Hemafogenous Photosensitization

A Mechanism for the Development of Age-Related Macular Degeneration

John D. Gottsch,* Sovirj Pou,† Leon A. Bynoe,* and Gerald M. Rosenf

Age-related macular degeneration (ARMD) is one of the leading causes of severe visual loss in the United States. Numerous risk factors have been investigated, but the pathogenesis of ARMD has remained elusive. The authors propose that ARMD develops as a direct result of photosensitization of the vascular endothelium of the choriocapillaris, Bruch’s membrane, and the retinal pigment epithelium (RPE) by superoxide anion and singlet oxygen generated by photoactive compounds in blood. Using electron-spin resonance spectrometry, the free-radical trap, 5,5-dimethyl-1-pyrroline-N-oxide, and the singlet-oxygen trap, 2-(9,10-dimethoxyanthracenyl)-t-butylhydroxylamine, the authors demonstrate that the photoactive compound, protoporphyrin IX (PP IX), a naturally occurring precursor molecule of hemoglobin found in erythrocytes and plasma, generates superoxide anion and singlet oxygen. The amount of reactive-oxygen species produced by this system is dependent on the concentration of PP IX and the intensity and wavelength of the light delivered. Furthermore the production of these photooxidants is significantly reduced by filtering the excitatory wavelengths of PP IX. These photogenerated oxidants could damage the vascular endothelium of the choriocapillaris, Bruch’s membrane, and the RPE, necessitating a reparative process. This could result in features characteristically seen in ARMD such as a thickened Bruch’s membrane, RPE atrophy, and hyperplasia. Prevention of phototoxic damage by this mechanism could involve enhancing protective enzymes, increasing scavenger substances, or supplying appropriate filters to eliminate the exciting wavelengths of light. Invest Ophthalmol Vis Sci 31:1674–1682, 1990

Protoporphyrin IX (PP IX) is a precursor molecule of hemoglobin, found naturally in erythrocytes. In erythropoietic protoporphyria, a disease in which cutaneous plaques develop in light-exposed areas, PP IX is detectable in erythrocytes and serum.1–4 The thickening noted in vascular basement membranes of the light-exposed skin of patients with erythropoietic protoporphyria5–7 is histopathologically similar to the changes noted in the endothelium of the choriocapillaris and Bruch’s membrane of patients with age-related macular degeneration (ARMD).8–11 Also the vascular basement membranes in the conjunctiva and cornea of a patient with congenital erythropoietic protoporphyria were reported to be similar to those vascular basement membranes found in the skin of patients with this disease.12 Recently, the ability of PP IX to photosensitize corneal endothelium has been demonstrated by ion-flux studies and scanning electron microscopy with ambient levels of light and physiologic concentrations of PP IX.13 Superoxide anion production by PP IX has been suggested as being responsible for producing the clinical manifestations of erythropoietic protoporphyria.14

In this study the role of PP IX as a photogenerator of the reactive-oxygen species, superoxide anion and singlet oxygen, which could potentially damage choriocapillary vascular endothelium, Bruch’s membrane, and retinal pigment epithelium (RPE) was investigated by electron-spin resonance (ESR) spectroscopy using spin-trapping and spin-labeling techniques. Of the available methods for the detection of free radicals, spin trapping has received the most attention.15 This technique consists of using a nitrone or nitroso compound to “trap” an initial unstable free radical as a “long-lived” nitroxide, which can be monitored at room temperature using commercial ESR spectrometers (Fig. 1).16–19 Information obtained from the hyperfine splitting of the spin-trapped adduct can aid in the identification of the original free radical (Fig. 2). Since the spin-trapped adduct accumulates, this technique is inherently more sensitive than procedures which measure only instantaneous or steady-state levels of free radicals. However, spin trapping is not without its limitations. For example, the reaction of superoxide anion with

From *The Wilmer Ophthalmological Institute and the †University of Maryland, School of Pharmacy, Department of Pharmacology and Toxicology, Baltimore, Maryland.

Reprint requests: John D. Gottsch, MD, The Wilmer Ophthalmological Institute, Johns Hopkins Hospital, Maumenee Building 305, 600 N. Wolfe Street, Baltimore, MD 21205.
Fig. 1. Illustration of the spin trapping of oxygen radicals by DMPO. The generation of DMPO-OH may result from the degradation of DMPO-OOH and does not require the generation of hydroxyl radical by the system.

the spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) is very slow, with a second-order rate constant of only 10 M⁻¹ sec⁻¹.²⁰ Because of this, high concentrations of DMPO are required. This may lead to untoward toxicities,¹⁹ which in some cases may limit the usefulness of this technique. These and other problems associated with spin trapping have been reviewed extensively¹⁸,¹⁹ and will not be discussed further, except to mention that, through appropriate experimental design, most limitations can be overcome. Recently a new ESR method was developed to detect singlet oxygen.²¹ This technique involves the reaction of nitroxide II (Fig. 3) with singlet oxygen to produce the endoperoxide III with its characteristic ESR spectrum,²¹ as shown in Figure 4.

Materials and Methods

Reagents

The compounds PP IX, diethylenetriaminepentaacetic acid (DTPA), superoxide dismutase (SOD), catalase (CAT), xanthine, and xanthine oxidase were purchased from Sigma Chemical Company (St. Louis, MO). Triethylamine, 1,4-diazabicyclo[2.2.2]octane (DABCO) and methylene blue were obtained from Aldrich Chemical Company (Milwaukee, WI). The spin trap DMPO was synthesized according to the method of Bonnett et al.²² The singlet oxygen trap 2-(9,10-dimethoxyanthracenyl)-t-butylhydroxylamine (DTBH, Fig. 3) was prepared as outlined by Keana et al.²¹ All buffers were passed through a Chelex-100 (Biorad, Richmond, CA) ion-exchange column to remove trace metal ion impurities.²³

Detection of Superoxide Anion and Singlet Oxygen

In a darkened room, varying amounts of PP IX were suspended in either: (1) 50 mM sodium phosphate containing 1 mM DTPA at pH 7.4 to which DMPO (0.1 M) was added for the detection of free radicals or (2) deionized water to which DTBH (200 μM) dissolved in dimethylsulfoxide (DMSO, 0.28 M) was included (Table 1). In some experiments, SOD, CAT, triethylamine, or DABCO were included. In all experiments, the final volume was 0.5 ml.

The reaction mixture was then transferred to a quartz flat cell, fitted into the cavity of an ESR spectrometer (Varian Associates Model E-9, Palo Alto, CA), and the signal was recorded at 20°C. Spectra were recorded at specific time intervals after continuous irradiation of the solution in the spectrometer with a 150-Watt halogen light source (Transilluminator Model OS 3000, Medical Instrument Research Associates, Waltham, MA). Narrow-band interference light filters (Edmund Scientific, Barrington, NJ)
were placed in front of the light beam to select specific wavelengths of light for irradiation of the solution. Light fluxes were measured with a spectroradiometer (E. G. & G., Gamma Scientific, Model DR 2550, San Diego, CA). The light source was placed 15 cm from the sample.

A number of experiments required a continuous flux of superoxide anion, which was generated by the aerobic action of xanthine oxidase on xanthine. The rate of superoxide anion production was calculated by following the SOD-inhibitable reduction of ferri-cytochrome c, at 550 nm, using the extinction coefficient of 21 mM$^{-1}$cm$^{-1}$.

Results

Spin Trapping With Continuous Irradiation

When PP IX (0.1 mM) was suspended in 50 mM sodium phosphate, pH 7.4, containing DTPA (1 mM) and DMPO (0.1 M) was irradiated (200 μE/m$^2$/sec), an ESR spectrum corresponding to 5,5-dimethyl-5-hydroperoxy-1-pyrrolidinyloxy (DMPO-OOH) was recorded (Figs. 1, 2, 5A). The ESR spectrum depicted in Figure 5A is representative of six independant studies, each of which gave the same result. In the absence of light, no spectrum was recorded. It should be noted that protoporphyrin is so sensitive to photoactivation that either incandescent or fluorescent lighting in the laboratory can lead to a "background" spin trapping of superoxide anion. Therefore it was essential to conduct all studies in the dark.

Effects of PP IX Concentration, Light Intensity, and Wavelength on Spin Trapping

The effects of light intensity and the concentrations of PP IX on the generation of superoxide anion were investigated. When the concentration of PP IX was held constant, increased intensity of light exposure produced larger ESR spectra, ie, more superoxide anion was generated (data not shown). When the light intensity was held constant at all concentrations of PP IX (0.5–100 μM), we were able to spin trap superoxide anion, even though at the physiologic concentration (0.5–1.5 μM), the ESR spectrum characteristic of DMPO-OH and not DMPO-OOH was recorded (Fig. 5B). The ESR signal was inhibited by the addition of SOD (30 U/ml) but not by CAT (300 U/ml) (Fig. 5C and data not shown). Furthermore, at these low concentrations of PP IX a longer period of irradiation (30 min) was necessary to detect the ESR spectrum shown in Figure 5B.

Finally when both the concentration of PP IX (0.1 mM) and light intensity (8 μE/m$^2$/sec) were held constant, light filtered at 405 nm (Fig. 6A) produced more superoxide anion detected as DMPO-OH than either light filtered at 525 nm or 650 nm (Figs. 6B and 6C, respectively).

Detection of Singlet Oxygen With DTBH

When PP IX (0.1 mM) was suspended in water containing DTBH (200 μM) and DMSO (0.28 M) and irradiated for 30 sec, the ESR spectrum corresponding to nitroxide II (Fig. 3) was observed (data not shown). However, continued irradiation for 5 min resulted in an ESR spectrum characteristic of nitroxide endoperoxide III (Fig. 7A). Superoxide...
Table 1. Detection of oxygen active species by photoactivation of PP IX

<table>
<thead>
<tr>
<th>Spin Trap</th>
<th>Buffer</th>
<th>PPIX</th>
<th>Light</th>
<th>Light intensity</th>
<th>Scavengers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection of superoxide</td>
<td>DMPO</td>
<td>50 mM phosphate pH 7.4 and 1 mM DPTA</td>
<td>0.25 μM to 100 μM</td>
<td>none filtered light and filtered at 405.525.650 nm</td>
<td>200 μE/m²/sec and 8 μE/m²/sec</td>
</tr>
<tr>
<td>Detection of singlet oxygen</td>
<td>DTBH</td>
<td>Deionized water containing 0.28 M DMSO</td>
<td>0.25 μM to 100 μM</td>
<td>none filtered light and filtered at 405.525.650 nm</td>
<td>200 μE/m²/sec and 8 μE/m²/sec</td>
</tr>
</tbody>
</table>

anion is known to mediate the oxidation of hydroxylamines to nitroxides. Therefore, a solution of DTBH in the presence of a superoxide anion flux (5–10 μM/min), generated by the aerobic action of xanthine oxidase on xanthine, was examined to determine what the ESR spectrum would be. We found to our surprise that there was no ESR spectrum. Furthermore, SOD (30 U/ml) and CAT (300 U/ml) had no effect on the ESR signal (Fig. 7A) formed by the action of light on PP IX in the presence of DTBH (data not shown). In contrast, addition of singlet-oxygen quenchers such as DABCO (10 mM) or triethylamine (0.28 M) in the illuminated PP IX solution eliminated the nitroxide signal corresponding to the endoperoxide III (Fig. 7B).

We were able to detect small characteristic ESR spectra produced from PP IX at physiologic concentration (0.25 μM) by comparing these spectra to those obtained from irradiation of control reaction solutions which contained no PP IX (data not shown). As in the case of the detection of superoxide anion, light centered at 405 nm (Fig. 8A) produced consistently more singlet oxygen than light centered at 525 nm and 650 nm (Figs. 8B and 8C, respectively). The ESR spectra (Fig. 8) consist of mixtures of nitroxides II and III. Irradiation of DTBH in the absence of PP IX did not yield any ESR signals.

Discussion

Superoxide-anion production has been suggested as the mechanism of tissue damage in the disease erythropoietic protoporphyria. Because ocular tissues might be similarly photosensitized, we undertook studies to determine what oxygen-reactive spe-
Fig. 7. (A) ESR spectrum following a 5 min irradiation of an aqueous solution of protoporphyrin (0.1 mM) in the presence of DTBH (200 µM in 0.28 M DMSO). (B) Same as in scan A with the presence of triethylamine (0.28 M). The light intensity was 200 µE/m²/sec. The ESR spectrometer settings were identical to those described in Figure 6.

Fig. 8. ESR spectra were obtained following a 5 min irradiation of protoporphyrin (0.1 mM) in the presence of filters. The light intensity was 8 µE/m²/sec. Scans A, B and C were the results obtained using 405 nm, 525 nm, and 650 nm filters. The ESR spectrometer settings were identical to those described in Figure 5.

The results of this study demonstrate that a naturally occurring constituent of blood—PP IX—produces the photooxidants superoxide anion and singlet oxygen. Since erythrocytes, through their ion channels, are permeable to superoxide anion it is possible that superoxide anion and hydrogen peroxide (the self-dismutation product of superoxide anion) may be delivered to and damage the vascular endothelium of the choriocapillaris, Bruch’s membrane, and the RPE.

In the United States ARMD is the most common cause of severe visual loss in the elderly. The prevalence of the disease in its mildest form, the presence of drusen with minimal pigmentation and retention of good vision, has been reported in about 25% of the general population. Those who develop visual loss of 20/30 or greater due to drusen or serous or hemor-
rhagic detachment of the retina constitute 1.2% of the population under 65 years of age and 19.7% of the population 75 years of age and older.29

Several theories of the pathogenesis of ARMD have been proposed based on histopathologic observations in which sclerosis and alterations of the choriocapillaris were noted by several investigators.30-32 However, a correlation between ARMD and high blood pressure (a cause of arteriosclerotic changes) or elevated low-density lipoproteins and cholesterol (a cause of atherosclerotic changes in the choriocapillaris) has not been documented.33

Primary dysfunction of the RPE has been suggested by some investigators as a cause of ARMD.34,35 They argue that with senescence the RPE may no longer be competent to perform its many functions and eventually degenerates. This hypothesis proposes that early histopathologic changes in the choriocapillaris and Bruch's membrane are secondary to altered RPE function, but it does not explain how dysfunctional RPE could cause these sclerotic changes throughout the choriocapillaris.

The role of light exposure has been investigated by many as causing retinal damage.36-45 However, all experiments on light toxicity have been done at high levels for short durations. As yet there have been no animal models of ARMD produced with low-level ambient illumination. Acute photic injury with high-intensity illumination causes damage to photoreceptors and the RPE.37,38,41,44 However, intense illumination rarely occurs naturally, and therefore it is more reasonable to postulate that if light stimulus is related to the development of ARMD, it occurs within the context of chronic exposure to ambient levels of illumination.

Because some ultraviolet radiation (UV) penetrates the ocular media to the retina, the possibility that ARMD is related to chronic high-level UV exposure has been investigated.46 Long-term exposure to UVB has been found to be associated with the development of cortical cataracts.47 However, in the same population, no association between chronic exposure to either UVA (320–400 nm) or UVB (290–320 nm) and ARMD was found.46

The development of ARMD proceeds with certain histopathologic characteristics. Hyalinization of Bruch's membrane and the choriocapillaris are the earliest manifestation of the disease.8-10,48 Gradual thickening initially occurs in the inner aspect of Bruch's membrane8-10 with an increase in periodic acid-Schiff (PAS)-positive material9,11 and collagen.9,11 The thickened area of Bruch's membrane is weakened which can lead to localized detachments,8,48 forming focal excrescences termed "dru-
anion and singlet-oxygen photogeneration can be greatly reduced by filtering the shorter wavelengths, particularly violet-to-blue light. Lipid peroxides may induce the molecular injury that occurs to capillary and Bruch’s membranes. Presumably the injury caused by free-radical penetration of these membranes would necessitate a reparative process. This injury may be the disturbance of the interaction of the choriocapillaris, Bruch’s membrane, and RPE that Tso\textsuperscript{50} believed to be a cause of drusen formation. Thickening of Bruch’s membrane is a consistent feature beneath RPE cells that have degenerated with the development of drusen.\textsuperscript{5,11,48,49} Specifically, PAS-positive material and collagen have been identified as aging changes in Bruch’s membrane.\textsuperscript{9,11} In erythropoietic protoporphyria, collagen and amorphous PAS-positive material have been found in blood vessel basement membrane.\textsuperscript{5-7} The thickened basement membranes seen in both patients with erythropoietic protoporphyria and ARMD may represent the same response to injury. In the eye, a thickened Bruch’s membrane could impair the exchange of nutrients and waste products to and from the RPE. The RPE could become progressively compromised so that it could no longer adequately support the outer retina. If this mechanism is correct, dysfunctional RPE would be expected to occur more frequently over the choriocapillaris network. This hypothesis is supported by observations in flat preparations of the choriocapillaris, Bruch’s membrane, and RPE that Tso\textsuperscript{50} believed to be a cause of drusen formation.

Retinopathy of prematurity (ROP) may be a disease that affects another population with compromised protective enzymes for photogenerated superoxide anion and singlet oxygen from photosensitizing compounds, such as PP IX, in blood. The proliferating endothelium of the preterm infant may be exquisitely sensitive to photooxidant products of blood, and because defensive enzyme systems may be immature, significant capillary damage with vascular closure may occur by this mechanism. Transmission electron microscopy visualized membrane changes, termed gap-junction formation,\textsuperscript{61,62} that may be evidence of membrane lipid peroxidation. The efficacy\textsuperscript{61,62} of vitamin E in ROP may be related to its ability to quench singlet oxygen. However, vitamin E is not always effective in preventing this disease.\textsuperscript{53,64} It is possible that other photooxidants such as superoxide anion are also produced which can continue to damage developing capillaries.

Oxygen toxicity has been correlated with the development of ROP.\textsuperscript{65,66} More recently there is evidence that light exposure may be a risk factor for the development of ROP.\textsuperscript{67} In addition, multiple transfusions\textsuperscript{68} produce an increased risk of ROP. The mechanism for this increased risk may be that with multiple transfusions, erythrocytic hemolysis occurs, increasing the serum concentration of PP IX and perhaps other photosensitizers. If photooxidants are involved in the development of ROP, perhaps the best prophylaxis would be to maintain the level of light exposure for the premature infant at that which would have existed in utero had the pregnancy gone to term: in other words no light exposure until term maturation occurs.

Photosensitization is undoubtedly a mechanism by which free radicals are generated which mediate biologic tissue injury. Light toxicity with the generation of free radicals has been proposed by others as a mechanism of injury to the eye.\textsuperscript{35-45} Photosensitization with PP IX to the vascular basement membranes of the skin of patients with erythropoietic protoporphyria is remarkably similar histopathologically to the changes noted in Bruch’s membrane in patients with ARMD. It is possible that any tissue that is exposed to light for significant periods of time in the presence of PP IX or other hematogenous photosensitizers may develop phototoxicity. Tissue damage by this mechanism may be prevented by inducing protective enzymes, using scavengers of free radicals and singlet oxygen, or filtering the appropriate excitatory wave lengths.

**Key words:** ARMD, ROP, free radicals, protoporphyria, ESR spectroscopy

**Acknowledgments**

The authors thank Drs. John Keana and Vaikumth Prabhu for providing the singlet-oxygen probe and Dr. Bruce Drum for providing the appropriate light filters.

**References**


42. McDonald HR and Irvine AR: Light-induced maculopathy from the operating microscope in extracapsular extraction and intraocular lens implantation. Ophthalmology 90:945, 1983.


