Advances in Brief

Involvement of Nitric Oxide during Phthalocyanine (Pc4) Photodynamic Therapy-mediated Apoptosis[1]

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Abstract

Photodynamic therapy (PDT), a new treatment modality, uses a combination of photosensitizing agent and visible light for the therapy of many solid malignancies. The hallmark of PDT is intracellular oxidative stress mediated by reactive oxygen species, which, through a cascade of events, results in a cell kill that induces apoptosis in some cells. To better understand the mechanism of apoptosis, we hypothesized the role of nitric oxide (NO), which is considered to be involved in a variety of physiological and pathological processes, during PDT. The model photosensitizer we have been working with is a silicon-phthalocyanine compound termed Pc4. Here, we investigated the involvement of NO during Pc4 PDT in PDT of apoptosis-resistant radiation-induced fibrosarcoma (RIF-1) cells and in PDT of apoptosis-sensitive human epidermoid carcinoma (A431) cells. Pc4 PDT resulted in a rapid increase in nitrite production in A431 cells, starting as early as 15 s post-PDT, and showed a progressive increase up to 15 min post-PDT. This increase in nitrite production was observed in cell lysates as well as in the cell culture medium. RIF-1 cells did not show an increase in nitrite production in either the cell lysates or the culture medium. At this time, a majority of the cells were viable. The Western blot analysis also showed a rapid increase in the expression of the constitutive form of NO synthase as early as 15 s post-PDT when compared to that of the controls. This response showed a dose dependency up to 5 min after Pc4 PDT. This observation was confirmed by a [^{3}H]L-citrulline assay, which also showed a similar pattern for constitutive NO-synthase activity. RIF-1 cells did not show any change in protein expression or enzyme activity after the same treatment. These data, for the first time, demonstrate the generation of NO during PDT and suggest that it may be involved in PDT-mediated apoptosis. This may have relevance in improving the therapeutic efficacy of PDT using pharmacological modulators of NO or NO synthase.

Introduction

PDT[3] is a promising treatment modality for the management of a variety of solid neoplasms. This modality was recently approved by the United States Food and Drug Administration for the treatment of tumors of the esophagus. Clinical trials using PDT for the treatment of cancers of the head and neck, brain, bladder, bronchus, skin, and other sites are being conducted. PDT is also showing promising results for the treatment of a variety of nonmalignant diseases (1–4). PDT is a bimodal therapy that possesses two components in an experimental protocol, chemical photosensitization and light irradiation. Both of these components are individually nontoxic but are tumoricidal in combination (5, 6). Typically, the treatment involves the uptake of a porphyrin-based photosensitizing chemical, preferentially in tumor tissue, followed by an irradiation of tumors with light of the visible or near IR region that is typically derived through a laser. The treatment results in a photoactivation that leads to the oxidative damage of a variety of cellular targets including the mitochondria, plasma membrane, and lysosome (7, 8) and to subsequent cell death, resulting in tumor ablation. The model photosensitizer we have been using for PDT is a silicon-phthalocyanine compound termed Pc4. The mechanism(s) of the PDT-mediated cytotoxic effect is not well defined; however, it is universally accepted that the generation of ROS is essential for PDT effects (9, 10). The ROS that have been implicated in the therapeutic or toxic response of PDT include singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical (5–8, 11). NO is an important bioactive signaling molecule that mediates a variety of physiological functions and is involved in the pathogenesis of many disorders including cancer (12–15). In the present study, we demonstrate the generation of NO during PDT and suggest that it may be involved in PDT-mediated apoptosis.

Materials and Methods

Photosensitizer and Reagents. The phthalocyanine photosensitizer Pc4 was provided by Drs. Ying-syi Li and Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University). Aprotinin, pepstatin A, and phenylmethylsulfonyl fluoride were purchased from Calbiochem (San Diego, CA); the Dowex AG 50W-X8 resin (200–400 mesh, sodium form) was purchased from Bio-Rad (Richmond, CA), and [^{14}C]L-arginine (specific activity, 320 mCi/mmol) was obtained from American Radiolabeled Chemical, Inc. (St. Louis, MO).

Cells. In present study, we used human epidermoid carcinoma cells (A431 cells) and radiation-induced fibrosarcoma cells (RIF-1 cells; obtained from American Type Culture Collection, Rockville, MD). The A431 cells were maintained in DMEM, whereas the RIF-1 cells were maintained in Eagle's MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained in an atmosphere of 95% air/5% CO_2 in a 37°C humidified incubator under standard conditions.

PDT. The cells (70–80% confluent) were treated with Pc4 (0.5 μM in DMEM or Eagle's MEM) overnight in a 100 × 20-mm Falcon disposable cell culture dish. The next morning, the cells were washed with HBSS (with Ca^{2+} and Mg^{2+}), irradiated (in HBSS with Ca^{2+} and Mg^{2+}) with 15 KJ/m^2 of light as measured by a digital photometer (Tektronix, Beaverton, OR) using a 300-W halogen lamp placed 30 inches below the surface of a glass exposure tray and standardized protocols in the laboratory (11). The delivered light was filtered through Lee primary red filter #106 (Vincent Lightning, Cleveland, OH) to remove light with wavelengths of <600 nm. The cells were then incubated at 37°C for selected times (15 s, 30 s, 1 min, 5 min, 15 min, 30 min, 60 min, and 180 min) post-PDT in a humidified incubator. The cells treated with vehicle (DMSO) only, Pc4 only, and light only served as controls. After the specified times, the medium was collected, and the cells were processed for preparation of the cellular lysate.

Preparation of Cell Lysate. The cells were treated with Pc4 and light as described above and harvested at different time points as specified above. The cells were washed with cold PBS [10 mM (pH 7.4)], and ice-cold lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na$_3$VO$_4$, 0.5% NP40, 1% Triton X-100, 1 mM phenylmethylsulfonyl

fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin (pH 7.4)] was added to the plates and placed over ice for 30 min. The plates were scraped, and the lysate was collected in a microfuge tube and passed through a 21-gauge needle to break up cell aggregates. The lysate was cleared by centrifugation at 14,000 × g for 15 min at 4°C, and the supernatant (total cell lysate) was collected and stored at −70°C. The protein concentration was determined by the DC Bio-Rad assay using the manufacturer's protocol (Bio-Rad) before using it for other biochemical assays.

Nitrite Assay. The NO production was quantified spectrophotometrically by measuring the nitrite concentration in the culture medium as well as the lysates using a NO detection kit (Oxford Biomedical Research, Oxford, MI) according to the manufacturer's protocol.

Determination of NOS Activity. The Ca^{2+}-dependent NOS activity was assayed by the method of Salter *et al.* (16) with slight modifications. Briefly, 0.2 ml of the cellular lysate was added with the enzyme substrate mixture containing calmodulin (2.0 µg/ml), NADPH (0.2 mM), L-arginine (0.02 mM), [^{14}C]L-arginine (0.5 µCi), $MgCl_2$ (2.0 mM), and $CaCl_2$ (0.2 mM) in HEPES [100 mM (pH 7.2)] in a total volume of 0.4 ml. This mixture was incubated at 37°C for 30 min with or without EGTA (1 mM). The reaction was terminated by the addition of 2.0 ml of 20 mM HEPES (pH 5.5) containing EDTA (5.0 mM), and the resulting mixture was run over a AG-50W-X8 resin column. L-Citrulline was eluted with three fractions of de-ionized water (3.0 ml), and ^{14}C radioactivity in the elute was measured. The Ca^{2+}-dependent constitutive NOS activity was obtained from the difference between the values obtained from the reaction mixture with and without EGTA.

Western Blot Analysis. The cell lysates (40–50 µg of protein) were resolved over 8% polyacrylamide-SDS gels and transferred to the nitrocellulose membrane. The blot was blocked in blocking buffer [5% nonfat dry milk and 0.1% Tween 20 in TBS (pH 7.6)] for 1 h at room temperature, incubated with anti-NOS-I rabbit polyclonal antibody (Santa Cruz Biochemicals, Santa Cruz, CA) in blocking buffer overnight at 4°C followed by incubation with antirabbit secondary antibody horseradish peroxidase conjugate (Amersham International), and detected by enhanced chemiluminescence (Amersham International) and autoradiography using XAR-5 Kodak film.

Results and Discussion

PDT has emerged as a promising modality for the treatment of many solid tumors as well as certain nonmalignant diseases (1–6). The involvement of ROS has been suggested as a prerequisite for PDT effect (8–11). In both *in vitro* and *in vivo* situations, PDT has been shown to cause apoptosis (17–20), which is a physiological and pathological cell deletion process that functions as an essential mechanism of tissue homeostasis and plays a crucial role in cancer. The mechanism of PDT-mediated apoptosis is not well defined. Using a cell culture system, the involvement of phospholipases A_2 and C, intracellular Ca^{2+}, and ceramide has been reported during PDT-mediated apoptosis (17). Recently, the involvement of caspases in PDT-mediated cell killing has been shown (18). In *in vivo* systems, we have shown the occurrence of apoptosis during the ablation of RIF-1-implanted tumors in C3H mice (19) and chemically induced squamous papillomas in mouse skin during Pc4 PDT (20). The mechanism of PDT-mediated apoptosis is not completely understood.

NO is an important signaling molecule that acts as a messenger molecule in cell-to-cell communication (12, 13) and thereby mediates many physiological functions. NO also plays an important role in the pathogenesis of many disorders including cancer (14, 15). We have previously shown an association of phorbol ester-mediated tumor promotion with a decrease in the constitutive form of NOS (21). Studies have also shown an up-regulation of constitutive NOS and nitrite generation during UVB radiation exposure in humans (22). Here we designed experiments to investigate the hypothesis that NO is involved in PDT-mediated apoptosis.

To best address this issue, we used A431 cells that undergo apoptosis due to Pc4 PDT and RIF-1 cells that are resistant to apoptosis (19). We believe that this pair of cell lines are an appropriate model

to study the involvement of NO during PDT-mediated apoptosis. We assessed the involvement of NO during PDT by comparing the effect of Pc4 PDT on nitrite generation in A431 and RIF-1 cells. Data in Fig. 1A showed a significant up-regulation of nitrite generation in the lysates of A431 cells after Pc4 PDT. This up-regulation of nitrite was rapid and was observed as early as 15 s post-PDT, showing a time-dependent increase that reached a maximum at 5 min post-PDT and reverted back to normal at 30 min post-PDT. Because nitrite and nitrate are soluble in aqueous medium, we considered the possibility of the release of soluble nitrite by cells into the medium. To assess this possibility, we also measured the nitrite concentration in cell culture medium after PDT. Data in Fig. 1A showed a time-dependent increase in the concentration of nitrite in culture medium. In RIF-1 cells, however, no significant change in the nitrite concentration was observed in either the cell lysates or culture medium (Fig. 1B).

In *in vivo* situations, NO is produced from L-arginine by a family of enzymes known as NOS via an oxygen-dependent reaction using NADPH as a cosubstrate (12–15). Three distinct forms of NOS are characterized: (*a*) the constitutive isoform in the endothelium of blood vessels (ec-NOS; 132-kDa protein); (*b*) the constitutive isoform in brain neurons (nc-NOS; 162-kDa protein); and (*c*) the inducible isoform that requires an induction (i-NOS; 130-kDa protein). The two constitutive forms are Ca^{2+} dependent and produce low levels of NO in response to receptor-mediated stimuli, whereas the inducible iso-
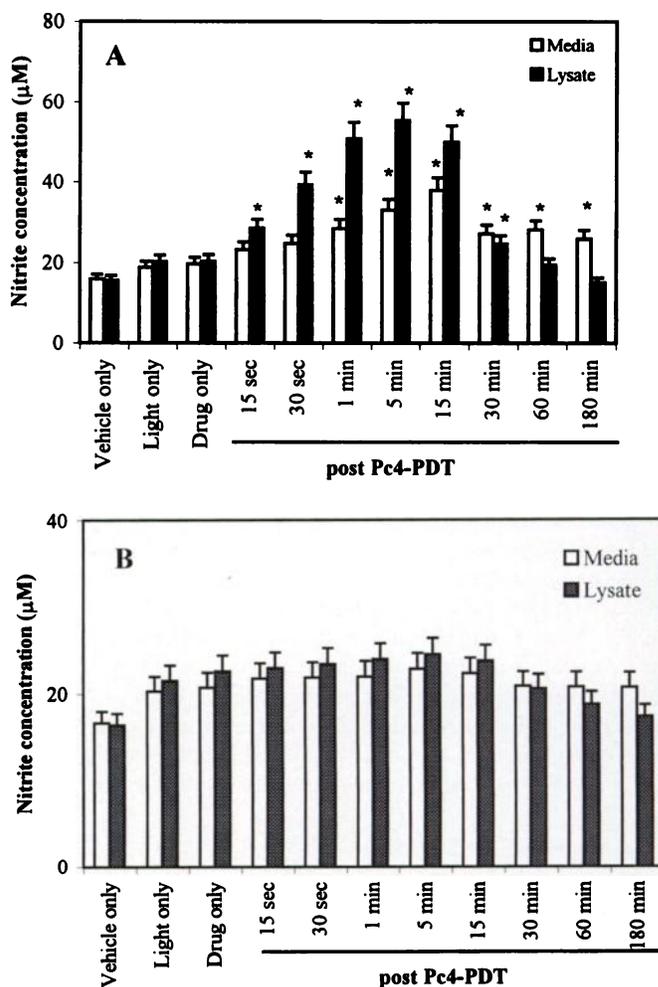

Fig. 1. Nitrite concentration in cell lysate and cell culture medium in (A) human epidermoid carcinoma A431 cells and (B) radiation-induced fibrosarcoma RIF-1 cells treated with Pc4 PDT at various time intervals. Each value is the mean of triplicate determinations + SE (*bars*). *, $P < 0.001$ when compared with the drug-treated control.

form is Ca^{2+} independent and produces much higher levels of NO after induction by cytokines/endotoxin (16). NO can react with a variety of substrates such as superoxide anion to form potentially dangerous species peroxynitrite. The final product of NO metabolism is usually nitrate.

Because NO generation depends on NOS, we also assessed the activity of NOS by following the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. As shown by data in Fig. 2A, Pc4 PDT also resulted in a rapid and significant increase in Ca^{2+}-dependent (constitutive) NOS at 15 min post-PDT. This response was time dependent up to 5 min and then showed a decreasing trend returning to almost normal levels at 60 min post-PDT. In RIF-1 cells, no significant change in the activity of NOS was observed (Fig. 1B). These results follow a pattern almost similar to that of nitrite, providing further support for the involvement of NO in PDT-mediated apoptosis.

To further strengthen our hypothesis using Western blot analysis, we next assessed the protein expression of different isoforms of NOS. As shown by data in Fig. 3A, a significant induction of ncNOS by PDT was observed in A431 cells. Pc4 PDT resulted in a time-dependent induction of ncNOS protein that was observed as early as 30 min post-PDT. In RIF-1 cells, Pc4 PDT had no effect on the protein expression of ncNOS (Fig. 3B). Pc4 PDT also did not show any change in the activity or protein expression of other forms of NOS, i.e. ecNOS and inducible NOS, in either A431 or RIF-1 cells. The data in Fig. 3 also show that Pc4 alone was capable of enhancing the expression of ncNOS. The significance of this drug effect remains to be examined.

Because PDT increased the generation of nitrite selectively in apoptosis-sensitive A431 cells but not in apoptosis-resistant RIF-1
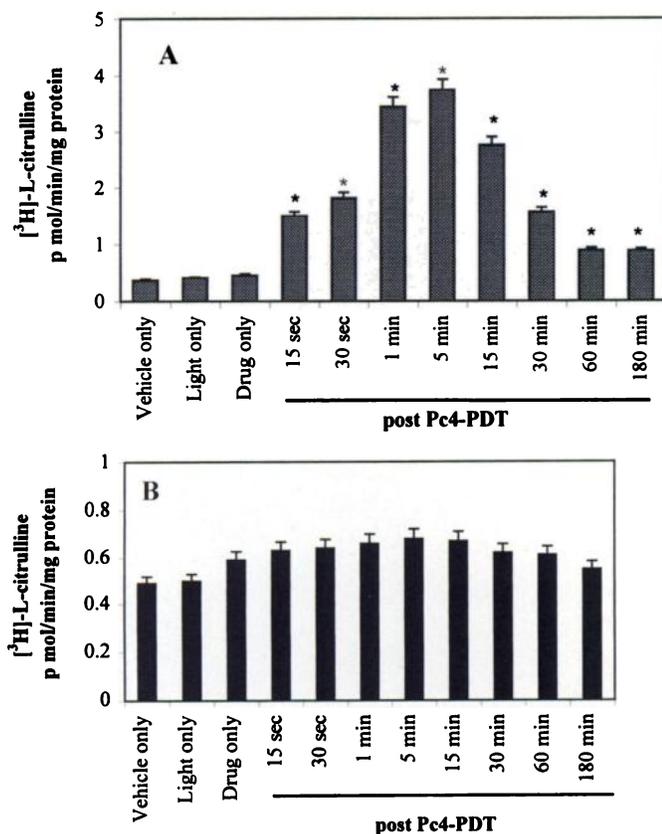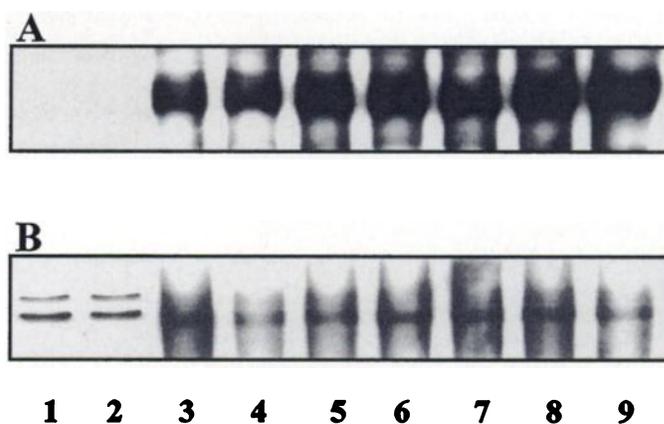

Fig. 3. Western blot analysis showing constitutive NOS (162 kDa) expression after Pc4 PDT treatment at various time intervals in (A) human epidermoid carcinoma A431 cells and (B) radiation-induced fibrosarcoma RIF-1 cells. Lane 1, no treatment; Lane 2, light only; Lane 3, drug only; Lane 4, 15 s; Lane 5, 30 s; Lane 6, 1 min; Lane 7, 5 min; Lane 8, 15 min; Lane 9, 30 min after Pc4 PDT. All cells were irradiated except control cells (Lane 1) and cells treated with drug only (Lane 3).

cells, these data indicate that NO may be involved in PDT-mediated apoptosis. Our data, for the first time, demonstrated the involvement of NO in PDT-mediated apoptosis. The induction of the Ca^{2+}-/calmodulin-independent (inducible) form of NOS in the production of NO that results in apoptosis has been documented in a variety of systems (13, 14, 23). The increase in Ca^{2+}-dependent NOS during Pc4 PDT in this study can be explained by the fact that PDT is known to cause a release of intracellular Ca^{2+} from its stores into the cells (24). This event may be responsible for triggering the formation of the Ca^{2+}-/calmodulin-dependent constitutive isoform of NOS. This may, in turn, result in the increased production of NO that is responsible for the induction of apoptosis in A431 cells. Because the pharmacological intervention using enhancers or inhibitors of NO or NOS may modulate apoptosis, our observations may have relevance in improving the therapeutic efficacy of PDT by pharmacologically modulating the in vivo concentration of NO. The immediate downstream targets of NO in PDT-mediated apoptosis remain to be identified, but the involvement of cell membrane receptor pathways such as the Fas-FAAD-pathway is an intriguing possibility in this direction.

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Fig. 2. Effect of Pc4 PDT on Ca^{2+}-dependent constitutive NOS (162 kDa) activity in (A) human epidermoid carcinoma A431 cells and (B) radiation-induced fibrosarcoma RIF-1 cells at various time intervals. Each value is the mean of triplicate determinations + SE (bars). *, $P < 0.001$ when compared with the drug-treated control.

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