In Vivo Quantitation of Peroxides in the Vitreous Humor by Fluorophotometry

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Purpose. To detect intravitreal peroxides in vivo by a new fluorophotometric method with a hydrogen peroxide (H₂O₂)-sensitive fluorescent dye.

Methods. The authors used a 2',7'-dichlorofluorescin (DCFH) assay to measure oxidative status in the rabbit vitreous. In the presence of H₂O₂ and lipid hydroperoxides, nonfluorescent DCFH in the vitreous is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF; excitation, 495 nm; emission, 520 nm) that is detectable by fluorophotometry. Reactions of DCFH with various concentrations of H₂O₂ were investigated in vitro and in vivo. An inhibitory effect of catalase also was monitored. Vitreous fluorophotometry with DCFH was performed immediately and at 3, 7, and 28 days after constant light exposure to the retina (1800 lux, 24 hours) as an oxidative stress.

Results. In vitro study revealed that H₂O₂ oxidized DCFH to DCF in a dose-dependent manner, ranging from 0.1 to 100 mmol/l in concentration. Catalase inhibited DCF production. Vitreous fluorophotometry demonstrated that H₂O₂ oxidized DCFH to DCF in vivo in a dose-dependent manner, ranging from 0.06 to 60 mmol/l in concentration. DCF production in the vitreous significantly increased immediately (P = 0.03) and at 3 days (P = 0.01) and 7 days (P = 0.01) after light exposure, and it returned to the pretreatment level by day 28.

Conclusions. The results suggest that this fluorophotometric method quantitatively can detect intravitreal peroxides in vivo. This method will be helpful to study the oxidative status in some experimental pathologic conditions. Invest Ophthalmol Vis Sci. 1996;37:1444-1450.
(O$_2^-$), in the retina. In addition, histochemical observations of H$_2$O$_2$ localization were reported by the cerium method. However, these methods were used in vitro and carry a possible risk for degradation of oxyradicals and their metabolites during extractive and analytic processes.

We used a 2',7'-dichlorofluorescin (DCFH) assay to measure H$_2$O$_2$ and lipid hydroperoxides directly in the vitreous humor in vivo. The DCFH assay was first described by Keston and Brandt. Briefly, nonfluorescent DCFH injected into the vitreous cavity is oxidized rapidly to highly fluorescent 2',7'-dichlorofluorescin (DCF) by H$_2$O$_2$ and lipid hydroperoxides. The fluorescence can be monitored in real time by fluorophotometry, because the excitation and emission spectra of DCF (excitation, 495 nm; emission, 520 nm) are similar to those of sodium fluorescein. In the current study, we developed a new method to quantify the level of intravitreal peroxides in vivo by fluorophotometry with an H$_2$O$_2$-sensitive fluorescent dye.

MATERIALS AND METHODS

Chemicals

The following materials were used in the study: 2',7'-DCFH diacetate (DCFH-DA) (Molecular Probe, Eugene, OR); 2',7'-DCF (Lambda, Grottenhofstr, Austria); catalase (Sigma Chemical, St. Louis, MO); and 30% H$_2$O$_2$ (Wako Chemical, Tokyo, Japan). The H$_2$O$_2$ concentration was determined by diluting the 30% stock solution with distilled water and measuring the absorbance at 230 nm using an extinction coefficient of 0.081 cm$^{-1}$ nM$^{-1}$.

DCFH-DA was dissolved in ethanol at a concentration of 5 mM and stored frozen as a stock solution. DCFH was prepared from DCFH-DA following the method described by Cathcart et al., with some modification. Briefly, a 0.5-ml volume of the stock solution was added to 2 ml of 0.01 N sodium hydroxide and allowed to stand at room temperature for 30 minutes. The hydrolysate, 0.5 ml, was neutralized with 12 ml of 5 mM sodium phosphate buffer, pH 7.2, and kept on ice in the dark until use. This solution was discarded each day after use.

In Vitro Studies

Calibration of the Fluorophotometric Technique. We used a scanning fluorophotometer (Fluorotron Master; Coherent, Palo Alto, CA) to measure oxidative products (DCF). To obtain a standard curve of fluorescence per concentration of DCF, the fluorescence of DCF in various concentrations (1 to 10,000 pmol/ml) in a cuvette was monitored on the fluorophotometer.

Reaction of DCFH With H$_2$O$_2$ In Vitro. To determine whether nonfluorescent DCFH reacts with H$_2$O$_2$ to form fluorescent DCF in a dose-dependent manner, DCFH was mixed with various concentrations of H$_2$O$_2$. A 0.5-ml volume of 40 mM DCFH solution was added to 8 ml of 40 mM Tris-HCl buffer, pH 7.4, including 0.1 to 100 mmol/l H$_2$O$_2$ in final concentration. Concentrations of each mixture were designed similarly to those used in the in vivo studies. Fluorophotometric readings were obtained 5 minutes after the mixture was made. Blank reactions (without H$_2$O$_2$) also were monitored and subtracted from reactions with H$_2$O$_2$. An inhibitory experiment was performed by adding 320 U/ml catalase in the incubation medium, including 10 mmol/l H$_2$O$_2$ (in final concentration) 5 minutes before the mixture to DCFH.

In Vivo Studies

Twenty-seven Dutch rabbits, each weighing between 2.5 and 2.8 kg, were used for the in vivo study. The animals were divided into three groups: group A (five eyes), to investigate the intravitreal kinetics of the dye; group B (16 eyes), to determine whether H$_2$O$_2$ actually can oxidize DCFH to DCF in the vitreous cavity; group C (31 eyes), to apply the method under the condition of oxidative stress through light exposure to the retina. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Before the preinjection scan, the rabbits were anesthetized with an intramuscular injection of ketamine (5 mg/kg) and xylazine (2 mg/kg). The pupils were maximally dilated with 10% phenylephrine hydrochloride and 1% cyclopentolate hydrochloride.

Intravitreal Kinetics of DCF (Group A). To estimate the intravitreal kinetics of nonfluorescent DCFH, DCF was substituted and injected into the vitreous, and its fluorescence was monitored for 6 hours because the molecular and chemical properties of DCF are similar to those of DCFH. At first, a preinjection scan was obtained to determine corneal, lens, and retinal autofluorescence. Subsequently, 0.1 ml of 40 mM DCF solution was injected by a 27-gauge needle inserted at the pars plana. Fluorophotometry was performed immediately, at 30 and 60 minutes, and at 3 and 6 hours after injection. The DCF concentration was calculated from the fluorescence using a standard calibration curve.

Reactions of DCFH With H$_2$O$_2$ In Vivo (Group B). After a preinjection scan was performed, 0.1 ml of 40 mM DCFH was injected into the rabbit vitreous. An initial scan was obtained 60 minutes after the injection to check a blank reaction (DCFH alone). Thereafter, intravitreal administration of 0.1 ml H$_2$O$_2$ in various concentrations (1, 10, 100, 1000 mmol/l) was performed. Subsequent scans were performed every 15
minutes for 60 minutes. For each concentration of H₂O₂, four eyes were used. DCF fluorescence in the vitreous was defined as the average fluorophotometric reading at 4 to 5 mm from the chorioretinal peak at 60 minutes after the H₂O₂ injection. The DCF concentration in the vitreous was calculated from the DCF fluorescence with a standard calibration curve.

**Constant Light Exposure to the Retina (Group C).** Fluorophotometry with DCFH after light exposure was designed to determine whether retinal irradiation by constant fluorescent light could increase intravitreal H₂O₂, lipid hydroperoxides, or both. Before the experiment, the rabbits were housed in cyclic illumination (12 hours of light, 12 hours of dark). The daytime illumination was 150 to 400 lux in front of the cages. The pupils were maximally dilated with 1% atropine sulfate with 10% phenylephrine hydrochloride, and the retina was exposed for 24 hours to constant white light emitted from a fluorescent tube. The head of the rabbit was fixed in a restrainer so that irradiation at the cornea could be maintained at approximately 1800 lux during the experiment. The cornea of the exposed eye was moistened continually with physiologic saline. Vitreous fluorophotometry was performed immediately (six eyes) and at 3 days (six eyes), 7 days (six eyes), and 28 days (three eyes) after exposure. For fluorophotometry, 0.1 ml of 40 μM DCFH was injected into the vitreous cavity after the preinjection scan. Measurement was made 60 minutes after the DCFH injection. Ten eyes of five other rabbits served as unexposed controls to examine a blank reaction (DCFH alone). Determinations of DCF fluorescence in the vitreous and calculation of DCF concentrations were performed as described previously. DCF production in exposed eyes was compared with that of unexposed control eyes using the Mann–Whitney test.

**RESULTS**

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**Calibration.** The intensity of fluorescence correlated well with the DCF concentration (r = 0.998). The linear dynamic range of DCF appeared to be from 1 to 10,000 pmol/ml by fluorophotometry (Fig. 1).

**Reaction of DCFH With H₂O₂ In Vitro.** Figure 2 shows the concentration-response curve of DCFH oxidation by H₂O₂. A blank reaction without H₂O₂, which means an auto-oxidation of DCFH, was always minimal and subtracted from those reactions with H₂O₂. The data demonstrated that H₂O₂ oxidized DCFH to fluorescent DCF in a dose-dependent manner, in the range of 0.1 to 100 mmol/l. The addition of 320 U/ml catalase to the incubation medium, which included 10 mmol/l H₂O₂ in final concentration, reduced H₂O₂-induced DCF formation from 140.8 ± 9.7 pmol/ml to 14.9 ± 2.3 pmol/ml (the data were obtained from five independent experiments and are expressed as the means ± SD). The inhibition rate was 89.4%.

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**Intravitreal Kinetics of DCF (Group A).** DCF uniformly diffused into the vitreous cavity within 60 min-
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FIGURE 3. Intravitreal concentration of dichlorofluorescein (DCF) obtained from vitreous fluorophotometry. (A) Preinjection scan, which demonstrates autofluorescence of the cornea, lens, and choroid-retina. (B) 0 minutes, (C) 30 minutes, (D) 60 minutes, (E) 3 hours, (F) 6 hours after DCF injection.

utes of injection, and its concentration was maintained for 6 hours (Fig. 3). The DCF concentration gradually declined, with a half-life of approximately 5.5 hours (Fig. 4). The elimination constant rate after 2 hours represented only approximately 10% of the peak.

Reactions of DCFH With H₂O₂ In Vivo (Group B). An initial scan, measured 60 minutes after administration of DCFH only (without H₂O₂), revealed a minimal increase in vitreal fluorescence. The addition of H₂O₂ resulted in DCFH oxidation to DCF in a dose-dependent manner, in the range of 1 to 1000 mmol/l in concentration (at the injection). The reaction appeared to reach completion at a concentration of 1000 mmol/l. Assuming that the volume of rabbit vitreous was 1.47 ml, the concentration of H₂O₂ in the vitreous cavity could be approximately 0.06 to 60 mmol/l. The range was almost similar to that found in the in vitro experiment (Fig. 5).

Constant Light Exposure to the Retina (Group C). A typical fluorophotometric scan registered 3 days after light exposure is presented in Figure 6. The preinjection scan (after light exposure and before DCFH injection) revealed normal autofluorescence of the cornea, lens, and choroid-retina (Fig. 6). The scan obtained 60 minutes after DCFH injection demonstrated increased DCF production in the posterior vitreous (Fig. 6). In untreated control eyes, vitreous fluorophotometry after DCFH injection revealed a minimal increase in DCF production (1.9 ± 1.1 pmol/ml). A significant increase in DCF production was detected immediately after the 24-hour light exposure (12.6 ± 10.1 pmol/ml; \( P = 0.034 \)) that peaked on day 3 (33.3 ± 11.8 pmol/ml; \( P = 0.011 \)) and remained elevated on day 7 (31.8 ± 20.6 pmol/ml; \( P = 0.011 \)). By day 28, the measurement returned to nearly the same level as in the untreated control eyes (2.3 ± 0.3 pmol/ml). There was no significant difference between eyes 28 days after treatment and control eyes (\( P = 0.396 \)) (Fig. 7).

DISCUSSION

In the current study, we used the DCFH fluorophotometric assay for in vivo quantitation of peroxides in the vitreous humor. The DCFH assay is based on the oxidation of nonfluorescent DCFH to highly fluorescent DCF by H₂O₂. Cathcart et al. reported that not only H₂O₂ but lipid hydroperoxides selectively oxidized DCFH to DCF and that nonreactive endoperoxides did not. Thus, the use of a DCFH assay provides the advantage of obtaining specificity for peroxides of biologic significance, and it can reduce false-negative and false-positive reactions for peroxides. Because the excitation (495 nm) and emission (520 nm) spectra of DCF are similar to those of sodium fluorescein, vitreous fluorophotometry enables real-time in vivo detection of DCF and may reduce the risk for possible degradation of oxyradicals and their metabolites during analytic procedures.

On calibration with the fluorophotometer, the fluorescence intensity correlated well with the DCF concentration. The dynamic range of DCF (1 to 10,000 pmol/ml) was similar to that of fluorescein sodium. These data showed that fluorophotometry

FIGURE 4. Clearance of dichlorofluorescein (DCF) from the vitreous. The data, which were obtained from five independent experiments, are expressed as the means ± SD.
FIGURE 5. Concentration-response curve of dichlorofluorescein (DCFH) oxidation by hydrogen peroxide (H2O2) in vivo. x-axis represents presumptive concentration of H2O2 in the vitreous cavity. The data, which were obtained from four independent experiments, are expressed as the means ± SD. DCF = dichlorofluorescein.

can measure DCF concentrations accurately in 1 to 10,000 pmol/ml. The kinetics and the clearance of DCFH in the vitreous are difficult to investigate by fluorophotometry because the dye is nonfluorescent. However, in vivo studies revealed that DCF spread uniformly within 60 minutes and that the elimination constant rate after 2 hours was no more than 10% of the peak. Because of the similarity in molecular structures and chemical characteristics between the two agents, intravitreal kinetics of DCFH is thought to be similar to that of DCF. It is suggested, therefore, that the outward movement of DCFH from the vitreous has little influence on vitreal concentration as long as the scan is performed within 2 hours of injection. In vitreous fluorophotometry, some artifacts affect vitreal fluorescence. The major one is the spread function of possibly an increased peak of retina and choroid.34,35 To minimize this artifact, we defined DCF fluorescence in the vitreous as the average fluorophotometric reading at 4 to 5 mm from the chorioretinal peak. In addition, biomicroscopic examination before each measurement confirmed the absence of cataract, which could attenuate vitreal fluorescence. Both in vitro and in vivo studies demonstrated that H2O2 oxidized DCFH to DCF in a dose-dependent manner. Furthermore, the addition of catalase suppressed the production of DCF in vitro. Based on these findings, we concluded that the fluorophotometric method with DCFH could yield reliable quantitative results.

Although the fluorescent dye was solubilized in ethanol, the solvent subsequently was diluted by sodium phosphate buffer to 0.8% at the injection. Assuming that the volume of rabbit vitreous was 1.47 ml, a presumptive concentration of ethanol in the vitreous cavity could be 0.05%.35 Therefore, the toxicity of ethanol to the ocular tissues could be negligible. Because this method requires an invasive procedure—intravitreal injection of the dye—minor compromise of the ocular tissue, such as inflammation and hemorrhage, may occur. These reactions may generate background oxyradicals and their metabolites, which can oxidize DCFH. However, in untreated control eyes, vitreous fluorophotometry after DCFH injection revealed minimal increase in DCF production. It is, therefore, suggested that the influence by the injection could be negligible regarding the oxidation of DCFH.

Vitreous fluorophotometry revealed a significant increase in DCF production immediately and 3 and 7 days after light exposure. There has been little study of the production of oxyradicals and their metabolites in the vitreous after light exposure to the retina. Constant light exposure to the retina reportedly increased the production of lipid hydroperoxides by peroxidation of rod outer segment polyunsaturated fatty acids.3 In addition, the acute light exposure over a 24-hour period may have caused severe retinal inflammation, resulting in a delayed maximal response at 3 days. Experimental uveitis has been reported to lead to retinal lipid peroxidation.7-9 The subsequent cell membrane damage may result in efflux of lipid hydroperoxides to the vitreous cavity. Moreover, rabbit vitreous contains a large quantity of fatty acids, including mod-

FIGURE 6. Fluorophotometric scans performed 3 days after light exposure. Preinjection scan demonstrates autofluorescence of the cornea, lens, and choroid-retina (A). Scan obtained 1 hour after the intravitreal injection of dichlorofluorescein (DCFH) (B). DCF = dichlorofluorescein.
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FIGURE 7. Dichlorofluorescin (DCF) formation in the vitreous after constant light exposure to the retina. All bars represent the means ± SD.

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In conclusion, we have studied a new method to detect the level of peroxides in the vitreous humor by vitreous fluorophotometry with an H2O2-sensitive dye. Because it is invasive and requires intravitreal injection before noninvasive measurement by vitreous fluorophotometry, it will be difficult to use this technique for clinical diagnostic purposes. However, it will be important as an experimental technique to study the oxidative status in the eye in some pathologic conditions.

Key Words
dichlorofluorescin, fluorophotometry, hydrogen peroxide, light exposure, oxyradical

References

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