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Effects of the Oxygenation level on Formation of Different Reactive Oxygen Species During Photodynamic Therapy

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Abstract

We examined the effect of the oxygenation level on efficacy of two photosensitizing agents, both of which target lysosomes for photodamage but via different photochemical pathways. Upon irradiation, the chlorin termed NPe6 forms singlet oxygen in high yield while the bacteriopheophorbide WST11 forms only oxygen radicals (in an aqueous environment). Photokilling efficacy by WST11 in cell culture was impaired when the atmospheric oxygen concentration was reduced from 20% to 1%, while photokilling by NPe6 was unaffected. Studies in a cell-free system revealed that rates of photobleaching of these agents, as a function of the oxygenation level, were correlated with results described above. Moreover, the rate of formation of oxygen radicals by either agent was more sensitive to the level of oxygenation than was singlet oxygen formation by NPe6. These data indicate that the photochemical process that leads to oxygen radical formation is more dependent on the oxygenation level than is the pathway leading to formation of singlet oxygen.

INTRODUCTION

The use of photosensitizing agents to sensitize neoplastic tissues to light is termed photodynamic therapy (PDT) (1,2). Success of PDT requires that the photosensitizing agent reach the appropriate loci and that there be sufficient light and oxygen available to sustain photochemical steps that lead to death of photosensitized cells and/or the shut-down of their blood supply. Since tissue oxygenation levels can vary widely, we examined the effects of oxygenation levels on efficacy of two photosensitizing agents with different photochemistries. The chlorin NPe6 is known to exhibit a high yield of singlet oxygen upon irradiation (3,4) while the bacteriopheophorbide WST11 forms only oxygen radicals upon irradiation in a relatively aqueous environment (5). Both agents localize in lysosomes (6, 7), permitting a comparison between two agents that differ in their photochemistry but have a common pathway to photokilling initiated by lysosomal photodamage (6). This pathway involves the release of lysosomal enzymes that ultimately initiate activation of procaspase-3 and apoptotic cell death (6,7). We also examined rates of photobleaching of NPe6 vs. WST11 along with fluorogenic interactions involving probes for specific reactive oxygen species (ROS) in a cell-free system.

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MATERIALS AND METHODS

Chemicals and supplies

NPe6 was provided by Dr. Kevin M Smith, Louisiana State University. WST11 was provided by Prof. Avigdor Scherz at the Weizmann Institute. All reagents were obtained from Sigma-Aldrich and were of the highest available purity. Gases were purchased from Cryogenic Gases [Detroit, MI] and from Praxair [Danbury, CT.] Fluorescent probes were provided by Molecular Probes/Invitrogen [Carlsbad, CA].

Cell culture and clonogenic assays

Growth of murine hepatoma 1c1c7 cells and procedures for clonogenic assays are described in Ref. 8. A hypoxia chamber [Biospherix, Lacona, NY] was used for maintenance of an environment consisting of 1% oxygen, 5% carbon dioxide, 94% nitrogen (37°C). Otherwise, cells were grown in a standard CO₂ incubator.

PDT protocols

Cultures were incubated with 1 μ M WST11 for 16 h, or 20 μ M NPe6 for 1 h. The medium was then replaced and the dishes irradiated using a 600-watt quartz-halogen source filtered with 10 cm of water to remove wavelengths of light greater than 900 nm. The bandwidth of the light beam was confined by interference filters (Oriel, Stratford CT) to 750 ± 10 nm for studies involving WST11, to 660 ± 10 nm for NPe6. Irradiation times were calculated based on clonogenic studies, so as to yield the desired effect on viability.

Photobleaching studies

These were carried out in 1×1×3 cm glass cuvettes with transparent sides and bottom. Solutions of NPe6 or WST 11 were prepared in 10 mM sodium phosphate buffer pH 7.0. Initial optical densities at 654 nm (NPe6) or 748 nm (WST11) were 0.100 ± 0.005 . Either of two fluorescent probes: aminophenyl fluorescein (APF) or singlet oxygen sensor green (SOSG) was present at a 2 μ M concentration, where specified. APF was designed to detect hydroxyl radical (9) while SOSG forms a fluorescent endoperoxide in the presence of ¹O₂ (10). Buffers were equilibrated with air (20% oxygen) or with nitrogen containing 1% or 0.01% oxygen. To minimize oxygen diffusion from the atmosphere, only PVC plastic tubing was used for connections.

Gas mixtures or air were bubbled into cuvettes during the irradiation process. The cuvettes were irradiated from beneath using a diode-laser/fiber optic system. Diode lasers (650 and 750 nm) were obtained from Intense, North Brunswick NJ. Power to the diode and thermoelectric cooler was provided by a Series 6000 laser diode controller (Newport, Irvine CA). A uniform power of 100 mW was used for all experiments. Spectra were acquired with a Shimadzu BioSpec-1601 spectrophotometer. Fluorescence emission of APF and SOSG (optimal wavelength = 520 nm) was measured using an Instaspec ISX CCD camera (Oriel/Newport) detector, using 500 nm excitation.

Measurements of dissolved oxygen levels

Samples of buffer equilibrated with air, 1% or 0.01% oxygen were analyzed for levels of dissolved oxygen using the Winkler procedure (11). Triplicate assays were carried out using reagent solutions that had been equilibrated with the specified oxygen mixtures.

RESULTS AND DISCUSSION

PDT efficacy in cell culture

Clonogenic studies revealed a loss of efficacy of photokilling by WST11 when the percentage of oxygen in the system was decreased from 20% to 1% (Fig. 1 panel A). A 50% loss of viability occurred with a light dose of approximately 170 mJ/cm² at 20% O₂ but this required 340 mJ/cm² in 1% O₂. In contrast, a similar decrease in the O₂ concentration had no detectable effect on photokilling by NPe6 (Fig. 1 B). A logistic regression model for testing the slopes of the two curves indicates that they are significantly different ($p = 0.0372$).

NPe6: photobleaching and ROS formation

The initial rate of NPe6 photobleaching was unaffected as the level of oxygenation was decreased, using light doses of 10 J/cm² or less (Fig. 2 panel A). This corresponds to photobleaching of 40% of the photosensitizer. This result was also reflected in rates of SOSG oxidation which were essentially identical at the lower light doses. The rate and extent of photobleaching at light doses higher than 10 J/cm² was proportional to the level of oxygenation. Total oxidation of the SOSG resulted in a reaction that yielded ~23,000 fluorescence units. Data shown in panel A indicate a sufficient level of oxygen remained, even in media degassed with 99.99% nitrogen, to oxidize nearly all of the 6 nmoles of SOSG present in the cuvette.

A different result was obtained with NPe6 using APF as the ROS probe. Results shown in Fig. 3 B indicate a correlation between the rate of probe oxidation vs. the level of oxygenation at light doses greater than 15 J/cm². Since NPe6 photobleaching rates were unaffected under these conditions, we conclude that [•]OH is not an important determinant of NPe6 photobleaching.

WST11: photobleaching and ROS formation

The rate of WST11 photobleaching was dependent on the oxygenation level even at the earliest time points (Fig. 2, panel C) as was the rate of APF conversion to a fluorescent product. Photobleaching of WST11 was also observed in deoxygenated buffer and continued even after APF fluorescence was no longer increasing (light doses greater than 17 J/cm²). This result suggests that WST11 photobleaching may be occurring independently of [•]OH formation. As predicted from data described in Ref. 5, we found only a barely detectable fluorogenic response of SOSG to irradiation of WST11 (not shown).

Photoproduct formation

A series of spectra were acquired as irradiation proceeded, using irradiation at 665 nm (NPe6) or 750 nm (WST11). The resulting spectra in medium equilibrated with 20% vs. 0.01% oxygen are shown in Fig. 3. As the absorbance of NPe6 at 650 nm decreased, a photoproduct with absorbance at 740 nm was formed (Fig. 3 A and B). Photobleaching was observed both in media in equilibrium under both normoxic and hypoxic conditions. A similar study involving WST 11 showed formation of a 630 nm photoproduct, even under hypoxic conditions (Fig. 3, C and D).

Dissolved oxygen levels

Analysis for dissolved O₂ indicated that buffer equilibrated with atmospheric oxygen contained 8.9 ± 0.3 mg/l of dissolved oxygen (280 μ M). Buffer equilibrated with 1% oxygen showed a level of 0.46 ± 0.02 mg/l of oxygen (14.5 μ M). Analysis of buffer degassed with 99.99% nitrogen indicated an oxygenation level of 0.031 ± 0.006 mg/l (480

nM). With extinction coefficients of 40,000 (NPe6) and 120,000 (WST11), the concentration of NPe6 and WST11 in a solution with an absorbance of 0.1 will be approximately 2.5 and 0.8 μM 's, respectively. The ratios of photosensitizer to oxygen molecules in solutions prepared in the oxygen-depleted buffer are therefore 5.2:1 (NPe6) and 1.7:1 (WST11).

CONCLUSIONS

Anti-tumor effects of photodynamic therapy are known to be dependent on the presence of oxygen (1,2). In this study, we examined the effect of hypoxia on rates of formation of singlet oxygen vs. oxygen radicals. Two photosensitizers with similar localization patterns (6,7) but different photochemistries were employed. NPe6 produces a high yield of $^1\text{O}_2$ upon irradiation, while WST11 produces only oxygen radicals in an aqueous environment. In this context, it should be noted that current clinical PDT protocols utilizing WST11 involve irradiation while photosensitizer levels in the circulation are high (12). It is therefore unlikely that the tissue oxygenation level will be a factor in WST11 efficacy. NPe6 is one of the older agents in the PDT inventory, and has been used for exploratory studies for many years (13). Like WST11, it also has profound effects on the tumor vasculature (14)

Phototoxicity studies showed NPe6, but not WST11 efficacy in cell culture was unaffected by lowering the oxygenation level from 20% to 1% (Fig. 1). Studies summarized in Fig. 2 indicate that the rate of $\cdot\text{OH}$ formation, indicated by a fluorogenic interaction with APF, was dependent on the degree of oxygenation for both photosensitizing agents. The correlation between NPe6 photobleaching and the increase in SOSG fluorescence (Fig. 2A), and of WST11 photobleaching and appearance of APF fluorescence (Fig. 2C) are interpreted to indicate the predominant involvement of $^1\text{O}_2$ in NPe6 photochemistry and of $\cdot\text{OH}$ in WST11 photochemistry. The lack of any correlation between APF fluorescence and NPe6 photobleaching is consistent with the proposal that $\cdot\text{OH}$ is not a major factor in NPe6 photobleaching.

Moan reported that the singlet oxygen formation and phototoxicity of the porphyrin-based photosensitizer 'HPD' both decreased by a factor of 2 when the oxygen level was reduced to 1% (15); Chapman et al reported a somewhat greater decrease in efficacy (16). The effect of hypoxia on HPD efficacy is therefore substantially greater than what is reported here for NPe6. The latter product may therefore represent a class of photosensitizers whose excited state has superior ability to interact with oxygen molecules. In this regard, Spikes reported that even 100 mM NaN_3 was unable to prevent NPe6 photobleaching (3). Other agents capable of photokilling in hypoxic loci have also been identified, e.g., as reported in Ref. 17. Such agents may be especially useful for photokilling of neoplastic cells when the level of oxygenation is impaired.

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Figure 1. Photokilling of murine hepatoma 1c1c7 cells by WST11 (A) and NPe6 (B) as determined by clonogenic assays. Incubations and irradiation were carried out in 1% (open circles) or 20% (closed circles) oxygen. Statistical data relating to these data are summarized in the text.

Figure 2.

Photobleaching and fluorogenic interactions. A, NPe6, probe = SOSG; B, NPe6, probe = APF; C, WST11, probe = APF. Closed circles represent photobleaching as determined by measuring the absorbance at 665 nm (NPe6) or 750 nm (WST11) as irradiation proceeded. Open circles show the fluorogenic effect of irradiation. Red = 20% oxygen; green = 1% oxygen; black = 0.01% oxygen. These data represent results from a typical experiment. Replicates differed by less than 5% of values shown.

Figure 3.

Absorbance spectra of NPe6 (A, B) or WST 11 (C, D) in 10 mM phosphate buffer during irradiation. Buffers were equilibrated with 0.01% (A, C) or 20% (B, D) oxygen, remainder nitrogen. These data represent results of spectra collected during the acquisition of results shown in Fig. 2.