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Protection of retinal pigment epithelium by OT-551 and its metabolite TEMPOL-H against light-induced damage in rats

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Abstract

OT-551 (1-hydroxy-4-cyclopropanecarboxyloxy-2,2,6,6-tetramethylpiperidine hydrochloride), is a novel small molecule with antioxidant and anti-inflammatory effects. Protective efficacy of OT-551 and its metabolite TEMPOL-H (TP-H) against light-induced degeneration of retinal pigment epithelium (RPE) was tested. Albino rats were intraperitoneally injected with OT-551, TP-H, or water approximately 30 min prior to a 6 h exposure to 2700 lux white fluorescent light. Retinal protection was evaluated histologically by counting the RPE cell nuclei and measuring the extent of RPE damage on the retinal sections as RPE damage index (%). Following light exposure, the number of RPE cell nuclei was significantly reduced in light-exposed eyes from water-treated animals, while the number was not significantly different between light-exposed and unexposed eyes from the animals treated with 100 mg/kg TP-H or with any dose of OT-551 in the inferior hemisphere, and with 100 mg/kg OT-551 in the superior hemisphere. RPE damage index was significantly lower in the animals treated with any dose of OT-551 compared to water-treated animals both in the inferior and superior hemispheres. Systemic administration of OT-551 and TP-H provides RPE cell protection against acute light damage, and the protection by OT-551 was greater than by TP-H.

Keywords

retinal light damage; retinal pigment epithelium (RPE); OT-551; TEMPOL-H

Acute light exposure causes photoreceptor and retinal pigment epithelial (RPE) cell damage (Noell et al., 1966; Tanito et al., 2005). Oxidative stress is likely to be involved in the pathogenesis of light-induced retinal damage, since the damage can be reduced by various

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types of antioxidants (Organisciak et al., 1985, 1992; Donovan et al., 2001; Tanito et al., 2002a, 2002b; Kaldi et al., 2003; Ranchon et al., 2003).

TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), a stable nitroxide free radical, and its diamagnetic hydroxylamine form TEMPOL-H (TP-H; 1,4-dihydroxy-2,2,6,6-tetramethylpiperidine) catalyze the dismutation of superoxide to H₂O₂ plus O₂ [superoxide dismutase (SOD)-like activity] and the oxidation of Fe²⁺ to Fe³⁺ (ferroxidase activity) (Mitchell et al., 1990). More recently several studies have shown that TEMPOL also demonstrates potent anti-inflammatory activity (Cuzzocrea et al., 2004). OT-551 (1-hydroxy-4-cyclopropanecarbonyloxy-2,2,6,6-tetramethylpiperidine hydrochloride), a new chemical entity that has both antioxidant and anti-inflammatory activity, is converted to TP-H in the body by ocular and systemic esterases (information from Othera Pharmaceuticals Inc., W. Conshohocken, PA.). Previous studies have suggested that TEMPOL and TP-H prevents cataract formation *in vivo* (Sasaki et al., 1998) and *in vitro* (Zigler, Jr. et al., 2003) and retinal ganglion cell damage in iron-loaded optic nerve crush model *in vivo* (Thaler et al., 2010). Previously, we have shown greater outer nuclear layer (ONL) thickness, larger electroretinogram (ERG) b-wave amplitudes, and lower retinal levels of 4-hydroxynonenal and 4-hydroxyhexenal, non-enzymatic oxidation end-products of n-6 and n-3 polyunsaturated fatty acids, respectively, in OT-551- and TP-H-treated rats compared with vehicle –treated rats after the intense light exposure (Tanito et al., 2007a). Thus, both drugs have been shown to be protective against light-induced photoreceptor cell damage *in vivo*, and inhibition of lipid peroxidation formation is likely to be involved in their protective mechanism.

Recently, Zhou et al. (2008) reported that TP-H suppresses short wavelength light-induced photo-oxidative cell damage in lipofuscin fluorophore-laden RPE cells *in vitro*. However, the potential protective effect of OT-551 in RPE cells has not been tested *in vivo*. Therefore, we tested the efficacy of OT-551 and TP-H in RPE cell protection using an *in vivo* model of light-induced retinal degeneration.

All procedures were carried out according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of Oklahoma Health Sciences Center (OUHSC) Guidelines for Animals in Research. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the OUHSC and the Dean A. McGee Eye Institute. In this report, we re-evaluated the retinal histology obtained in our previous work (Tanito et al., 2007a) to measure the RPE cell damage. Accordingly, details of the experimental methods such as drug administration, light exposure regimen, and preparation of retinal histology were described previously (Tanito et al., 2007a). Both OT-551 and TP-H were provided by Othera Pharmaceuticals Inc. (W. Conshohocken, PA). Sprague-Dawley (SD) rats (6-7 weeks of age) kept under dim cyclic light (5 lux, 12 h on/off) were intraperitoneally injected with OT-551 (25, 50, or 100 mg/kg dissolved in sterile water at the concentration of 50 mg/ml), TP-H (100 mg/kg in sterile water at the concentration of 50 mg/ml), or an equivalent volume of sterile water 30 minutes before light exposure. Unanesthetized rats were exposed to 2700 lux diffuse, cool, white fluorescent light for 6 h in clear plastic cages with wire tops. During the light exposure, the right eye of each rat was covered with a black-painted polypropylene eye cup attached to the right face skin, and served as the non-light-damaged control (covered eye). The left eye of each rat was left uncovered and was considered the light-damaged eye (uncovered eye). The rats were returned to the dim cyclic light environment following light exposure and 5-7 days later the animals were euthanized, and the eyes were enucleated, fixed, and embedded in paraffin. Sections (5-μm thick) were taken along the vertical meridian to allow for comparison of all regions of the retina in the superior and inferior hemispheres, stained with hematoxylin-eosin (H&E), and digitized images of the entire retina were captured using a

VB-7010 digital imaging system (Keyence, Osaka, Japan) at 20x magnification with 4080 × 3072 pixels. To cover the entire retina, 12-14 images were obtained from each section.

After light exposure, a remarkable loss of ONL was observed in retinal sections of uncovered eyes from water-treated animals, while no apparent decrease of ONL thickness was observed in retinal sections of uncovered eyes from 100 mg/kg OT-551-treated animals and those of covered fellow eyes from water- or OT-551-treated animals (Figure 1A, upper panels), indicating that OT-551 inhibits the photo-oxidative stress-induced photoreceptor cell damage and that the administered doses of OT-551 do not cause the loss of photoreceptor cells, as reported previously (Tanito et al., 2007a). When observed in higher magnification (Figure 1A, lower panels), there was a remarkable loss of RPE cell nuclei in uncovered eyes from water-treated animals, often leaving cone photoreceptor cell nuclei (white arrows) and choroidal cell nuclei (black arrow heads) adjacent to each other, while virtually intact RPE cell nuclei (black arrows) were observed in sections of uncovered eyes from 100 mg/kg OT-551-treated animals and those of covered fellow eyes from water- or OT-551-treated animals (Figure 1A, lower panels), indicating the possible protection of OT-551 against photo-oxidative stress-induced damage in RPE cells. To quantify the protective effect of OT-551 and TP-H on RPE damage, the number of RPE cell nuclei was counted on digitized images opened using Photoshop software version 10 (Adobe systems, San Jose, CA) at 6 locations (0-1 mm, 1-2 mm, and 2-3 mm inferior or superior from the ONH) (Figure 1B). In water-treated animals, the number of RPE cell nuclei was significantly lower both in the inferior and the superior hemispheres from uncovered eyes compared to covered eyes, while the number was not significantly different between uncovered and covered eyes in the inferior hemisphere from the animals treated with 100 mg/kg TP-H and with any dose of OT-551, and in the superior hemisphere from the animals treated with 100mg/kg OT-551.

The extent of RPE damage was evaluated as the RPE damage index in each inferior and superior hemisphere. The digitized images of retinal sections were opened using Photoshop software version 10 and the length of damaged RPE was measured at 2 locations (0-3 mm inferior or superior from the ONH) (Figure 2A). Damaged RPE is defined as thinning or complete loss of the RPE layer. The RPE damage index was then calculated by dividing the length of damaged RPE by 3.0 mm and multiplying by 100. It is important to note that the RPE damage index was greater in the superior hemisphere than the inferior hemisphere; which correspond wells with the geography of simultaneous photoreceptor cell damage (Tanito et al., 2008) and with the collapse of the choroidal circulation several months after light stress (Tanito et al., 2007b). Thus, the RPE damage index is likely to reflect well the RPE cell/tissue damage induced by photo-oxidative stress. In the uncovered eyes, RPE damage index was significantly lower in the animals treated with any dose of OT-551 compared to water-treated animals both in inferior (Figure 2B, right panel) and superior (Figure 2B, left panel) hemispheres, and was significantly lower in the animals treated with 100 mg/kg OT-551 compared to 100 mg/kg TP-H-treated animals in the superior hemisphere (Figure 2B, right panel). In the covered eyes, RPE damage index was 0 % in any animal groups (data not shown). Collectively, the results clearly indicate that both drugs protect the morphology of the RPE cell layers from light-induced damage, and the protection is greater with OT-551 than TP-H (at equivalent doses). Reason for the greater protection with OT-551 than TP-H is currently unknown. The difference of drug bioavailability such as penetration of cellular/tissue membrane or drug clearance may explain the phenomenon, but needs to be clarified.

In conclusion, systemic administration of OT-551 and TP-H provides RPE cell protection against acute light stress.

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Figure 1. Effect of light stress on the survival of RPE cells

(A) Representative H&E stained retinal sections at 1 mm superior from the optic nerve head from covered and uncovered eyes of water- and 100 mg/kg OT551-treated animals. Images with lower (upper panels) and higher (lower panels) magnifications are shown. Black arrows, white arrows, and black arrowheads indicate RPE cell nuclei, cone cell nuclei, and choroidal cell nuclei, respectively. RPE, retinal pigment epithelium; ONL, outer nuclear layer; and INL, inner nuclear layer.

(B) The mean (+SD) number of RPE cell nuclei at 6 retinal locations are shown (n=9 rats for the 50 mg/kg OT-551 group and n=10 rats for all other groups). The *, **, and *** indicate $p<0.05$, $p<0.01$, and $p<0.001$, respectively, by paired t-test between covered and uncovered eyes in each animal groups.

Figure 2. RPE damage index

(A) Schematic diagram to explain the calculation of RPE damage index. Extent of RPE damage is measured in each inferior and superior hemisphere, and expressed as RPE damage index (%) in each hemisphere. ONH, optic nerve head.

(B) The mean (+SD) of RPE damage index of inferior (left panel) and superior (right panel) hemispheres in uncovered eyes is shown (n=9 rats for the 50 mg/kg OT-551 group and n=10 rats for all other group). The #, ##, and ### indicate $p<0.05$, $p<0.01$, and $p<0.001$, respectively, by 1-way ANOVA followed by Scheffe's posthoc test.