Light-Induced Retinal Vascular Damage by Pd-porphyrin Luminescent Oxygen Probes

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PURPOSE. The phosphorescence lifetime of certain metalloporphyrins dissolved in a physiological medium provides an optical signature for local oxygen concentration (\(P_O2\)). This effect is used for measuring physiological \(P_O2\) levels in various tissues. However, the phosphorescence quenching of certain metalloporphyrin triplet states by oxygen also creates singlet oxygen, which is highly reactive and capable of inducing tissue damage. In the current study, the Pd-meso-tetra(4-carboxyphenyl)porphyrin dye (PdTCPP) was simultaneously used as an oxygen sensor and a photosensitizer. Phototoxicity was assessed in the eye fundus and correlated with tissue oxygenation, drug-light dose, and severity of tissue damage.

METHODS. The kinetics of photochemical oxygen depletion during PdTCPP excitation was measured in vivo on the optic disc of piglets by phosphorescence lifetime imaging. Blood-retinal barrier breakdown and tissue damage were assessed by confocal and electron microscopy.

RESULTS. For a retinal irradiance of 5 mW/cm² at 532 nm and an injected PdTCPP dose of 20 mg/kg, the mean phosphorescence lifetime measured at the optic disc increased from 100 to 600 μs within 8 minutes of continuous illumination. This corresponds to a decrease of \(P_O2\) from 25 to 0 mm Hg, induced by a light dose of only 2.4 J/cm². An exposure time of 6 minutes (1.8 J/cm²) generated an increase in phosphorescence lifetime from 100 to 400 μs, corresponding to a decrease in \(P_O2\) from 25 to 4 mm Hg. This caused edema in all retinal layers, whereas irradiation of 2 minutes (0.6 J/cm²) damaged blood vessels and induced edema in the inner nuclear layer only. Heavy redistribution of occludin occurred after a 30-minute exposure time (9 J/cm²).

CONCLUSIONS. PdTCPP is potentially photoxic under certain experimental conditions and can induce damage in peripapillary retina and optic nerve head after light exposure. The severity of tissue damage correlates with the phosphorescence measurements. (Invest Ophthalmol Vis Sci. 2005;46:956–966) DOI:10.1167/iovs.04-0500

Compromised delivery of oxygen (\(O_2\)) to the tissues of the eye fundus has been implicated in a variety of ocular diseases, such as proliferative retinopathies and possibly glaucoma.1,2 Therefore, many studies have been conducted using \(O_2\)-sensitive microelectrodes to examine retinal tissue oxygenation.3,5 However, the invasiveness of this technique limited its application to animal models or human surgery.6 An optical technique using reflectance spectroscopy for probing the difference in light absorption between oxy- and deoxyhemoglobin7–9 was proposed for noninvasively measuring the \(O_2\) content in the ocular fundus. This approach appeared experimentally difficult, because light absorption by the ocular pigments has to be carefully calibrated and corrected to obtain reproducible and reliable results.10–14 Recently, an optical technique was developed for noninvasively measuring the intravascular partial pressure of oxygen (\(P_O2\)) in the ocular fundus.15 It locally deduces the \(P_O2\) by measuring the phosphorescence emission of an injected dye.16,17 In detail, \(O_2\) molecules present in blood interact with excited triplet states resulting from a luminous excitation of the dye. This interaction, referred to as “quenching,” depopulates the triplet state, consequently shortening the phosphorescence lifetime (\(\tau\)). The \(P_O2\) is then related to the phosphorescence emission through the Stern-Volmer relationship:

\[
\tau_0/\tau = 1 + k_qP_O2,
\]

where \(\tau_0\) (in seconds) is the phosphorescence lifetime in the absence of \(O_2\), \(\tau\) (in seconds) is the phosphorescence lifetime for the local \(P_O2\) (in mm Hg) level, \(I_0\) and \(I\) are the steady state phosphorescence intensities at zero \(P_O2\) and local \(P_O2\), respectively. The quenching constant \(k_q\) (s⁻¹ · mm Hg⁻¹) is a diffusion-limited, second-order rate constant.

Among several possible phosphorescent dyes, the Pd-meso-tetra(4-carboxyphenyl) porphyrin (PdTCPP) complex has been used to measure the \(O_2\) partial pressure in various biological samples16,19 and, more specifically, in the ocular fundus of various animal species.15,20–22 Except for the intravenous administration of the dye, the quenching technique is noninvasive. In some contributions it has been mentioned, however, that sustained \(O_2\) measurements by phosphorescence quenching could transiently or permanently reduce the blood oxygenation or perfusion, reduce the blood-retinal barrier’s integrity, and even alter the tissue’s integrity.23,24 These side effects can be attributed to the singlet oxygen molecules resulting from the quenching of PdTCPP triplet states by \(O_2\).25 Indeed, the high reactivity of the singlet oxygen could lead to vascular and tissue damages, depending on the agent’s concentration and the applied light dose,26 as a well known effect in photodynamic therapy (PDT) used to occlude subfoveal choroidal neovascularization sec-

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Supported by the Swiss National Science Foundation, Grants 3100-065093, 3100-55106, 2053-061685; and the Swiss Federal Office for Education and Science, Grant 00.0047.2.

Submitted for publication May 5, 2004; revised August 28 and October 19, 2004; and accepted October 29, 2004.

Disclosure: T.K. Stepinac, None; S.R. Chamot, None; E. Rungger-Brändle, None; P. Ferrez, None; J.-L. Munoz, None; H. van den Bergh, None; C.E. Riva, None; C.J. Pournaras, None; G.A. Wagnières, None

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ondary to age-related macular degeneration.\textsuperscript{27} This suggests that the O\textsubscript{2} sensor PdTCPP is phototoxic and could induce PDT-like damage in tissue. This hypothesis has never been clearly demonstrated nor studied in vivo but can be supported through in vitro experiments.\textsuperscript{28–30}

With the purpose of quantifying PdTCPP phototoxicity in the eye fundus and of measuring how the induced photodamage relates to tissue oxygenation and drug/light dose, we used this dye simultaneously as an O\textsubscript{2} sensor and a photosensitizer. To our knowledge, this is the first report of photo-oxidative tissue damage and O\textsubscript{2} measurement performed simultaneously using the same agent.

**METHODS**

The experiments consisted of measuring the pO\textsubscript{2} by phosphorescence quenching before, during, and after a quasicontinuous photoactivation of the PdTCPP dye and comparing the administered light dose to the histologic alterations at the retinal tissue level. An optical instrument performing the frequency-domain lifetime measurement was used to induce tissue damage and simultaneously to measure the blood pO\textsubscript{2} in a portion of the treated area. The alterations of the blood pO\textsubscript{2} after dye photoactivation were also studied by means of a second optical instrument performing the time-domain lifetime measurement.

**Animals**

Experiments were performed on four young piglets (\(\text{\sim} 10\) kg). All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. After intramuscular injection of a tranquilizer for pigs (Azaperone, Stresnil; Janssen-Cilag AG, BA, Switzerland), anesthesia was induced with 25 to 30 mg of metamidate chloride hydrochloride (Hypnolid; Janssen-Cilag AG) injected into the ear vein. After arterial and venous catheterization, the pigs were curarized (1 mg/10 kg tubocurarine chloride; Abbott AG, BA, Switzerland), intubated, and artificially ventilated. During the experiment, anesthesia was maintained by continuous perfusion of pentobarbital (Nembutal; Abbott AG) and tubocurarine chloride. The animals were ventilated at approximately 18 strokes per minute, with a continuous flow of 20% O\textsubscript{2} and 80% N\textsubscript{2}O by a variable-volume respirator (Siemens Volumeter; Siemens-Elema, Zurich, Switzerland). The head was secured to prevent movements. The mean arterial blood pressure was monitored via the femoral artery by transducer (Mingograph; Siemens-Elema). The systemic arterial pO\textsubscript{2}, pCO\textsubscript{2}, and pH were measured intermittently from the same artery with a blood gas analyzer (AVL automatic gas system 940; AVL Scientific Corp., Roswell, GA). Adjustments of ventilation rate, stroke volume, and the composition of the inspired gas maintained arterial pCO\textsubscript{2} at 30 to 35 mm Hg and arterial pO\textsubscript{2} at 100 to 110 mm Hg. A rectal thermometer was used to measure the animal’s temperature, which was maintained between 36°C and 38°C by means of a thermal blanket with a temperature control unit.

**Phosphorescent Dye**

The phosphorescent probe PdTCPP (Oxygen Enterprises, Philadelphia, PA) was bound to albumin before injection. To achieve this binding, blood was drawn from the animal (\(\text{\sim} 4\) mL/kg), and the serum was extracted by centrifugation at 8000 rpm for 10 minutes. The probe (20 mg/kg of animal weight) was mixed with the serum, the pH was adjusted to 7.4, and the serum–probe solution was filtered with 0.2-mm sterile filter. The solution was infused intravenously (\(\text{\sim} 100\) mL/h), allowing equilibration with the blood before the measuring started. Forming a PdTCPP–albumin complex ensures that the parameters \(\tau_0\) and \(k_q\) (cf., equation 1) are stable over the range of physiological pH and temperatures. This binding also improves the probe sensitivity to O\textsubscript{2} and reduces self-quenching. Finally, it confines the phosphorescent dye to the intravascular space, since albumin does not diffuse across the wall of the retinal blood vessels (blood–tissue barrier formed by tight endothelial junctions).

In phosphorescence solution, PdTCPP absorbs maximally at 412 and 528 nm, and its emission spectrum is centered at 706 nm. In the present study, we used the values of \(\tau_0\) and \(k_q\) corresponding to a pH of 7.4 and a temperature of 38°C (i.e., 637 \(\mu\)s and 381 s\(^{-1}\)·mm Hg\(^{-1}\), respectively).\textsuperscript{31}

Oxygen being the only quenching agent present in significant concentrations in blood, the phosphorescence lifetime measurements provide an evaluation of pO\textsubscript{2}. Thus, the values of \(k_q\) and \(\tau_0\) determined in vitro hold for measurements in vivo.\textsuperscript{31,32} Phosphorescence lifetime measurements also have the advantage that they are practically unaffected by changes in the absorbance of pigments present in the sample. The lifetime measurements are independent of the concentration of the probe and of the intensity of the excitation light in our conditions.

**Instrumentation**

A fundus camera (Carl Zeiss Meditec, Göttingen, Germany) was modified for imaging the phosphorescence of the eye fundus in both frequency and time domains (Fig. 1). A bundle of four ultrabright yellow diodes (peak emission = 590 nm, full-width at half maximum [FWHM] = 25 nm: SL905ICE, Sloan, Ventura, CA) replaced the conventional tungsten lamp used for fundus imaging to allow camera alignment prior to phosphorescence measurements. This wavelength minimized the photosensitizing effects, since PdTCPP absorption is not significant in the yellow spectral band.

The frequency-domain phosphorescence lifetime imaging (FD-PLIM)\textsuperscript{33,34} instrument\textsuperscript{35–39} and its measurement principle\textsuperscript{35–39} have been widely discussed in the literature. It consists of modulating the excitation light and measuring the phase shift (\(\phi\)) or the demodulation (\(m\)) between phosphorescence and excitation, from which the respective lifetimes \(\tau_0\) and \(\tau_m\) can be deduced by

\[
\tau_0 = \frac{1}{2nf} \tan(-\phi) \quad (2)
\]

\[
\tau_m = \frac{1}{2nf} \frac{\text{FWHM}}{\sqrt{2}} - 1. \quad (3)
\]
that allowed the determination of the phase shift and demodulation between excitation and detection.

The time-domain phosphorescence lifetime imaging (TD-PLIM) instrument used in this study has been described in detail elsewhere. The output of a flash lamp (Oxygen Enterprises) delivering 2 μs light pulses (FWHM) was filtered (peak transmittance = 530 nm, FWHM = 25 nm; XM-530; Corion; Spectra Physics) and fed to the fundus camera through an optical fiber bundle. Each flash delivered a light dose of ~20 μJ/cm² at the retina. A long-pass glass filter in the observation path spectrally selected the phosphorescence emission (λ > 630 nm; model RG 630; Schott, Mainz, Germany), which was then imaged (~7° retinal field) with an intensified CCD camera (model ISG-250-R-3; Xybion, San Diego, CA). The phosphorescence decay was measured in the time-domain by recording a set of 11 intensity images (512 × 486 pixel) at various delays (Δt1, ..., Δt11) after the excitation flash. The Δt used were 20, 30, 40, 50, 60, 100, 200, 300, 400, 600, and 2500 μs. The analyses of a set of phosphorescence intensity images proceeded as follows. The background image (Δt1 = 2500 μs) was subtracted from the 10 other images (Δt1 - Δt10). For each pixel, a data set was formed with its intensity value in each of the 10 phosphorescence images. This was then transformed by taking the natural logarithm of these intensities, and the best fit to a linear function was applied to the resultant data. This fit was then used as an initial estimate for a new fit, using the Marquardt-Levenberg algorithm. This operation provided the resultant data. This fit was then used as an initial estimate for a new fit, using the Marquardt-Levenberg algorithm. This operation provided the resultant data. This fit was then used as an initial estimate for a new fit, using the Marquardt-Levenberg algorithm.

Electron Microscopy

Tissue was processed essentially as previously described with minor modifications. Briefly, enucleated eyes were opened at the ora serrata and the posterior eyecup immersed in fixative solution I (1.5% glutaraldehyde, 2% formaldehyde, 2 mg/mL tannic acid, 0.1 M phosphate buffer [pH 7.4]) overnight at 4°C. During the washing steps in 0.1 M cacodylate buffer (pH 7.4, 4°C), the peripapillary retina was detached from the underlying pigmented epithelium and the optic nerve cut through. The optic nerve head was thoroughly isolated from the surrounding retina and bisected. The remaining retina (approximately 1 cm²) was cut into small pieces. After osmification, the tissue blocks were stained with uranyl acetate, dehydrated through a graded series of ethanol and propylene oxide, and embedded in Epon. Semithin sections were stained with methylene blue and thin sections with uranyl acetate and lead citrate. Photographs were taken with an electron microscope (model CM10; Philips, Eindhoven, The Netherlands).

Immunolocalization of Occludin

Posterior eyecups were extracted with 0.2% Triton X-100 in 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid) buffer (PIPES; pH 6.9), containing 5 mM EGTA and 2 mM MgCl₂ (2 minutes, 0°C), before fixation with 3% freshly prepared formaldehyde in PIPES buffer without Triton X-100 (45 minutes, room temperature). Before isolation of the peripapillary retina, the fixed eyecup was rinsed several times in TBS (20 mM Tris-HCl [pH 8.0], and 150 mM NaCl), containing 0.9% Triton X-100. Part of the retinal tissue was cryoprotected in 30% sucrose and embedded in mounting resin (Tissue Tek; Sakura Finetek, Torrance, CA). Cryostat sections were mounted on glass slides and, without further processing, photographed with Nomarski optics.

After fixation, the tissue was glycinated for cryoprotection. After storage in 55% glycerol at ~20°C, the tissue was rehydrated in TBS-0.5% Triton X-100 and preincubated in 0.2% bovine serum albumin. Incubation in polyclonal rabbit anti-occludin antibody (dilution 1:100; Zymed Laboratories, South San Francisco, CA) was in preincubation buffer over 3.5 days at 4°C. Incubation in anti-rabbit IgGs coupled to Texas red (dilutions 1:200; Jackson ImmunoResearch, Milan Analytica, LaRoche, Switzerland) was performed for 2 days. Retinas were mounted, vitreous side up, in a mixture of 3:7 of 0.1 M Tris-HCl (pH 9.5) and glycerol containing 50 mg/mL n-propyl gallate.

Specimens were viewed with a confocal microscope (model LSM 410; Carl Zeiss Meditec) using a 63× oil objective. Optical sections...
were taken at a wavelength of 543 nm at intervals of 0.5 μm. Extended focus images from vessels close to the vitreous aspect were processed on computer (Imaris software; Bitplane AG, Zurich, Switzerland).

**Protocols**

Each eye of the four animals measured with the FD-PLIM instrument received a mean retinal irradiance of 5 mW/cm² at 532 nm for various durations, which is an optimal compromise to avoid thermal effect with reasonable illumination time (e.g., 2 minutes). This value was calibrated with a power meter (model H410; Scientech Inc., Boulder, CO) through a lens mimicking a piglet eye. This illumination covered a 30° retinal field centered at the optic nerve. This light was amplitude modulated at a frequency of 500 Hz, to allow simultaneous measurements of O₂ concentration with the FD-PLIM technique. A 30-minute irradiation was performed on both eyes of pigs 1 and 2. The left eye was irradiated before dye injection, whereas the right eye was irradiated afterward. For pigs 3 and 4, both eyes were irradiated after dye injection for 6 and 2 minutes, respectively. For comparison with the FD-PLIM results, phosphorescence lifetime measurements were obtained before and after photoirradiation with TD-PLIM in all eyes except 1a and 1b, which did not receive any dye before irradiation. These two eyes were measured after photoirradiation with TD-PLIM. At the end of the experiments, the eyes were enucleated under deep anesthesia and the animals were killed. The enucleated eyes were then photographed with a standard operating microscope (Carl Zeiss Meditec) and examined for histologic alterations.

**RESULTS**

**Vascular Damage to the Optic Disc**

All experimental conditions were repeated twice and led to similar results. The optic disc of eyes 4a and 4b that had been irradiated with 9 J/cm² after dye injection appeared altered.
possibly hyperemic, on photographs taken under white light illumination after enucleation (Fig. 2). This was probably due to vascular hyperpermeability causing extravasation of the dye and circulating blood cells (cf., Fig. 9D).

Electron microscopy of the optic nerve head of eye 4b revealed an important interstitial edema, seen as thinning of the endothelial cells and swelling of the mitochondria, spacing of the individual smooth muscle cells layers, and loosening of the collagen bundles (Fig. 3A). The presence of polymorphonuclear leukocytes in the subendothelial space and the granular appearance of the interstitial fluid indicated leakage and extravasation, early signs of an inflammatory process. In the irradiated but noninjected control eye 1b, the cells of the vascular wall appeared normal and the collagen fibers in the underlying matrix were densely packed (Fig. 3B).

To assess structural effects at the blood-retinal barrier, occludin was immunolocalized in retinal wholemounts (Fig. 4). In the absence of circulating PdTCPP, irradiation at 9 J/cm² did not induce changes in the staining pattern of either arteries (Fig. 4A) or veins (Fig. 4D). Irradiation in the presence of the dye, however, caused local redistribution of occludin, blurring the characteristic reticular pattern (Figs. 4B, 4C, 4E, 4F). This blurring was most conspicuous in small arteries and arterioles (Figs. 4B, 4C), whereas arteries of larger caliber appeared virtually normal (Fig. 4B). Redistribution along a vessel was locally restricted (Fig. 4C). Contrary to arteries, irradiation in the presence of PdTCPP also led to an irregular staining pattern in large-caliber veins (Fig. 4E) with local clumping or absence of staining (Fig. 4F) suggesting heavy damage of the endothelial cell layer. With electron microscopy, we indeed detected retraction of endothelial cells, particularly in occluded vessels, leaving extended stretches of the vessel circumference bare of cellular elements (not shown).

**Damage in the Peripapillary Retina**

Figure 5 shows transverse cryostat sections from eyes that had been irradiated for various lengths of time in the absence or presence of PdTCPP. In the absence of the photosensitizer, the irradiated control retina showed good preservation of the laminar structure (Fig. 5A), whereas irradiation in the presence of the dye caused edema to various degrees (Figs. 5B–D). Likewise, a 2-minute irradiation resulted in vacuolization, particularly of the inner nuclear and outer plexiform layers (Fig. 5B). At 6 minutes, vacuolization had extended into the inner plexiform and ganglion cell layers (Fig. 5C). Irradiation for 30 minutes led to severe damage in virtually all retinal layers and thus to a complete loss of laminar organization (Fig. 5D). When studied with electron microscopy, irradiation for 30 minutes in the absence of the photosensitizer led to the formation of lamellar bodies throughout the retina, but the overall ultrastructural pattern of retinal parenchyma and vessels appeared normal (Fig. 6A). However, in the presence of PdTCPP, irradiation for 2 minutes caused occlusion of small vessels (Fig. 6B), thrombus formation (Fig. 6C), and heavy damage to the vascular wall (Figs. 6B, 6C). The neuropil of the inner plexiform layer looked normal (Fig. 6A), but damage in the inner nuclear layer was prominent with marked thinning of the glia limitans and swelling of Müller cells but not of the basement membrane (Fig. 6C). In regions of minor edema, Müller cells contained remnants of red blood cells (not shown). This indicates that, under certain circumstances, these glial cells are able to function as phagocytes. Taken together, the volume increase of the retina is due to both interstitial and cellular edema, the basement membrane contributing little if at all to fluid absorption. This situation is unlike the one in the optic nerve head, where the abundant extracellular matrix absorbs exudated fluid quite efficiently (cf. Fig. 3A).

**Phosphorescence Lifetime Imaging with the Time-Domain Device before and after Photoirradiation**

Phosphorescence lifetime images were taken before and after photo-irradiation with the time-domain device and are shown in Figures 7 and 8. In eyes 3a and 3b, an increase in phosphorescence lifetime (Figs. 7E, 7F) and a leakage of PdTCPP out of blood vessels were observed after illumination (Fig. 7B), whereas there was almost no visible difference before and after illumination in eye 2. After irradiation, we noticed early leakage, indicated by two spots that had a longer phosphorescence lifetime than the rest of the image (Fig. 7H, arrows). In eyes 4a and 4b that had received 9 J/cm², the change of phosphorescence lifetime and pO₂ during the interval before and after
Irradiation was dramatic (Fig. 8) and a decrease in phosphorescence lifetime and leakage of PdTCPP was observed after illumination. The mean phosphorescence lifetimes measured in retinal vessels and at the optic disc before and after photoradiation are summarized in Table 1.

Phosphorescence Lifetime Monitoring with the Frequency-Domain Device during the Generation of Photodynamic Tissue Damage

The evolution of phosphorescence lifetime and thus of pO$_2$ during the irradiation/photoirradiation process was continuously monitored with the FD-PLIM device. The phosphorescence lifetime calculated with the modulation was up to 30% higher than the phosphorescence lifetime calculated with the phase. This phenomenon is well known and indicates a non-monoexponential phosphorescence lifetime. We have therefore considered the “apparent” lifetime as representing the average between the “modulation” lifetime and the “phase” lifetime.

Figure 9 shows the phosphorescence lifetime images of PdTCPP with the FD-PLIM device during the irradiation of ONH of eye 3a. The lifetime images were taken at several time intervals after onset of irradiation. The sequences of images show that the phosphorescence lifetime increases during the irradiation process. This increase is more marked in extravascular tissue and at the beginning of irradiation.

The “apparent” phosphorescence lifetime was plotted versus the time elapsed since the beginning of irradiation for a region of the image taken on a blood vessel and an adjacent extravascular compartment. Figure 10 shows that the phosphorescence lifetime increased from $1/25$ mm Hg to approximately 600 $\mu$s (corresponding to a pO$_2$ of $\leq 1$ mm Hg) within the first 8

Figure 4. Immunolocalization of occludin in arteries (A–C) and veins (D–F) after irradiation with 9 J/cm$^2$ in the absence (A, D) or presence (B, C, E, F) of PdTCPP. Confocal microscopy of flatmounted retina. The regular reticulate pattern (A, D) outlining the endothelial cells was locally disrupted in the presence of the drug (B, C, E, F). Local displacement (arrows) and absence of staining (double arrows) suggest heavy damage of the blood–retina barrier. Scale bars, 20 $\mu$m.

Figure 5. Influence of duration of irradiation on the integrity of the peripapillary retina, irradiated in the absence (A) or presence (B–D) of PdTCPP. Micrographs of unstained transverse cryostat sections obtained with Nomarski optics. (A) Control retina after 30 minutes of irradiation showed good preservation of laminar organization. (B–D) Prominent swelling, vacuolization, and disruption of the tissue with increasing duration of irradiation in the presence of PdTCPP. Arrows: vacuoles in the OPL formed at 2 minutes (0.6 J/cm$^2$) of irradiation (B) and at 6 minutes (1.8 J/cm$^2$) also present in the INL, IPL, and GCL (C). At 30 minutes (9 J/cm$^2$) all layers were affected (D). OS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; ONFL, optic nerve fiber layer. Scale bar, 100 $\mu$m.
minutes of irradiation (corresponding to a light dose of 2.4 J/cm²), reaching a plateau at ~600 μs. The increase in phosphorescence lifetime (i.e., the depletion of O₂) is slightly faster in the tissue than in the vascular bed. Similar results were obtained in a second animal treated under identical conditions.

**DISCUSSION**

Oxygen quenching of metalloporphyrin triplet states creates singlet oxygen that is highly reactive in biological systems. These O₂-consuming reactions can lead to vascular and tissue damage and perturbing tissue oxygenation, depending on...
the agent’s concentration and the applied light dose, a well-known effect in PDT. Our results suggest that the O\textsubscript{2} sensor PdTCPP is potentially phototoxic under certain experimental conditions and could induce PDT-like damage in tissue.

Kinetics of photochemical O\textsubscript{2} depletion and tissue damage during the illumination of a Pd-porphyrin O\textsubscript{2} probe were measured in vivo at the optic disc of piglets. Both TD- and FD-PLIM systems coupled to a fundus camera were used for this purpose. At a retinal irradiance of 5 mW/cm\textsuperscript{2} at 532 nm and an injected dose of Pd-porphyrin of 20 mg/kg, the mean phosphorescence lifetime measured at the optic disc increased from 100 to 600 s within 8 minutes, corresponding to a light dose of 2.4 J/cm\textsuperscript{2}, to then reach a plateau level of 600 s. The general aspect of the curves representing the phosphorescence of PdTCPP as a function of the irradiation time (cf., Fig. 10) is similar to what has been reported earlier.\textsuperscript{41}

The vascular effects of PDT have been recently reviewed.\textsuperscript{26,27,42,43} PDT induces hypoxia as a result of decreased perfusion of the affected tissue. Endothelial cell damage leads to the establishment of thrombogenic sites within the vessel lumen that initiate a physiological cascade of responses, including platelet aggregation, release of vasoactive molecules, leukocyte adhesion, increases in vascular permeability, and vessel constriction. These effects combine to produce blood-flow stasis\textsuperscript{20,42,43}, however, the increase in phosphorescence lifetime and intensity observed in our experiments cannot be explained simply by reduced perfusion. Under normal conditions, at the beginning of our experiments, the dye is located within the blood vessels, due to its linkage to albumin,\textsuperscript{44,45} and O\textsubscript{2} is sensed exclusively within the vasculature. The emission of oxidative substances during quenching of phosphorescence by O\textsubscript{2} causes damage to the vascular wall and entails leakage of the dye. As a consequence, the intensity of the phosphorescence deriving from the extravascular compartment increases during irradiation. Because the pO\textsubscript{2} outside the vessel is lower than inside, the phosphorescence lifetime increases. This correlation was indeed observed in our study. Furthermore, O\textsubscript{2} is consumed during the photoirradiation,\textsuperscript{29} further contributing to an increase in phosphorescence lifetime.

Several studies report alterations of the vascular endothelium during irradiation of tissues in vivo, photosensitized with porfimer sodium, verteporfin, and the purpurins.\textsuperscript{26,43,46,47} At short drug–light intervals in vivo,\textsuperscript{26,27} endothelial dysfunction and blood flow stasis followed by vessel occlusion appear to be the predominant mechanisms of damage. Moreover, a direct relationship between the vascular changes and the amount of photosensitizer in circulation during PDT has been suggested.\textsuperscript{20} This may hold true also for PdTCPP linked to albumin, the concentration of which remains nearly constant over the experimental time (<6 hours).\textsuperscript{26} Thus, the drug–light in-
interval is not likely to have an impact on the outcome of the treatment.

In both arteries and veins of the irradiated peripapillary retina, our histologic analysis revealed local redistribution of occludin in the presence of the photosensitizer, corroborating earlier observations on site-restricted leakage of vessels in the presence of porfimer sodium. In our study, displacement of occludin was evidence of the breakdown of the blood–retinal barrier, leading to the formation of edema in the retinal parenchyma, noted first in the inner nuclear and outer plexiform layers after an exposure time as short as 2 minutes. Longer exposure time caused edema in virtually all retinal layers. Neurons did not seem to be heavily affected, whereas the glia limitans ensheathing the capillary network was vacuolized. The presence of such cellular edema in Müller cells suggests that glial cells take up and accumulate

![Figure 9](https://www.arvojournals.org/doi/10.1167/iovs.05-0698)

**Figure 9.** Phosphorescence lifetime images of PdTCPP in two vessels of the ONH of eye 3 calculated after the modulation as a function of the elapsed time since the beginning of the irradiation. The diameter of the image is approximately 1 mm (~5° field). The color bar next to each figure indicates the color code used to display the phosphorescence lifetime. The average phosphorescence lifetime calculated on the whole image and the elapsed time since the beginning of illumination is at the top of each image. The phosphorescence lifetime increased faster during photolysis in the extravascular space. The images on the top and bottom right show the intensity of backscattering and the phosphorescence intensity, respectively.

![Figure 10](https://www.arvojournals.org/doi/10.1167/iovs.05-0698)

**Figure 10.** Phosphorescence lifetime and pO$_2$ plotted versus irradiation time and light dose. Values were reported for a large vessel and an area of the optic disc in two different eyes (3a and 4a). The values of the phosphorescence lifetime are the average between the “phase” lifetime and the “modulation” lifetime obtained with the FD-PLIM instrument. The increase of phosphorescence lifetime—that is, the depletion of O$_2$—was more marked in the tissue than in the blood vessels. After approximately 10 minutes (3 J/cm$^2$), the lifetimes in both optic nerve tissue and vessel stabilized above 500 μs, which corresponds to a pO$_2 < 1$ mm Hg.
fluid. This buffer function may protect neurons from irradiation damage.

Histology also revealed a striking difference between damage in the peripapillary retina and the optic nerve head. Although the optic disc was the direct target of irradiation, cell damage in the optic nerve head was surprisingly discrete. It thus appears that extravasated fluid is absorbed efficiently by the abundant extracellular matrix surrounding the vessels, thus preventing severe cell damage outside the vessel wall.

Fingar et al. studied the role of microvascular damage in PDT in animals photosensitized with porfimer sodium. Doses of 10 and 25 mg/kg porfimer sodium (Photofrin; QLT Phototherapeutics, Basel, Switzerland) caused a dose-related constriction of arterioles that was observed within the first minutes of illumination at the higher drug dose. Porfimer sodium doses that produce arteriole constriction also caused an increase in venule permeability to albumin, which occurred shortly after the start of light treatment and was progressive with time. Leakage began at specific sites along the venule wall but became uniform along the entire length of the venule within 1 hour after treatment.

At the drug–light dose we applied, a vessel leakage and a decrease in pO2 were noticed within minutes. With the frequency-domain device used in this study, the time needed for a single measurement is typically 20 seconds, corresponding to a light dose to the retina of 100 ml/cm2. The time-domain device delivered to the retina a light dose per measurement of approximately 11 ml/cm2 (20 µJ/cm2 per flash × 50 flashes × 11 time intervals). No adverse events where observed in previous work with that device for typically less than 10 repeated measurements. However, the light-collimating ability and detection sensitivity of our frequency-domain device could be further optimized, thus reducing the needed light dose by more than one order of magnitude.

Shonat and Knight have reported that after a PdTCPP injection dose of 10 mg/kg and an accumulated light dose of 2.5 J/cm2 at 524 nm, no immediate vessel damage (leaking) was observed in mice. Because the conditions (drug dose and animals) are different from ours, these findings are difficult to compare.

Differences in the cytotoxic effect of PDT between pulsed and CW excitations exist, which could contribute to the difference in tissue damage generated between our time- and frequency-domain instruments. It has been shown that the cytotoxic effect of PDT using pulsed light may be significantly less than that using CW light when the lifetime of the excited photosensitizer is longer than the pulse duration and the light intensity is high (typically, short pulses at a low repetition rate). It has also been shown that during PDT, cells exposed to pulsed light consume O2 more slowly, resulting in a lower amount of O2 consumption compared with PDT using CW light. In accordance with O2 consumption, the pulsed light induces significantly less photobleaching of the photosensitizer than the CW light. These results indicate that the efficiency of PDT using pulsed light is less than that of CW light, probably because of reduced O2 consumption during pulsed-light irradiation.

When PDT is used in clinical therapy, tissue damage usually occurs a few hours or days after treatment, whereas the severity and rapidity of the tissue alterations observed under our experimental conditions show that the drug and/or light doses used were within one or two orders of magnitude stronger than should be used in PDT. Reducing the drug dose, typically by a factor of 10, to 2 mg/kg, and applying a light dose of 1 to 2 J/cm2 could generate a phototoxic effect several days after photoradiation in young piglet eyes.

A more complete study of the phototoxicity of this dye is currently being conducted in our group in a choroidal-vascular membrane (CAM) model, which represents a good model for evaluating the vascular effect of phototoxic substances or new photosensitizer and easily allows their comparison.

In conclusion, our study showed that the O2 measurement with certain phosphorescent sensors could be less innocuous than previously stated and that the light and drug dose should be kept low. Further studies are necessary to evaluate the extent to which PdTCPP induces perturbations of the biological systems investigated. According to our results, PdTCPP is phototoxic under certain experimental conditions and can induce tissue alterations after light exposure.

Acknowledgments

The authors thank Nicole Gilodi and Alain Conti for skilled technical assistance.

References


20. Blumenroeder S, Augustin AJ, Koch FH. The influence of intravascular pressure and systemic oxygen tension on the intravascular...


