Photochemical Oxygen Consumption Sensitized by a Porphyrin Phosphorescent Probe in Two Model Systems

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ABSTRACT Phosphorescence quenching of certain metalloporphyrins is used to measure tissue and microvascular pO_2. Oxygen quenching of metalloporphyrin triplet states creates singlet oxygen, which is highly reactive in biological systems, and these oxygen-consuming reactions are capable of perturbing tissue oxygenation. Kinetics of photochemical oxygen consumption were measured for a Pd-porphyrin in two model systems in vitro over a range of irradiances (1.34–134 mW cm^{-2}). For a given irradiance, and, after correction for differing porphyrin concentrations, rates of oxygen consumption were similar when the Pd-porphyrin was bound to bovine serum albumin and when it was taken up by tumor cells in spheroids. At irradiances comparable to those used in imaging superficial anatomy, rates of oxygen consumption were sufficiently low (2.5 $\mu M\ s^{-1}$) that tissue oxygenation would be reduced by a maximum of 6%. An irradiance of 20 mW cm^{-2}, however, initiated a rate of oxygen consumption capable of reducing tissue pO_2 by at least 20–40%. These measured rates of consumption impose limitations on the use of phosphorescence quenching in thick tissues. The irreversible photobleaching of the Pd-porphyrin was also measured indirectly. The bleaching branching ratio, 23 M^{-1}, is significantly lower than that of porphyrin photodynamic agents.

INTRODUCTION

Remarkably high yields of room-temperature triplet state phosphorescence have been demonstrated for a number of metalloporphyrins (Eastwood and Gouterman, 1970; Gouterman et al., 1975; Vanderkooi et al., 1987). Porphyrin triplets are efficiently quenched by molecular oxygen ($^3O_2$), which is a ground state triplet, through the scheme credited to Kautsky (Foote, 1968), $T + {}^3O_2 \rightarrow S + {}^1O_2$, where T and S are the porphyrin triplet state and ground singlet state, respectively, and $^1O_2$ is singlet oxygen, the lowest lying electronic excited state of dioxygen. Thus, the lifetime of the metalloporphyrin phosphorescence provides an optical reporter of the oxygen concentration of the ambient medium. The use of phosphorescence quenching in biological systems was introduced by Vanderkooi et al. (1987), and it has been implemented by that group and by several other investigators to measure the oxygen tension in a variety of biological systems in vitro and in vivo. Examples of the use of this method in vivo include pO_2 measurements in subcutaneous rodent tumor models (Wilson and Cerniglia, 1992; Cerniglia et al., 1997), exposed organs (Rumsey et al., 1988; McIlroy et al., 1998), the retina of the eye (Shonat et al., 1992), and in thin window chamber tissue preparations (Torres Filho and Intaglietta, 1993; Torres Filho et al., 1994; Buerk et al., 1998).

Recently, Vinogradov and his colleagues have developed and characterized a series of longer-wavelength absorbing and emitting metalloporphyrins to take advantage of the more favorable optical properties of tissue in the red and near infrared spectral regions (Vinogradov and Wilson, 1995; Vinogradov et al., 1996). Between ~600 and 1300 nm, absorption of soft tissue is significantly reduced compared to that on either side of this window (Wilson and Jacques, 1990). Light scattering by tissue, which follows a weaker wavelength dependence, is also reduced in this region compared to shorter wavelengths (for example, Parsa et al., 1989). The phosphor Green 2W, with an absorption maximum at 636 nm and a phosphorescence emission maximum at 790 nm (Vinogradov et al., 1996), is well designed to allow exploitation of these favorable optical properties in the attempt to image oxygenation of relatively deep tissue structures, such as malignant tumors of the breast and of the extremities, for example, in which hypoxia has been linked to a poor response to ionizing radiation therapy and to increased probability of appearance of metastases (Okunieff et al., 1993; Brizel et al., 1996).

Although light at these wavelengths can penetrate several centimeters in tissue, it is rapidly attenuated, and efforts to extract optical signals from phosphorescent probes in thick tissues must address this practical reality. Using typical values of soft tissue absorption and transport scattering coefficients, 0.01 and 1.0 mm^{-1}, respectively, the value of the characteristic effective attenuation length in the diffusion approximation is approximately 5.7 mm. Because both the excitation light and the luminescence emission will be attenuated as they propagate from and to the tissue surface, higher excitation irradiances will be needed for signal recovery from deeper structures. This fact has been acknowledged by Vinogradov et al. (1996), who suggested that

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excitation lamp intensities could be "increased 10- to 100-fold" in efforts to extend phosphorescence-quenching measurements to "tissue thicknesses of 5 cm or greater." We have also suggested the feasibility of using metalloporphyrin phosphorescence to detect regions of hypoxia in tumors residing several centimeters from the skin surface (Hull et al., 1998). In considering implementation of higher excitation irradiances in such measurements, it is necessary to evaluate carefully the consequences of the photochemical kinetics. Because $^1O_2$ is highly reactive in biological systems, the quenching of metalloporphyrin triplets by $^3O_2$ can, under conditions of sufficiently high irradiance, result in significant rates of photochemical oxygen consumption, which could potentially perturb the oxygen concentration that is being measured. These $^1O_2$ reactions are also capable of inducing undesirable biological damage (Weishaupt et al., 1976) and may lead to the self-sensitized photobleaching of the metalloporphyrin (Moan and Berg, 1991). Little experimental work has been reported that investigates the possible limitations that these phenomena may impose on the application of the phosphorescence quenching technique. The purpose of this study was to perform detailed oxygen-consumption measurements during irradiation of a widely studied metalloporphyrin in environments relevant to the in vivo situations in which luminescence-quenching measurements are performed. In one series of experiments, the metalloporphyrin was bound to bovine serum albumin (BSA) in aqueous solution to mimic the environment encountered during intravascular pO_2 measurements. In the second, multicell tumor spheroids were incubated with porphyrins to approximate the situation in which the phosphorescent probe leaves the blood vessels and is taken up by the surrounding cells. We also evaluated the rates and possible mechanism of photobleaching of this compound.

METHODS

Chemicals

Pd-meso-tetra(4-carboxyphenyl)porphine (PdTCPP) was obtained from Porphyrin Products Inc. (Logan, UT) and was used as received. To simulate two types of environments in which metalloporphyrin phosphorescence has been used to report O_2 concentrations, O_2 consumption experiments were performed on aqueous solutions of PdTCPP bound to BSA in cuvettes and on multicell tumor spheroids preincubated with PdTCPP. Cell culture media, fetal calf serum, and Hank's balanced salt solution were purchased from GIBCO (Grand Island, NY). Unless otherwise noted, other chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO).

Solution experiments

To simulate intravascular O_2 concentration measurements, PdTCPP (0.7 mg ml^{-1}) was added to a solution of BSA (60 mg ml^{-1}) in physiological saline buffered to pH 7.4. The PdTCPP concentration we used was determined on the basis of in vivo experiments reported by Wilson and Cerniglia (1992) and Cerniglia et al. (1997), in which 4 mg PdTCPP were administered to tumor-bearing rats via intravenous tail vein injection. The rat blood plasma volume is ~5–6 ml, thus the intravascular concentration in vivo of the metalloporphyrin was approximately 0.7 mg ml^{-1}. Metalloporphyrin solution, 150 μl, was placed in a UV quartz cuvette (NSG Precision Cells Inc., Farmingdale, NY) with inside dimensions of 3×3×24 mm (height). A Clark-style microelectrode (Diamond General, Ann Arbor, MI) with a tip diameter of ~10 μm and a response time of <1 s was immersed into the sample solution. Sample irradiation was performed using the 514-nm output of a cw argon ion laser (Coherent Inc., Santa Clara, CA). The laser light was delivered to the surface of the cuvette through an optical fiber terminated with a gradient index lens (General Fiber Optics, Fairfield, NJ). The optical density of the sample at 514 nm attenuated the incident beam 10-fold across the 3-mm cuvette, producing a gradient of photochemical oxygen consumption. Thus, to ensure reproducibility, care was taken to position the electrode tip inside the cuvette as closely as possible to the surface at which the laser beam was incident. The incident irradiation spot size was 4.9 cm^2, so that the laser beam overfilled the area of the cuvette occupied by the sample. The electrode current, which is directly proportional to the O_2 concentration, was recorded during the irradiation period. We observed a monotonic decrease in O_2 concentration of the solution with the onset of irradiation. As indicated in Fig. 1, this O_2 depletion results from the irreversible reaction of $^1O_2$ with substrates, which, in the case of the solution experiments, are amino acids of BSA. These reactions compete with the monomolecular decay of $^1O_2$ to the ground triplet state, resulting in a progressive depletion of O_2 from the medium as irradiation proceeds. The reported O_2 consumption rates are averages calculated from three separate experiments at each of five incident irradiances. In each experiment, the O_2 consumption rate was determined from the initial slope of the electrode data.

Spheroid experiments

To determine O_2 consumption rates under conditions of intracellular metalloporphyrin distribution, experiments were performed with multicell tumor spheroids. Our electrode technique is a time-dependent extension of the method developed by Mueller-Klieser (1984). The details of the method have been described previously (Nichols and Foster, 1994). Briefly, EMT6/Ro spheroids (500-μm diameter) were incubated in a 100-mm suspension-tissue culture dish containing 20 ml Eagle's basal medium with 10% fetal calf serum and 10 μg ml^{-1} PdTCPP at 37°C in a humidified 5% CO_2 and 95% air atmosphere for approximately 24 hrs. After the incubation period, a single spheroid was selected and placed in an open dish containing 20 ml of Hank's balanced salt solution. The spheroid was then immobilized with a thin glass needle on top of a pedestal, which has an O_2 permeable membrane. The tip of the Clark-style microelectrode was placed at the edge of the spheroid using a micropositioning device. A lensed optical fiber coupled to an argon ion laser (Ion Laser Technology, Salt Lake City, UT) delivered 514 nm light to the surface of the pedestal. The spheroids were irradiated using three different irradiances, and the results presented are averages computed from three separate experiments at each irradiance. The microelectrode current represents the changes in the O_2 concentration taking place in the spheroid. The irradiation was continuous for approximately 500 s. Consistently, we observed a rapid initial decrease in the O_2 concentration followed by a slight increase due to metalloporphyrin photobleaching. The initial O_2 consumption rate, Γ_0, and the ratio of two photophysical rate constants, k_p/k_{ot}, were determined by fitting numerical solutions to a pair of diffusion-with-reaction equations (see below) to the first 40 s of data using a Levenberg–Marquardt nonlinear least squares fitting algorithm. After these two parameters were determined from the initial O_2 transients, the entire time-dependent data were analyzed to obtain the ratio $k_{os}/k_{oa}[A]$, as described below. All rate constants are defined in the next section and are depicted in Fig. 1.

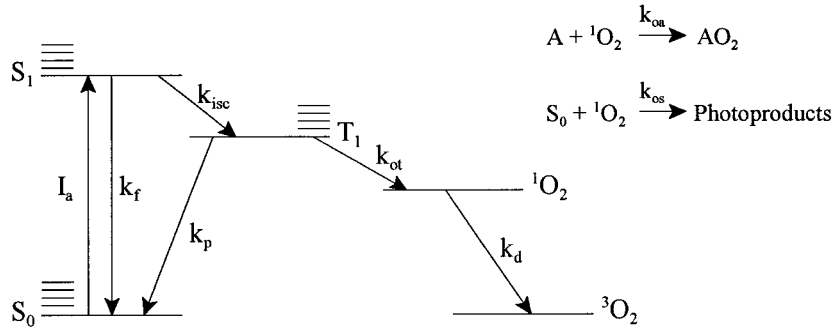

FIGURE 1 Energy level diagram depicting the photophysical and photochemical processes relevant to metalloporphyrin phosphorescence quenching, photochemical oxygen consumption, and photobleaching. The porphyrin-excited singlet state, S_1, is populated at the rate I_a through absorption of light by the ground state, S_0. Rate k_f denotes the direct de-excitation of S_1 to S_0. The porphyrin triplet state, T_1, is populated via intersystem crossing at the rate k_{isc}. T_1 may decay directly to the ground state at the rate k_p, or it may be quenched by ground state molecular oxygen, $^3O_2$, at the rate k_{ot}. Radiative decay of T_1 is phosphorescence. Quenching of T_1 by $^3O_2$ results in energy transfer to $^3O_2$ and in the formation of $^1O_2$. $^1O_2$ may relax directly to the ground state at rate k_d, or it may undergo irreversible chemical reaction with one of a variety of biological targets, denoted A in the diagram, at the rate k_{oa} or with the porphyrin itself at the rate k_{os}, which can result in the irreversible photobleaching of the porphyrin. $^3O_2$ is depleted from the system through either of the irreversible reaction pathways.

Spheroid data analysis

Details of the method developed to analyze time-dependent O_2 concentrations in spheroids during photodynamic therapy have been published elsewhere (Nichols and Foster, 1994; Georgakoudi et al., 1997). Briefly, the spatial and temporal distribution of the O_2 concentration within and in the proximity of a sensitized multicell spheroid is described by solutions to the time-dependent diffusion equations,

$$\frac{\partial[^3O_2](r,t)}{\partial t} = D_d\nabla^2[^3O_2](r,t) \quad R_s < r \le R_d, \qquad (1)$$

$$\frac{\partial[^3O_2](r,t)}{\partial t} = D_s\nabla^2[^3O_2](r,t) - \Gamma(r,t) \quad 0 \le r \le R_s, \qquad (2)$$

where D_d and D_s are the diffusion coefficients of O_2 in the medium and in the spheroid, respectively, R_s is the spheroid radius, and R_d is the radius of the O_2 depletion zone in the medium near the spheroid. $\Gamma(r,t)$ is the total rate of O_2 consumption within the spheroid, which, during irradiation, includes contributions from both photodynamic and metabolic processes. Therefore, for a sensitized spheroid during irradiation,

$$\Gamma(r,t) = \Gamma_{PDT}(r,t) + \Gamma_{met}, \qquad (3)$$

where Γ_{met} is the rate of O_2 consumption due to cellular metabolism, and $\Gamma_{PDT}(r,t)$ represents the rate of photochemical O_2 consumption. The explicit functional form of Γ_{PDT} is derived from the kinetic equations governing the photophysical processes, as described in detail by Nichols and Foster (1994) and by Georgakoudi et al. (1997). Anticipating our experimental result, for the case in which the metalloporphyrin undergoes irreversible photobleaching predominantly via self-sensitized $^1O_2$ reactions, Γ_{PDT} is written as

$$\Gamma_{PDT}(t) = \Gamma_0\left(\frac{k_{ot}[^3O_2](t)}{k_{ot}[^3O_2](t) + k_p}\right)\exp\left\{-\frac{k_{os}}{k_{oa}[A]}\int_0^t \Gamma_{PDT}(t)\,dt\right\}, \qquad (4)$$

where

$$\Gamma_0 = S_\Delta\,\Phi_t\,I_a(0)\left(\frac{k_{oa}[A]}{k_d + k_{oa}[A]}\right), \qquad (5)$$

S_Δ denotes the fraction of triplet quenching collisions with $^3O_2$ that result in $^1O_2$ formation, Φ_t denotes the photosensitizer triplet yield, $I_a(t)$ denotes the rate of photon absorption, k_{ot} is the bimolecular rate of triplet quenching by $^3O_2$, k_{oa} is the rate of chemical reaction between $^1O_2$ and unspecified substrate [A], k_{os} is the rate of chemical reaction between $^1O_2$ and the metalloporphyrin, and k_d and k_p are the monomolecular decay rates of $^1O_2$ and the metalloporphyrin triplet, respectively. Γ_0 is a constant that, in our experiments, describes the initial rate of photochemical O_2 consumption, i.e., the rate of consumption before the onset of significant O_2 depletion or photobleaching. We note that treating Γ_0 in this way implies an assumption of a uniform distribution of the metalloporphyrin throughout the spheroid. In the event that there is a radial concentration gradient, the value of Γ_0 that we extract will underestimate the rate of O_2 consumption near the outer rim of the spheroid, and it will overestimate this rate near the center of the spheroid. On the basis of the above definitions and the energy level diagram of Fig. 1, the ratio $k_p/(k_{ot}[^3O_2])$ defines a branching ratio for the metalloporphyrin triplet state. When this ratio is equal to unity, the triplets are as likely to undergo monomolecular decay to the ground state as they are to be quenched through collisions with $^3O_2$. Thus, the ratio k_p/k_{ot}, which has dimensions of a concentration, is a measure of the $^3O_2$ dependence of the dye-sensitized $^1O_2$ formation process. At O_2 concentrations higher than k_p/k_{ot}, quenching by O_2 is the predominant decay route for the triplets, whereas, at lower concentrations, the quenching efficiency is limited by O_2 availability. As noted above, numerical solutions to Eqs. 1 and 2 are fitted to the first 40 s of the data to obtain best estimates of Γ_0 and k_p/k_{ot}. Then, solutions are fit to the entire data set (approximately 500 s of recordings) to obtain the ratio $k_{os}/k_{oa}[A]$, which provides a measure of the self-sensitized photobleaching.

Selection of irradiances

The rationale for the specific irradiances used in these studies was based on the published literature, with particular emphasis on phosphorescence-quenching experiments performed in vivo. The lowest value was chosen to match the average irradiance used in imaging studies of superficial anat-

omy. Shonat et al. (1992) used flash-lamp pulse fluences of 39 μJ cm^{-2} for blue light excitation and 52 μJ cm^{-2} for green excitation. Typically, for each data acquisition delay time, 8 frames are averaged, and the total time required to obtain an image is approximately 1 s (Wilson and Cerniglia, 1992; Vinogradov et al., 1996; Cerniglia et al., 1997). Thus, the time between successive flash-lamp excitations is 125 ms, and the average irradiance incident on the tissue surface is ~0.3 mW cm^{-2}. The extinction coefficient of PdTCPP is much greater in the blue region than it is at 514 nm, where our experiments were performed. Using the measured absorption spectrum of PdTCPP (see Fig. 5) and accounting for the difference in photon energies at the two wavelengths, we determined the 514-nm intensity that would match the rate of photon absorption that resulted from 0.3 mW cm^{-2} delivered at 418 nm. These considerations combine such that the appropriate 514-nm irradiance is ~1.3 mW cm^{-2}. The higher irradiances were selected because of the recent availability of longer wavelength absorbing and emitting metalloporphyrins (Vinogradov and Wilson, 1995; Vinogradov et al., 1996), which have created interest in the possibility of imaging oxygen at depths of several centimeters in tissue. On the basis of the suggestion of Vinogradov et al. (1996) that the flash intensity could be increased 10–100-fold, we selected a maximum irradiance of 134 mW cm^{-2}.

Porphyrin concentration in spheroids

To make a quantitative comparison between the spheroid and BSA solution oxygen-consumption rates, it is necessary to measure the concentration of metalloporphyrin in the spheroids. To accomplish this, 20 spheroids (500 μm diameter) were incubated with 10 μg ml^{-1} PdTCPP for 24 hrs as described above. After incubation, the spheroids, along with the media, were placed in a 15-ml tube and centrifuged for 5 min. After centrifugation, the medium above the spheroid pellet was aspirated, and the spheroids were then dissociated and the cells dissolved using 25% Scintigest in 10× trypsin without phenol red. We then measured the absorption spectrum of this sample. The metalloporphyrin concentration was calculated from these absorption measurements using a calibration curve generated from the absorbance of known PdTCPP concentrations in the same solvent.

RESULTS

In the solution experiments, the choice of the lowest irradiance, 1.34 mW cm^{-2}, was based on an approximation to the average incident irradiance used in the in vivo experiments described by Shonat et al. (1992). As described in the Methods section, this average was computed on the basis of the fluence per pulse and the pulse repetition rate reported by those authors. Examples of the photochemically-induced changes in the $^3O_2$ concentration observed using the microelectrode during sample irradiation with this and with several other irradiances are shown in Fig. 2. From three experiments performed with separate samples at the lowest irradiance, the $^3O_2$ consumption rate was found to be 2.5 ± 0.1 μM s^{-1}. Typical image-acquisition times reported in papers that describe results of in vivo phosphorescence imaging of superficial anatomy are 1–1.5 s (Wilson and Cerniglia, 1992; Cerniglia et al., 1997). Oxygen tensions in normal tissue are typically in the range of 40–50 torr (~60–80 μM). Thus, it may be concluded that the conditions under which the phosphorescence-quenching measurements are performed for superficial anatomy produce a modest reduction in the tissue $^3O_2$ concentration of 6% or less, provided that there is sufficient time between image acquisitions to allow for $^3O_2$ recovery.

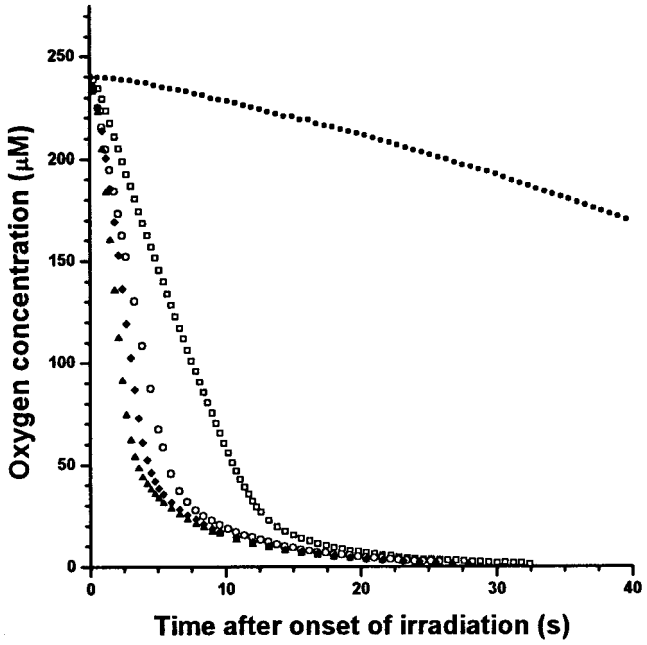

FIGURE 2 Oxygen concentrations recorded using a microelectrode immersed in a cuvette containing an aqueous solution of PdTCPP (0.7 mg ml^{-1}) bound to BSA during 514 nm irradiation at five different irradiances: 1.34 mW cm^{-2} (●●●●); 20 mW cm^{-2} (□□□□); 60 mW cm^{-2} (○○○○); 100 mW cm^{-2} (◆◆◆◆); and 134 mW cm^{-2} (▲▲▲▲).

As described earlier, the rationale for the particular choices of the higher irradiances was based on suggestions published by Vinogradov et al. (1996) and on our own experimental experience with detection of sources of luminescence buried in scattering media (Hull et al., 1998). With irradiances of 20, 60, 100, and 134 mW cm^{-2}, the O_2 consumption rates were found to be considerably higher, as shown in Fig. 2 and summarized in Fig. 3. The rates reported in Fig. 3 were determined from the initial slopes of the curves in Fig. 2 and represent means and standard deviations of three separate experiments. At 20 mW cm^{-2}, assuming the same range of tissue O_2 concentrations and image-acquisition times as mentioned above, the data indicate that the measurement would perturb the O_2 concentration by a minimum of 20% and by as much as 40%. Of course, higher irradiances would have an even greater effect. The curves of Fig. 2 illustrate that the rate of depletion is diminished at the lowest O_2 concentrations. This diminished rate occurs as the concentration approaches that described by the ratio k_p/k_{ot} (see Methods) and results from the O_2 dependence of the rate of $^1O_2$ formation. Thus, the magnitude of the perturbation imposed by the phosphorescence-quenching technique depends in part on the ambient O_2 concentration.

Whereas the solution experiments, where the metalloporphyrin is bound to BSA, represent a reasonable approxima-

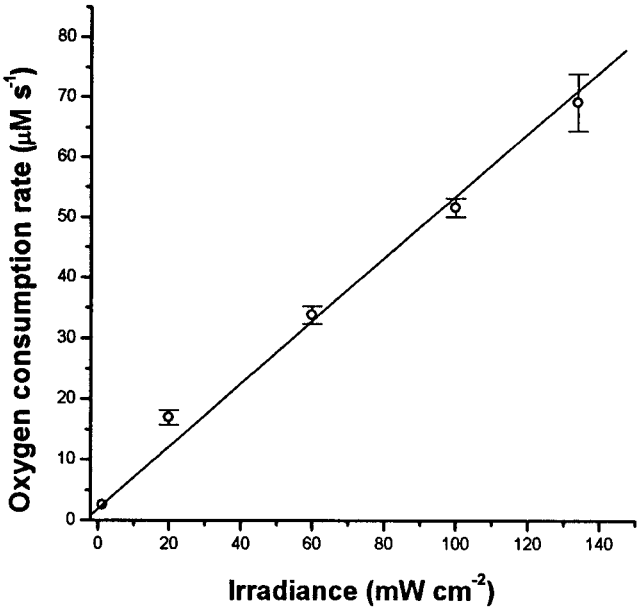

FIGURE 3 The maximal rates of photochemical oxygen consumption versus irradiance measured in the PdTCPP-BSA solution experiments (0.7 mg ml^{-1} PdTCPP, 514 nm irradiation), which were determined from the initial slopes of the data plotted in Fig. 2. In order of increasing irradiance, the oxygen consumption rates (in $\mu M\ s^{-1}$) are 2.5 ± 0.1, 16.9 ± 1.8, 33.4 ± 1.4, 53.2 ± 1.5, and 68.9 ± 4.8 (means ± SD), computed from three independent experiments performed at each of the five irradiances. Where not shown, error bars are smaller than the plotted symbol.

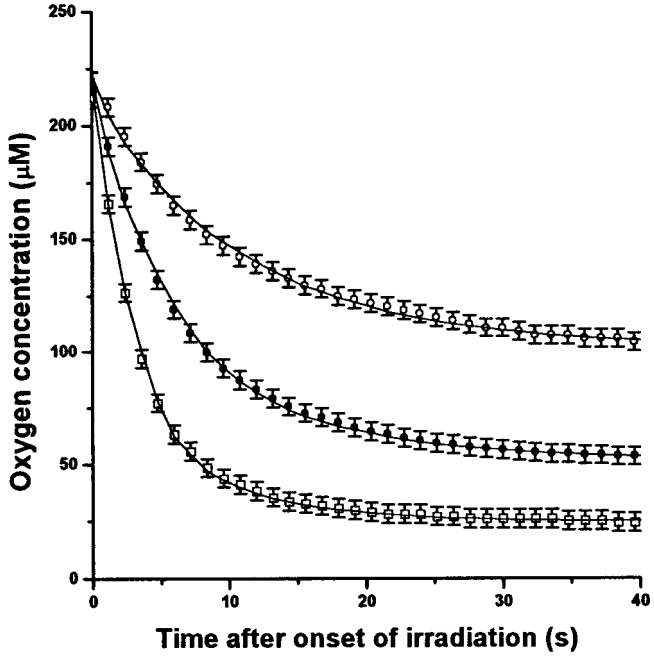

FIGURE 4 Microelectrode measurements of oxygen depletion versus time at the edge of a PdTCPP-sensitized spheroid during the first 40 s of 514 nm irradiation at three different irradiances: 20 mW cm^{-2} (○○○○); 40 mW cm^{-2} (●●●●); and 60 mW cm^{-2} (□□□□). The solid lines represent the best fits to the data using numerical solutions to Eqs. 1 and 2.

tion to the experiments in vivo that seek to measure intravascular O_2 concentrations, the spheroid provides a test of the O_2 consumption that would be expected under conditions where the phosphorescent probe leaks from the vasculature into the surrounding tissue. Examples of the initial changes in the $^3O_2$ concentration that we record at the edge of PdTCPP-sensitized spheroids for irradiances of 20, 40, and 60 mW cm^{-2} are shown in Fig. 4. The solid lines through the curves represent the best fits of numerical solutions of Eqs. 1 and 2 to the data. In this analysis, both Γ_0 and k_p/k_{ot} are extracted as fitting parameters. From a total of nine experiments ($n = 3$ for each irradiance), we find that the ratio of k_p/k_{ot} for PdTCPP is 8.3 ± 3.8 μM. Figure 5 summarizes the values of the means and standard deviations of Γ_0 determined from the same nine experiments.

A comparison of Figs. 3 and 5 indicates that, under the conditions of these experiments, the photochemical O_2 consumption rate in spheroids was significantly greater than that observed in solutions containing BSA at the same irradiance. To compare these results on a quantitative basis, we determined the concentration of the metalloporphyrin in a spheroid after the same 24-hr incubation period used in the O_2 consumption experiments. From a series of three independent solubilization and optical absorption measurements, we calculated the PdTCPP concentration in the spheroids to be approximately 1.1 mg ml^{-1}. The absorption spectrum of PdTCPP in aqueous solution of trypsin and the solubilizing agent Scintigest is shown in Fig. 6 along with the calibration curve used to determine the metalloporphyrin concentration. The porphyrin concentration in the spheroids was greater than that used in the solution experiments (0.7 mg ml^{-1}). When the O_2 consumption rates measured in BSA solution are corrected for this difference in porphyrin concentration, they approach those measured in the spheroids.

When porphyrins are subjected to sustained irradiation in biological systems, irreversible photobleaching is observed (Mang et al., 1987; Moan et al., 1988). Bleaching may be measured indirectly with O_2 electrodes through the gradual increase in the O_2 concentration that results as the rate of light absorption by the porphyrin decreases (Georgakoudi et al., 1997). To determine the rate and mechanism of PdTCPP bleaching, we analyzed time-dependent O_2 concentration data recorded continuously for approximately 500 s at the edge of spheroids irradiated using three different fluence rates. Representative data are shown in Fig. 7. The best fits of solutions to Eqs. 1 and 2 to the data are depicted by the solid lines. The particular form used for the rate of photochemical O_2 consumption, Γ_{PDT}, was derived on the basis of a bleaching mechanism in which the porphyrin is degraded through self-sensitized $^1O_2$ reaction. Fits to the data allow determination of a ratio, $k_{os}/k_{oa}[A]$, which is a measure of the relative bleaching efficiency. From the nine experiments

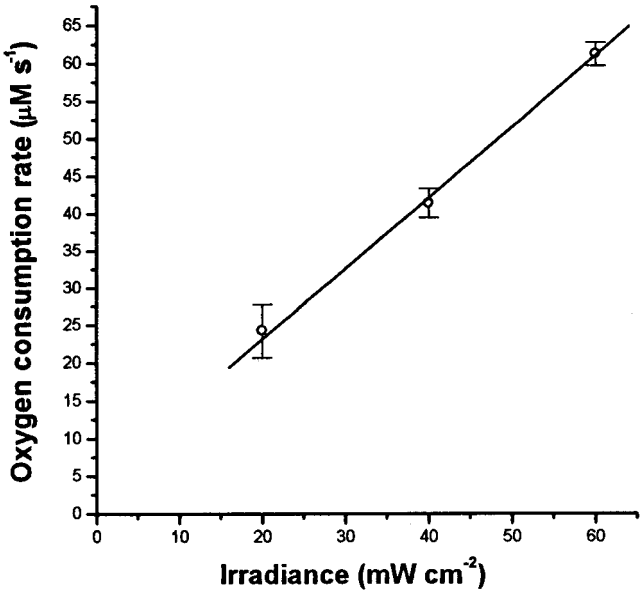

FIGURE 5 The maximal rate of photochemical oxygen consumption (Γ_0 in Eqs. 4 and 5) versus irradiance determined from the spheroid experiments depicted in Fig. 4. In order of increasing irradiance, the values of Γ_0 (in μM s^{-1}) are 24.2 ± 3.6, 41.4 ± 1.9, and 61.2 ± 1.2 (means ± SD). Means and uncertainties were determined from three independent experiments performed at each of the three irradiances.

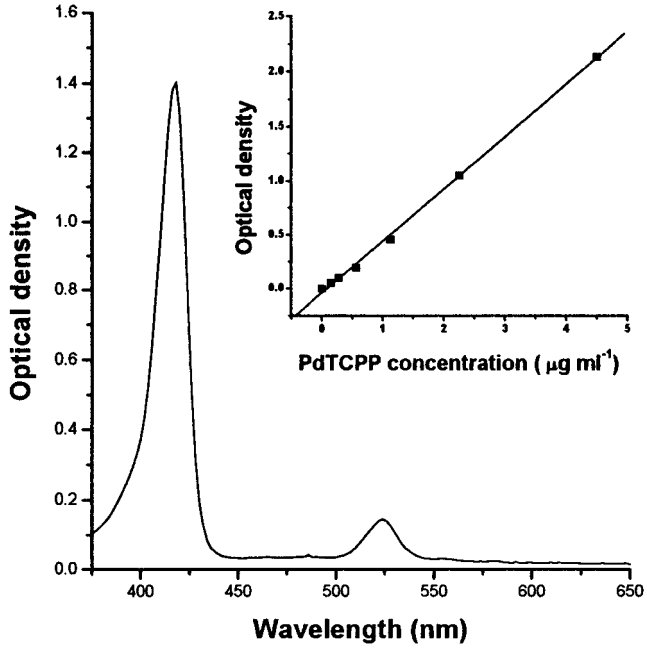

FIGURE 6 Absorption spectrum of PdTCPP in 25% scintigest and trypsin. The inset shows the calibration curve (optical density at 418 nm versus concentration) used to determine metalloporphyrin concentrations in multicell spheroids, which was generated from known PdTCPP concentrations in the same solvent.

conducted using the three fluence rates mentioned above, we find that the value of this ratio for PdTCPP is 23.0 ± 7.0 M^{-1} (mean ± SD). This value is significantly lower than that measured previously by Georgakoudi et al. (1997) for the photosensitizer Photofrin (76 ± 12 M^{-1}) and by Georgakoudi and Foster (1998) for δ-aminolevulinic acid-induced protoporphyrin IX (90 ± 15.9 M^{-1}) using this method.

DISCUSSION

The findings of this study illustrate potential limitations imposed by O_2 consumption during measurement of tissue pO_2 by the metalloporphyrin phosphorescence-quenching technique. We have demonstrated the severity of the problem by determining the O_2 consumption rates when the metalloporphyrin PdTCPP was irradiated in two environments in vitro. In the first case, the PdTCPP was bound to BSA in solution, with the metalloporphyrin concentration chosen to be as close as possible to that used in the intravascular pO_2 measurements in vivo of Cerniglia et al. (1997) and Wilson and Cerniglia (1992). In these in vivo experiments, the metalloporphyrin is bound to BSA, so that the probe is confined to the blood volume and so that the calibration parameters calculated from measurements in vitro are relevant in vivo (Sinaasappel and Ince, 1996; Vinogradov et al., 1996). In the second case, multicell spheroids were incubated with PdTCPP to approximate the environment encountered when the porphyrin leaks out of the vessels into the tissue (Torres Filho et al., 1994; Buerk et al., 1998).

The results of our solution experiments demonstrate that, for most if not all published reports in which phosphorescence quenching has been used to measure O_2 concentrations in superficial structures in vivo and in applications involving intravital microscopy, photochemical oxygen consumption is of minimal importance. However, if the average excitation irradiance is increased to just 20 mW cm^{-2}, the perturbation of the local oxygen concentration would be significant, even if one assumes that the total image acquisition time is held to the 1–1.5 s range typically used in imaging superficial anatomy. Because the detection of luminescence signals originating at depths of several centimeters in an attenuating medium will require both relatively higher irradiances and longer data-acquisition times, our results suggest that great caution must be exercised in applying the phosphorescence-quenching technique to the imaging of oxygenation in all but the most shallow layers of tissue. In our own experience with imaging highly fluorescent targets buried in a scattering emulsion using a sensitive, cooled CCD camera and 160 mW cm^{-2} incident irradiance, signal-acquisition times varied from 10 s for source depths of approximately 6 mm to 245 s for source depths of approximately 44 mm (Hull et al., 1998).

The plots of Fig. 2 show that the rate of photochemical oxygen consumption depends on the O_2 concentration of the

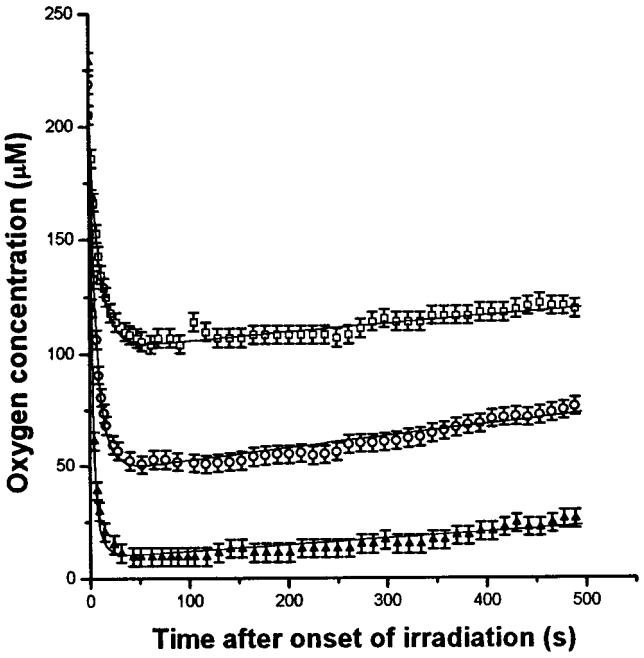

FIGURE 7 Time-dependent oxygen concentrations recorded using a microelectrode at the edge of a PdTCPP-sensitized spheroid during 500 s of 514 nm irradiation at three different irradiances: 20 mW cm^{-2} (□□□□); 40 mW cm^{-2} (○○○○); and 60 mW cm^{-2} (▲▲▲▲). The solid lines are best fits to the data using the singlet oxygen-mediated photobleaching model expressed in Eq. 4.

medium, with the rate being diminished at lower concentrations. This phenomenon follows simply from the fact that, as the O_2 concentration is lowered, the probability increases that the metalloporphyrin triplet will undergo direct, monomolecular decay to the ground state. Thus, relatively fewer triplets are quenched by oxygen, leading to a reduced rate of $^1O_2$ formation. The characteristic O_2 concentration at which this effect becomes appreciable is described by the ratio of porphyrin triplet state rate constants, k_p/k_{ot}, which were previously defined. This ratio identifies the O_2 concentration at which triplet monomolecular decay and quenching by $^3O_2$ are equally likely. Our analysis of the electrode recordings obtained at the edge of irradiated spheroids indicates that, for the PdTCPP compound in the intracellular environment, this characteristic concentration is approximately 8 μM. A practical and perhaps somewhat counterintuitive consequence of this aspect of the photochemistry is that phosphorescence quenching is more likely to perturb tissue oxygenation at relatively higher tissue oxygen tensions. From the perspective of imaging thick tissue, then, it appears unlikely that a scheme could be devised to correct for the effects of photochemical oxygen consumption without prior knowledge of the detailed oxygen distributions.

When the data from the two in vitro systems that we investigated are corrected for the difference in their PdTCPP concentrations, the rates of photochemical oxygen consumption are found to be comparable. It is well known that the intracellular environment presents many targets for $^1O_2$ reaction, and these data demonstrate quantitatively that BSA provides a similar effective concentration of substrate. This finding is consistent with those of Reddi et al. (1984), who showed that the tryptophan, tyrosine, and histidine residues of human serum albumin were readily photo-oxidized in the presence of hematoporphyrin derivative. Indeed, in the paper that introduced the phosphorescence-quenching method, Vanderkooi and her colleagues (1987) described BSA "a sink" for reactive oxygen species. On the basis of our results, therefore, one may conclude that the potential problems posed by photochemical oxygen consumption are at least as likely to occur during intravascular phosphorescence-quenching measurements as they are in those situations where the porphyrin is allowed to distribute out of the intravascular space.

The limitations imposed by photochemical oxygen consumption on the applications of metalloporphyrin phosphorescence quenching here appear to be fundamental to the method rather than specific to the PdTCPP compound. Therefore, it is not immediately apparent what, if any, means could be used to diminish or overcome this effect. No significant photochemical differences should be anticipated through the use of the longer wavelength-absorbing metalloporphyrin Green 2W. The peak of the phosphorescence emission of this compound was reported by Vinogradov et al. (1996) to be 790 nm. Thus, the Green 2W triplet state is sufficiently energetic to sensitize the formation of $^1O_2$, which is 1270 nm above the ground state. It is true that, for a source of phosphorescence buried several centimeters from the tissue surface, the incident irradiance would be attenuated significantly, and this will reduce the rate of photochemical O_2 consumption at that site. However, because the phosphor cannot be expected to localize perfectly in the target tissue volume, the oxygen concentration in the superficial tissue would be perturbed significantly. As mentioned previously, without detailed prior knowledge of the oxygen distribution in tissue calculation of a correction, does not seem feasible. Other imaging strategies not based on triplet-state quenching could be considered. For example, oxygen quenching of singlet-state fluorescence has been reported (Chong and Thompson, 1985). Although fluorescent probes are certainly useful in a variety of fundamental studies, their lifetimes are sufficiently short that it is unlikely that they could be useful reporters of physiologically relevant hypoxia in tissue. Therefore, we are left to conclude that, although the very long-lived porphyrin triplet-state luminescence provides a sensitive measurement of tissue pO_2, the mechanism through which the triplet reports the O_2 concentration imposes important and quite fundamental limits to the situations in which phosphorescence quenching can be applied.

An interesting recent study by Buerk et al. (1998) compared the tissue oxygen tensions reported by 5-μm tip-recessed electrodes and the phosphorescence-quenching method in a hamster skinfold model. They concluded that, under the conditions of their intravital microscopy experiment, the phosphorescence technique lowered the oxygen concentration only minimally, even when the irradiation was performed for as long as 1 min. Although the pulse energies used in that study were not reported, it is likely that their findings are not inconsistent with our results. In several respects, intravital microscopy represents the most favorable situation in vivo for the application of the phosphorescence method. The thicknesses of tissue that are interrogated are minimal, typically 200–300 μm, thereby enabling the use of very low excitation pulse energies and short acquisition times. In the particular case of the Buerk et al. study, in which avascular regions of the hamster skin fold were investigated, the metalloporphyrin concentration that was illuminated was also very low, because only a small amount of the albumin-bound compound will extravasate. Further, because the illuminated field diameter is restricted to approximately 140 μm in these experiments (Torres Filho and Intaglietta, 1993), any oxygen depletion during irradiation is replenished from outside the irradiated area.

The photobleaching of porphyrins in biological systems is a well-established phenomenon. Although the phosphorescence-quenching technique is based on emission lifetimes rather than amplitudes, the bleaching kinetics and mechanism of the metalloporphyrins are nevertheless of interest, especially in the context of proposals to use the method to image in thick systems. Photobleaching during irradiation reduces the rate of light absorption and would thereby progressively reduce the rate of photochemical oxygen consumption. Also, if photobleaching is mediated through a predominantly oxygen-dependent mechanism, as has been reported for various porphyrins (Krieg and Whitten, 1984; Spikes, 1992), one might imagine a scenario in which a period of preirradiation preferentially bleached the compound in relatively well-oxygenated regions, thereby possibly enhancing the optical detection of hypoxic malignant tumors.

Photobleaching is reported indirectly in the spheroid microelectrode measurements as a gradual increase in the measured oxygen concentration with prolonged irradiation (Georgakoudi et al., 1997). Analysis of the traces obtained during irradiation of spheroids incubated with PdTCPP demonstrates that this compound is significantly more stable with respect to bleaching than the other porphyrins that we have studied. As noted earlier, this relative stability is reported through the ratio $k_{os}/k_{oa}[A]$, which defines the reciprocal of the porphyrin concentration at which $^1O_2$ reactions with the porphyrin are as likely as those with other nearby targets. This property, along with the efficiency with which it sensitizes the formation of reactive oxygen species, may make PdTCPP an interesting candidate for biological and medical studies of photodynamic action.

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