Fluorophotometric Quantitation of Oxidative Stress in the Retina In Vivo

Taiji Takanashi, Yuichiro Ogura, Hogara Taguchi, Mototane Hashizoe, and Yoshihito Honda

**Purpose.** To establish a new fluorophotometric method to quantitate oxidative stress in the retina in vivo with a hydrogen peroxide (H₂O₂)-sensitive fluorescent dye.

**Methods.** For in vitro fluorophotometric study, nonfluorescent 2',7'-dichlorofluorescin (DCFH) was incubated with H₂O₂ (10 pM to 100 nM), and the production of fluorescent 2',7'-dichlorofluorescein (DCF) was measured with fluorophotometric analysis. The inhibitory effect of catalase was also examined. For in vivo fluorophotometric study, rabbit eyes received vitrectomy and were perfused with 5 μM 2',7'-dichlorofluorescein diacetate (DCF-DA) or 2',7'-dichlorofluorescein diacetate (DCFH-DA). For oxidative stress, 300 μM H₂O₂ was infused after perfusion of DCFH-DA. Fluorophotometric measurements of the chorioretinal peak were performed. The eyes were enucleated for fluorescent microscopic examination to determine the localization of DCF fluorescence.

**Results.** H₂O₂ converted DCFH to DCF in a dose-dependent manner, which was inhibited by catalase dose dependently. In vivo fluorophotometric study showed DCF-DA and DCFH-DA caused production of 2006 ± 274 picomole/ml (mean ± SD, n = 5) and 8.35 ± 1.11 picomole/ml (n = 5), respectively, in the chorioretinal peak. DCFH-DA with stimulation by H₂O₂ induced 30.7 ± 13.1 (n = 4) picomole/ml DCF. Fluorescent microscopy showed DCF production in the retina was significant in the eye treated with DCF-DA and minimal in the eye treated with DCFH-DA. Moderate DCF production in the nerve fiber layer was observed in the eye treated with DCFH-DA and H₂O₂.

**Conclusions.** This new fluorophotometric method with DCFH-DA may be useful in quantitatively evaluating oxidative stress in the retina in vivo. Invest Ophthalmol Vis Sci. 1997; 38:2721–2728.

Reactive oxygen species have been reportedly involved in the pathogenesis of various retinal diseases, such as phototoxic retinal damage, ischemic injury, malignant tumor, and retinopathy of prematurity. A widely used method of detecting reactive oxygen species has been to quantitate conjugated dienes or thiobarbituric acid substances, which indicates the production of lipid peroxides in the retina. Recently, staining with nitro blue tetrazolium and cerium has been used to analyze morphologically the production of free radicals in the retina. These methods can be applied only in vitro.

We used a 2',7'-dichlorofluorescin diacetate (DCFH-DA) method to determine the intracellular hydrogen peroxide in the retina in vivo. In brief, DCFH-DA (Fig. 1A) is diffused into the cell and is deacetylated by esterases inside the cell to nonfluorescent 2',7'-dichlorofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of hydrogen peroxide (H₂O₂) and peroxidase. The DCF-associated fluorescence in the retina could be monitored by ocular fluorophotometry, because the excitation and emission spectra are similar to those of sodium fluorescein.

We report here the usefulness of DCFH as an H₂O₂-sensitive fluorescent probe and demonstrate a

---

From the Department of Ophthalmology and Visual Sciences, Graduate School of Medicine, Kyoto University, Kyoto, Japan
Supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of the Japanese Government.
Submitted for publication October 28, 1996; revised April 7 and July 21, 1997; accepted July 23, 1997.
Propriety interest category: N.
Reprint requests: Yuichiro Ogura, Department of Ophthalmology, Nagoya City University Medical School, 1 Kawanami, Misashicho, Misashiku, Nagoya, Aichi 467, Japan.
new fluorophotometric method to assess quantitatively the oxidative stress in the retina in vivo.

MATERIALS AND METHODS

Reagents

The DCFH-DA was purchased from Molecular Probe (Eugene, OR). DCF and 2',7'-dichlorofluorescein diacetate (DCF-DA) were obtained from Lambda (Grottenhofstr, Austria). Catalase was purchased from Sigma Chemical (St. Louis, MO). Other chemicals were obtained from Wako Chemical (Tokyo, Japan). The DCF-DA and DCFH-DA were dissolved in ethanol for stock solutions at a concentration of 5 mM. The H_2O_2 concentration was determined by freshly diluting the 30% stock solution to 100-fold into distilled water and by measuring absorbance at 230 nm, using an extinction coefficient of 0.081 cm^{-1} mM^{-1}.

Characterization of DCF as a Fluorescent Probe

Excitation and emission spectra were determined for reagent DCF using a Shimadzu RF 5000 spectrofluorometer (Shimadzu, Kyoto, Japan). A 5 mM stock solution of reagent DCF was diluted in sodium phosphate buffer, pH 7.4, to a final concentration of 200 picomole/ml and placed in a 3-ml cuvette with a magnetic stirring flea at 37°C. To calibrate the output of a Fluorotron Master fluorophotometer (Coherent, Palo Alto, CA) into DCF concentration units, the fluorescence of 1 to 10,000 picomole/ml DCF was measured using a 3-ml cuvette.

Conversion of DCFH to DCF in Response to H_2O_2 In Vitro

DCFH was mixed with various concentrations of H_2O_2 to determine whether nonfluorescent DCFH reacts with H_2O_2 to form fluorescent DCF. DCFH was prepared using the methods described by Cathcart et al. and LeBel et al. Briefly, 0.5 ml of stock DCFH-DA was added to 2.0 ml of 0.01 N NaOH and allowed to stand at room temperature for 30 minutes. The deacetylated hydrolysate containing DCFH was then neutralized with 10 ml 25 mM sodium phosphate buffer, pH 7.4, and stored on ice. Next, 0.5 ml of this solution was mixed with 12 ml sodium phosphate buffer containing 19.2 μg/ml hematin, which has peroxidase activity. Then, 1 ml of the resulting solution was mixed with 1 ml of 10 mM phosphate buffer containing 19.2 μg/ml hematin, and stored on ice. The final concentrations of DCFH and hematin were 2.5 nanomole/ml and 1.2 μg/ml, respectively. Fluorophotometric measurement of DCF production was performed using a 3-ml cuvette. To examine the inhibitory effect of catalase, 0.32 to 3200 U/ml catalase supplemented the mixture of DCFH and hematin. One unit of catalase scavenges 1 micromole of H_2O_2 per minute at 25°C and pH 7.4. The resulting solution was mixed with 1 mM H_2O_2 for reaction for the same duration, followed by fluorophotometric measurements.
Quantitation of Oxidative Stress in Retina

Animals and Anesthesia

We used 14 pigmented rabbits, weighing 2.5 to 2.8 kg each. The animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Operative procedures were performed after animals were anesthetized with intramuscular ketamine (5 mg/kg) and xylazine (2 mg/kg), and with a topical instillation of 4.0% oxybuprocaine hydrochloride. Additional anesthesia was administered every 30 minutes. The pupils were maximally dilated for surgery and observation with topical 1% tropicamide and 2.5% phenylephrine hydrochloride.

Procedures of In Vivo Experiments

Two-port vitrectomy was performed. A sclerotomy was made in the superonasal quadrant 2 mm from the limbus with a 20-gauge knife, where an infusion tube was fixed with a 5-0 Dacron suture (Alcon, Fort Worth, TX). Another sclerotomy was made in the superonasal quadrant 2 mm from the limbus, where an Ocutome vitreous cutter (Alcon) was inserted into the vitreous cavity. Core vitrectomy was performed within 1 minute to minimize the damage of the surgical procedures. The perfusate was a balanced salt solution (BSS) containing 125 mM NaCl, 5 mM KCl, 2 mM Na₂HPO₄, 20 mM NaHCO₃, 1 mM CaCl₂, 2 mM MgCl₂, and 10 mM glucose. The pH and osmolarity ranged from 7.42 to 7.65 and from 275 to 289, respectively. The flow rate was 1 ml/min.

After vitrectomy, the rabbits were placed in front of the fluorophotometer for fluorophotometric measurements. Fluorophotometry was performed with a commercial computerized fluorophotometer. The main transmittance of the exciting filter was 440 to 480 nm, and that of the barrier filter was 500 to 630 nm. Fluorescence of the chorioretina was measured posteriorly along the ocular axis. Scans were performed with data points averaged for 100 milliseconds every 0.25 mm, and the results were expressed in equivalents to fluorescein sodium concentration (ng/ml Eq). The peak value of choroid-retina was taken from a fluorophotometric profile and used for the analysis. Before each experiment, autofluorescence of choroid-retina was measured as a background fluorescence. After experiments, the obtained fluorophotometric readings of the chorioretinal peak were manually subtracted by that of autofluorescence of the choroid-retina, and the resultant value was converted to DCF concentration (picomole/ml) with the use of the calibration curve. This value was defined as retinal DCF production. Comparative statistics were performed using the Mann-Whitney test because of the unequal variances in the comparison groups.

The 14 eyes were divided into three groups. Group A (five eyes) underwent perfusion with 5 μM DCF-DA in BSS for 20 minutes, followed by 30 minutes of a washout with BSS only. Nonfluorescent DCF-DA was deacetylated by esterases inside the cell to fluorescent DCF. This experiment was designed to assess the dynamics of intracellular DCF from DCF-DA in the retina. This enabled us to simulate the dynamics of intracellular DCFH derived from DCFH-DA, because the molecular structure of DCF-DA is very similar to that of DCFH-DA. Fluorophotometric scans were performed at 0, 5, 10, 20, 35, and 50 minutes to measure fluorescence intensity of the chorioretinal peak.

Group B (five eyes) was perfused with 5 μM DCFH-DA for 20 minutes, followed by 30 minutes of washout with BSS. Fluorophotometric scans were performed at 0, 5, 10, 20, 30, 40, and 50 minutes. This experiment evaluated whether vitrectomy itself causes an oxidative response.

In Group C (four eyes), as an oxidative stress, 300 μM H₂O₂ in BSS was perfused for 5 minutes after a washout of DCFH-DA, followed by fluorophotometric scans.

Localization of DCF Fluorescence in the Retina

To determine the localization of DCF fluorescence in the retina, three rabbits in each group were killed. The eyes were rapidly enucleated and fixed in ice-cold 0.1 M phosphate-buffered saline, pH 7.4, containing 0.3% glutaraldehyde and 4% paraformaldehyde for 15 minutes. The eyeballs were cut at the equator and the anterior segments and vitreous were removed. Remaining eyecups were postfixed overnight in 0.1 M phosphate-buffered saline with 4% paraformaldehyde, followed by cryoprotection in 15% sucrose. The fixed blocks were cut into sections (20 μm thick) on a cryostat (−18°C) (Cryotom; Nakagawa Seisakusho, Tokyo, Japan) and mounted onto glass slides coated with gelatin–chrome–alum. The sections were dried and observed on an Axiovert 100 inverted microscope (Zeiss, Oberkonchen, Germany). The sections were excited by 50 W halogen light passing through an excitation filter BP 450–490 (peak excitation, 490 nm) and an ND filter (ND6). The emitted fluorescence passing through an emission filter BP 520–560 was projected to the face of a Star 1 cooled CCD camera (Photometrics, Munchen, Germany). Captured digital images of 640 × 480 pixels were transferred to a Macintosh Centris 650 personal computer (Apple Computer Co., Cupertino, CA) and were analyzed with IP-Lab software (Signal Analytics, Vienna, VA).

RESULTS

Characterization of DCF as a Fluorescence Probe

We determined excitation and emission spectra for reagent DCF. Excitation spectrum was monitored at
FIGURE 2. Calibration curve of a fluorophotometer using reagent 2',7'-dichlorofluorescein (DCF). The fluorophotometric reading is linear with the DCF concentration except at low and high concentrations. The y axis represents the fluorescence of sodium fluorescein equivalent.

520 nm emission. Emission spectrum was monitored with 490 nm excitation. The peaks of excitation and emission were 495 and 520 nm, respectively (Fig. 1B). They were similar to those of sodium fluorescein.

We next measured the output of the fluorophotometer with various concentrations of DCF. Figure 2 shows the calibration curve of the fluorophotometer. The concentration of DCF correlated well with that of sodium fluorescein. The linear dynamic range of DCF appeared to be 1 to 10,000 picomole/ml. The dynamic range of DCF was similar to that of sodium fluorescein.

**Conversion of DCFH to DCF in Response to H₂O₂ In Vitro**

DCFH was incubated with 10 pM to 100 nM H₂O₂ in the presence of hematin. H₂O₂, in the range of 10 pM to 10 nM, converted DCFH to DCF in a dose-dependent manner (Fig. 3A). Catalase inhibited the production of DCF by H₂O₂ dose dependently (Fig. 3B).

**Fluorophotometry in Eyes Treated With DCF-DA (Group A)**

Figure 4 shows a typical fluorophotometric scan of the eye perfused with 5 μM DCF-DA for 20 minutes. The increase in DCF production was 2006 ± 274 picomole/ml in choroid-retina (Table 1). The chorioretinal peak increased remarkably after perfusion with 5 μM DCF-DA for 5 minutes and reached a plateau after 20 minutes. After washout with BSS, the trapped DCF was maintained for at least 30 minutes (Fig. 4B).

**Fluorophotometry in Eyes Treated With DCFH-DA (Group B)**

Figure 5 shows a typical scheme of fluorophotometric scans in eyes treated with DCFH-DA for 20 minutes. A minimal increase in DCF production (8.35 ± 1.11 picomole/ml) (see Table 1) was observed. After washout with BSS, the chorioretinal peak was maintained (Fig. 5B).

**Fluorophotometry in Eyes Treated With DCFH-DA With H₂O₂ Stimulation (Group C)**

Figure 6 shows a typical scheme of fluorophotometric scans in eyes treated with 300 μM H₂O₂ for 5 minutes.

**FIGURE 3.** (A) Dose-dependent curve of the 2',7'-dichlorofluorescein (DCF) production from 2',7'-dichlorofluorescein in reaction to various concentrations of hydrogen peroxide. (B) Inhibitory effect of catalase on the production of DCF.
FIGURE 4. (A) Scheme of fluorophotometric scannings in the eye treated with 2',7'-dichloro-fluorescein diacetate (DCF-DA). Dashed line indicates before DCF-DA treatment, showing autofluorescence of choroid-retina, lens, and cornea. Solid line indicates after DCF-DA treatment, showing a marked increase in the chorioretinal peak. (B) Time course curve of the chorioretinal peak in the eye treated with DCF-DA. The chorioretinal peak rapidly reached a plateau and was maintained for 30 minutes.

$H_2O_2$ caused a significant increase in DCF production ($30.7 \pm 13.1$ picomole/ml) (see Table 1), as compared with eyes perfused with DCFH only ($P = 0.0013$).

Localization of Fluorescent Products in the Retina

In a control eye that received vitrectomy, moderate autofluorescence was observed in the retina, retinal pigment epithelium (RPE), choroid, and sclera (Fig. 7A). In a specimen treated with DCF-DA (Fig. 7B), all retinal layers and RPE were strongly fluorescent. The choroid and sclera showed no fluorescence increase. In retina treated with DCFH-DA, the fluorescence did not increase, as compared with that of the control (Fig. 7C). In eyes treated with DCFH-DA and $H_2O_2$, the nerve fiber layer was significantly fluorescent compared with that treated with only DCFH-DA (Fig. 7D).

DISCUSSION

We have demonstrated the characteristics of DCFH as an $H_2O_2$-sensitive probe. The DCFH reacted with $H_2O_2$ dose dependently and was inhibited by catalase in vitro. The detection limit of $H_2O_2$ concentration was on the order of picomoles. Cathcart et al.22 reported that the sensitivity of DCFH is at the picomole level, whereas that of thiobarbituric acids is on the order of nanomoles. Furthermore, DCFH reportedly reacts with various kinds of lipid hydroperoxides, such as cholesterol hydroperoxide, linoleic hydroperoxide, and t-butyl hydroperoxide, but not with endoperoxides.22 It was possible to detect not only $H_2O_2$ but also lipid hydroperoxide with the use of DCFH. We first confirmed the characteristics of DCF as a fluorescent probe. The peak excitation and emission wavelengths were 495 nm and 520 nm, respectively. The excitation and emission spectra of DCF were similar to those of sodium fluorescein, which has been widely used in ophthalmology. Furthermore, the intensity of DCF-associated fluorescence was almost the same as that of sodium fluorescein.

First described by Keston and Brandt,19,20 DCFH-DA diffuses into the cell, is deacetylated to DCFH by esterase, and is trapped inside the cell. In the presence of $H_2O_2$ and peroxidase, DCFH is converted to fluorescent DCF. Various studies have used DCFH-DA as a marker of intracellular oxidative reaction.24,25 Using DCFH-DA and a fluorophotometric device, intracellular hydroperoxide formation in the retina in vivo can be detected.

In the eyes treated with DCF-DA, more than 2 nanomoles/ml of DCF was produced in the retina and maintained for at least 30 minutes. In addition, morphologic study confirmed that DCF was trapped in the retina and RPE, not in the choroid. The fluorescence in the chorioretinal peak was supposed to be

A

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>DCF (pmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 5)</td>
<td>5 $\mu$M DCF-DA</td>
<td>2006 ± 274</td>
</tr>
<tr>
<td>B (n = 5)</td>
<td>5 $\mu$M DCFH-DA</td>
<td>8.35 ± 1.11</td>
</tr>
<tr>
<td>C (n = 4)</td>
<td>5 $\mu$M DCFH-DA + 0.5 $\mu$M $H_2O_2$</td>
<td>30.7 ± 13.1*</td>
</tr>
</tbody>
</table>

Values are mean ± SD; n = number of eyes.

* $P = 0.0013$; calculated by Mann-Whitney test comparing groups B and C.

DCF = 2,7'-dichlorofluorescin; DCFH = 2,7'-dichlorofluorescein; DCFH-DA = DCFH diacetate.
produced in the retina and RPE. A moderate amount of increased DCF also was observed in the vitreous, lens, anterior chamber, and cornea (see Fig. 4A). Theoretically, negatively charged DCF was trapped inside the cell, but some leakage of DCF from the retina might occur. So DCF that was once trapped in the retina might diffuse into the vitreous cavity.

In the eyes treated with DCFH-DA, a minimal increase in DCF production was detected with fluorophotometry. From the study of the eyes treated with DCF-DA, we speculated that adequate amounts of DCFH were trapped in the retina when perfused with DCFH-DA because the molecular structure and chemical properties of DCF-DA and those of DCFH-DA are similar. Performing vitrectomy and perfusion might have induced oxidation of the retina that resulted in the production of a small amount of DCF.

The oxidative stress caused by 300 μM \( H_2O_2 \) demonstrated a significant increase in DCF production in

**FIGURE 5.** (A) Scheme of fluorophotometric scannings in the eye treated with 2',7'-dichlorofluorescein diacetate (DCFH-DA). Dashed line indicates before DCFH-DA treatment, showing autofluorescence of choroid–retina, lens, and cornea. Solid line indicates after DCFH-DA treatment, showing a minimal increase in the chorioretinal peak. (B) Time course curve of the chorioretinal peak in the eye treated with DCFH-DA. The chorioretinal peak showed minimal increase throughout the experiment.

**FIGURE 6.** Scheme of fluorophotometric scannings in eyes treated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) and with both DCFH-DA and hydrogen peroxide. Dashed line indicates the eye perfused only with DCFH-DA. Solid line indicates the eye loaded with both DCFH-DA and hydrogen peroxide, showing a significant increase in the chorioretinal peak.

**FIGURE 7.** Fluorescent microscopic images. (A) Control eye that underwent vitrectomy. Moderate autofluorescence was observed in the retina, retinal pigment epithelium (RPE), choroid, and sclera. (B) Eye treated with 2',7'-dichlorofluorescein diacetate (DCF-DA). All retinal layers but the photoreceptor layer and RPE are strongly fluorescent. (C) Eye treated with 2',7'-dichlorofluorescein diacetate (DCFH-DA). The fluorescence did not increase compared with that of control. (D) Hydrogen peroxide-loaded eye. The nerve fiber layer is significantly fluorescent, as compared with that treated only with DCFH-DA.
the retina. In our previous study, we used intravitreal injection of DCFH, which reacts with peroxides in the vitreous humor to form fluorescent DCF, which was quantitatively measured by fluorophotometry. We demonstrated that the stimulation with a 100-micromole order of H$_2$O$_2$ in the vitreous humor is the lower limit of detection. The dose of H$_2$O$_2$ used in the present study for the oxidative stimulation was determined from those results. It is difficult to assess whether this dose of H$_2$O$_2$ is clinically relevant. It should be studied in the future under experimental pathologic conditions that might induce oxidative stress.

Morphologic study also confirmed the DCF production in the retina. Fluorescent microscopy, especially, revealed moderately increased fluorescence in the nerve fiber layer. It is reasonable to assume that most of the superficial layer in contact with the H$_2$O$_2$ solution suffers the strongest oxidative stress. Another speculation is that the retinal ganglion cell is susceptible to oxidative stress. Many investigators have reported that the retinal photoreceptor cell and RPE are rich in antioxidative agents and enzymes. Few studies, however, report the presence of a scavenging system in the ganglion cell.

The method we used in this study has some limitations. First, surgical removal of the vitreous and intravitreal administration of the dye are necessary; a method that requires no surgical manipulation is desirable. When the dye was systemically injected, the concentrations reached were not high enough for fluorophotometric measurement. It was speculated that the dye did not penetrate adequately into the retina because of the blood–retinal barrier and the low plasma concentration of the dye. Another limitation is that two-dimensional evaluation of oxidative lesions is difficult with fluorophotometry because the fluorophotometer used in this study scans only a small area along the optical axis. The third drawback is that clear optical media are essential for fluorophotometry of the retina. Opacities of the cornea, lens, and vitreous might interfere with the output of the fluorophotometer.

In conclusion, we demonstrated the usefulness of DCFH as an H$_2$O$_2$-sensitive fluorescent probe and developed a new method to detect hydroperoxides in the retina in vivo.

**Key Words**

dichlorofluorescin diacetate, fluorophotometry, hydrogen peroxide, oxidative stress, retina

**References**


