Cytoprotective Effects of Rebamipide and Carteolol Hydrochloride against Ultraviolet B–Induced Corneal Damage in Mice

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PURPOSE. To analyze whether rebamipide (REB) and carteolol hydrochloride (CH) protect against UVB-induced corneal damage in mice.

METHODS. BALB/c mice topically pretreated with REB (1 and 10 mM) or CH (1, 10, and 100 mM) were exposed to ultraviolet (UV) B light at 416 μW/cm². To evaluate corneal damage, mire irregularity was graded, and the haze index was estimated by using digitized corneal images. The formation of oxidized DNA in the corneal epithelium resulting from UVB exposure was estimated by using quantitative immunohistochemistry for 8-hydroxy-2-deoxyguanosine (8OHdG index). To analyze the mechanism of cytoprotection by REB and CH against UVB-induced cell damage, the UV absorption spectrum in these agents was evaluated by spectrophotometry, and their hydroxyl radical scavenging effect was evaluated by the electron spin resonance (ESR) spin trapping technique with Fenton system hydroxyl radical generation.

RESULTS. Seventy-two hours after UVB exposure, the severity of mire irregularity, haze index, and 8OHdG index were significantly lower in mice pretreated with 10 mM (P < 0.05, P < 0.05, and P < 0.01, respectively) of REB and in mice pretreated with 10 mM (P < 0.05, P < 0.01, and P < 0.01, respectively) and 100 mM (P < 0.01, P < 0.01, and P < 0.01, respectively) of CH compared with mice treated with vehicle. The absorption spectrum of REB overlapped with the UVB wavelength, and that of CH overlapped partially. The ESR spin signal corresponding to the hydroxyl radical was reduced by the addition of REB or CH.

CONCLUSIONS. REB and CH attenuate UVB-induced corneal damage, which may be partly responsible for their sunscreensing and hydroxyl radical scavenging effects. (Invest Ophthalmol Vis Sci. 2003;44:2980–2985) DOI:10.1167/iovs.02-1043

The mammalian cornea protects the interior ocular tissues by providing a robust physical barrier and by absorbing ultraviolet (UV) light in the range of 290 to 320 nm. When threshold levels of UV light absorption are exceeded, the corneal effects of UV light exposure may include photokeratitis, destruction of the epithelium, edema, and stromal opacity, and a number of biochemical changes, including DNA photodamage, protein cross-linking, enzyme inactivation, and the production of free-radical oxygen and related species such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Previous reports suggested that antioxidants are crucial in the host defense against UVB-induced cell damage in skin and cornea.

Rebamipide (REB; (2-(4-chlorobenzoylamino)-3-[2-(1H)-quinolinone-4-yl] propionic acid), is widely used as an antiaglar ulcer drug. Pretreatment with REB significantly inhibits gastric mucosal injury induced by the increase in free radicals after treatment with platelet-activating factor or diethylthiolumbarbamate, which chelates copper from superoxide dismutase. Using the electron spin resonance (ESR) spin trapping technique, Yoshikawa et al. found that REB scavenges hydroxyl radicals, and Naito et al. showed that the quinolinone structure was an important molecular structures associated with the hydroxyl radical scavenging capability. Carteolol hydrochloride (CH; (3-tert-butylamino-2-hydroxypropoxy)-3,4-dihydro-2-(1H)-quinolinone monohydrochloride), is a β-adrenergic blocker used to treat cardiac arrhythmia and myocardial hypokinesis and glaucoma/ocular hypertension. Although CH also has a quinolinone structure, the antioxidative effect of CH has not been studied.

The purpose of this study was to analyze whether REB and CH protect against UVB-induced corneal damage. To test this, mice pretreated with various concentrations of REB or CH were exposed to UVB light, and the severity of the corneal damage was evaluated, including the severity of mire irregularity, corneal haze, and the formation of oxidized DNA, 8-hydroxy-2-deoxyguanosine (8OHdG) in the cornea. To analyze the possible mechanism of cytoprotection by REB and CH against UVB-induced cell damage, the absorption spectrum of UV in these agents was evaluated by spectrophotometry, and their hydroxyl-radical–scavenging effect was evaluated by the ESR spin trapping technique.

MATERIALS AND METHODS

Animals

All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eight-week-old male BALB/c mice were obtained from Japan SLC (Shizuoka, Japan) and maintained in the colony room of our institution for 1 to 3 days before the experiments. The temperature in the colony room was maintained at 25°C, and the day-night cycle was 12 hours each (8 AM–8 PM; 8 PM–8 AM).

UVB Exposure

The corneas of the animals were exposed to UVB light according to the method of Downes et al., with slight modification. After deep anes-
thesis was exposed to intraperitoneal injection of pentobarbital, the right eye was exposed to 416 μW/cm² of UVB light (UVGL-58; UVP Inc., San Gabriel, CA) for 3 minutes. The wavelength of the light source peaked at 302 nm (range, 280–315 nm). The energy output was measured with a digital radiometer (UVX) with a 300-nm sensor (UVX-31; both from UVP, Inc.). One hour before and immediately before UVB exposure, 10 μL of 1 or 10 mM REB dissolved in 0.00001 N NaOH or 10 μL of 1, 10, or 100 mM CH dissolved in distilled water was applied topically to the right eye. In control mice, 0.00001 N NaOH or distilled water was applied 1 hour before and immediately before UVB exposure. REB and CH were provided by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan).

**Evaluation of Corneal Damage**

Seventy-two hours after UVB exposure, the digitized color images (1600 × 1200 pixels) of the mouse corneas were obtained with a dissection microscope (SZ-PT; Olympus, Tokyo, Japan) equipped with a digital imaging system (PDMC Ie; Olympus) at ×6 magnification. To obtain the mire image of the cornea, a ring-shaped light source (LG-PS2; Olympus) was attached to the dissection microscope, and the light was projected to the center of the cornea when the images were obtained. To evaluate corneal surface irregularity caused by UVB exposure, mire irregularity, which is thought to reflect corneal surface integrity, was classified into four grades: none (grade 0), mild (grade 1), moderate (grade 2), and severe (grade 3). This was judged by one author (MT), using a digitized color image. To evaluate the corneal opacity caused by UVB exposure, the following formula was used: haze index (%) = area of corneal opacity (pixels)/area of entire cornea (pixels) × 100.

Digitized color images described previously were opened on computer (Macintosh; Apple Computer, Cupertino, CA) in image-analysis software (Photoshop ver. 5.5 software: Adobe Systems, Inc., San Jose, CA). The contour lines of opacity and entire cornea were drawn manually, and the areas of opacity and the entire cornea were obtained by using a histogram command.

**Preparation of Corneal Tissue Sections**

After deep anesthesia was induced by intraperitoneal injection of pentobarbital, phosphate-buffered saline (PBS; pH, 7.4) first was perfused through the left cardiac ventricle followed by freshly prepared 4% paraformaldehyde containing 0.25% glutaraldehyde in PBS. The eyes were removed. All tissues were fixed in the same fixative as described previously for 12 hours at 4°C, embedded in paraffin, and cut into 4-μm sagittal sections through the center of the cornea. A 7-0 silk suture was placed as a landmark at the temporal side of the eye. Tissue sections were collected on glass slides.

**Quantification of 8OHdG Corneal Immunostaining**

Immunostaining for 8OHdG was performed with the alkaline-phosphatase (APAAP) technique. After the sections were deparaffinized and microwave in 10 mM citrate buffer (pH 6.0) at 1 hour. The stained sections were washed with 0.01 M of Tris buffer, pH 7.8, and the 8OHdG levels determined by an HPLC/electrochemical detector (ECD) method had been confirmed by Toyokuni et al. 13,14

The absorption spectrum of UV (range, 200–400 nm) was analyzed in 0.1 mM REB dissolved with 0.001 N NaOH and in 0.1 mM CH dissolved with distilled water, by using a UV-visible spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan).

**ESR Spin Trapping for Hydroxyl Radicals**

The hydroxy radical scavenging effects of REB and CH were analyzed by using a spin trapping technique. The spin trapping agent used in the experiments was 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; Sigma-Alrich, St. Louis, MO), which forms secondary radicals (spin adduct) with hydroxyl radicals. The Fenton system, containing diethylenetriaminepentaacetic acid (DETAPAC; Wako Pure Chemicals, Osaka, Japan), ferrous iron (Nakalai Tesque, Kyoto, Japan), and hydrogen peroxide (Nakalai Tesque), was used as a hydroxyl radical generating system. As previously described, 10.05 mM of ferrous iron, 0.125 mM of DETAPAC, the test sample, and 1.0 or 5.0 mM of DMPO were added to a 50 mM phosphate buffer solution (pH 7.8). The reaction was started by the addition of 1 mM hydrogen peroxide. An aliquot of the reaction mixture was then transferred into a flat cell, and ESR spectra were recorded by spectrometer (JES FR-50; JEOL, Tokyo, Japan). Measurements were performed under the following conditions: magnetic field, 341.0 ± 10.0 mT; power, 4.0 mW; frequency, 9420 MHz; modulation frequency, 100 kHz; modulation width, 0.25 mT; sweep time, 10 mT/min; time constant, 0.1 second; and received gain, ×100.

**Statistical Analysis**

The mean mire grade, the haze index, and the 8OHdG index were compared among groups by one-way analysis of variance followed by Dunnett post hoc tests. All statistical analyses were performed on computer (StatView, ver. 5.0; SAS, Cary, NC).

**RESULT**

**Effects of REB and CH on UVB-Induced Corneal Damage**

Seventy-two hours after UVB exposure, marked corneal damage (Fig. 1), that is—irregular mire and corneal haze—was observed in mice pretreated with 0.00001 N NaOH (Fig. 1B) and distilled water (Fig. 1E) compared with mice not exposed to UVB (Fig. 1A). In the mice pretreated with REB (Figs. 1C, 1D) and CH (Figs. 1F, 1G, 1H), corneal damage after UVB exposure was not severe compared with mice pretreated with 0.00001 N NaOH and distilled water. The mire grade and the haze index are summarized in Figs. 11 and 1J, respectively. Both the mire grade and the haze index were substantially lower in mice pretreated with REB or CH compared with the values in the mice pretreated with the vehicles.
Effect of REB and CH on Oxidized DNA Formation after UVB Exposure in the Corneal Epithelium

In UVB-unexposed mice, the nuclei of the corneal epithelial, stromal, and endothelial cells showed trace nuclear immunostaining of 8OHdG (Fig. 2A). Seventy-two hours after UVB exposure, the 8OHdG staining was remarkably enhanced in the epithelial cells and the stromal cells near the corneal surface in mice pretreated with 0.00001 N NaOH (Fig. 2B) and distilled water (data not shown). Because the 8OHdG staining was predominantly located in the corneal epithelium, the 8OHdG index in the corneal epithelial layer was compared among mice pretreated with REB (Fig. 2C) or CH (Fig. 2D). Seventy-two hours after UVB exposure, the 8OHdG index was significantly lower in mice pretreated with 10 mM REB ($P < 0.01$) than in mice treated with 0.00001 N NaOH and significantly lower in mice pretreated with 10 mM ($P < 0.01$) and 100 mM ($P < 0.01$) CH than in mice pretreated with distilled water (Fig. 2E).

Absorption Spectrum of UV in REB and CH

To test the sunscreening ability of REB and CH against the UV spectrum, we checked the absorption spectrum ranging from 200 to 400 nm. In REB, the peak wavelength of absorption occurred at 200, 226, and 324 nm, and the absorption spectrum overlapped with the UVB spectrum (280–315 nm; Fig. 3).
solid line). In CH, the peak wavelength of absorption occurred at 214 and 250 nm, and the absorption spectrum partially overlapped with the UVB spectrum (Fig. 3, dotted line).

**Effects of REB and CH on the ESR Signal of the DMPO-OH Spin Adduct**

In the sample with DMPO only, no ESR signal was seen (Fig. 4A). With the hydroxyl radical generation system, quartet ESR signal patterns were observed that were specific for the hydroxyl radical trapped by DMPO (Fig. 4B). Hyperfine splitting constants at nitrogen ($a_N$) and hydrogen ($a_H$) for the spin adduct (Fig. 4B) were analyzed with values for $a_N = 1.49$ mT and $a_H = 1.49$ mT, suggesting this component of the spectrum was assigned to the DMPO-OH adduct according to reported values. In the presence of REB (20 mM, Fig. 4C) or CH (1-50 mM, Figs. 4E–I), the ESR signal was attenuated. When the concentration of DMPO was increased, the ESR signal increased in the samples with REB (Fig. 4D) and CH (Fig. 4J) compared with the samples with a regular concentration of DMPO and REB (Fig. 4C) and with a regular concentration of DMPO and CH (Fig. 4G). This suggests that both REB and CH are direct scavengers of hydroxyl radicals, rather than inhibitors of the generation system of hydroxyl radicals.

**DISCUSSION**

Corneal exposure to UVB (416 μW/cm², 3 minutes) caused damage, such as corneal surface irregularity and opacity after 72 hours in mice pretreated with 0.00001 N NaOH and distilled water (Fig. 1), which agrees with a previous murine study. 8OHdG, a major DNA-base-modified product, is induced by hydroxyl radical, singlet oxygen, or photodynamic action and is an established marker of oxidative stress-induced DNA damage. Ahmed et al. reported that UVB exposure induces 8OHdG in human epidermis. In the present study, remarkable
upregulation of 8OHdG was observed in the corneal epithelium 72 hours after UVB exposure (Figs. 2A, 2E), suggesting that DNA damage also occurs in the cornea after UVB exposure. We showed that in mice topically pretreated with 10 mM REB and 10 and 100 mM CH, the severity of the mire irregularity (Fig. 1), the area of corneal opacity (Fig. 1), and formation of oxidized DNA (Fig. 2) were attenuated compared with that in mice treated with vehicle after UVB exposure, suggesting that these agents have a cytoprotective effect against UVB-induced corneal damage.

UVB is directly absorbed by cellular macromolecules, including DNA and protein, and causes DNA photodamage and mutagenesis. Sunscreens that absorb the UVB wavelength effectively inhibit cell or tissue damage resulting from UVB. The absorption spectrum of REB (peak wavelength of absorption, 200, 226, and 324 nm) overlapped with the UVB spectrum (280–315 nm) and that of CH (peak wavelength, 214 and 250 nm) overlapped partially (Fig. 3), suggesting that the sunscreen effect is a mechanism of inhibition of 8OHdG formation in mice treated with REB and CH. The difference in the absorption spectrum against UVB may be a reason for the lower 8OHdG index in mice treated with 10 mM REB compared with mice treated with 10 mM and 100 mM CH (Fig. 2E). Despite the difference in UVB absorption, the degree of attenuation of corneal damage after UVB provided by REB and CH was similar (Figs. 1I, 1J), suggesting another mechanism of cytoprotection by these agents.

An excessive level of UVB generates hydroxyl radicals in skin fibroblasts and generated hydroxyl radicals can react with DNA, proteins, and lipids. Oxidation of lipids can result in lipid peroxide, which persists much longer in the cell, may initiate radical chain reactions, and thus enhances the oxidative damage caused by UVB. Using the ESR spin trapping technique, we showed that CH and REB, reported to be scavengers of hydroxyl radicals, directly scavenge the hydroxyl radicals (Fig. 4), suggesting that scavenging of hydroxyl radicals is a mechanism of cytoprotection of REB and CH against UVB damage and that the hydroxyl radical is an important cause of UVB damage in the cornea. The 3,4 double-bond of 2(1H)-quinolinone is responsible for the hydroxyl radicals scavenging activity of REB. Because CH contains the same structure, the quinolinone structure may contribute to the hydroxyl radicals scavenging activity of CH.

Cell or tissue damage after UVB exposure might be mediated by the upstream products of hydroxyl radicals—that is, superoxide radicals and hydrogen peroxide. Therefore, the cytoprotective effect of REB and CH can be attributed to its effect on superoxide radicals and hydrogen peroxide. In fact, REB inhibits the luminol-dependent chemiluminescence of neutrophils activated by formyl-methionyl-leucyl-phenylalanine, the myeloperoxidase-hydrogen peroxide system. However, the reduction of cytochrome c by superoxide radicals in the xanthine-xanthine oxidase system was unaltered by REB. Seo et al. recently reported the cytoprotective effect of REB against oxidative stress-induced injury in pancreatic acinar cells. In their report, superoxide dismutase also effectively inhibited cell damage. The effect of REB or CH on superoxide radicals and hydrogen peroxide must be further analyzed.

In conclusion, topical application of REB and CH attenuated UVB-induced corneal damage in mice, which may be partly responsible for the sunscreens and hydroxyl-radical-scavenging effects of these agents. Because both REB and CH attenuate the UVB-induced corneal surface irregularity (mire grade) and the DNA oxidation in the corneal epithelial layer (8OHdG index), the protective effects of REB and CH against UVB-induced corneal damage are mainly mediated by their effects on the corneal epithelial cells. Shimmura et al. reported that hydroxyl radicals are generated by excimer laser.
photoablation and may cause corneal fibroblastic cell apoptosis; thus, oxygen radicals including hydroxyl radicals seem to be targets to prevent corneal diseases other than UV-induced keratopathy. Clinical experience with REB uncovered few adverse effects in humans, and topical CH₂, with a clinical dosage of 2% eye drops that corresponds to 61 mM of CH₂, is the drug of choice to manage glaucoma. These agents may be useful therapies to prevent corneal diseases related to UV exposure and oxidative stress.

References