SirT1 and STAT3 protect retinal pigmented epithelium cells against oxidative stress

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Abstract. It has been previously demonstrated that there are interactions between sirtuin 1 (SirT1) and signal transducer and activator of transcription 3 (STAT3), which have versatile roles in various microenvironments. However, whether or not there is crosstalk between these two molecules during oxidative stress, and what mechanism of crosstalk occurs in retinal pigmented epithelium cells (RPEs), the protection of which may delay the process of age-related macular degeneration (AMD), has required further elucidation. The present study aimed to investigate the interactions between SirT1 and STAT3 in RPEs, following exposure to oxidative stress. The rates of proliferation and apoptosis, levels of intracellular reactive oxygen species and cell senescence of RPEs, induced by oxidants [H₂O₂ and oxidized low density lipoprotein (oxLDL)], were evaluated. The results revealed a downregulation of SirT1 expression, and an upregulation of STAT3 expression during oxidative stress. Further investigation indicated that SirT1 protected RPEs from oxidative stress-induced damage. Furthermore, gain- and loss-of-function experiments indicated that SirT1 had negative effects on the regulation of STAT3 expression in RPEs during oxidative stress. Notably, STAT3 directly protected the cells from oxidative stress, rather than depending on SirT1. Additionally, the protective effects of STAT3 had no association with the modulation of cell senescence during oxidative stress. In conclusion, SirT1 had negative effects on the regulation of STAT3 expression during oxidative stress. However, SirT1 and STAT3 demonstrated protective roles against oxidative stress in RPEs. These results therefore suggested that there was an equilibrium mechanism between SirT1 and STAT3 against oxidative stress, meaning that an equilibrium mechanism is required to be considered when combined application of STAT3 and SirT1 were performed to treat AMD.

Introduction

Age-related macular degeneration (AMD) is the most prevalent cause of central vision loss amongst elderly individuals (1,2). It has been reported that smoking cigarettes is the greatest environmental risk factor for the development of AMD (3). The mechanism underlying the effect of smoking on the incidence and severity of AMD remains to be elucidated. However, it is clear that cigarettes contain a large number of oxidants (4). The Age-Related Eye Disease Study Group demonstrated that antioxidants are able to reduce the risk of individuals proceeding to advanced AMD (5). Furthermore, proteomic analysis identified numerous proteins in the drusen, which developed as a result of oxidative damage (6). It was therefore hypothesized that oxidative stress may be involved in AMD pathogenesis, and that oxidative injury contributes to the pathogenesis of AMD (1,7). According to postmortem analyses, patients with AMD exhibit extensive free radical expression and damage to the proteins, lipids, DNA and mitochondria of retinal pigmented epithelial cells (RPEs) (8), further supporting this hypothesis. RPEs are a single layer of epithelial cells, which are located between the light-sensing photoreceptor cells and the choriocapillaris. Damage to the RPEs is known to be an early event in AMD (9). Oxidative stress is implicated in the development of age-associated RPE cell degeneration, dysfunction and loss (10,11). Therefore the elucidation of effective methods for protecting RPEs from oxidative stress in order to delay the onset and/or progression of AMD is required (12).

Cells have certain protective strategies, which minimize the effects of oxidative damage. The most well-documented of these defense mechanisms is the production of endogenous antioxidants, including superoxide dismutases (SODs), carotenoids and vitamins (7). Additionally, specific cytoprotective proteins participate in combating oxidative stress, for example sirtuin 1 (SirT1) and signal transducer and activator of transcription 3 (STAT3). SirT1, a class III protein deacetylase, is a longevity factor and an aldehyde dehydrogenase-dependent protein deacetylase, the activity of which has regulatory
effects on cellular stressors, including genotoxic, oxidative and proteotoxic stress (13,14). It has been reported that moderate overexpression of SirT1 protects the mouse cardiac muscle against oxidative stress and attenuates the development of age-associated cardiomyopathy involving hypertrophy (15). SirT1 is also able to inhibit certain transcription factors, which regulate cellular redox balance. For example, inhibition of the transactivation capacity of nuclear factor-like 2 (Nrf2) in HepG2 cells (16). However the detailed mechanisms underlying the function of SirT1 against oxidative stress, particularly in RPEs, remain to be elucidated. STAT3, one of the seven STATs, is regulated by a signaling regulator, comprising interleukin (IL)-6 and IL-10 family members, and has multiple functions in mediating cell survival, apoptosis, migration and differentiation (17). Regarding oxidative stress, previous studies have demonstrated the crosstalk between STAT3 and oxidative metabolism and its involvement in cancer and heart disease (18,19). Based on the known roles of SirT1 and STAT3 in oxidative stress, it was hypothesized that there is crosstalk between the two molecules during oxidative stress. Interactions between SirT1 and STAT3, which have versatile roles in various microenvironments, had previously been identified (20,21). However, whether or not there was crosstalk between the two molecules during oxidative stress in RPEs and what the mechanism of crosstalk was, remained to be elucidated.

In the present study, the proliferation, apoptosis, intracellular ROS and senescence of RPEs induced by oxidants, as well as the expression of SirT1 and STAT3 during oxidative stress were evaluated. Subsequently, the effects of SirT1 on proliferation, apoptosis, intracellular ROS and senescence of RPEs during oxidative stress was examined. Finally, the SirT1/STAT3 interaction in RPEs upon exposure to oxidative stress was investigated.

Materials and methods

Cell culture. The ARPE-19 human RPE cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA), 100 µg/ml streptomycin and 100 U/ml penicillin (Harbin Pharmaceutical Group Co., Ltd., Harbin, China) in 5% CO₂ at 37°C. The cells were passaged approximately every 3 days and subcultured at ~90% confluence.

Cell proliferation and apoptosis assay. ARPE-19 cells were grown to 80% confluence, at ~2000 cells/well in 96-well plates. Cells were subsequently treated with oxidized low density lipoprotein (oxLDL; 200 µg/ml; Kalen Biomedical, Montgomery Village, MD, USA) or H₂O₂ (50 µM) for 24 h. Proliferation and apoptosis were assessed using a Cell Titer 96® AQueous One Solution reagent (Promega Corporation, Madison, WI, USA) and an Annexin V-fluorescein isothiocyanate apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, 1-5x10⁴ cells were resuspended in 0.5 ml binding buffer and incubated with annexin V-fluorescein isothiocyanate and propidium iodide for 10 min in the dark at room temperature. A FACScan flow cytometer (BD Biosciences) equipped with a FITC signal detector FL1 (excitation 488 nm) and a phycoerythrin emission signal detector FL3 (excitation 585 nm) was used to analyze cellular apoptosis. The results were calculated using the CellQuestTM Pro software (BD Biosciences) and expressed as the percentage of apoptotic cells from the total cells.

Analysis of intracellular reactive oxygen species (ROS) levels. Intracellular ROS levels were measured using an intracellular ROS assay kit (Cell Biolabs, San Diego, CA, USA). Briefly, ARPE-19 cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in the culture medium for 30 min at 37°C. Subsequently, the pre-loaded cells were treated with oxLDL (200 µg/ml) or H₂O₂ (50 µM) for 24 h or as described in the figure legends. DCFH fluorescence of the cell lysate was observed by using fluorescence microscope (OLYMPUS, Japan). Images were captured using a Digital SLR camera (Canon, Beijing, China). The fluorescence intensity of each reaction mixture was determined and quantified using Image J software v1.4 (National Institutes of Health, Bethesda, MA, USA).

Cell senescence assay. Cell senescence was measured using a cellular senescence assay kit associated β-gal (SA-β-gal) assay (Cell Biolabs, San Diego, CA, USA), assay according to the manufacturer's instructions. Briefly, ARPE-19 cells were cultured to ~2000 cells/well in 96-well plates, and then exposed to oxLDL (200 µg/ml) or H₂O₂ (50 µM) for 24 h in a 37°C incubator. Following washing and collection, cells were treated with freshly prepared fluorometric substrate for 2 h at 37°C in the dark. The fluorescence intensity of each reaction mixture was determined and quantified using Image J software (NIH). The average fluorescence intensity was analyzed from five fields for each treatment.

Cell treatments. Cells were incubated with H₂O₂ (50 µM) for 24 h. Subsequently, H₂O₂ was detoxified by the addition of catalase (100,000 µl; Worthington Biochemical Corp., Lakewood, NJ, USA) at the end of the incubation (22). oxLDL (200 µg/ml) was added to other samples to induce pathological stress in ARPE-19 cells following 24 h treatment (23). To further evaluate the effects of SirT1, resveratrol (RSV; 10 mM) and nicotinamide (NA; 5 mM) (Sigma-Aldrich) were applied to incubated cells prior to oxidant treatments (24). The pRC/CMV-ST3 and pcRC/CMV expression vectors (STAT3OV and C-STAT3OV) were acquired from Promega Corporation and used to evaluate the effects of STAT3 overexpression. SirT1 and STAT3 knockdown were performed using small interfering (si)RNA for SirT1 (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and short hairpin (sh)RNA STAT3/Puro (STAT3KO; Sigma-Aldrich) lentiviral vectors, respectively. shRNAi Mission Non-Target shRNA Control/Puro (Sigma-Aldrich) was used as a negative control of gene knockdown (C-SirT1KO and C-STAT3KO). Cell transfection was performed using Lipofectamine® 2000 reagent (Invitrogen Life Technologies) according to the manufacturer's instructions.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using RNeasy kits (Qiagen, Valencia, CA, USA) and first-strand complementary DNA was synthesized using...
SuperScript III (Invitrogen Life Technologies). RT-qPCR was performed on an ABI 7500 (Applied Biosystems Life Technologies, Foster City, CA, USA) with a SYBR Premix Ex Taq™ kit (Takara Bio, Inc., Otsu, Japan). GAPDH was used as the internal control. Data were normalized using the 2^−∆∆Ct method for relative quantification as shown in (E) SirT1 and (F) STAT3. Values were expressed as the mean ± standard deviation of three independent experiments. Analysis of variance was performed to analyze the differences statistically. *P<0.05, **P<0.01 vs. control. OxLDL, oxidized low density lipoprotein; RPEs, retinal pigmented epithelium cells; ROS, reactive oxygen species; mRNA, messenger RNA; SirT1, sirtuin 1; STAT3, signal transducer and activator of transcription 3.

Western blot analysis. Cell lysates were acquired from the collected cells cell lysis reagent (Promega, Madison, WI, USA), containing Complete Mini protease inhibitor cocktail (Roche). The protein concentration was determined by using Bradford Reagent (Bio-Rad) and the protein samples were subjected to 10% SDS-PAGE. The proteins were subsequently transferred onto nitrocellulose membranes and the membranes were blocked with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% Tween-20 (TBST), containing 5% non-fat milk or 5% bovine serum albumin (Sigma-Aldrich) for 30 min. The membranes were subsequently incubated with rabbit monoclonal primary antibodies against STAT3 (1:500; Minneapolis, MI, USA), phosphospecific STAT3 (Tyr705) (1:500; Cell Signaling Technology, Inc., Danvers, MA, USA), SirT1 (1:500; Sigma-Aldrich) and mouse β-actin (1:1,000; Sigma-Aldrich) overnight at 4°C. Subsequently, goat anti-mouse immunoglobulin G (IgG) or goat anti-rabbit IgG (Pierce Biotechnology, Inc., Thermo Fisher Scientific, Rockford, IL, USA) were utilized to visualize the results, which were detected using enhanced chemiluminescence detection systems (Super Signal West Femto; Pierce Biotechnology, Inc.).

Statistical analysis. Values are presented as the mean ± standard deviation. Student's t-test was used to analyze differences between two groups. The experimental results were analyzed with one-way analysis of variance to compare the differences between three or more groups. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were conducted using SPSS 17.0 software (SPSS, Chicago, IL, USA). All experiments were performed in triplicate.
Results

Oxidative stress induces RPE damage and dysregulation of SirT1 and STAT3. In order to mimic the oxidative damage that occurs in AMD, H₂O₂ and oxLDL have been previously employed as experimental oxidants, with which to induce pathological stresses in RPEs (25,26). Following treatment with H₂O₂ (50 µM) or oxLDL (200 µg/ml) for 24 h, the proliferation, apoptosis, intracellular ROS and senescence of ARPE-19 cells were analyzed. It was revealed that H₂O₂ and oxLDL significantly elevated cellular proliferation (Fig. 1A; P=0.020 and P=0.026, respectively) and significantly enhanced the apoptotic rate (Fig. 1B; P=0.013 and P=0.003, respectively), compared with that of the control group. Due to the association of an accumulation of intracellular ROS with AMD (27), the intracellular ROS levels were also evaluated. H₂O₂ and oxLDL significantly increased the intracellular ROS levels in RPEs (P=0.040 and P=0.016, respectively), compared with those of the control group (Fig. 1C). Cellular senescence has a critical function in contributing to AMD, and the biological ageing process is associated with an increase in senescence (25). The cellular senescence of RPEs induced by H₂O₂ or oxLDL was evaluated using an SA-β-gal assay. As indicated in Fig. 1D, oxLDL and H₂O₂ promoted cell senescence, compared with the control group, with a significant difference following treatment with H₂O₂ (P=0.045).

To analyze the effects of SirT1 and STAT3 on oxidative stress, the expression of SirT1 and STAT3 in RPEs was assessed. Notably, the expression of SirT1 mRNA was found to be downregulated in RPEs treated with H₂O₂ and oxLDL, when compared with that in the control group (P=0.005 and P=0.039, respectively). Conversely, the expression of STAT3 mRNA was demonstrated to be upregulated (P=0.012 and P=0.020, respectively), as shown in Fig. 1E and F. These results demonstrated that oxidative stress induced RPE damage and resulted in the dysregulation of SirT1 and STAT3 mRNA expression.

SirT1 influences the damage of RPEs induced by oxidative stress. In order to evaluate the effects of SirT1 in oxidative stress, RPEs were pretreated with the SirT1 inhibitor, NA, or the SirT1 activator, RSV, prior to treatment with H₂O₂ (50 µM) or oxLDL (200 µg/ml) for 24 h. The proliferation, apoptosis, intracellular ROS and senescence of RPEs were subsequently evaluated. NA enhanced the effects of H₂O₂, markedly decreasing the proliferation rate (P=0.049), stimulating apoptosis (P=0.028), as well as increasing intracellular ROS and senescence in RPEs. Conversely, RSV was able to resist the effects of H₂O₂.

To examine whether SirT1 had analogous effects on RPEs treated with oxLDL, identical experiments were performed following oxLDL treatment. Furthermore, the SirT1 activator, RSV, exerted opposite effects on the regulation of RPEs, when compared with the effects of NA, as indicated in Fig. 2.

SirT1 negatively influences the expression of STAT3 during oxidative stress. In the present study, an overexpression of STAT3, which was involved in STAT3 activation during oxidative stress in RPEs, was identified. In order to better understand
the protective roles of SirT1 in RPEs during oxidative stress, the association between STAT3 activation and SirT1 required elucidation. Differences in the expression of STAT3 following SirT1 knockdown, or overexpression in RPEs during oxidative stress were evaluated. As shown in Fig. 3A, the SirT1 activator, RSV, significantly decreased the expression of STAT3 (P=0.048), while a significant increase in STAT3 expression was detected in RPEs following SirT1 knockdown (P=0.025), compared with that in the control, during H₂O₂ treatment. Furthermore, SirT1 knockdown was also found to promote the expression of STAT3 protein and phosphorylated STAT3 protein during oxidative stress (Fig. 3B), whereas RSV and SirT1 knockdown did not influence the expression of STAT3 in the absence of oxidative stress (Fig. 3A and B). The results presented in Fig. 3B demonstrate the efficiency of SirT1 knockdown. To further investigate the association between STAT3 and SirT1, the expression of SirT1 following STAT3 knockdown (STAT3KO group) or overexpression (STAT3OV group) was evaluated in RPEs during oxidative stress. The expression of STAT3 protein was determined in each group by western blot analysis, as shown in Fig. 3C. Notably, overexpression of STAT3 induced a decrease in the expression of SirT1, when compared with that in the C-STAT3OV group (P=0.022), during oxidative stress. The expression of SirT1 was significantly enhanced in the STAT3KO group (Fig. 3D; P=0.015). These results indicated that STAT3 exerts negative effects on the expression of SirT1 in RPEs during oxidative stress.

**Discussion**

Oxidative stress is considered an important contributing factor to the development of AMD, and has been implicated in the pathophysiology of AMD (1,7). The protection of RPEs against oxidative damage presents a potential therapeutic strategy.
for AMD (12). H$_2$O$_2$ has been extensively used to mimic oxidative stress in vitro. H$_2$O$_2$ and oxLDL were therefore added to cultured RPEs in order to mimic oxidation-induced AMD injury (26,27,29). However, the detailed mechanisms underlying the injuries induced by H$_2$O$_2$ and oxLDL, and a comparison between the effects of H$_2$O$_2$ and oxLDL, which were used as oxidants to mimic oxidative stress in RPEs, remained to be elucidated. In the present study, a model to test oxidative stress susceptibility and antioxidant efficiency in RPEs was constructed. It was found that two oxidants (H$_2$O$_2$ and oxLDL) induced damage to RPEs, including attenuation of proliferation, increased apoptosis, increased intracellular ROS and promotion of cell senescence.

SirT1 exerts multiple, pleiotropic effects in oxidative metabolism (30-32). In addition to post-translational modifications induced by oxidative stress (33), SirT1 has been demonstrated to protect cells against oxidative stress via the upregulation of antioxidant genes and the modulation of Nrf2 (16). SirT1 expression and activity levels are decreased during lung inflammation caused by oxidative stress, and in human endothelial cells and macrophages, following exposure to oxidants (34,35). However, whether SirT1 levels remain decreased in RPEs under oxidative stress required experimental investigation. In the present study, it was shown that H$_2$O$_2$ and oxLDL decreased SirT1 levels in RPEs. STAT3, which was activated at a basal level in the RPEs, was upregulated and activated following oxidative stress-induced RPE injury (36,37). The results of the present study were in accordance with those of previous studies (36,37). Dysregulation of SirT1 and STAT3 implied that the two molecules may have important roles in the modulation of oxidative stress in RPEs.

It has previously been reported that moderate overexpression of SirT1 may have protective roles in the heart, following oxidative stress-induced injury (15). In addition, SirT1 was previously reported to exert significant protective effects against ocular diseases in animal models, including cataracts, retinal degeneration, optic neuritis and uveitis (38). Regarding the protective roles of SirT1 in RPEs, it has been demonstrated that SirT1 may be a significant regulator in the protection of RPEs against ultraviolet B-induced injury, and may abrogate amyloid-β-induced inflammation (39,40). In the present study, SirT1 was demonstrated to significantly attenuate the effects induced by oxidants. These results indicated that SirT1 may provide protection against oxidative stress in animal models and RPEs.

Berner et al (22) reported negative regulation of STAT3 protein-mediated cellular respiration by SirT1 in murine embryonic fibroblast cells (21), and a previous study provided further support for these results (41). As previously demonstrated, pSTAT3 stimulated expression of the STAT3 gene [unphosphorylated STAT3 (U-STAT3)], which exerted persistent activity (42). The STAT3 gene is driven by pSTAT3 (phosphorylated STAT3) (1). pSTAT3 lead to dramatically increased U-STAT3 (Unphosphorylated STAT3) which persists for many days. Nevertheless pSTAT3 is sustained for a short period of time (2,3). In the present study, the effects of pSTAT3 on SirT1 were not investigated; instead, the expression levels of total STAT3 mRNA, which reflected the expression of U-STAT3 and pSTAT3, were analyzed. The level of total STAT3 may be in response to the degree of STAT3 activation. The use of gain- and loss-of-function experiments revealed that SirT1 exerted negative effects on the regulation of STAT3 expression during oxidative stress in RPEs (Fig. 3).

STAT3 is an essential mediator of Toll-like receptor 3-regulated protection of RPEs against oxidative stress (20). STAT3 was shown to be a directly protective regulator in the present study, and the protective effects of SirT1 against oxidative stress in RPEs were also demonstrated. It is therefore hypothesized that SirT1 and STAT3 may exert synergistic effects against oxidative stress. SirT1 attenuated STAT3 activation according to the results of a previous study (43), and those of the present study. The present study examined how SirT1 regu-
lated STAT3, in order to protect RPEs against oxidative stress. The results of the present study revealed that the protective effects of STAT3 were increased in the presence of the SirT1 inhibitor NA; although, no alterations in cellular senescence were detected. These results suggested that SirT1 had crucial roles in protection against oxidative stress in the presence of SirT1 activator, while STAT3 was an essential protective regulator when the effect of SirT1 was inhibited. There may therefore be an equilibratory mechanism between SirT1 and STAT3. Alcendor et al (15) demonstrated that moderate (7.5-fold) overexpression of SirT1 attenuated age-dependent increases in oxidative stress. By contrast, high level (12.5-fold) overexpression of SirT1 resulted in increased levels of apoptosis and hypertrophy and decreased cardiac function. These observations indicated that there may be a negative regulator to resist the effects of SirT1. The elucidation of whether or not high levels of SirT1 result in destruction of the equilibrium mechanism requires further investigation.

In conclusion, SirT1 exerted negative effects on the regulation of STAT3 expression during oxidative stress. However, SirT1 and STAT3 each had protective roles against oxidative stress in RPEs. The results suggested that there may be an equilibratory mechanism between SirT1 and STAT3 against oxidative stress. This suggested that SIRT1 and STAT3 may be promising drugs for the treatment or prevention of AMD. However, there is equilibrium mechanism which must be considered when combined application of SIRT1 and STAT3 were performed.

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