Substance P promotes the recovery of oxidative stress-damaged retinal pigmented epithelial cells by modulating Akt/GSK-3β signaling

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Purpose: Senescence of the retina causes an accumulation of reactive oxygen species (ROS). Oxidative stress associated with ROS can damage RPE cells, leading to neovascularization and severe ocular disorders, including age-related macular degeneration (AMD). Thus, the early treatment of the damage caused by oxidative stress is critical for preventing the development of ocular diseases such as AMD. In this study, we examined the role of substance P (SP) in the recovery of RPE cells damaged by oxidative stress.

Methods: To induce oxidative stress, RPE cells were treated with H2O2 at various doses. Recovery from oxidative stress was studied following treatment with SP by analyzing cell viability, cell proliferation, cell apoptosis, and Akt/glycogen synthase kinase (GSK)-3β activation in RPE cells in vitro.

Results: H2O2 treatment reduced cellular viability in a dose-dependent manner. SP inhibited the reduction of cell viability due to H2O2 and caused increased cell proliferation and decreased cell apoptosis. Cell survival under oxidative stress requires the activation of Akt signaling that enables cells to resist oxidative stress-induced damage. SP treatment activated Akt/GSK-3β signaling in RPE cells, which were damaged due to oxidative stress, and the inhibition of Akt signaling in SP-treated RPE cells prevented SP-induced recovery. Pretreatment with the neurokinin 1 receptor (NK1R) antagonist reduced the recovery effect of SP on damaged RPE cells.

Conclusions: SP can protect RPE cells from oxidant-induced cell death by activating Akt/GSK-3β signaling via NK1R. This study suggests the possibility of SP as a treatment for oxidative stress-related diseases.

RPE cells form a monolayer that performs important functions as a compact barrier between photoreceptors and the choroid, a nutrient supplier of photoreceptors, and a disposer of shed photoreceptor outer segments by phagocytosis [1,2]. In diseases such as age-related macular degeneration (AMD) or retinitis pigmentosa, excessive oxidative stress occurs, resulting in the accumulation of reactive oxygen species (ROS), causing damage to RPE cells [3,4]. If RPE cells are damaged, choroidal neovascularization or inflammation occurs and induces whole retinal degeneration and potential vision loss. Therefore, protection and regeneration of the RPE cells under oxidative stress are essential for the prevention of retinal disease development.

To treat injured RPE cells in the clinic, anti-inflammatory agents or inhibitors of vascularization have been administered, but their undesirable effects have limited their use [5-7]. Transplantation of mesenchymal stem cells (MSCs) was revealed to delay ocular disease progression [8-11]. In addition, transplantation of RPE cells into the vitreous has been attempted to repair damaged RPE cells [12], but the efficacy was less than expected because of the poor attachment of the RPE layer.

To remove the causative factor of RPE cellular damage, elimination of oxidative stress was considered. This was anticipated to halt the damage of RPE cells at the initial stage of disease onset [13-15]. However, because it is difficult to inhibit the generation of oxidative stress, damage due to oxidative stress is inevitable. Thus, upon damage to RPE cells, the enhancement of recovery is key to interrupting neovascularization and/or inflammation and consequently, the progression of retinal diseases such as AMD.

To respond to the harsh conditions associated with oxidative stress, cell survival signaling needs to be activated, to enable the cell to survive. The phosphoinositide 3-kinase (PI3K)/Akt pathway is a prosurvival pathway regulated by ROS. When oxidative stress is exerted on cells, Akt is
phosphorylated in a PI3K-dependent manner, inducing subsequent phosphorylation and consequent inactivation of proapoptotic factors, including glycogen synthase kinase (GSK)-3 [16,17]. Thus, the activation of the Akt pathway would be expected to be crucial for cellular survival under oxidative stress. However, activation of this survival signal can be maintained for only a short duration; constant stimulation of oxidative stress renders the survival signaling inactive, ultimately bringing about cell death.

Substance P (SP) is an 11-amino acid neuropeptide that preferentially binds to the neurokinin 1 receptor (NK1R) and is related to neuroinflammation, cell proliferation, anti-apoptosis, and wound healing [18-21]. In previous studies, SP was found to stimulate cell proliferation by activating the extracellular signal-regulated kinases 1 and 2 (ERK1/2) or Akt, and by translocating β-catenin to cell nuclei [19,22,23]. Given the known functions of SP, it was likely that SP would be capable of recovering the oxidative stress-damaged RPE cells, possibly by promoting cell proliferation and suppressing apoptosis through the activation of cell survival signaling.

To explore the potential recovery role of SP in RPE cells injured due to oxidative stress, ARPE-19 cells, a human retinal pigment epithelium cell line, were used. The cells were treated with H2O2 at various concentrations to cause oxidative damage. Subsequently, SP was added to the damaged ARPE-19 cells. The effect of SP was assessed by evaluating cell viability, cell proliferation, apoptosis, and Akt/GSK-3β signaling. To study whether the effect of SP was mediated by NK1R, an NK1R antagonist was introduced before SP treatment.

**METHODS**

*Materials:* SP, 5′-bromo-2′-deoxyuridine (BrdU), Triton X-100, phenylmethylsulfonyl fluoride (PMSF), Akt1/2 kinase inhibitor, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin/streptomycin, 0.25% trypsin–EDTA solution, and PBS (200 mg/l KCl, 200 mg/l KH2PO4, 8 g/l NaCl, 2.16 g/l Na2HPO4·7H2O, pH 7.0, Osmolality 271 – 299) were provided by Welgene (Daegu, Korea). Fetal bovine serum (FBS) and Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) were purchased from Gibco (Grand Island, NY). Anti-GAPDH antibody (Abcam, Cambridge, MA), CP-96345 (TOCRIS; Bristol, UK), and WST-1 (Roche; Indianapolis, IN) were used. Cell lysis buffer, anti-Akt antibody, anti-phospho-Akt antibody, anti-GSK-3β antibody, and anti-phospho-GSK-3β antibody were purchased from Cell Signaling Technology (Danvers, MA). ARPE-19 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA).

**Cell culture:** ARPE-19 cells (ATCC cell line, passages 23–25) were cultured in DMEM-F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO2. The medium was changed every alternate day. The ARPE-19 cells were verified by the ATCC cell line authentication service with short tandem repeat analysis using an amelogenin gender-determining locus (Promega, Madison, WI; Appendix 1).

**MTT assay:** A total of 2 × 104 cells were seeded in each well of a 48-well plate and incubated for 24 h. After serum starvation for 24 h, H2O2 was added for 24 h followed by 100 nM SP (final concentration) for 24 h with 500 nM CP-96345 (NK1R antagonist) added 30 min before treatment with SP. Next, 50 μl of MTT solution was added into each well, and the plate was incubated for 3 h at 37 °C with 5% CO2. The MTT solution was replaced with HCl/isopropyl alcohol to dissolve formazan. The optical density (OD) of the wells was measured at a wavelength of 540 nm.

**Preparation of cell extracts and western blot analysis:** At each time point after SP treatment, cells were rapidly washed with chilled 1× PBS and lysed with 1× lysis buffer. Cells were scraped, and supernatants were collected by centrifugation (rotor radius: 70 mm) at 13,500 ×g for 10 min. Protein concentrations of lysates were determined using bicinchoninic acid (BCA) reagents (Thermo Fisher; Rockford, IL). The lysates were denatured and electrophoresed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. Blocking of the membrane was performed with 5% skim milk or bovine serum albumin (BSA) for 1 h. After blocking, the membranes were incubated with primary anti-Akt, phospho-Akt, GSK-3β, phospho-GSK-3β, and GAPDH antibodies, followed by anti-immunoglobulin G (IgG) horseradish peroxidase-conjugated secondary antibody. The blots were visualized using chemiluminescence (GE Healthcare, Buckinghamshire, UK).

**TUNEL assay:** The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the instructions provided by the manufacturer (Roche; Indianapolis, IN). In brief, 3 × 104 ARPE-19 cells were cultured on glass coverslips. The cells were treated with H2O2 for 24 h. This was followed by treatment with SP for 24 h. The cells were washed twice with PBS and fixed with 3.7% formaldehyde for 20 min. After washing in PBS, the cells were incubated in 0.1% Triton X-100 for 20 min to permeabilize the cells. The coverslips were rinsed twice with PBS, and the TUNEL reaction mixture was incubated for 1 h at 37 °C. Coverslips were mounted using VECTASHIELD with
4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), and apoptotic cells were observed using the Nuance multiplex biomarker imaging system (Cambridge Research Instrumentation, Mornburn, MA).

BrdU incorporation: At 2 h before cell fixation, 10 μM BrdU was added into each well. To fix the cells, the medium was removed, and the cells were incubated with absolute methanol for 10 min. For DNA denaturation, fixed cells were incubated with 2 N HCl for 60 min at 37 °C and subsequently neutralized by adding 0.1 M borate buffer, pH 8.5. The cells were incubated with anti-BrdU antibody (Roche) for 60 min and with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Vector Laboratories) for 60 min. Cells were counterstained with propidium iodide (PI; Sigma). BrdU-incorporated cells were observed using the Nuance multiplex biomarker imaging system (Cambridge Research Instrumentation). Quantification was performed by counting the BrdU(+) cells among the total PI(+) cells and expressing them as percentages.

WST-1 assay: For the assay, 20 μl of WST-1 solution was added to each well at 10% the total volume of the medium, and the plate was incubated for 30 min at 37 °C in 5% CO₂. After incubation, the OD values for each well were measured at a wavelength of 450 nm using an enzyme-linked immunosorbent assay (ELISA; The EMax Endpoint ELISA Microplate Reader; Molecular Devices, Sunnyvale, CA) reader.

Statistical analysis: Data are presented as the mean ± standard deviation (SD) of three independent experiments. P values of less than 0.05 were considered statistically significant. Statistical analysis of all data was carried out by an unpaired, two-tailed Student t-test.

RESULTS

Oxidative stress induced cellular damage and reduced the viability of the ARPE-19 cells [24,25]. To induce oxidative stress in vitro, H₂O₂ has been widely used in a variety of cell types [26,27]. The cellular responses against H₂O₂ may differ, depending on the cell type. To determine the effect of H₂O₂ on ARPE-19 cells, H₂O₂ was treated at various concentrations, and then cellular morphology and viability were examined (Figure 1A). Figure 1B,C indicate the cellular morphology and viability of ARPE-19 cells with H₂O₂ for 24 h. A concentration of 100 μM H₂O₂ did not visibly affect cell viability. However, the 200 μM concentration resulted in decreased cellular activity, resulting in 65% of viable cells compared to the control. In addition, 300 μM and 400 μM H₂O₂ resulted in cell viability of 38% and 12%, respectively (Figure 1C). Treatment with H₂O₂ for 48 h further decreased cell viability, reaching a value of 66% at 100 μM, 50% at 200 μM, 23% at 300 μM, and 7.3% at 400 μM (Figure 1D,E).

These data demonstrated that H₂O₂ provoked cellular damage and decreased cell viability. Based on these data, the dose of H₂O₂ for the subsequent studies on oxidative stress was determined to be 200 μM.

SP increases cell viability by increasing cell proliferation and blocking cell death in ARPE-19 cells under oxidative stress: SP has been shown to stimulate cell proliferation and prevent cell apoptosis [27-30]. To evaluate the effect of SP on RPE cells under oxidative stress, ARPE-19 cells were treated with 200 μM H₂O₂ for 24 h, and SP was added. After 24 h, cell viability was assessed (Figure 2A). When H₂O₂ was added, cellular morphology was transformed, and a detached cell pool was also observed; these effects were slightly alleviated with the SP treatment (Figure 2B). H₂O₂ treatment reduced cell viability to approximately 52% compared to that of the untreated control, whereas SP treatment maintained cell viability at 80% compared to that of the untreated control (Figure 2C). On addition of a higher dose of H₂O₂ (400 μM), SP treatment induced the recovery of cell viability; however, the effect was minimal (Appendix 2). SP-mediated signaling is preferential via NK1R, stimulates cell proliferation and migration, and prevents apoptosis [13,25]. To investigate whether the increase in the viability of the ARPE-19 cells with the SP treatment was mediated by NK1R, the cells were pretreated with CP (NK1R antagonist), following which the effect of SP was evaluated. Figure 2C shows that although SP augmented the viability of ARPE-19 cells under oxidative stress, this effect was suppressed by the CP treatment.

Next, after confirming the recovery effect of SP on damaged ARPE-19 cells, we measured changes in cell proliferation and apoptosis after the SP treatment in the presence of H₂O₂. The effect of SP on oxidative stress-induced cell death was evaluated with the TUNEL assay. Figure 3A reveals that H₂O₂ significantly elevated cell apoptosis, while the SP treatment inhibited this cell death. Meanwhile, the CP treatment reversed the effect of SP on cell apoptosis. To detect the effect of SP on cell proliferation, the BrdU incorporation assay was performed. As shown in Figure 3B, the cell proliferation pool decreased in the presence of oxidative stress. The pool of proliferating cells was increased after SP treatment, and this SP effect disappeared upon CP treatment, as predicted.

Collectively, these data suggest that SP is capable of rescuing ARPE-19 cells from oxidative damage, which might be attributed to the increase in cell proliferation and the decrease in apoptosis of ARPE-19 cells. In addition, the effects of SP on ARPE-19 cells were mediated via NK1R.
SP improves cell survival by activating Akt signaling: It is well-known that PI3K/Akt signaling is associated with cell proliferation and antiapoptotic effects [31]. Notably, cell survival under oxidative stress requires PI3K/Akt signaling [32]. Previous studies have revealed that SP binds to the NK1 receptor, the signaling of which is related to survival [33-35]. To examine whether the increase in ARPE-19 cell viability by SP was accompanied by activation of Akt signaling, cells were treated with H$_2$O$_2$ for 24 h, and SP was then added for 5, 10, and 15 min. The activation of Akt in ARPE-19 cells was then investigated. Figure 4A demonstrates that cells treated with H$_2$O$_2$ failed to maintain phosphorylated Akt, whereas SP treatment promoted phosphorylation of Akt, resulting in the peak at 15 min in the treatment. Additionally, GSK-3β, a downstream molecule of Akt signaling and a proapoptotic molecule, was also examined. Phosphorylation of GSK-3β results in an inactive state that lacks an apoptotic effect. Consistent with the phosphorylation of Akt, GSK-3β was obviously phosphorylated, relative to the H$_2$O$_2$-only treated cells, with SP treatment for 15 min. The expression levels of phospho-Akt and phospho-GSK-3β were quantified relative to the levels of total Akt and GSK-3β and are represented as a fold difference in comparison to the untreated control (Figure 4B,C).

To check whether the effect of SP on Akt/GSK-3β phosphorylation was mediated via NK1R, CP was used as a pretreatment, and SP was then added. After 15 min, the levels of phosphorylated Akt/GSK-3β in the ARPE-19 cells were determined. SP caused phosphorylation of Akt and GSK-3β under oxidative stress conditions, but CP treatment suppressed SP-induced Akt/GSK-3β phosphorylation. Phosphorylation of Akt and GSK-3β was expressed as a fold difference, compared to H$_2$O$_2$-only treated cells (Figure 5A–C).
Next, we investigated whether the increased viability of ARPE-19 cells by SP is diminished when Akt activation is inhibited. To inhibit activation of Akt, cells were pretreated with A6730 (Akt1/2 kinase inhibitor) before SP was added. The effect of SP was then evaluated. As shown in Figure 5D, H₂O₂-induced reduction in cell survival was reversed by SP treatment, but treatment with A6730 almost eliminated the effect of SP. Taken together, these results demonstrate that SP can activate Akt/GSK-3β signaling via NK1R binding, which contributes to SP-induced recovery of ARPE-19 cells damaged due to H₂O₂.

**DISCUSSION**

To block progression of retinal diseases, including AMD, various therapies have been used, including verteporfin photodynamic therapy, stem cell transplantation, laser photocoagulation treatment, and steroid injection. However, their effects were equivocal, and conventional treatments have side effects [36-40]. Therefore, an advanced therapy is needed. Above all, improvements in cell survival under oxidative stress can be considered an early treatment option.

In the current study, oxidative stress was induced by treating ARPE-19 cells with H₂O₂. H₂O₂ treatment caused a reduction in the survival of ARPE-19 cells that was mitigated by SP treatment. The recovery of damaged ARPE-19 cells by SP was accompanied by enhanced cell proliferation and decreased apoptosis. To survive under oxidative stress, a cell requires the activation of survival signaling that includes Akt. Activation of PI3K/Akt signaling has been shown to provoke antiapoptotic effects against oxidative stress-induced damage in various cell types [41-43]. The inhibition of Akt signaling can promote H₂O₂-induced cell death. That is, the Akt signaling pathway serves as a protective mechanism to prevent oxidant-mediated RPE cell death. However, persistent or severe stress ultimately fails to activate Akt signaling, leading to cell death. A previous study demonstrated that short-term H₂O₂ treatment provoked transient activation of Akt signaling in RPE cells within 3 h, which might be a survival signal [32]; however, our data showed that H₂O₂ treatment for 24 h could not sustain activated Akt signaling in ARPE-19 cells, and cell viability subsequently decreased. In contrast, SP treatment caused activation of Akt signaling within 15 min after the SP was added, which led to the inactivating phosphorylation of GSK-3β, a proapoptotic signal. Therefore, blockage of Akt phosphorylation by the Akt1/2 kinase inhibitor prevented the SP-induced recovery of the damaged ARPE-19 cells. That is, SP-activated Akt signaling might contribute to the accelerated recovery of RPE cells under oxidative stress. In addition, the effects of SP on the
Figure 3. SP increases cell viability by inhibiting apoptosis and promoting the proliferation of RPE cells damaged by oxidative stress. RPE cells were damaged by H$_2$O$_2$, and then treated with substance P (SP) for 24 h. The effects of SP on cell apoptosis and proliferation were evaluated. A: The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to quantify the number of apoptotic RPE cells. B: The 5′-bromo-2′-deoxyuridine (BrdU) incorporation assay was performed to determine the proliferating cell pool. Representative images for BrdU-incorporated RPE cells. Yellow arrow: BrdU (+) cells. C: BrdU (+) cells were quantified by counting BrdU (+) cells from the total PI (+) cells and expressing them as a percentage. Scale bar: 100 μm. P values of less than 0.05 were considered statistically significant (*p<0.05, **p<0.01, ***p<0.001). The data are expressed as the mean ± standard deviation (SD) of three independent experiments.

Figure 4. SP activates the Akt/GSK-3β signaling in RPE cells under oxidative stress. RPE cells were treated with 200 μm H$_2$O$_2$ for 24 h, and substance P (SP) was added for 5, 10, and 15 min. A: The levels of phospho-Akt and phospho-glycogen synthase kinase (GSK)-3β were detected with western blotting. B, C: Phospho-Akt and phospho-GSK-3β protein expression levels, relative to the total Akt and GSK-3β, were quantified using the Image J program. The expression level was represented relative to that of the untreated control. P values of less than 0.05 were considered statistically significant (*p<0.05, **p<0.01, ***p<0.001). The data are expressed as the mean ± standard deviation (SD) of three independent experiments.
activation of signaling molecules and cell survival were mediated via NK1R. Furthermore, ERK1/2 activation can be associated with cell proliferation under oxidative stress. When SP was used to treat damaged ARPE-19 cells, activation of ERK1/2 was also detected, but its effect was less than that of SP on Akt/GSK-3β (Appendix 3).

In conclusion, this study demonstrated that SP could stimulate the recovery of RPE cells under oxidative stress, possibly by promoting cell proliferation and inhibiting apoptosis through the activation of Akt/GSK-3β signaling. As we treated severely damaged ARPE-19 cells with SP, it remains unclear whether SP can affect early responses immediately after ROS accumulation. However, the therapeutic effect of SP on injured RPE cells was adequately shown in this study. Further studies on the effects of SP on oxidative stress–induced ocular disease will be undertaken to examine the mechanism of action of SP in vivo.

APPENDIX 1. STR ANALYSIS.
To access the data, click or select the words “Appendix 1.”

APPENDIX 2. THE EFFECT OF SP ON RPE UNDER OXIDATIVE STRESS.
To access the data, click or select the words “Appendix 2.”

APPENDIX 3. THE EFFECT OF SP ON ERK ACTIVATION.
To access the data, click or select the words “Appendix 3.”

ACKNOWLEDGMENTS
This study was supported by a Korean Health Technology R&D Project grant (HI13C1479) from the Ministry of Health and Welfare (Sejong, Republic of Korea) and by the Bio & Medical Technology Development Program (NRF-2012M3A9C6050499) of the National Research Foundation.

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