activity assay of NEP, α-secretase, BACE and γ-secretase

Hundred microliters DMEM containing 20 μg total cell lysates of freshly isolated 2M RPE and 24M RPE were respectively added in duplicate to black CulturPlate-96. Ten micrometers (final concentration) fluorogenic peptide substrate (Mca-RPPGF-SAFK[Dnp]-OH; R&D Systems, Cat. No. ES-005) for NEP, 10 μM (final concentration) fluorogenic peptide substrate (Nma-GGVVLATVL[DNP]-D-A-D-A-D-A-NH2; Calbiochem, Cat. No. 565764) for γ-secretase, 40 μM (final concentration) BACE1 substrate and γ-secretase substrate that provided in BACE1 and γ-secretase activity assay kits were respectively added to each corresponding well and incubated at 37 °C. Fluorescence intensities were read on a fluorescent ELISA plate reader every 30 min of 120 min. The excitations were done at 320 nm for NEP, 355 nm for γ-secretase, 490 nm for α-secretase and BACE. The emissions were done at 405 nm for NEP, 440 nm for γ-secretase, 520 nm for α-secretase and BACE. Because the substrate for NEP is also an excellent substrate for endothelin-converting enzyme-1 (ECE-1), to determine the specificity of the NEP activity, an inhibition study was performed. The RPE cell lysates used for NEP activity assay were preincubated with 100 nM thiorphan; an inhibitor of ECE-1 cleavage of the substrate [29], for 10 min at room temperature before addition of substrate and then processed as described above. All experiments were performed at least four times.

2.3. SiRNA inactivation of BACE2 gene expression

Predesigned siRNA specific for mouse BACE2 was purchased from Dharmacon (Lafayette, CO). Transfection of the siRNA into cultured RPE cells was done with lipofectamine 2000 and OPTI-MEM according to the manufacturer's instructions. In brief, primary RPE cells were allowed to reach 40% confluence on 10 cm plate till the day of transfection. One milliliter of OPTI-MEM containing 10 μM siRNA was incubated with lipofectamine 2000 for 20 min at room temperature. This mixture was then added to the medium of the RPE cells which contained 4 ml OPTI-MEM. Cells were incubated for 24 h at 37° C prior to changing the medium to DMEM supplemented with 10% FCS. A second transfection was carried out after dividing the transfected RPE cells into 12 well plates and after the cells had reached 40% confluence. After the second transfection, the cells were allowed to reach confluence in the DMEM supplemented with 10% FCS. The medium was then changed to serum free DMEM, and incubated for another 48 h before the supernatants were collected for ELISA analysis of Aβ. The inhibition of BACE2 gene expression was confirmed by real time PCR. Non-target siRNA was used as negative control. All the experiments were performed at least six times.

2.4. Measurements of BACE1 by ELISA

The level of BACE1 protein expressed by 24M RPE and 2M RPE was determined by a commercial ELISA kit (Wuhan, China). Twenty micrograms total cell lysates of freshly isolated RPE cells, diluted in standard diluent to a final volume 100 μl, was added to wells pre-coated with the BACE1 antibody. The absorbance was measured at 450 nm in Bio-Rad Model 450 microplate reader (Bio-Rad Laboratories, Hercules, CA). All experiments were performed at least six times.

2.5. Statistical analysis

Mann–Whitney *U* test or analysis of variance (ANOVA) was used to determine whether the differences existed between experimental mean values. A P value < 0.05 was considered significant. All statistical analysis was done with StatView software (SAS Institute, Cary, NC).

3. Results

3.1. RPE cells isolated from senescent mice produce more Aβ than young mice

The concentration of $A\beta_{1-40}$ was 65.5 ± 10.8 pmol/ml in the supernatants of 24M RPE which was significantly higher than the 33.6 ± 6.5 pmol/ml in the supernatants of 2M RPE (Fig. 1A, $n = 6$, $P = 0.012$). The concentration $A\beta_{1-42}$ was 8.4 ± 0.8 pmol/ml in 24M RPE which was significantly higher than the 4.2 ± 0.3 pmol/ml in the 2M RPE (Fig. 1B, $n = 6$, $P = 0.008$).

3.2. Activity and mRNA levels of NEP decreased in RPE cells isolated from senescent mice than young mice

RT-PCR results showed that mRNAs of APP, NEP, α-secretase, BACE (BACE1 and BACE2) and γ-secretase (PS1 subunit) were constitutively expressed in primary mouse RPE cells (Fig. S1). The mRNA levels of APP were not significantly different between 2M RPE and 24M RPE (Fig. S2). However, the mRNA levels of NEP were significantly decreased to 0.06 ± 0.02 in 24M RPE compared to 1.00 ± 0.17 in 2M RPE (Fig. 2A, $n = 6$, $P = 2.35 \times 10^{-7}$). Total RPE cell lysates (20 μg) diluted in 100 μl DMEM was incubated with

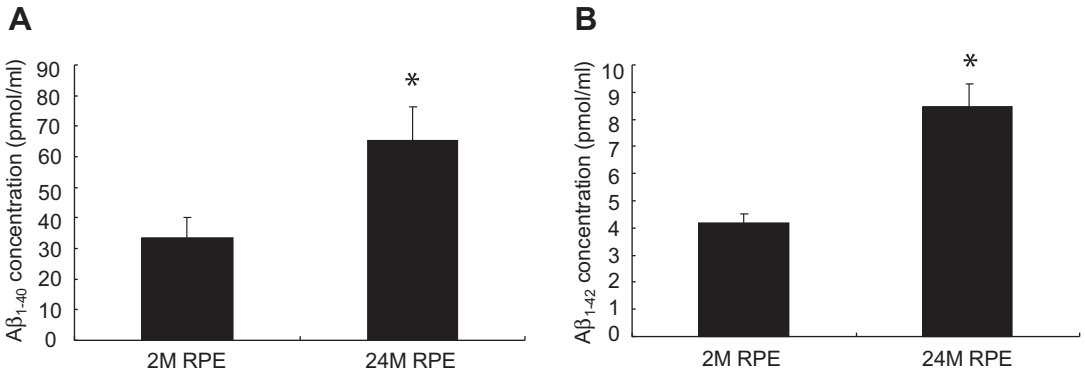

Fig. 1. Concentrations of $A\beta_{1-40}$ (A) and $A\beta_{1-42}$ (B) in culture supernatants of retinal pigment epithelial (RPE) cells isolated from 2-month old (2M RPE) and 24-month-old (24M RPE) mice. After reaching cellular confluence, the medium was changed to serum free DMEM and RPE cells were cultured for another 48 h. Supernatants were then collected and used for ELISA analysis. Values are expressed as the means ± SEMs, $n = 6$, $^*P < 0.05$.

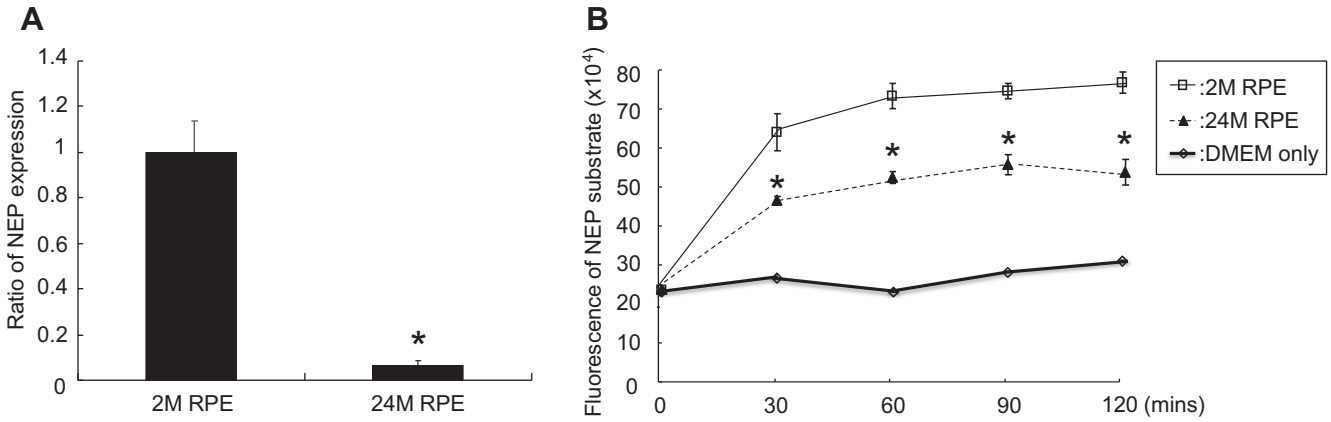

Fig. 2. Comparison of gene expression and activity of Aβ degrading enzyme; neprilysin (NEP) between retinal pigment epithelial (RPE) cells isolated from 2-month-old (2M RPE) and 24-month-old (24M RPE) mice. (A) Comparison of NEP gene expression by real time PCR analysis. The relative changes in gene expression are calculated by subtracting the threshold cycles of the target genes from the internal control gene GAPDH. Values are expressed as the means ± SEMs, $n = 6$, $^*P < 0.05$. (B) Comparison of NEP activity by NEP-specific fluorogenic substrate. NEP-specific fluorogenic substrate was respectively added to corresponding wells containing 20 μg total cell lysates of 2M RPE (thin line) and 24M RPE (dotted line). DMEM containing no cell lysates (bold line) was used as negative control. Fluorescence of catalyzed substrate was measured every 30 min of 120 min. Values are expressed as the means ± SEMs, $n = 4$, $^*P < 0.05$.

NEP-specific fluorogenic substrate for 120 min. After the incubation, the increased fluorescence intensity of the substrate was $2.57 \pm 0.37 \times 10^5$ in the 24M RPE which was significantly lower than the $5.32 \pm 0.31 \times 10^5$ in the 2M RPE (Fig. 2B, $n = 4$, $P = 7.26 \times 10^{-6}$).

3.3. BACE2 is the main isoform of BACE in RPE cells but BACE2 does not contribute to Aβ production

RPE cells expressed two kinds of BACE; BACE1 and BACE2 (Fig. S1). Real time PCR results showed that the mRNA level of BACE2 was 37.3 ± 6.5 times higher than BACE1 in RPE cells (Fig. 3A, $n = 4$, $P = 2.47 \times 10^{-5}$). Treatment of RPE cells with siRNA specific to BACE2 significantly inhibited BACE2 gene expression to 0.15 folds compared to non-treated RPE cells (Fig. 3B, $n = 6$, $P = 5.81 \times 10^{-6}$), however, did not affect $A\beta_{1-40}$ or $A\beta_{1-42}$ protein amounts in RPE culture supernatants (Fig. 3C and D).

3.4. BACE1 protein expression did not differ between RPE cells isolated from young and senescent mice, but BACE1 activity increased in RPE cells isolated from senescent mice

BACE1 protein expression in RPE cell lysates measured by ELISA were 26.4 ± 6.6 pg/μg in 2M RPE, which was not significantly different from 29.9 ± 8.7 pg/μg in 24M RPE (Fig. 4A, $n = 6$). Increased fluorescence intensity of BACE1-specific substrate was $3.65 \pm 0.25 \times 10^5$ in 24M RPE, significantly higher compared to $2.19 \pm 0.15 \times 10^5$ in 2M RPE (Fig. 4B, $n = 4$, $P = 2.46 \times 10^{-5}$).

3.5. Neither activities of α-secretase nor γ-secretase differ between RPE cells from young and senescent mice

Increased fluorescence intensities of catalyzed α-secretase specific substrate (Fig. S3A) and γ-secretase specific substrate (Fig. S3B) did not differ between 24M RPE and 2M RPE after 120-min incubation of the substrates with RPE cell lysates (Fig. S3, $n = 4$).

4. Discussion

Our results showed that the level of Aβ was significantly higher in 24M RPE than in 2M RPE, and this was accompanied by decreased expression and activity of NEP and increased activity of BACE1. Although earlier studies reported the increased Aβ accumulation in the human brain during the normal aging process [30–32], PubMed search extracted only one article reporting an increase in Aβ deposition in the retina during normal aging [28]. Hoh Kam and colleagues [28] reported an accumulation of Aβ -rich extracellular

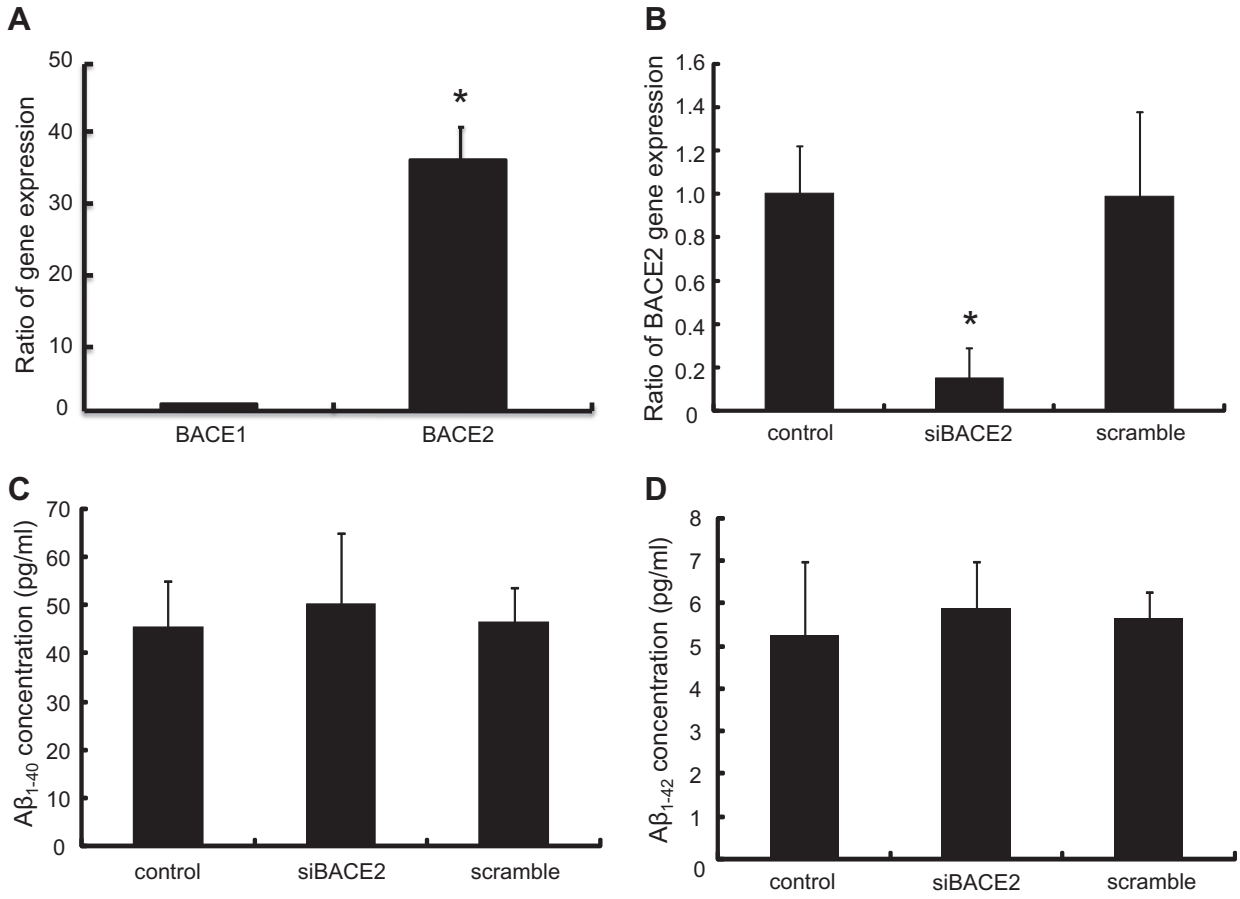

Fig. 3. Comparison of gene expression of β-secretase isoforms; BACE1 and BACE2 in retinal pigment epithelial (RPE) cells isolated from 2-month-old (2M RPE) mice and determination of Aβ amounts in the culture supernatants of RPE cells whose BACE2 gene was inactivated by siRNA. (A) Real time PCR comparison of BACE1 and BACE2 gene expression in 2M RPE. (B) Real time PCR examination of the effect of BACE2 siRNA on BACE2 gene expression in 2M RPE. (C) ELISA measurement of $A\beta_{1-40}$ concentration in culture supernatants of 2M RPE treated with/without BACE2 siRNA. (D) ELISA measurement of $A\beta_{1-42}$ concentration in culture supernatants of 2M RPE treated with/without BACE2 siRNA. Values are expressed as the means ± SEMs, $n = 6$, $^*P < 0.05$.

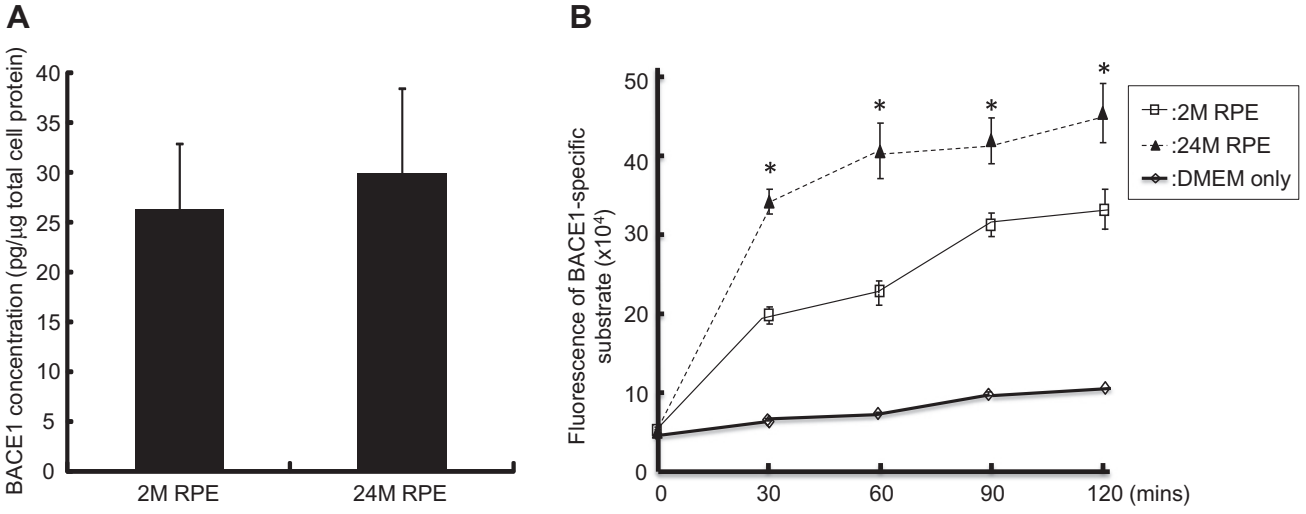

Fig. 4. ELISA analysis of BACE1 protein amount and activity assay analysis of BACE1 activity in cell lysates of retinal pigment epithelial (RPE) cells isolated from 2-month-old (2M RPE) and 24-month-old (24M RPE) mice. (A) BACE1 protein amounts in 2M RPE and 24M RPE culture supernatants determined by ELISA. After reaching cellular confluence, RPE cells were cultured in serum free DMEM for another 48 h. Cells were then lysed and 20 μg total cell lysates (diluted in standard diluents, final volume 100 μl) were respectively added to corresponding BACE1 antibody-coated wells. All procedures were performed according to manufacturer's instructions. Values are expressed as the means ± SEMs, $n = 6$, $^*P < 0.05$. (B) BACE1 activity assay using cell lysates of 2M RPE and 24M RPE. BACE1-specific fluorogenic substrate was added to corresponding wells containing 20 μg total cell lysates of 2M RPE (thin line) and 24M RPE (dotted line). DMEM containing no cell lysates (bold line) was used as negative control. The reaction mixtures were incubated at 37 °C. Fluorescence of catalyzed substrates was measured every 30 min of 120 min. Values are expressed as the means ± SEMs, $n = 4$, $^*P < 0.05$.

deposition along Bruch's membrane and also in the outer segments of the photoreceptors in both human and mouse retinas with normal aging, and they hypothesized that this accumulation was related to the increased number of macrophages bloated with Aβ. Results in the present study suggest another mechanism that age-related alterations of NEP and BACE1 are key factors that modulate the accumulation of Aβ in retina by producing more Aβ.

Age-associated alterations of Aβ synthesis and degradation enzymes have been reported in the brain [11,22–27,33–35], but there have been no such studies in the eye. Hellstrom-Lindahl and associates [33] reported that NEP protein levels decreased with increasing age in temporal and frontal cortex of normal brain. A significant positive correlation between insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ with age was found in cortex of normal brain, and they [33] concluded that the observed age-dependent decline in NEP may be related to increased Aβ levels during normal aging. Caccamo et al. [34] reported that the protein levels of NEP decreased in the hippocampus with increasing age in both mice and humans but the levels in the cerebellum increased or remained unaltered. In the present study, we demonstrated a significant lower level of NEP gene expression in RPE cells isolated from senescent mice than from young mice. This finding indicated an age-related decrease of NEP may also be involved in Aβ accumulation in the subretinal space. However, some of other studies reported the conflicting results in NEP protein levels during aging [11,24,27,35]. The reasons for these discrepancies need to be investigated in the future.

BACE1 is the main isoform of β-secretase in the brain, while BACE2 is more prevalent in peripheral tissues like colon, kidney, pancreas, placenta, prostate, stomach, trachea [14]. The results in the present study showed that BACE2 mRNA expression was 37 times higher than BACE1 in 2M RPE, which indicates that BACE2 is the main BACE isoform in RPE cells. BACE2 cleaves APP at the same site with BACE1 and also within the Aβ region of APP [36,37], the function of BACE2 on Aβ production was not consistent among the tissues [13,38]. Over-expression of BACE2 Markedly reduced Aβ synthesis in primary neurons from APP transgenic mice [38], however, BACE2 enhanced Aβ synthesis in astrocytes [13]. In the current study, BACE2 inactivation using siRNA did not affect Aβ production in RPE cells, suggesting that BACE2 was not involved in Aβ production in RPE cells.

The BACE1 activity but not protein level was significantly higher in 24M RPE cells than in 2M RPE cells which is compatible with the results in the brain [24]. Fukumoto et al. [22] reported that BACE1 activity, but not the protein level, increased with age in normal human brain. They suggested that the BACE1 activity levels may be modulated by factors other than the BACE1 protein levels. Similar mechanism might be applied to NEP expression and NEP activity, although the NEP protein levels were not measured in the current study.

The mechanism of age-related alteration of NEP and BACE1 activities has not been clarified. Several factors, like endoproteolysis [39], oxidative stress [40], membrane cholesterol composition [41], interactions with nicastrin [42], or chronic inflammation [43] have been reported to affect the activity of NEP or BACE1. Thus, the effects of aging on the above factors which affect NEP and BACE activities should be investigated in the future.

In conclusion, the present study showed a significant age-dependent decrease in NEP mRNA expression and activity in mouse RPE cells. In contrast, the activity of BACE1 was increased with age in mouse RPE cells. These findings suggest that these age-dependent changes might contribute to subretinal accumulation of Aβ in AMD.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.085.

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