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MECHANISMS OF THE HARMFUL ACTION OF FLUORESCENT DYES ON THE RETINA

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The method of fluorescence angiography is widely used in ophthalmologic practice [14, 2]. It has recently been shown that angiographic investigations with fluorescent dyes may be associated with injury to the retina, especially if high-intensity light sources are used [3, 5].

To overcome the limitations imposed on the method of fluorescence angiography by these side effects, their mechanisms must be elucidated. It can be tentatively suggested that the photic injury to the retina produced by fluorescent dyes is based on a phenomenon of photosensitized generation of singlet oxygen ($*O_2$) and subsequent accumulation of lipid peroxidation (LPO) products, as has been demonstrated recently in the membranous structures of the retina [7, 4], in accordance with the scheme:

$$S + h\nu \longrightarrow S^*$$
$$S^* + O_2 \longrightarrow S + *O_2$$
$$*O_2 + RH \longrightarrow ROOH,$$

where S and S* denote the basic and excited state respectively of the dyes, $*O_2$ the singlet-excited state of O_2, and RH and ROOH represent molecules of exogenous substrates and their peroxidation products respectively.

In connection with the facts described above an investigation was undertaken to study mechanisms of the light-induced injurious action of a fluorescent dye (methylene blue — MB) — on the retina and its membranous structures in different model systems: in a suspension of the outer segments of the rods (OSR) of the frog's retina and on preparations of the isolated frog retina, and also *in vivo*, on the retina of chinchilla rabbits.

EXPERIMENTAL METHOD

The OSR fraction was obtained from the retinas of dark-adapted frogs (*Rana temporaria*) by centrifugation in a sucrose density gradient by methods described previously [1, 10]. Criteria of purity of the OSR suspension varied from 2.1 to 2.4 ($A_{280/500}$). Thermal denaturation of rhodopsin was determined and the thermodynamic parameters calculated by the method in [9]. Proteins of OSR were separated by gel-electrophoresis in Na-SDS-polyacrylamide gel [11]. Protein was determined by Lowry's method [12]. Lipids were isolated by the method in [8]. The level of LPO products was determined spectrophotometrically by measuring absorption at 232 nm, characteristic of diene conjugates [6]. The content of carbonyl compounds — secondary LPO products — was determined by the reaction with 2-thiobarbituric acid [14]. The isolated frogs' retinas and rabbits' eyes were illuminated by means of the illuminating system of the MPS50 L spectrophotometer, with a high-pressure xenon lamp. MB was excited by a photic flux with $\lambda \geqslant 620$ nm (0.3 mW/cm^2) and retinal by a photic flux with $360 \leqslant \lambda \leqslant 380$ nm, the spectral regions being isolated by cutoff filters. Preliminary bleaching of rhodopsin in the

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TABLE 1. Accumulation of MDA and Changes in Thermostability of Rhodopsin during Oxidation of OSR of Frog Retina Photosensitized by MB (M ± m)

Experimental conditions	MDA, nmoles/mg protein	$K_{td} \cdot 10^{-4}$, sec^{-1}
Control	2.6 ± 0.1	2.7 ± 0.1
Illumination of OSR ($\lambda \geqslant 620$ nm) without MB (30 min)	2.6 ± 0.1	2.7 ± 0.1
MB + dark OSR (30 min)	2.6 ± 0.1	2.7 ± 0.1
MB + 15 min illumination	13.0 ± 0.2	9.7 ± 0.1
MB + 30 min illumination	28.0 ± 0.2	14.0 ± 0.2
MB + 15 min illumination + α-naphthol (10^{-4} M)	2.6 ± 0.1	2.6 ± 0.2
MB + 15 min illumination + 4-methyl-2,6-di-tert-butylphenol (10^{-4} M)	2.6 ± 0.1	2.7 ± 0.1
MB + 15 min illumination + α-tocopherol (10^{-4} M)	5.2 ± 0.1	7.8 ± 0.1
MB + 15 min illumination + DABCO (10^{-4} M)	10.4 ± 0.1	—

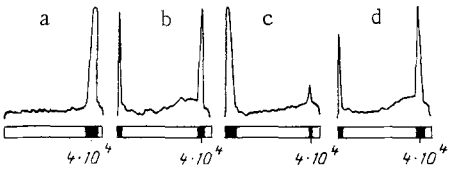

Fig. 1. Changes in protein composition of OSR of frog retina under the influence of singlet oxygen, photosensitized by MB ($5 \cdot 10^{-6}$ M). Densitograms and schemes of gels after Na-SDS (0.1%)-polyacrylamide gel (9%) electrophoresis of OSR membranes: a) control; b) after illumination for 15 min ($\lambda \geq 620$ nm) in presence of MB ($5 \cdot 10^{-6}$ M); c) after illumination for 30 min in presence of MB; d) after illumination for 30 min in presence of MB and α-tocopherol (10^{-4} M).

isolated retina was carried out by flashes of white light (flash energy 1.7 kJ). The electroretinogram (ERG) from the isolated frog retina immersed in Ringer's solution and from the rabbit's eye *in vitro* were recorded with a UBP-1-02 biopotentials amplifier and N-327 automatic writer. MB (10 mg/kg, from Chemapol, Czechoslovakia) was injected into the rabbit's auricular vein 10 min before exposure to light. 1,4-Diazobicyclo(2,2,2)octane (DABCO), from Merck, West Germany, and α-naphthol, from Reokhim, were injected into the animals 1 h before exposure to light in a dose of 250 mg/kg intramuscularly. α-Tocopherol (from Serva, West Germany), and 4-methyl-2,6-di-tert-butylphenol (BHT) also were used.

EXPERIMENTAL RESULTS

During exposure of a suspension of OSR from frog's retina to a photic flux with $\lambda \geqslant 620$ nm in the presence of MB injury took place to the protein and lipid components of the photoreceptor membrane (PRM). This injury was expressed as disappearance of the monomeric form of the visual pigment rhodopsin (the basic protein of the PRM), which has a molecular weight of 40 kilodaltons, and with the simultaneous appearance of rhodopsin oligomers (Fig. 1). This effect was potentiated by an increase in the duration of exposure to light (Fig. 2).

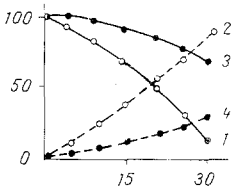

Fig. 2. Changes in content of monomeric and oligomeric forms of rhodopsin in OSR membranes of frog retina after oxidation of photosensitized MB. 1, 3) Monomeric forms, 2, 4) oligomeric forms, 4) illumination in presence of α-tocopherol (10^{-4} M). Abscissa, duration of illumination (in min); ordinate, rhodopsin content (%).

Modification of the polypeptide chain of rhodopsin also was revealed by a study of its thermal denaturation. The results (Table 1) show that as a result of exposure of the OSR suspension to light the thermostability of the rhodopsin was reduced; an increase in the length of exposure in this case also led to an increase in the thermal denaturation constant (K_{td}). In the lipid phase of the PRM the action of light caused accumulation of LPO products recorded as an increase in the content of diene conjugates and of malonic dialdehyde (MDA, Table 1). In the absence of MB irradiation of the OSR suspension with a photic flux of the same spectral composition ($\lambda \geqslant 620$ nm) did not cause the above-mentioned changes in the lipid and protein components of the PRM.

If the modification of PRM produced by the fluorescent dye was due to the action of singlet oxygen, it will be evident that quenchers of $^*O_2$ ought to have a protective effect. In fact, as the results show, DABCO and α-tocopherol, which are quenchers of $^*O_2$, did largely prevent the photoinduced effects of injury to the visual pigment rhodopsin and the lipid components of PRM (Table 1; Figs. 1, 2, and 3). It is essential to note that inhibitors of free-radical oxidation of lipids (4-methyl-2,6-di-tert-butylphenol, α-naphthol), which are not effective quenchers of $^*O_2$, also have the same protective action. Hence it can be concluded that the injury to PRM due to the action of light sensitized by MB is based on a reaction of free-radical lipid oxidation initiated by $^*O_2$.

During irradiation of isolated frog retinas under identical conditions (in the presence of MB, $\lambda \geqslant 620$ nm) electrical activity was depressed, as shown by a decrease in amplitude of the a wave on the ERG. This inhibitor effect also depended on the duration of exposure to light, it was accompanied by accumulation of LPO products, and it could be largely prevented both by quenchers (DABCO) and by the antioxidants α-naphthol and 4-methyl-2,6-di-tert-butylphenol (Fig. 2).

Finally, experiments *in vitro* demonstrated that during exposure to light ($\lambda \geqslant 620$ nm) of the eyes of rabbits receiving MB previously by injection into the auricular vein, depression of ERG waves took place, which was not observed in the case of similar exposure to light in the absence of the fluorescent dye. In this case also the protective action against light-induced damage sensitized by MB was exhibited both by quenchers of $^*O_2$ and by antioxidants (Fig. 4).

It can thus be concluded from the experimental results that during the action of light on the retina and in the presence of fluorescent dyes $^*O_2$ is generated, the lipid and protein

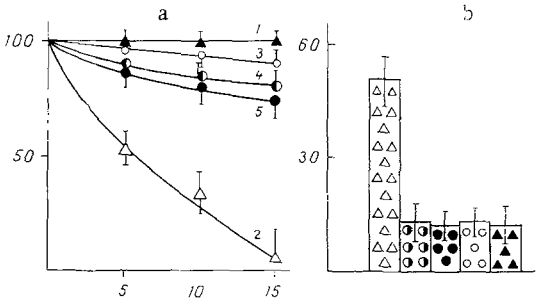

Fig. 3. Changes in electrical activity of isolated frog retina (ERG) and accumulation of LPO products during singlet oxygen generation photosensitized by MB. 1) Without illumination in presence of MB ($5 \cdot 10^{-6}$ M), 2) after illumination in presence of MB without inhibitors, 3) after illumination in presence of MB and DABCO (10^{-5} M), 4) after illumination in presence of MB and α-naphthol (10^{-5} M), 5) after illumination in presence of MB and BHT (10^{-5} M). Abscissa, duration of illumination (in min); ordinate: a) change in amplitude of *a* wave on ERG (%), b) MDA level (in nmoles/mg protein).

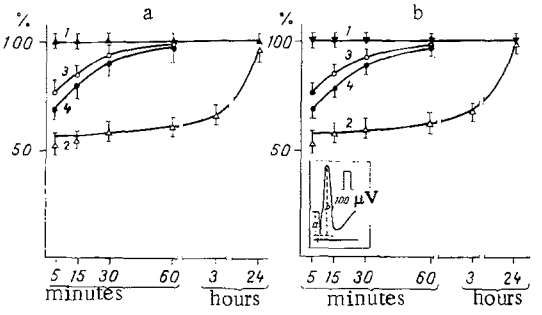

Fig. 4. Changes in amplitude of ERG waves of rabbit during singlet oxygen generation photosensitized by MB *in vivo*. a) *a* wave on ERG; b) b wave on ERG. 1) Without illumination but with injection of MB (10 mg/kg); 2) after illumination ($\lambda \geq 62$ nm, 30 min) after injection of MB without inhibitors; 3) after illumination + injection of MB and DABCO; 4) after illumination + injection of MB and α-naphthol. Abscissa, time; ordinate, change in amplitude of waves (in %).

components of the membranous structures of the visual cells are modified, and electrical activity of the retina is depressed. These light-dependent harmful effects of fluorescent dyes can be prevented or limited by two classes of compounds of different chemical nature: quenchers of $^*O_2$ and inhibitors of free-radical lipid oxidation. The effects thus demonstrated are a key component in injury to the retina produced by light in the presence of fluorescent dyes. The results are evidence that quenchers of $^*O_2$ and antioxidants can be used for chemical prevention of injuries arising during fluorescence angiography.

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