SIRT1 negatively regulates amyloid-beta-induced inflammation via the NF-κB pathway

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Abstract

Chronic inflammation induced by amyloid-beta (Aβ) plays a key role in the development of age-related macular degeneration (AMD), and matrix metalloproteinase-9 (MMP-9), interleukin (IL)-6, and IL-8 may be associated with chronic inflammation in AMD. Sirtuin 1 (SIRT1) regulates inflammation via inhibition of nuclear factor-kappa B (NF-κB) signaling, and resveratrol has been reported to prevent Aβ-induced retinal degeneration; therefore, we investigated whether this action was mediated via activation of SIRT1 signaling. Human adult retinal pigment epithelial (RPE) cells were exposed to Aβ, and overactivation and knockdown of SIRT1 were performed to investigate whether SIRT1 is required for abrogating Aβ-induced inflammation. We found that Aβ-induced RPE barrier disruption and expression of IL-6, IL-8, and MMP-9 were abrogated by the SIRT1 activator SRT1720, whereas alterations induced by Aβ in SIRT1-silenced RPE cells were not attenuated by SRT1720. In addition, SRT1720 inhibited Aβ-mediated NF-κB activation and decrease of the NF-κB inhibitor, IκBα. Our findings suggest a protective role for SIRT1 signaling in Aβ-dependent retinal degeneration and inflammation in AMD.

Key words: Amyloid-beta; SRT1720; Age-related macular degeneration; Barrier integrity; Tight junction; Matrix metalloproteinase-9

Introduction

Age-related macular degeneration (AMD) is a principal cause of irreversible blindness in people over 65 years of age in industrialized countries. AMD is a multifactorial disease and its pathogenesis remains unexplored; however, recent evidence confirms that inflammation plays an important role in its pathology (1). Drusen, tiny yellow or white accumulations of extracellular material within the retina peculiar to AMD, have been suggested as high-risk factors for the development of chronic inflammation. (1,2). Amyloid-beta (Aβ), a known constituent of drusen, is thought to contribute to development of AMD (3-5).

The retina is an immune-privileged site protected by a local blood-retinal barrier (BRB), but it is not known how immune cells can pass through this barrier and cause chronic inflammation. A layer of retinal pigment epithelial (RPE) cells forms the outer BRB, and the inner BRB is formed by blood vessels of the inner retina. Alterations of the BRB play a crucial role in the development of AMD (6,7). The function of the outer BRB is dependent on well-developed tight junctions (TJs) between RPE cells, which are mediated by the transmembrane proteins occludin and the claudin family, and junctional adhesion molecules, along with the scaffolding zonula occludens protein-1 (ZO-1). Abnormal expression or localization of occludin and ZO-1 in RPE cells leads to barrier dysfunction (6,9). The antioxidant resveratrol has been shown to protect ARPE-19, a spontaneously transformed RPE cell line, from Aβ-induced barrier dysfunction (10). Our previous study found that matrix metalloproteinase-9 (MMP-9) mediated Aβ-induced barrier disruption, suggesting that MMP-9 contributes to chronic inflammation in AMD (11). Breakdown of the epithelial barrier would thus be both a stimulus for inflammation in tissue injury and a component of normal inflammatory processes that permit leukocyte influx into areas of tissue damage.

Colocalization of Aβ and activated complement immunoreactivity in AMD patients provide evidence for Aβ deposition in the local inflammatory events contributing to pathogenesis of AMD (12), and suggest that Aβ-induced complement activation may be involved in chronic inflammation in AMD (13). Microarray analysis of gene expression in ARPE-19 revealed that Aβ stimulation...
significantly upregulated interleukin (IL)-8 gene expression (14). Our previous study found that Aβ stimulation increased IL-6, IL-8, and MMP-9 expression in human adult RPE cells (11). Increased intracellular concentrations of IL-6, IL-8, and MMP-9 have also been found in AMD patients, and the levels were significantly associated with the severity of the disease (15). However, the mechanisms by which Aβ upregulates inflammation-associated cytokines is largely unknown.

Nuclear factor-kappa B (NF-κB), a key regulator of the inflammation response, is modulated by post-translational modifications, including reversible acetylation of the NF-κB RelA/p65 subunit (16). Full transcriptional activity of RelA/p65 requires acetylation of Lys310, which can be deacetylated by sirtuin 1 (SIRT1) (17). Resveratrol, an activator of SIRT1 (18), inhibits NF-κB signaling by promoting deacetylation of Lys310 of RelA/p65 (16). Thus, SIRT1 may be a key regulator of inflammation in mammalian cells via inhibition of NF-κB activation. In this study, we explored the inhibitory role of SIRT1 in Aβ-induced proinflammatory cytokine production and BRB disruption in human adult RPE cells by activating SIRT1 with SRT1720 or silencing SIRT1 with small-interfering RNA (siRNA). We further demonstrated that Aβ-induced expressions of MMP-9, IL-8, and IL-6 were regulated by the SIRT1/NF-κB pathway.

Material and Methods

Aβ1-42 oligomerization and detection of Aβ1-42 oligomers

Oligomerized Aβ (OAβ) was synthesized as previously described (11). Briefly, lyophilized Aβ1-42 peptide (Sigma-Aldrich, USA) was dissolved in 1.5 M hexafluoroisopropanol (HFIP) on ice and aliquoted at -20°C. Aβ monomers were spin-vacuumed just prior to use, diluted to 250 μM in HFIP solution, and kept at room temperature for 3 days to synthesize Aβ oligomers. Several lines of evidence have demonstrated that soluble Aβ oligomers may be better correlated with the severity of the disease than are monomers or insoluble amyloid fibrils (3,19). Therefore, the oligomeric form of Aβ1-42 was verified using Western blot with the anti-Aβ monoclonal antibody 6E10 (1:500, Covance, USA).

Ethics

Informed consent for human tissue donation was obtained from relatives, and the study protocol was approved by the Ethics Committee of the Tenth People’s Hospital affiliated to the Tongji University and adhered to the tenets of the Declaration of Helsinki for experiments involving human tissue.

Isolation of human RPE cells

Five human donor eyes were obtained from the eye bank of the Eye & ENT Hospital of Fudan University, Shanghai, China. The donors were between 30 and 40 years of age and none had a history of eye disease. Human RPE cells were harvested as described previously (11). In brief, whole eyes were cleaned in 0.9% NaCl solution, immersed in 5% polyvinylpyrrolidone iodine, and rinsed again in NaCl solution. The anterior segment was removed from each eye and the neural retina was peeled away from the RPE choroid sclera. The eyecup was rinsed with Ca²⁺- and Mg²⁺-free Hank’s balanced salt solution, and treated with 0.25% trypsin for 1 h at 37°C. The trypsin was aspirated and replaced with DMEM/F12 (HyClone Laboratories, USA) supplemented with 20% fetal calf serum. The RPE cells isolated from the five donor eyes were combined, yielding about 10⁵ cells per milliliter.

Silencing of SIRT1 by RNA interference

To confirm the exact role of SIRT1 on MMP-9, IL-8, and IL-6 expression by OAβ-stimulated cells, gene expression of SIRT1 was silenced by an RNA interference strategy. The SIRT1 silencer and negative siRNA were synthesized by GenePharma (China). The sequences were: SIRT1 siRNA, sense: 5′-GAUGAGAGUUUGACCCUCUCATT-3′, anti-sense: 5′-UGAGGGAGUCACUUCUAUCC-3′; and negative siRNA (siRNA-N), sense: 5′-CCUACGCCACAUUUCGU-3′, anti-sense: 5′-ACGAAUUGUGCGGUAGG-3′. siRNA at a final concentration of 25 nM was combined with 10 μL Lipofectamine 2000 (Invitrogen, USA) in 500 μL DMEM/F12 and allowed to complex by incubation for 20 min. The RPE cells were incubated with the transfection mixture for 24 h, and the cells were transferred to culture medium as described above and incubated for 72 h before treatment.

Cell viability assay

Human adult RPE cells (2 × 10⁴/well) seeded on 96-well plates were treated with 0.001-12.0 μM OAβ for 24 h or treated with 0.3 μM OAβ for 4-24 h. To determine its protective effect, cells were treated with 5 μM SRT1720 (Cayman Chemical, USA) 1 h before or 1 h after treatment with 0.3 μM OAβ. In other experiments, RPE cells exposed to the transfection mixture (25 nM, 24 h) were treated with OAβ with or without SRT1720. Cell viability was measured by addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, USA) for 3 h at 37°C. The absorbance was measured spectrophotometrically at 490 nm with a microplate reader (ELx800, Bio-Tek, USA).

Real-time quantitative-PCR

Total RNA was isolated using Trizol (Invitrogen) and reverse-transcribed to cDNA with RT Master Mix (TaKaRa, China). Expression of IL-8 and IL-6 was measured using a PCR mix (TaKaRa). The PCR primers were: IL-8, sense: 5′-GACAATTCCCCACATTTCCGC-3′, antisense: 5′-GAACTTGCTCCACACCTGTG-3′; IL-6, sense: 5′-CACTCACCTCTTTAGAAT-3′, antisense: 5′-TTGTACTCAGTGCACAGT-3′. An ABI7500
Samples were examined after incubation for 12 b for 24 h in the presence or for 4 h. Total for 24 h, with or without oligomers and morphology.

**Gelatin zymography**

Gelatin zymography was carried out as previously described (11). Briefly, supernatant was collected after treatment and subjected to 10% SDS-PAGE with 1 mg/mL gelatin. After electrophoresis, gels were incubated in 2.5% Triton X-100 (1 h, 37°C) followed by overnight incubation in 50 mM Tris-HCl, pH 7.8, 5 mM CaCl₂, 0.02% NaN₃, and 0.02% Brij. Gels were stained with 2.5% Coomassie blue R-250 (Bio-Rad, USA) for 45 min followed by destaining in deionized water with 10% acetic acid and 20% methanol. Gels were scanned and band densities were measured using Photoshop CS4.0 (Adobe Systems, USA).

**Cell morphology and immunofluorescence staining**

To confirm the protective role of SIRT1 on RPE barrier integrity, normal RPE cells or siRNA-transfected RPE cells were treated with 0.3 μM Aβ for 24 h, with or without pretreatment with 5 μM SRT1720 for 1 h. After 3 days, the cells were fixed in 4% paraformaldehyde for 30 min and blocked with 1% bovine serum albumin in Tris-buffered saline for 1 h, then incubated with rabbit anti-occludin antibody (1:250), mouse anti-ZO-1 antibody (1:250; Abcam, China), or rabbit anti-ezrin antibody (1:250; Anbo Biotechnology, USA) for 1 h. After washing, they were incubated with AlexaFluor 488-conjugated or AlexaFluor 594-conjugated secondary antibody (1:500; Invitrogen) and DAPI nuclear stain (1:1000) for 1 h. Slides were then viewed on a Leica TCS SP5 scanning confocal microscope (Leica Microsystems, Germany). For immunofluorescence analysis of nuclear translocation of NF-κB/p65, the cells were incubated with rabbit polyclonal anti-p65 primary antibody (1:500, Abcam) for 1 h and incubated with anti-rabbit AlexaFluor 488-conjugated secondary antibody and DAPI (1:1000) for 1 h.

**Measurement of transepithelial electrical resistance (TER)**

Normal or siRNA-transfected RPE cells were grown on filters to form monolayers. TER was measured using a Millicell electrical resistance system (Millipore, USA) and calculated by subtracting the value of a blank filter from cells within the experimental value. Final resistance × area products (Ω × cm²) were obtained by multiplying TER by the effective growth area. Fifteen days after TER stabilization, RPE monolayers were exposed to 0.3 μM OAb for 24 h in the presence or absence of 5 μM SRT1720 for 1 h. The culture medium was then changed, restoring normal conditions, and TER was measured after 3 days of incubation. Measurements were repeated at least four times for each filter, and each experiment was repeated at least four times.

**Permeability assay**

Permeability assays were performed by measuring passive permeation of fluorescein isothiocyanate (FITC)-dextran (4 kDa; Sigma-Aldrich) across confluent cells grown on filters. Fifteen days later, the monolayers were treated as described above, and 500 mg/mL FITC-dextran was added to the upper chamber after treatment. Samples (100 μL) were taken from the upper and lower chambers 24 h after addition of FITC-dextran. The concentration of FITC-dextran in the samples was measured with a microplate reader (ex1800; Biotek, USA). Each experiment was repeated four times.

**Protein extraction and Western blot analysis**

To explore the protective mechanism of SRT1720, protein levels of NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBa), a specific inhibitor of NF-κB activity, and SIRT1 were analyzed by Western blot. Briefly, RPE cells were pretreated with 5 μM SRT1720 for 60 min, then the medium was changed immediately with 0.3 μM Aβ for 4 h. Total proteins were obtained using radio-immunoprecipitation assay buffer (Abcam) containing protease inhibitor, and nuclear proteins were extracted following the manufacturer’s protocol. Protein content was determined by a bicinchoninic acid assay ( Pierce, USA). Equal amounts of total cellular protein were loaded per lane onto 10% SDS-PAGE for electrophoresis and then transferred onto PVDF membranes. The membranes were blocked, and incubated with the primary antibody, rabbit polyclonal antibodies against IkBa (Abcam, 1:2000), NF-κB p65/RelA (1:1000), or SIRT1/Sir2 (1:2000), and rabbit polyclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (1:2000) or TATA binding protein (1:1000), overnight. The membranes were then washed and incubated with horseradish peroxidase-coupled secondary antibodies for 2 h. Blots were washed and developed with chemiluminescent reagent. Membranes were exposed to ImageQuant LAS 4000, and densitometry was performed using Photoshop CS4.0.

**Statistical analysis**

Results are reported as means±SE. The unpaired t-test or ANOVA was used for statistical analysis (SPSS 15.0, USA) and differences were considered to be significant at P<0.01 or P<0.05.

**Results**

**Characterization of Aβ42 oligomers and morphology of human adult RPE cells**

Aβ samples were examined after incubation for 12 or 72 h. Spherical oligomers, including trimers and
tetramers, were initially absent from freshly prepared Aβ(1-42) solutions or monomers (aggregated for less than 24 h). Western blots with anti-Aβ antibody (6E10) further confirmed the presence of Aβ(1-42) as a monomer, trimer, and tetramer in the oligomerized Aβ sample, whereas only monomers were detected in the fresh non-oligomerized Aβ sample (Figure 1A). These results indicated that the Aβ samples incubated for 72 h had aggregated, forming oligomers.

Structure integrity is the basis of good function. To confirm that the isolated human adult RPE displayed classic morphology (uniform hexagonal arrays of cells), confluence, and uniform pigmentation typical of native tissue, the morphology of the cells was evaluated by fluorescence microscopy. Our previous data confirmed that isolated human adult RPE cells were heavily pigmented and had the hexagonal epithelial morphology seen in native tissue (11). Ezrin is a cytoskeleton protein that has been identified exclusively in RPE cell apical processes and has been associated with polarization of RPE cells (20). Apical localization of ezrin in cultured human adult RPE cultures was confirmed by fluorescence microscopy (Figure 1B).

SIRT1 was required to protect RPE cells from Aβ-induced cytotoxicity

The effects of 0.001-12.0 μM OAβ on cell viability were observed at 24 h (Figure 2A); mean cell viability was 95% at 0.1 μM but fell to 30% at 12.0 μM. The effect of 0.3 μM OAβ on the viability of cells was assayed at 4-24 h; cell survival gradually dropped from 87.6% at 4 h to 68.4% at 24 h (Figure 2B). This indicated that a time- and dose-dependent decrease in cell viability occurred in OAβ-stimulated cells. Treatment with 5 μM SIRT1720 1 h before or 1 h after 0.3 μM OAβ treatment for 24 h increased cell viability by 33% (P<0.05) or 26% (P<0.01), respectively, compared with the OAβ-treated group (P<0.01). However, inhibition of SIRT1 with SIRT1-specific siRNA resulted in an increased susceptibility of RPE cells to OAβ stimulation and significantly attenuated SIRT1720’s protection against OAβ (Figure 2C). This suggested that SIRT1720 might protect RPE cells from OAβ-induced cytotoxicity via the SIRT1 pathway. To confirm this result, we examined SIRT1 expression in Western blots. As shown in Figure 2D, SIRT1 expression was significantly decreased 2-fold in the OAβ insult group compared with controls (P<0.05). Meanwhile, the expression of SIRT1 was markedly increased when RPE cells were incubated with SRT1720 (P<0.01; Figure 2D). In addition, knockdown of SIRT1 completely blocked the effect of SRT1720 on SIRT1, and transfection of cells with siRNA-N did not abolish the positive effect of SRT1720 on SIRT1 activation (Figure 2D). These data suggested that SIRT1 activation was essential for protection of RPE cells against OAβ injury.

SIRT1 negatively regulates OAβ-induced inflammation and MMP-9 expression

We have previously shown that OAβ could increase IL-8, IL-6, and MMP-9 expression in human adult RPE cells (11). SRT1720, a potent SIRT1 agonist, has been reported to have anti-inflammatory properties (21). Therefore, we used SRT1720 to investigate the anti-inflammation function of SIRT1. For inhibition studies, SIRT1 expression was silenced by RNA interference. To demonstrate the anti-inflammation effects of SIRT1, we measured the expression levels of IL-8, IL-6, and MMP-9 using RT-PCR and gelatin zymography. Treatment with SRT1720 significantly attenuated OAβ-induced upregulation of IL-6, IL-8, and MMP-9, whereas the inhibitory effects of SRT1720 on OAβ-induced upregulation of IL-6, IL-8, and MMP-9 were attenuated in the cells in which SIRT1 expression was knocked down (Figure 3). To test the time course of inflammation inhibition, SRT1720 was administered 1 h before or 1 h after OAβ treatment. Inflammation was not significantly inhibited following SRT1720 administration 1 h after OAβ stimulation compared with SRT1720 pretreatment (Figure 3). These results suggest that SIRT1 is required for blocking the OAβ-induced proinflammatory effect.

SIRT1 is required to attenuate OAβ-induced disruption of barrier integrity

Structures of occludin and ZO-1 are crucial to the development and maintenance of RPE morphology and function. Ezrin, one of the first proteins to be polarized, is localized mainly on the apical side of well-polarized human RPE cells (20). A recent study found that the
SIRT1 activator resveratrol could attenuate OAβ-induced RPE barrier disruption (10). MMP-9 has been reported to modify barrier function by disrupting TJ proteins (22-24), and our previous study demonstrated that silence of MMP-9 partially abrogated the damaging effect of OAβ on the RPE barrier (11). It has been demonstrated that SIRT1 is a negative regulator of MMP-9 expression in human monocytic cells in vitro (25). Based on the aforementioned result that demonstrated that SIRT1 activation could inhibit OAβ-induced expression of MMP-9 (Figure 3), we asked whether SIRT1 could protect RPE cells from OAβ-induced barrier disruption. Normal or siRNA-transfected cells treated with OAβ or SRT1720. SRT1720 attenuated OAβ-induced decrease of cell viability, and knockdown of SIRT1 inhibited the protective effect of SRT1 on cell viability. D, Western blot analysis of SIRT1 expression. The SIRT1 expression of OAβ-stimulated cells was lower than that of control, and SRT1720 treatment significantly increased the SIRT1 expression in OAβ-stimulated cells. Transfection with SIRT1 siRNA blocked SIRT1 expression in cells, but transfection with negative siRNA did not affect the inhibitory effect of SRT1720 on OAβ-induced decrease of SIRT1 expression. Data are reported as means ± SE from 4 measurements. Each panel shows a representative experiment repeated three times with similar results. SRT: cells treated with SRT1720; SIRT1 siRNA: cells transfected with SIRT1 siRNA; siRNA-N: cells transfected with negative control of siRNA. *P<0.05, **P<0.01 (unpaired t-test and ANOVA).

SIRT1 activator resveratrol could attenuate OAβ-induced RPE barrier disruption (10). MMP-9 has been reported to modify barrier function by disrupting TJ proteins (22-24), and our previous study demonstrated that silence of MMP-9 partially abrogated the damaging effect of OAβ on the RPE barrier (11). It has been demonstrated that SIRT1 is a negative regulator of MMP-9 expression in human monocytic cells in vitro (25). Based on the aforementioned result that demonstrated that SIRT1 activation could inhibit OAβ-induced expression of MMP-9 (Figure 3), we asked whether SIRT1 could protect RPE cells from OAβ-induced barrier disruption. Normal or siRNA-transfected RPE cells were exposed to OAβ with or without SRT1720 pretreatment (1 h). In the control cells (Figure 4A), no morphological change was observed, the distribution of occludin and ZO-1 was continuous and regular with no breaks around the cell borders, and ezrin was localized mainly on the apical side. Exposure to Aβ (Figure 4B) caused a diffuse cytoplasmic distribution of ZO-1 and occludin, and the immune staining of ezrin was dispersed on both sides of the cells. Pretreatment with SRT1720 (Figure 4C) inhibited the deleterious effects of Aβ on RPE integrity. Knockdown of SIRT1 significantly abolished the protective effect of SRT1720 on OAβ-induced barrier disruption (Figure 4D) compared with siRNA-N-transfected cells. These results suggested that SRT1720, with its anti-inflammatory properties might reverse the deleterious effects of Aβ on RPE barrier structure. However, activation or inhibition of SIRT1 expression could affect the localization of ezrin, and this result implied that ezrin expression or localization was not regulated by SIRT1.

Barrier functional studies, including TER detection and permeability assays, were performed to confirm the protective effect of SIRT1 against the deleterious effect of OAβ on the RPE barrier. TER (Figure 5A) was recorded to determine the stability of TJ proteins, and the transepithelial diffusion rate of FITC-dextran (Figure 5B) was measured to evaluate the permeability of the monolayers. A mean TER of $160\pm 23 \Omega \times cm^2$ was recorded in control cells. OAβ decreased TER by $83 \pm 18 \Omega \times cm^2$, but pretreatment with SRT1720 partially

Figure 2. MTS assay of cell viability. A, MTS assay of cell viability after stimulation with different concentrations of OAβ. Upon exposure to 0.001 μM OAβ, no reduction of MTS activity was detected in cells, while a significant reduction was observed in response to 0.1-12 μM OAβ treatment after 24 h. B, MTS assay for cell viability after stimulation with 0.3 μM OAβ for different times. RPE cells stimulated with 0.3 μM OAβ demonstrated a time-dependent reduction of MTS activity dropping from 87.6% at 4 h to 68.4% at 24 h. C, MTS assay on normal or siRNA-transfected cells treated with OAβ or SRT1720. SRT1720 attenuated OAβ-induced decrease of cell viability, and knockdown of SIRT1 inhibited the protective effect of SRT1 on cell viability. D, Western blot analysis of SIRT1 expression. The SIRT1 expression of OAβ-stimulated cells was lower than that of control, and SRT1720 treatment significantly increased the SIRT1 expression in OAβ-stimulated cells. Transfection with SIRT1 siRNA blocked SIRT1 expression in cells, but transfection with negative siRNA did not affect the inhibitory effect of SRT1720 on OAβ-induced decrease of SIRT1 expression. Data are reported as means ± SE from 4 measurements. Each panel shows a representative experiment repeated three times with similar results. SRT: cells treated with SRT1720; SIRT1 siRNA: cells transfected with SIRT1 siRNA; siRNA-N: cells transfected with negative control of siRNA. *P<0.05, **P<0.01 (unpaired t-test and ANOVA).

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reversed this effect, and transfection with SIRT1 siRNA significantly exacerbated the Aβ-induced decrease of TER (Figure 5A). A significantly increased diffusion rate of FITC-dextran was observed when the cells were stimulated with Aβ, but pretreatment with SRT1720 abolished this effect, and SIRT1 knockdown induced a greater permeability than Aβ stimulation alone (Figure 5B). These results suggested that activation of SIRT1 could prevent Aβ-induced barrier dysfunction, including decrease of TER and permeability dysfunction.

Expression of IκBa and NF-κB in RPE cells

In the present study, SRT1720 had an obvious effect, protecting RPE cells from Aβ-induced cytotoxicity and inflammation and dysfunction of barrier integrity in RPE cells (Figures 2-5). To explore the underlying mechanisms of its beneficial effects, RPE cells were pretreated with SRT1720 for 60 min before Aβ was added. Expression of NF-κB (Figure 6A) and IκBa (Figure 6C) proteins was analyzed by Western blot. Nuclear translocation of NF-κB/p65, an index of NF-κB activation, was measured using immunofluorescence (Figure 6B). Treatment of RPE cells with Aβ induced a significant 12.8-fold increase in the amount of NF-κB/p65 in the nucleus (Figure 6A), positive immunostaining of p65 translocated into the nucleus (Figure 6B), and a significant decrease in IκBa protein content (Figure 6C). Pretreatment with SRT1720 decreased the amount of NF-κB/p65 in the nucleus (Figure 6A and B) and increased the amount of IκBa compared with stimulation with Aβ (Figure 6C). Knockdown of SIRT1 with siRNA not only attenuated the inhibitory effect of SRT1720 on Aβ-induced nuclear translocation but also induced a higher amount of NF-κB/p65 in the nucleus than the Aβ insult group (Figure 6A and B). With the altered expression levels of SIRT1 (Figure 2D), these results are consistent with the theory that SIRT1 activation has an anti-inflammatory effect via inhibition of NF-κB activation. In addition, we found that the expression level of IκBα, an inhibitor of NF-κB, was increased in SIRT1-siRNA-transfected cells, which implied that the inhibitory effect of SIRT1 on NF-κB was not fully dependent on IκBα activation.

Figure 3. Effects of Aβ, SRT1720 and knockdown of SIRT1 on IL-8, IL-6 and MMP-9 expression in RPE cells. A, PCR analysis of IL-8 mRNA expression. B, PCR analysis of IL-6 mRNA expression. C, Gelatin zymography assay of MMP-9 expression. Stimulation with 0.3 μM OAβ for 24 h induced significant increases in mRNA expression of IL-6, IL-8 and MMP-9 in RPE cells. Pretreatment with SRT1720 markedly attenuated OAβ-induced increases of IL-6, IL-8 and MMP-9 expression. Knockdown of SIRT1 gene expression by SIRT1 siRNA significantly intensified the proinflammatory effects of OAβ. Data are from three representative experiments. *P<0.05, **P<0.01 (unpaired t-test and ANOVA).
Discussion

OAβ1,42 can induce inflammation and barrier disruption in RPE cells (11), but the underlying mechanism is largely unknown. The main results of this study are as follows: 1) SIRT1 is required to protect RPE cells from Aβ-induced cytotoxicity; 2) SIRT1 negatively regulates Aβ-induced inflammation and MMP-9 expression; 3) SIRT1 is required to attenuate Aβ-induced disruption of barrier integrity; 4) the protective effects of SIRT1 on inflammation and barrier disruption depend on its inhibition of NF-κB.

The present study showed that exposure of human adult RPE cells to Aβ induced a dose- and time-dependent decrease in cell viability (Figure 2A and B). The protective role of SIRT1 on cell viability was further confirmed by pharmacological activation of SIRT1 with SRT1720 and by the knockdown of SIRT1 gene expression with an RNA interference strategy (Figure 2C). Interestingly, we found that Aβ could inhibit SIRT1 expression (Figure 2D). Accumulated evidence suggests that resveratrol, an SIRT1 activator, can attenuate Aβ-induced toxicity (26). Aβ-induced MTS reduction as the result of decreased mitochondrial membrane potential is a marker of the production of excessive reactive oxygen species (ROS).
and mitochondrial dysfunction (27). The NAD\(^+\)-dependent deacetylase SIRT1 can increase mammalian FOXO3 transcription factor through direct binding or deacetylation to induce cell resistance to oxidative stress (28), which implies that Aβ-induced inhibition of SIRT1 expression may contribute to the reduction of MTS in cells as a result of the excessive generation of ROS. Our results are consistent with a recent report (29) that suggested Aβ-mediated SIRT1 inhibition may contribute to decreased cell viability, but the mechanism by which Aβ regulates SIRT1 expression is largely unknown.

This study demonstrated that Aβ-induced IL-8, IL-6, and MMP-9 expressions were attenuated in cells pretreated with SIRT1 activators, and the Aβ-induced proinflammatory effects were exacerbated by knockdown of SIRT1 expression (Figure 3). These results suggested that SIRT1 could negatively regulate Aβ-induced inflammation. A recent study also reported that the SIRT1 activator resveratrol prevents the proinflammatory effect of Aβ on macrophages (30).

Exposure of RPE cells to Aβ resulted in a disruption of ZO-1 and occludin (Figure 4), loss of TER (Figure 5A), and increased permeability (Figure 5B). Furthermore, the SIRT1 activator SRT1720 inhibited deleterious effects of Aβ on morphology (Figure 4) and barrier function of RPE monolayers (Figure 5), and knockdown SIRT1 expression aggravated Aβ-induced RPE barrier disruption (Figures 4 and 5). Aβ-induced MMP-9 expression may contribute to barrier disruption (11,31); therefore, transcriptional inhibition of MMP-9 by SIRT1, which is a negative regulator of MMP-9 (25), may protect RPE cells from Aβ-induced RPE barrier disruption via inhibition of MMP-9 expression. Aβ induced a diffuse ezrin staining at both sides of RPE cells, but activation of SIRT1 did not attenuate the effect of

![Figure 5. Effects of Aβ, SRT1720 and knockdown of SIRT1 on the barrier integrity of RPE monolayers. A, Measurement of trans-epithelial resistance (TER) of retinal pigment epithelial (RPE) cells. B, Analysis of transepithelial permeability by measuring the passive permeation of FITC-dextran. Normal or siRNA-transfected RPE cells were cultured for 15 days after TER stabilization, and then left untreated (control) or exposed to 0.3 μM Aβ for 24 h with or without 5 μM SRT1720 pretreatment for 1 h. Aβ induced a significant decrease in TER and increased permeability compared with the control. SRT1720 markedly attenuated Aβ-induced low TER and high permeability, whereas transfection with SIRT1 siRNA led to a lower TER and a higher permeability than the Aβ insult group. Data are from 3 representative experiments. *P<0.05, **P<0.01 (unpaired t-test and ANOVA).](image1)

![Figure 6. Detection of NF-κB/p65 and IκBa expression. A, Western blot analysis of NF-κB/p65 expression in the nucleus. B, Immunofluorescence images for nuclear translocation of activated NF-κB/p65 subunit. C, Western blot analysis of IκBa expression. Exposure to Aβ induced a significant increase of NF-κB/p65 protein level in the nucleus and markedly increased nuclear NF-κB/p65 staining. In addition, the protein level of IκBa was decreased in Aβ-stimulated cells. SRT1720 significantly abrogated the effect of Aβ on NF-κB/p65 expression or nuclear translocation, and IκBa expression. Silencing SIRT1 expression by siRNA blocked the inhibitory effect of SRT1720 on Aβ-induced NF-κB/p65 expression and activation, but it also significantly increased the protein level of IκBa. Relative protein expression was quantified by calculating the mean gray density value. Green fluorescence indicates the location of the p65 subunit; nuclei are stained with DAPI (blue). Sr: cells treated with SRT1720; Si: cells transfected with SIRT1 siRNA; Ni: cells transfected with negative control siRNA; TBP: TATA binding protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. *P<0.05, **P<0.01 (unpaired t-test and ANOVA).](image2)
Aβ on localization of ezrin (Figure 4). This result implied that Aβ stimulation could destroy the well-developed polarization of normal RPE monolayers, but it was independent of SIRT1 inhibition. Although diffuse immune staining of ezrin has been reported in atrophic and hyperplastic RPE cells of patients (20), the essential factor that controls localization of ezrin or polarization of RPE is largely unknown.

Western blot analysis of NF-κB and IkBa as well as immunofluorescence of NF-κB/p65 suggested that SIRT1720 protected RPE cells from the Aβ-mediated breakdown of barrier integrity via its activation of SIRT1 (Figure 6). SIRT1 then suppressed NF-κB activation (16,17), which is required for transcriptional regulation of IL-6, IL-8, and MMP-9.

Therefore, we conclude that SIRT1 activation attenuated Aβ-induced inflammation by suppressing NF-κB activation, the transcription of which regulates expression of IL-6, IL-8, and MMP-9 (Figure 7A). The prototypical NF-κB complex, including a heterodimer of p50 and p65 subunits, is chiefly sequestered in the cytoplasm through its association with IkBa (32). The I kappa B kinase (IKK) complex, including two catalytic subunits, IKKa and IKKβ, as well as a regulatory subunit IKKγ (NF-κB essential modulator), lies at the confluence of the different NF-κB signaling cascades: Toll-like receptors, T-cell receptor, or ataxia telangiectasia mutated protein. Stimulus-induced phosphorylation of the IKK complex mediates IkBa activation or phosphorylation, this response in turn triggers the rapid ubiquitination and subsequent degradation of IkBa, and the newly liberated NF-κB heterodimer then rapidly translocates into the nucleus (33). Then, p300/CREB-mediated RelA acetylation increases transcriptional activity of NF-κB (33), which then transcriptionally regulates genes encoding cytokines and MMPs. However, acetylated forms of RelA are deacetylated through specific interaction with SIRT1 (17). Deacetylation of RelA promotes its effective binding to IkBα and leads to IkBα-dependent nuclear export of NF-κB (33); therefore, SIRT1 could attenuate NF-κB activation-mediated inflammation via deacetylation of RelA/p65. On the one hand, Aβ could bind Toll-like receptor 4 (30) to activate the classical NF-κB signaling pathway; and, on the other hand, Aβ could increase NF-κB activation via inhibition of SIRT1 (Figure 7A). Then, NF-κB activation induced by Aβ transcriptionally regulates IL-6, IL-8, and MMP-9 (34,35). In general, Aβ, detected in drusen of AMD (3-5), may trigger inflammatory responses in the RPE/choroidal layers of the eye for the following reasons (Figure 7B): 1) The retina is an immune-privileged site where inflammatory responses are suppressed, but opening of epithelial barriers by MMP-9 may be a mechanism that allows passage of plasma proteins and inflammatory cells into this privileged compartment. 2) The aqueous humor of AMD patients contains higher concentrations of IL-6 and IL-8 (15), which play pivotal roles in NK cell and neutrophil recruitment. It has been demonstrated that the two kinds of immune cells could accelerate tissue damage via release of ROS or directly

Figure 7. Schematic illustration of the effect of OAβ on the NF-κB/SIRT1 pathway in cells and the role of OAβ in the chronic inflammation of AMD in tissue. A. Aβ induces NF-κB signaling via inhibition of SIRT1. 1: Binding of Aβ to an extracellular receptor, such as TLR4, leads to phosphorylation of IKK complex subunits (NEMO, IKKα and IKKβ), which subsequently phosphorylate IkBα and lead to its proteosomal degradation. This then releases NF-κB into the nucleus. 2: Newly liberated NF-κB can be acetylated for full-transcriptional activation. However, the acetylated NF-κB can also be deacetylated by SIRT1. This action of SIRT1 promotes IkBα binding and nuclear export of the deacetylated NF-κB complex, which terminates the NF-κB response and replenishes the cytoplasmic pool of latent NF-κB/IkBα complexes. B. Aβ-induced expression of IL-8, IL-6 and MMP-9 contributes to chronic inflammation in age-related macular degeneration. Figure adapted from Refs. 11,33,38.
inducing apoptosis of cells (36,37). 3) Continued presence (sometimes over many years) of proinflammatory factors and immune cells in the retina may cause chronic inflammation. Together with these observations, our present results demonstrated that Aβ-induced inflammation and RPE barrier disruption were regulated by the SIRT1/NF-κB pathway. Maintenance of barrier integrity in the RPE by blocking the action of Aβ or by activation of SIRT1 may thus represent a new approach to the treatment of AMD.

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References

1. Buschini E, Piras A, Nuzzi R, Vercelli A. Age related macular degeneration and drusen: neuroinflammation in the retina. Prog Neurobiol 2011; 95: 14-25, doi: 10.1016/j.pneurobio.2011.05.011.

2. Johnson LV, Forest DL, Banna CD, Radeke CM, Maloney MA, Hu J, et al. Cell culture model that mimics drusen formation and triggers complement association associated with age-related macular degeneration. Proc Natl Acad Sci U S A 2011; 108: 18277-18282, doi: 10.1073/pnas.1109703108.

3. Luibl V, Isaas JM, Kayed R, Glabe CG, Langen R, Chen J. Drusen deposits associated with aging and age-related macular degeneration contain nonfibrillar amyloid oligomers. J Clin Invest 2006; 116: 378-385, doi: 10.1172/JCI25843.

4. Mullins RF, Russell SR, Anderson DH, Hageman GS. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. FASEB J 2000; 14: 835-846.

5. Isaas JM, Luibl V, Johnson LV, Kayed R, Wetzel R, Glabe CG, et al. Soluble and mature amyloid fibrils in drusen deposits. Invest Ophthalmol Vis Sci 2010; 51: 1304-1310, doi: 10.1167/iovs.09-4027.

6. Jo DH, Kim JH, Kim JH. How to overcome retinal neuropathy: the fight against angiogenesis-related blindness. Arch Pharm Res 2010; 33: 1557-1565, doi: 10.1007/s12272-010-0107-6.

7. Cunha-Vaz J, Bernardes R, Lobo C. Blood-retinal barrier. Eur J Ophthalmol 2011; 21 (Suppl 6): S3-S9, doi: 10.5301/ejjo.2010.6049.

8. Bailey TA, Kanuga N, Romero IA, Greenwood J, Luthert PJ, Cheetham ME. Oxidative stress affects the junctional integrity of retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2004; 45: 675-684, doi: 10.1167/iovs.03-0351.

9. Peng S, Gan G, Rao VS, Adelman RA, Rizzolo LJ. Effects of proinflammatory cytokines on the claudin-19 rich tight junctions of human retinal pigment epithelium. Invest Ophthalmol Vis Sci 2012; 53: 5016-5028, doi: 10.1167/iovs.11-8311.

10. Bruban J, Glotin AL, Dinet V, Chalour N, Sennlaub F, Jonet L, et al. Amyloid-beta-(1-42) alters structure and function of retinal pigmented epithelial cells. Aging Cell 2009; 8: 162-177, doi: 10.1111/j.1474-9726.2009.00456.x.

11. Cao L, Wang H, Wang F. Amyloid-beta-induced matrix metalloproteinase-9 secretion is associated with retinal pigment epithelial barrier disruption. Int J Mol Med 2013; 31: 1105-1112.

12. Johnson LV, Leitner WP, Rivest AJ, Staples MK, Radeke MJ, Anderson DH. The Alzheimer’s A beta-peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration. Proc Natl Acad Sci U S A 2002; 99: 11830-11835, doi: 10.1073/pnas.192203399.

13. Wang J, Ohno-Matsui K, Yoshiida T, Kojima A, Shimada N, Nakahama K, et al. Altered function of factor I caused by amyloid beta: implication for pathogenesis of age-related macular degeneration from Drusen. J Immunol 2008; 181: 712-720.

14. Kurji KH, Cui JZ, Lin T, Hariman D, Prasad SS, Kojic L, et al. Microarray analysis identifies changes in inflammatory gene expression in response to amyloid-beta stimulation of cultured human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2010; 51: 1151-1163, doi: 10.1167/iovs.09-3622.

15. Jonas JB, Tao Y, Neumaier M, Findeisen P. Cytokine concentration in aqueous humour of eyes with exudative age-related macular degeneration. Acta Ophthalmol 2012; 90: e381-e388, doi: 10.1111/j.1755-3768.2012.02414.x.

16. Chen L, Fischle W, Verdin E, Greene WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science 2001; 293: 1653-1657, doi: 10.1126/science.1062374.

17. Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, et al. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J 2004, 23: 2369-2380, doi: 10.1038/emboj.2004.245.

18. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, et al. Small molecule activators of sirtuins extend lifespan. Science 2003; 300: 191-196, doi: 10.1038/nature001960.

19. Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, et al. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science 2003; 300: 486-489, doi: 10.1126/science.1079469.

20. Kivela T, Jaaskelainen J, Vaheri A, Carpen O, Ezrin, a membrane-organizing protein, as a polarization marker of the retinal pigment epithelium in vertebrates. Cell Tissue Res 2000; 301: 217-223, doi: 10.1007/s004410000225.

21. Villalta JM, Alcain FJ. Sirtuin activators and inhibitors. Biofactors 2012; 38: 349-359, doi: 10.1002/biof.1032.

22. Feng S, Cen J, Huang Y, Shen H, Yao L, Wang Y, et al. Matrix metalloproteinase-2 and -9 secreted by leukemic cells increase the permeability of blood-brain barrier by disrupting tight junction proteins. PLoS One 2011; 6: e20599, doi: 10.1371/journal.pone.0020599.

23. Martins T, Baptista S, Goncalves J, Leal E, Milhazes N, Borges F, et al. Methamphetamine transiently increases the

www.bjournal.com.br
blood-brain barrier permeability in the hippocampus: role of tight junction proteins and matrix metalloproteinase-9. *Brain Res* 2011; 1411: 28-40.

24. Bauer AT, Burgers HF, Rabie T, Marti HH. Matrix metalloproteinase-9 mediates hypoxia-induced vascular leakage in the brain via tight junction rearrangement. *J Cereb Blood Flow Metab* 2010; 30: 837-848, doi: 10.1038/jcbfm.2009.248.

25. Nakamaru Y, Vuppussetty C, Wada H, Milne JC, Ito M, Rossios C, et al. A protein deacetylase SIRT1 is a negative regulator of metalloproteinase-9. *FASEB J* 2009; 23: 2810-2819, doi: 10.1096/fj.08-125468.

26. Li F, Gong Q, Dong H, Shi J. Resveratrol, a neuroprotective supplement for Alzheimer’s disease. *Curr Pharm Des* 2012; 18: 27-33, doi: 10.2174/138161212798919076.

27. Canevari L, Abramov AY, Duchen MR. Toxicity of amyloid beta peptide: tales of calcium, mitochondria, and oxidative stress. *Neurochem Res* 2004; 29: 637-650, doi: 10.1023/B:NERE.0000014834.06405.af.

28. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004; 303: 2011-2015, doi: 10.1126/science.1094637.

29. Feng X, Liang N, Zhu D, Gao Q, Peng L, Dong H, et al. Resveratrol inhibits beta-amyloid-induced neuronal apoptosis through regulation of SIRT1-ROCK1 signaling pathway. *PLoS One* 2013; 8: e59888, doi: 10.1371/journal.pone.0059888.

30. Capiralla H, Vingtdeux V, Zhao H, Sankowski R, Al-Abed Y, Davies P, et al. Resveratrol mitigates lipopolysaccharide- and Abeta-mediated microglial inflammation by inhibiting the TLR4/NF-kappaB/STAT signaling cascade. *J Neurochem* 2012; 120: 461-472, doi: 10.1111/j.1471-4159.2011.07594.x.

31. Kook SY, Hong HS, Moon M, Ha CM, Chang S, Mook-Jung I. Abeta(1-42)-RAGE interaction disrupts tight junctions of the blood-brain barrier via Ca(2+)-calcineurin signaling. *J Neurosci* 2012; 32: 8845-8854, doi: 10.1523/JNEUROSCI.6102-11.2012.

32. Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 1996; 14: 649-683, doi: 10.1146/annurev.immunol.14.1.649.

33. Chen LF, Greene WC. Regulation of distinct biological activities of the NF-kappaB transcription factor complex by acetylation. *J Mol Med* 2003; 81: 549-557, doi: 10.1007/s00109-003-0469-0.

34. Park SY, Jin ML, Kim YH, Kim Y, Lee SJ. Anti-inflammatory effects of aromatic-turmerone through blocking of NF-kappaB, JNK, and p38 MAPK signaling pathways in amyloid beta-stimulated microglia. *Int Immunopharmacol* 2012; 14: 13-20, doi: 10.1016/j.intimp.2012.06.003.

35. Walker DG, Lue LF, Beach TG. Gene expression profiling of amyloid beta peptide-stimulated human post-mortem brain microglia. *Neurobiol Aging* 2001; 22: 957-966, doi: 10.1016/S0197-4580(01)00306-2.

36. Nathan CF. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *J Clin Invest* 1987; 80: 1550-1560, doi: 10.1172/JCI113241.

37. Lowin B, Peitsch MC, Tschopp J. Perforin and granzymes: crucial effector molecules in cytolytic T lymphocyte and natural killer cell-mediated cytotoxicity. *Curr Top Microbiol Immunol* 1995; 198: 1-24, doi: 10.1007/978-3-642-79414-8_1.

38. Tilstra JS, Clauson CL, Niedermoher LF, Robbins PD. NF-kappaB in aging and disease. *Aging Dis* 2011; 2: 449-465.