Lipid Peroxidation and Antioxidant Enzymatic Systems in Rat Retina as a Function of Age

C. Castorina, A. Campisi, C. Di Giacomo, V. Sorrenti, A. Russo, and A. Vanella

(Accepted November 7, 1991)

In the present study, we have assayed the enzymatic activity of Cu,Zn-SOD, Mn-SOD, GSH-Px, GSH-Red, Cat, and G6PD in rat retina as a function of age. Conjugated diene levels and MDA formation were also determined. The conjugated diene levels in rat retina were found to increase significantly with age, accompanied by a marked decrease in GSH-Px and Cat activities. No age-related change in MDA levels and in GSH-Red and G6PD activity was found, whereas a significant increase in SOD activity was observed between 1 and 4 months. Decreased GSH-Px and Cat activity is related to increased lipid peroxidation with age.

KEY WORDS: Lipoperoxidation; antioxidant enzymatic systems; retina; aging.

INTRODUCTION

The retina is characterized by an extremely aerobic metabolism, its oxygen consumption being the highest of all body tissues (1). This elevated oxygen utilization leads to the production of toxic oxygen derivatives, such as superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (·OH) (2-4). Although both O$_2^-$ and H$_2$O$_2$ are potentially cytotoxic, most of the oxidative damage is caused by the ·OH radical, which is generated by the O$_2^-$ and H$_2$O$_2$ reaction in the presence of transition metal ions (5). The retina possesses several defences including both low molecular weight scavengers, such as α-tocopherol, cysteine, β-carotene, reduced glutathione, ascorbic acid (4, 6-10) and enzymatic systems, such as superoxide dismutase (SOD), catalase (Cat), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Red) and glucose-6-phosphate dehydrogenase (G6PD), which protect it from oxidative damage (11-20). However, small amounts of oxygen free radicals can escape the antioxidant defenses and cause damage to cellular constituents. This damage could be exiguous, but constant and, if unrepaired, could accumulate with age. Several studies have examined the age-related changes of SOD in different mammalian tissues. In view of these considerations we have assayed the activities of Cu,Zn-SOD, Mn-SOD, Cat, GSH-Px, GSH-Red, and G6PD in rat retina as a function of age (1, 4, 12 and 24 months). Conjugated diene levels and malondialdehyde formation (MDA), as markers of lipid peroxidation, were also determined.

EXPERIMENTAL PROCEDURE

Treatment of Animals. Experiments were performed with male Wistar rats at different ages (1, 4, 12, and 24 months). The animals were kept under constant environmental conditions (temperature 22 ± 1°C; relative humidity 60 ± 5%; exposure to natural light and fed normal laboratory diet with water ad libitum). Animals were killed by decapitation and retinas from animals of the same age were rapidly removed in the cold room, pooled, washed with cold NaCl (0.9%) and homogenized in appropriate buffer; lipid peroxidation was estimated by measuring conjugated diene levels, and malondialdehyde pro-
duction; antioxidant enzymatic activities were evaluated as described below.

**Enzymatic Assays.** The retinas were pooled (10 retinas for 1 month old rats; 6 retinas for 4 months; 4 retinas for 12 months and 2 retinas for 24 months) and homogenized in 50 mM potassium phosphate buffer, pH 7.0, and the supernatant obtained by centrifugation for 45 min at 20,000 g was used for enzymatic assays.

**Superoxide Dismutase** (superoxide: superoxide oxidoreductase, EC. 1.15.1.1, SOD). Cu,Zn-SOD activity was measured using the supernatant obtained by centrifugation for 45 min at 20,000 g. Mn-SOD activity was measured using the mitochondrial pellet resuspended in 50 mM potassium phosphate buffer, pH 8.6, and lysed by digitonin 2.5 mg/ml; the lysate then was centrifuged at 12,000 g for 15 min and the supernatant was employed for enzymatic assay. Superoxide dismutase activity was measured by the method of McCord and Fridovich (21). 0.5 ml of assay mixture contained: 50 mM potassium phosphate buffer, pH 8.6, 1 mM sodium azide, 0.1 mM EDTA, 0.5 mM hypoxanthine, 12 mM xanthine oxidase (specific activity 1 U/mg of protein), 19 μM cytochrome c and supernatant containing about 5-20 μg of protein. The reduction of cytochrome c was measured at λ = 550 nm at 25°C. The results are expressed as a U/mg of protein. Proteins were determined by the method of Lowry et al. (22). Under these conditions 1 unit of SOD activity was defined as the amount of enzyme which inhibits the rate of ferriytochrome c reduction by 50%.

**Glutathione Peroxidase** (glutathione: hydrogen peroxide oxidoreductase, EC. 1.1.1.9, GSH-Px). Glutathione peroxidase activity was measured by the method of Paglia and Valentine (23). 1 ml of assay mixture contained: 50 mM potassium phosphate buffer, pH 7.0, 5 mM reduced glutathione, 1 unit glutathione reductase (specific activity 1 U/mg of protein), 200 μM NADPH, 70 μM hydrogen peroxide, 3.75 mM sodium azide (inhibitor of catalase) and supernatant containing 300-500 μg of protein. The oxidation of NADPH was measured at λ = 340 nm at 25°C. Enzymatic activity was expressed as mU/mg of protein; 1 mU of activity was defined as the amount of enzyme which caused the oxidation of 1 nmol of NADPH per min.

**Glutathione Reductase** (NAPDH: oxidized-glutathione oxidoreductase, EC. 1.6.4.2, GSH-Red). Glutathione reductase activity was measured by following NADPH oxidation at λ = 340 nm, according to Horn’s method (24). 1 ml of assay mixture contained: 50 mM potassium phosphate buffer, pH 6.8, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1.3 mM GSSG, 200 μM NADPH and supernatant containing 300-500 μg of protein. The enzyme activity was expressed as mU/mg of protein; 1 mU of activity was defined as the amount of enzyme which caused the oxidation of 1 nmol of NADPH per min.

**Catalase** (hydrogen-peroxide: hydrogen-peroxide oxidoreductase, EC. 1.11.1.6, Cat). Catalase activity was assayed by the method of Holmes and Masters (25) with hydrogen peroxide as substrate; 1 ml of assay mixture contained: 20 mM potassium phosphate buffer, pH 7.0, 20 mM hydrogen peroxide and supernatant containing 300-500 μg of protein. Enzyme activity was expressed as U/mg of protein; a unit was defined as the amount of enzyme breaking down 1 mmol of H₂O₂ per min.

**Glucose-6-Phosphate Dehydrogenase** (d-glucose-6-phosphate: NADP oxidoreductase, EC. 1.1.1.49, G6PD). Glucose-6-phosphate dehydrogenase activity was determined spectrophotometrically at λ = 340 nm as described by Kornberg and Horecker (26). 1 ml of assay mixture contained: 50 mM Tris-HCl, 5 mM EDTA, pH 7.6, 0.3 mM NADP, 0.5 mM glucose-6-phosphate and supernatant containing 100-300 μg of protein. Enzymatic activity was expressed as mU/mg of protein.

**Measurement of Conjugated Dienes.** Conjugated diene levels were assayed by Recknagel’s method (27). The retinas were pooled (10 retinas for 1 month old rats; 6 for 4 months; 4 for 12 months and 2 for 24 months) and homogenized in 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA. Lipids were extracted from 1 ml of homogenate using 5 ml of 2:1 (V/V) chloroform/methanol and this extract was evaporated to dryness under a stream of nitrogen at ambient temperature. The dried extract was then redissolved in cyclohexane of spectroscopic quality and its absorbance at λ = 234 nm measured against a solvent blank, by using the ε₅₃₂ = 2.52 × 10⁴ M⁻¹ cm⁻¹; results are expressed as nmol of lipohydroperoxide/mg protein or mg lipid. Lipids were determined by the method of Henry (28).

**MDA Assay.** MDA formation was measured by thioarbitruric acid (TBA) colored reaction (29). The retinas were pooled (10 retinas for 1 month old rats: 4 for 4 months; 2 for 12 months and 24 months) and homogenized in 1.15 KCl 10% (W/V). 2 ml of assay mixture contained: 14 mM SDS, 1.25 M acetic acid, 0.5 mM butylated-hydroxytoluene (BHT), 21 mM TBA and homogenate containing 500-800 μg of protein; the mixture was then heated at 95°C for 20 min; after cooling, MDA was extracted with a mixture of n-butanol and pyridine (15:1 V/V) and the absorbance was measured at λ = 532 nm. Results are expressed as nmol of MDA/mg of protein using the ε₅₃₂ = 1.56 × 10⁵ M⁻¹ cm⁻¹.

**RESULTS**

MDA and conjugated diene levels were regarded as “markers” of lipid peroxidation. While no change in MDA formation was found during aging (Table I), there was a significant increase in conjugated diene levels (Figure 1). Consistent with increased conjugated diene levels in older rats, a marked decrease in GSH-Px and Cat activities was detected (Figures 2 and 3). No age-related change in other antioxidant enzymatic systems such as GSH-Red and G6PD (Table II) was found; a significant increase in Cu,Zn-SOD and Mn-SOD activities was observed in rat retinas between 1 and 4 months, whereas no change in SOD activity was detected among other ages considered (Figure 4).

**DISCUSSION**

Evidence indicates that oxidative damage to the photoreceptors and/or the Retinal Pigment Epithelium (RPE), induced by Vitamin E deficiency, iron, pro-

### Table I. Endogenous MDA in Rat Retina at Different Ages

<table>
<thead>
<tr>
<th>Months</th>
<th>MDA (μmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>2.33 ± 0.12</td>
</tr>
<tr>
<td>4 months</td>
<td>2.24 ± 0.25</td>
</tr>
<tr>
<td>12 months</td>
<td>2.41 ± 0.20</td>
</tr>
<tr>
<td>24 months</td>
<td>2.40 ± 0.15</td>
</tr>
</tbody>
</table>

Experimental procedures are as described in the text. Results are expressed as nmol MDA/mg prot. Each value represents the mean ± SD of 6 experiments.