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NITRIC OXIDE-MEDIATED RESISTANCE TO PHOTODYNAMIC THERAPY IN A HUMAN BREAST TUMOR XENOGRAFT MODEL: IMPROVED OUTCOME WITH NOS2 INHIBITORS

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Abstract

Many malignant tumors employ iNOS-derived NO to resist eradication by chemotherapeutic agents or ionizing radiation. In this study, we determined whether human breast carcinoma MDA-MB-231 cells *in vitro* and *in vivo* as tumor xenografts would exploit endogenous iNOS/NO to resist the cytotoxic effects of 5-aminolevulinic acid (ALA)-based photodynamic therapy (PDT). Broad band visible irradiation of ALA-treated cells resulted in a marked after-light upregulation of iNOS protein which persisted for at least 24 h. Apoptotic killing of ALA/light-challenged cells was significantly enhanced by iNOS inhibitors (1400W, GW274150) and a NO trap (cPTIO), implying that stress-induced iNOS/NO was acting cytoprotectively. We found that cells surviving the photostress proliferated and migrated more rapidly than controls in 1400W- and cPTIO-inhibitable fashion, indicating iNOS/NO involvement. Female SCID mice bearing MDA-MB-231 tumors were used for animal model experiments. ALA-PDT with a 633 nm light source caused a significant reduction in post-irradiation tumor growth relative to light-only controls, which was further reduced by administration of 1400W or GW274150, whereas 1400W had little or no effect on controls. Immunoblot analyses of tumor samples revealed a progressive post-PDT upregulation of iNOS, which reached >5-times the control level after six days. Correspondingly, the nitrite/nitrate level in post-PDT tumor samples was substantially higher than that in controls. In addition, a 1400W-inhibitable upregulation of pro-survival/progression effector proteins such as Bcl-xL, Survivin, and S100A4 was observed after *in vitro* and *in vivo* ALA-PDT. This is the first known study to demonstrate iNOS/NO-induced resistance to PDT in an *in vivo* human tumor model.

Keywords

Nitric Oxide; Photodynamic therapy; Breast cancer; Human tumor xenograft model

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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1. Introduction

Photodynamic therapy (PDT) is a unique minimally invasive anti-tumor modality involving a photosensitizing agent (PS), PS-exciting light, and molecular oxygen. [1–3]. Developed over 40 years ago and gaining FDA approval for selected clinical applications in 1996, PDT is based on photochemical reactions occurring at sites accessible to PS, *viz.* the tumor *per se* or tumor-supporting vasculature [1–3]. A prominent reaction involves energy transfer from excited state PS to O₂, giving singlet oxygen (¹O₂), a reactive oxygen species (ROS) that can (i) kill cells by irreversibly damaging vital molecules (proteins, lipids, nucleic acids), or (ii) initiate relatively subtle death signaling cascades [4,5]. Unlike chemotherapy and radiotherapy, PDT has few, if any, light-independent side effects and is target-specific, i.e. limited to the tumor site at which light is directed (typically via fiber optic channels). A number of different PSs have been developed and tested pre-clinically, those absorbing light in the far-visible to near-infrared range being preferred because of deeper light penetration [2,3]. The hematoporphyrin oligomer Photofrin[®] was introduced relatively early as a PDT sensitizer and was FDA-approved for esophageal tumors in 1996, but since then has been used for many other solid tumors, including bladder, breast, prostate, and brain malignancies [1–3]. In some instances, the pro-PS 5-aminolevulinic acid (ALA) or an ester thereof is preferred for PDT. Unlike Photofrin[®] or some other pre-existing PS, ALA is metabolized to protoporphyrin IX (PpIX), the active PS in this case, via the heme biosynthetic pathway [6,7]. An attractive feature of ALA-PDT is that PpIX tends to accumulate preferentially in tumor cells [7,8].

The efficacy of PDT, like that of many other anti-cancer therapies, is typically sub-optimal due to many different factors, including pre-existing or stress-induced resistance mechanisms [9]. We propose that nitric oxide (NO) generated endogenously by inducible nitric oxide synthase (iNOS) in malignant tumors accounts for considerable resistance to PDT. This proposal is supported by our recent studies showing that several cancer cell lines (including breast, prostate, glioma) can exploit iNOS/NO to resist photodynamic cytotoxicity on the one hand and increase surviving cell aggressiveness on the other [10–14].

In this paper, we provide the first known evidence for tumor NO-mediated resistance to PDT in a human tumor model, namely severe combined immunodeficient (SCID) mice bearing human breast carcinoma MDA-MB-231 tumor xenografts. Our evidence is based on the following key observations: (i) the strong increase in iNOS level and NO-derived nitrite level in tumor samples after ALA-PDT, and (ii) the significant improvement in anti-tumor efficacy when iNOS inhibitors were present during and after PDT. These *in vivo* findings were entirely consistent with those obtained with MDA-MB-231 cells *in vitro*, e.g. (i) iNOS/NO upregulation by several fold after an ALA/light challenge, (ii) NO-mediated protection against apoptotic photokilling; and (iii) a growth spurt in surviving cells. As suggested by results of these *in vitro* and *in vivo* studies, clinical PDT outcomes might be significantly improved through use of iNOS inhibitors as pharmacologic adjuvants. One such inhibitor, GW274150, which was used in our xenograft experiments, has already been tested in a clinical trial unrelated to cancer or PDT, and with no untoward side effects [15].

2. Materials and methods

2.1. General chemicals and reagents

Cayman Chemicals (Ann Arbor, MI) supplied the iNOS inhibitor N-[3-(aminomethyl)benzyl]acetamide (1400W), the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a kit for determining NO-derived nitrite/nitrate (NO_x) via the Griess assay, and an Annexin V-FITC plus propidium iodide (PI) kit for detecting apoptosis vs. necrosis. The iNOS inhibitor GW274150 was obtained from GlaxoSmithKline LLC (Research Triangle Park, NC) via a material transfer agreement. Sigma-Aldrich (St. Louis, MO) supplied the 5-aminolevulinic acid (ALA), 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), Dulbecco's Modified Eagles' Ham's Nutrient F-12 (DME/F12) growth medium, and antibiotics (penicillin, streptomycin).

2.2. Cell line and cell culture

Human breast adenocarcinoma MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), which provided the necessary authentication details for this line. Cells were grown under routine culture conditions, using DME/F12 medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml). Proliferating cells received fresh medium every 2–3 days, but this was always done 24 h before beginning a new experiment. All *in vitro* experiments were carried out on cells that had been passaged fewer than 10 times. Other details were as described previously [10–14].

2.3. Cell sensitization and irradiation

MDA-MB-231 cells at 40–50% confluency in 35-mm or 10-cm dishes, were metabolically sensitized with PpIX by incubating with 1.0 mM ALA in phenol-red- and serum-free DME/F12 medium for 30 min in the dark at 37 °C. When effects of an iNOS competitive inhibitor (1400W, GW274150) or NO scavenger (cPTIO) were assessed, the intended agent was added to the medium 30 min before ALA and held at a given concentration during irradiation and throughout post-irradiation incubation. After ALA treatment, cells were washed, overlaid with fresh medium that either lacked or contained one of the above agents, and irradiated on a translucent platform above a set of four 40W cool-white fluorescent tubes emitting at a fluence rate of ~1.1 mW/cm². The latter was measured with a YSI radiometer (Yellow Springs, OH). A typical irradiation period of 15 min corresponded to a delivered light fluence of ~1.0 J/cm² at the bottom surface of a culture dish. Temperature remained at 25–27 °C throughout. Light-only and ALA-only controls were prepared alongside and analyzed similarly to ALA/light-treated cells.

2.4. Evaluation of in MDA-MB-231 cell death in vitro

Approximately 15 min after irradiation, any detached cells were removed by aspiration and remaining cells in 35-mm dishes were overlaid with 10% FBS-containing medium and returned to the incubator. At various time intervals or after a single 20 h period, the viable cell fraction was determined by thiazolyl blue (MTT) assay, as described previously [10,16].

Early state apoptosis, typically assessed 4 h after irradiation, was determined by Annexin V-FITC fluorescence assay [16,17]; PI was included to detect any necrosis. A 96-well plate reader (Biotek Synergy-MX, Winooski, Vt) was used for recording fluorescence (485 nm excitation; 535 nm emission). Experimental measurements are expressed as a percentage of the signal obtained with 25 μ M camptothecin, which induced complete apoptosis.

2.5. Evaluation of proliferative and invasive properties of surviving cells

Twenty-four hours after MDA-MB-231 cells were ALA/light-challenged in the absence vs. presence of an iNOS inhibitor or NO trap, then washed to remove dead/dying cells, remaining attached cells were overlaid with 10% FBS-containing medium and returned to the incubator. After various time periods, the medium was removed and replaced with 1.0 ml of fresh medium containing MTT (0.5 mg/ml). After a 4 h incubation period, cells were solubilized in 1.0 ml of acidified isopropanol, after which formazan absorbance at 563 nm was recorded as a measure of live proliferating cells.

A 96-place trans-well chamber (Model MBA96) from NeuroProbe (Gaithersburg, MD) was used for assessing the invasiveness of cells surviving ALA/light-induced stress. For these measurements, cells were grown on 100-mm dishes to provide sufficient amounts for analysis. A 225 μ l aliquot of 10% FBS-containing medium was added to each lower well of the invasion chamber, over which was placed a matrigel-infused polycarbonate filter with 8 μ m pores. After ALA/light treatment and removal of any detached cells, attached cells were gently scraped into serum-free medium and a 225 μ l aliquot was layered into each upper well of the invasion chamber. The chamber was sealed and placed in the incubator and after a given time period, e.g. 24 h, residual cells in the upper well were gently removed. Those that had migrated to the lower surface of a filter were centrifuged off (500x g, 10 min) in 10% FBS-medium, allowed to attach, then photographed and quantified by MTT assay. Other details were as described previously [13].

2.6. Generation of MDA-MB-321 tumors in immunodeficient mice

Female severe combined immunodeficient (SCID) mice at four weeks of age (strain NOD.CG.prkdc(sud)il2rg) were obtained from Jackson Laboratories (Bar Harbor, ME). After arrival, mice were housed in a pathogen-free room in the Biomedical Resource Center (BRC) of the Medical College of Wisconsin (MCW). All protocols to be described were approved by the MCW Institutional Animal Care and Use Committee (IACUC). After a one-week acclimation period, mice were anesthetized with 5% isoflurane and injected subcutaneously into the mammary fat pad with $\sim 10^6$ (100 μ l) MDA-MB-231 cells. These cells (<3 passages) had been collected by gentle scraping, washed twice with PBS, and suspended in PBS/matrigel (1:1 by vol) immediately before injection. Injected mice were returned to their cages and monitored every other day. Tumors were allowed to grow to 300–400 mm³, which took approximately three weeks. At this point, animals were injected intraperitoneally (i.p.) with ALA (100 mg/kg) in PBS. Some animals received a subsequent injection of 1400W (10 mg/kg) or GW274150 (25 mg/kg). Injected mice were then shaved to expose only the tumor area and returned to their cages for 4 h. During this holding period, cages were shielded from direct room light.

2.7. In vivo PDT protocols and assessment of treatment outcomes

After the 4 h sensitization period, animals were again anesthetized with isoflurane and inserted into extra small opaque restraining cones (Harvard Apparatus, Holliston, MA). A hole was carefully cut into each cone so as to expose the tumor area; a small nose opening was also present. Restrained mice were then positioned on an Omnilux-Revive LED source (PhotoTherapeutics Inc., London, UK), which emits at 633 ± 6 nm with an irradiance (fluence rate) of ~ 105 mW/cm². Up to two animals could be irradiated simultaneously with this source. The irradiation time was typically 15 min, which corresponds to a delivered light fluence of ~ 95 J/cm². The ambient temperature increased <1 °C during light exposure. After irradiation, animals were removed from restraining bags, returned to their cages, and maintained under subdued room lighting for the next 24 h. When used, an iNOS inhibitor (1400W or GW274150) was re-injected at the same initial dose at consecutive 24 h post-irradiation intervals for at least seven days. Tumor volumes were measured every three days after irradiation, using a traceable carbon fiber caliper and the expression: $\text{vol} = (\text{length}^2 \times \text{width})/2$. Control mice not treated with ALA, but irradiated in the absence vs. presence of an iNOS inhibitor were examined similarly. At appropriate times dictated by tumor burden, animals were euthanized by CO₂ inhalation according to guidelines specified by the MCW BRC, and tumors were harvested and stored at -80 °C for various subsequent analyses.

2.8. Determination of nitrite/nitrate in tumor samples

Mice bearing MDA-MB-231 tumor xenografts were subjected to ALA-PDT in the absence vs. presence of an iNOS inhibitor as described in the preceding section, non-ALA controls being irradiated similarly. After at least two post-irradiation time intervals, animals were euthanized; tumors were excised, flash-frozen in liquid nitrogen, and stored at -80 °C. Immediately before NO_x analysis, tumors were minced and homogenized in ice cold PBS buffer (pH 7.4) for 2–3 min, using a Micro-Vial Homogenizer (Wilmad LabGlass, Vineland, NJ). After centrifugation at $10,000 \times g$ for 20 min, recovered supernatants were ultra-filtered using a 30 kDa cut-off filter. Filtrates were analyzed for NO-derived nitrite (NO₂[−]) and nitrate (NO₃[−]) by Griess assay, using a protocol recommended by the reagent supplier (Cayman Chemical Co.). This protocol included reduction of any NO₃[−] in the samples to NO₂[−], using nitrate reductase. Absorbance of the azo dye product at 540 nm was recorded in a plate reader and quantification of total tumor NO₂[−]/NO₃[−] was based on a NO₂[−] standard curve.

2.9. Western blot analyses

The expression and/or activity status of iNOS and several pro-survival/pro-expansion effector proteins (Akt, Survivin, Bcl-xL, S100-A4) in cells after ALA/light treatment and in tumors after ALA-PDT was assessed by Western blotting, using β -actin as an internal standard. Lysates of photostressed MDA-MB-231 cells, along with non-stressed controls were prepared as described [10–13], analyzed for total protein, and subjected to Laemmli SDS-PAGE, using appropriate percentages of acrylamide/bis-acrylamide. Separated proteins were transferred to a PVDF membrane and, after blocking, the membrane was incubated overnight at 4 °C with a given primary antibody at the supplier-recommended dilution. The polyclonal antibody against human iNOS was obtained from Cayman Chemicals (Ann

Arbor, MI). Cell Signaling Technology (Danvers, MA) supplied the polyclonal antibodies against human Bax and Bcl-xL, the monoclonal antibodies against human Akt (pan), p-Akt, Survivin, S100-A4 and β -actin, and the peroxidase-conjugated IgG secondary antibodies. After primary and secondary antibody treatment, a SuperSignal West Pico chemiluminescence kit (Thermo Scientific, Rockford, IL) was used to detect the protein of interest.

Tumors recovered after ALA-PDT were minced immediately and homogenized in ice-cold RIPA buffer (2–3 min). Homogenates were mixed by rocking at 4 °C for ~30 min and then centrifuged at 13,000 $\times$ g for 15 min. Recovered supernatants were centrifuged again and the resulting final supernatants were aliquoted and frozen for Western blot determinations of iNOS and other proteins, using the general methodology described above.

2.10. Statistical analyses

All *in vitro* and *in vivo* experiments were carried out at least in triplicate, with resulting data expressed as means $\pm$ SEM. The two-tailed Student's *t*-test was used for determining the significance of perceived differences between experimental values, $P < 0.05$ being regarded as statistically significant.

3. Results

3.1. iNOS upregulation in photodynamically stressed MDA-MB-231 cells

Prior to determining how iNOS/NO might influence the outcome of ALA-PDT in MDA-MB-231-bearing mice, we studied the effects of analogous treatment at the *in vitro* level. Cells at ~40% confluence were sensitized with ALA-induced PpIX, exposed to ~1 J/cm² of broad band visible light, then analyzed for the level of iNOS protein after increasing periods of dark incubation. As shown by the Western blot in Fig. 1, ALA-treated cells kept in the dark for 24 h expressed a low level of iNOS. However, irradiated cells exhibited a progressive increase in iNOS during post-irradiation incubation, its level reaching >8-times that of the dark control after 24 h. The other NOS isoforms, nNOS and eNOS, were barely detectable in these cells, and no significant changes occurred after ALA/light treatment (not shown). Also, a light control (no ALA) displayed the same low iNOS signal as the dark control (ALA-only) after 24 h (not shown). Thus, the iNOS induction observed in Fig. 1 was most likely a response to photodynamic stress.

3.2. iNOS/NO-mediated resistance to cell photokilling

To determine whether stress-induced iNOS might alter the susceptibility of MDA-MB-231 cells to photokilling, we studied the effects of two different iNOS competitive inhibitors, 1400W and GW274150. As shown in Fig. 2A, MTT-assessed viability at 20 h post-irradiation decreased with increasing light fluence, the LD₅₀ being ~2 J/cm². When 1400W was present during and after irradiation, the LD₅₀ decreased to ~1.3 J/cm², suggesting that iNOS-derived NO was signaling for increased cell resistance. Under the conditions used for cell photosensitization (Sect. 2.3), most of the ALA-induced PpIX and PpIX-sensitized photooxidative stress would have been localized to mitochondria [7,10–14]. This suggests that intrinsic (mitochondrion-centered) apoptosis probably played a major role in cell

photokilling. Using Annexin V-FITC/PI to assess apoptosis, we found that ~25% of the cells were apoptotic 4 h after an ALA/light challenge (Fig. 2B), whereas no significant necrosis was detected. Importantly, apoptosis increased to 40–45% when 1400W or GW274150 was present during and after the ALA/light challenge (Fig. 2B). Since iNOS was already upregulated more than 2-fold 4 h after irradiation (Fig. 1), these results suggest that NO from the induced enzyme was playing a significant cytoprotective role. When included in the reaction system, the NO scavenger cPTIO also caused a significant boost in the apoptotic cell count (not shown). Collectively, these results are consistent with the notion that photostress-upregulated iNOS/NO played a key role in cell resistance to photodynamic cytotoxicity.

3.3. Increased aggressiveness of cells surviving a photochallenge

When sensitized cells were exposed to a typical light fluence of $\sim 1 \text{ J/cm}^2$, the stress experienced must have been relatively modest because at least 70% of the cells remained viable after 20 h (Fig. 2A). We asked whether these surviving cells retained their original growth and mobility characteristics or whether these had changed, and if so, iNOS/NO's possible involvement. At 24 h after ALA/light treatment, surviving attached cells were overlaid with fresh 10% FBS-medium, returned to the incubator, and extent of proliferation over the next 24 h was monitored. We found that the growth rate of these cells was more than twice that of ALA-only controls (Fig. 3A). Moreover, iNOS inhibition with 1400W resulted in a significant decrease in growth rate, implying that iNOS-derived NO was signaling for growth enhancement. We also found that cells treated with 1400W alone grew at the same relatively low rate as ALA-only controls (Fig. 3A). Since neither 1400W nor ALA alone (Fig. 1) caused any change in iNOS from basal expression, these results indicate that stress-upregulated iNOS/NO was primarily responsible for the observed growth spurt.

In examining the possible effects of photodynamic stress on MDA-MB-231 invasiveness, we found cells surviving an ALA/light challenge invaded ~70% more rapidly than dark (ALA-only) controls (Fig. 3B). When present in the system throughout, i.e. before, during, and after irradiation, 1400W or cPTIO abolished this accelerated invasiveness (Fig. 3B), whereas neither agent had any significant effect on the invasion rate of control cells (not shown). These findings established that photostress-enhanced invasion, like proliferation, was driven mainly by NO from stress-induced iNOS.

3.4. Altered status of pro-survival/pro-expansion effector proteins in photostressed cells

Having observed greater NO-dependent resistance and survivor aggressiveness in photostressed MDA-MB-231 cells (Figs. 2 and 3), we asked how the post-irradiation expression/activity of proteins associated with pro-death vs. pro-survival responses might have been affected. The following proteins were selected for monitoring: Bcl-xL, Bax, Survivin, S100S4, and Akt. As shown by the Western blot in Fig. 4A, anti-apoptotic Bcl-xL was transiently upregulated relative to a control at ~3 h after irradiation (Fig. 4A), whereas pro-apoptotic Bax, in striking contrast, was transiently down-regulated at 3–6 h (Fig. 4B). Survivin, an inhibitor of apoptosis protein [18], was progressively overexpressed over a 24 h post-irradiation period (Fig. 4C). S100A4, a key promoter of tumor cell growth and migration/invasion [19], underwent a very robust upregulation after photodynamic

challenge, increasing ~4-fold from a weak background level after 24 h (Fig. 4D). Importantly, all of these responses to photodynamic stress were at least partially (Bcl-xL, Bax) or completely (S100A4) reversed by GW274150 (Fig. 4 A–D), implying that iNOS/NO was signaling for increased survival and progression, at least during early post-irradiation periods. Finally, Akt, a key promoter of tumor growth/progression [20], exhibited a transient phosphorylation-activation (p-Akt appearance) 2–4 h after ALA/light treatment, which subsided thereafter, returning to the control level by 24 h (Fig. S1). However, GW274150 had no significant effect on this activation (Fig. S1). This is consistent with previous evidence obtained with another breast cancer line [11], which indicated that Akt activation occurs upstream of iNOS induction.

3.5. Antagonistic effects of iNOS/NO on ALA-PDT in a tumor xenograft model

We inoculated female immunodeficient (SCID) mice with MDA-MB-231 cells as described in Sect. 2.6 and when tumors were fully established, subjected the animals to ALA-PDT, using an Omnilux-Revive LED source for irradiation. This unit emits red (633 nm) light for optimal tissue penetration and absorption by the longest wavelength Q-band of the photosensitizer, PpIX [6,7]. A cut-out in the opaque restraining cone allowed only the projecting tumor area to be irradiated (Fig. 5A, B). In an initial ALA-PDT experiment, we observed a significant reduction in MDA-MB-231 tumor growth relative to that in light-only control animals over a 25-day post-irradiation period (Fig. S2). A single dose of 1400W or GW274150 administered after ALA did not further reduce tumor growth over that observed with PDT alone (Fig. S2). We considered the possibility that a single dose of either iNOS inhibitor might not allow an optimal steady state level to be reached in the tumor area. Therefore, in follow-up experiments, we used multiple administrations of each inhibitor spaced 24 h apart, the initial one before irradiation, and the succeeding ones for at least 9 days after irradiation.

Using this protocol, we first tested the effects of 1400W on light-only controls, i.e. animals that had not been sensitized with ALA-induced PpIX prior to irradiation. This was done to account for any constitutive (pre-existing) iNOS/NO that might be signaling for tumor resistance or progression. As shown in Fig. 6A, mean tumor volume in mice treated with light alone increased steadily over a 12-day post-irradiation period. Pre- and post-irradiation administration of 1400W resulted in a barely significant decrease in tumor volume relative to control 3 days after irradiation, but no significant difference from 6 to 12 days. Since light alone did not affect iNOS expression, these results indicate that constitutive iNOS/NO had little or no stimulatory effect on tumor progression. These *in vivo* findings are consistent the *in vitro* findings of Fig. 3A showing the non-effects of 1400W on control cell growth rate. When actual ALA-PDT was carried out with vs. without multiple injections of an iNOS inhibitor, the results were strikingly different. In agreement with the Fig. S2 results, ALA-PDT by itself significantly reduced tumor expansion relative to that in light-only controls, beginning at ~600 mm³. More importantly, either 1400W or GW274150, administered before and after PDT, caused a highly significant further reduction in tumor growth, particularly after 6 days (Fig. 6B). These findings recapitulate and reinforce those in Figs 2 and 3, demonstrating that the anti-PDT effects of iNOS/NO observed for MDA-MB-231 cells *in vitro* also apply *in vivo*.

Additional supporting evidence for the results shown in Fig. 6B was sought by examining iNOS levels in MDA-MB-231 tumors after ALA-PDT. As shown by the Western blots in Fig. 7, animals that were irradiated without prior ALA administration expressed iNOS at the same basal level a throughout post-irradiation holding period of at least 6 days. In striking contrast, the iNOS level in tumors from animals subjected to full ALA-PDT increased steadily over this period, going from ~2-times the initial (day-0) amount by day-2 to nearly 5-times by day-6. Clearly, therefore, the induction of iNOS in ALA/light-stressed MDA-MB-231 cells *in vitro* (Fig. 1) could be recapitulated at the *in vivo* level, using tumor xenografts of these cells. It is important to point out that responses to treatment in the Fig. 7 experiment were consistent with those shown in Fig. 6, *viz.* a significant slowdown in tumor growth after ALA-PDT compared with light treatment alone, e.g. ~15% lower tumor volume at 6 h.

We used the Griess assay on tumor homogenate samples to determine whether iNOS upregulation after ALA-PDT would be reflected in higher NO levels, detected as NO-derived $\text{NO}_2^-/\text{NO}_3^-$. Prior to analysis, any NO_3^- in our samples was reduced to NO_2^- with nitrate reductase, so that only total NO_2^- would be represented. As shown in Fig. 8, the NO_2^- level in tumors treated with light alone started low (~2 $\mu\text{M}/\text{mg}$) and remained so over a 6-day post-irradiation period. However, in ALA/light-treated tumors, NO_2^- increased to ~8 $\mu\text{M}/\text{mg}$ after two days, and to ~14 $\mu\text{M}/\text{mg}$ after six days. These increases were significantly reduced by 40–50% when 1400W was administered before and after irradiation, demonstrating that photostress-upregulated iNOS was a major source of elevated NO levels in our MDA-MB-231 tumors after ALA-PDT treatment.

3.6. Altered expression of effector proteins in MDA-MB-231 tumors after ALA-PDT

We found that the NO-mediated post-photostress changes in effector protein expressions observed *in vitro* (Sect. 3.4) could, for the most part, be recapitulated *in vivo*. For example, Bcl-xL was upregulated nearly 3-fold two days after ALA-PDT (Fig. 9A), whereas Bax was down-regulated by ~40% after two days and ~60% after six days (Fig. 9B). The level of Survivin increased by nearly 4-fold and ~2-fold at two and six days, respectively, after PDT (Fig. 9C). In addition, there was a nearly 3-fold upregulation in S100A4 six days after PDT (Fig. 9D). As observed for ALA/light-treated cells *in vitro* (Fig. 4), these *in vivo* protein upregulations could be significantly blunted by the iNOS inhibitor GW274150. For example, Bcl-xL was reduced to ~1.5-times its control level by two days post-PDT (Fig. 9A) and Bax was approximately back to its control level by six days (Fig. 9B). Similarly, GW274150 at least partially reversed the upregulation of Survivin (Fig. 9C) and S100A4 (Fig. 9D) caused by ALA-PDT. These findings begin to pinpoint the effects that PDT-induced iNOS/NO can have on expression of pro-survival *vs.* pro-death regulators.

4. Discussion

This is the first study to describe the antagonistic effects of endogenous iNOS/NO on *in vivo* PDT using a human tumor xenograft SCID mouse model. In all previous *in vivo* studies dealing with NO's impact on PDT, syngeneic mouse models were used, i.e. immune-normal mice implanted with murine tumors. One of the earliest of these studies with syngeneic

systems was carried out by Henderson *et al.* [21], who showed that Photofrin-sensitized PDT outcomes could be improved by NOS inhibition. For example the PDT cure rate for radiation-induced fibrosarcoma (RIF) tumors was significantly increased when the non-specific inhibitor L-NNA was administered before and after irradiation [21]. About the same time, Korbelik *et al.* [22,23] reported that syngeneic tumors with high NO output, e.g. RIF and squamous cell carcinoma (SCCVII), were more resistant to Photofrin-PDT than those with relatively low output, e.g. mammary sarcoma EMT6 and fibrosarcoma FsaR. Accordingly, administration of a NOS inhibitor such as L-NAME or L-NNA produced a greater improvement in PDT outcome for RIF and SCCVII tumors than for EMT6 and FsaR [23]. More recently, Reeves *et al.* [24,25], using mice with syngeneic RIF and EMT6 tumors sensitized with ALA-induced PpIX, confirmed that endogenous NO exerted a negative effect on PDT efficacy. In the cited studies [21–25], it was postulated that measurement of constitutive tumor NO output might be used to predict PDT efficacy and whether administration of NOS inhibitors might improve PDT outcomes. Each of these studies [21–25] attributed NO's anti-PDT activity mainly to microvasculature effects, i.e. that vasodilation due to endothelium (eNOS)-generated acted in opposition to the well-known vasoconstrictive effects of PDT which cut off the vital supply of O₂ and nutrients. These early studies were groundbreaking in terms of advancing from the relatively simple *in vitro* level, e.g. cells in two-dimensional culture or spheroids, to the *in vivo* animal model level, they left many unanswered questions. These include: (a) whether antagonistic NO derives from the tumor cells themselves or whether stromal cells such as endothelial cells and tumor-associated macrophages might contribute, either before or after PDT stress develops; (b) which NOS isoform is most important as an endogenous NO donor; (c) whether pre-existing NO at some low steady state level is sufficient for PDT resistance or whether NOS/NO induction by photodynamic stress plays a more important role; (d) whether NO might act cytoprotectively by scavenging damaging intermediates generated by PDT, e.g. oxyl/peroxyl radicals from membrane lipid peroxidation [26,27]; and (e) the types of pro-survival signaling pathways that may be activated by pre-existing or stress-upregulated NO [28–30]. Some of these crucial questions have been addressed in the present study.

One of our most significant findings was that ALA/light treatment resulted in a dramatic upregulation of iNOS protein in MDA-MB-231 breast carcinoma cells, both *in vitro* and *in vivo* as tumor xenografts (Figs. 1 and 7). Irradiation alone in both settings had no effect on iNOS level, indicating that photodynamic action mediated by ¹O₂ and/or other reactive oxidants arising from photoexcitation of ALA-induced PpIX was necessary. Based on a previous study with another breast tumor cell line [10], we assume that MDA-MB-231 iNOS induction was mainly due to transcriptional activation, but this needs to be confirmed by RT-PCR determinations. The post-photostress induction of iNOS appears to be a general response, since we have now observed it in several different cancer lines, including breast, prostate, and glioma [10–14]. Another key observation in the present study is that two highly specific competitive inhibitors of iNOS, 1400W and GW274150, not only stimulated ALA/light-induced tumor cell killing *in vitro* (Fig. 2), but also *in vivo*, as evidenced by a significantly greater reduction in tumor growth after ALA-PDT (Fig. 6B). The latter finding could be attributed to a reduction in NO-mediated resistance to photokilling, although restriction of NO-stimulated cell growth after ALA-PDT might have contributed to the

greater tumor regression in the presence of iNOS inhibitors. A 1400W-inhibitable growth spurt in MDA-MB-231 cells was observed after an ALA/light challenge (Fig. 3A), and this could well have occurred at the tumor level. Thus, endogenous NO may have played a cytoprotective (anti-apoptotic) role as well as a pro-growth role for cells surviving PDT, and together these could explain the observed anti-PDT effects of iNOS/NO (Fig. 7). Of added importance is our finding that iNOS upregulation in MDA-MB-231 tumors after ALA-PDT was accompanied by a progressive 1400W-inhibitable increase in NO level, measured as NO_2^- over a six day post-irradiation period (Fig. 8). Another crucial observation in this study is that the low level of pre-existing iNOS/NO in MDA-MB-231 cells had little (if any) effect on survival or proliferation of light-only or ALA-only controls (Fig. 3A). Similarly, tumor growth in control animals subjected to irradiation without ALA administration was not significantly inhibited by 1400W (Fig. 6A). Thus, although MDA-MB-231 cells are known to be rather aggressive in terms of basal proliferation and migration [31], this did not appear to significantly iNOS/NO-dependent in our system, either at the *in vitro* or *in vivo* level. This realization highlights the importance of photostress-induced iNOS/NO, not only in cell resistance to ALA/light-induced stress, but also stimulating surviving cell proliferation and invasion (Fig. 3B). In addition to the above findings, we obtained *in vitro* and *in vivo* evidence for activation/induction of pro-survival vs. pro-death effector proteins and trends were similar at both levels. For example, in both cases, a transient GW274150-inhibitable down-regulation of pro-apoptotic Bax and upregulation of anti-apoptotic Bcl-xL was observed, consistent with resistance signaling by iNOS-derived NO. Also observed were post-photostress activation of Akt *in vitro* and overexpression of Survivin and S100A4 *in vitro* and *in vivo*, each response exhibiting at least partial iNOS-dependency. The strong similarity of the *in vitro* and *in vivo* responses described in this section with regard to iNOS/NO-dependency (ALA/light effects) vs. non-dependency (light-only effects) suggests that the stress conditions that developed in our tumor xenografts after ALA-PDT closely recapitulated those that developed in isolated cells. Therefore, it appears that endogenous tumor iNOS was mainly (if not solely) responsible for the NO-mediated resistance to PDT that we observed *in vivo*. This recapitulation of the relatively simple *in vitro* situation may reflect, at least in part, the fact that immunodeficient SCID mice were used. Such mice have been widely employed as human tumor-bearing models, one highly relevant example being animals transplanted with human melanoma which exhibited iNOS/NO-dependent progression [32]. In another relevant recent example, animals with MDA-MB-231 tumor xenografts were found to respond better to ALA-PDT after administration of vitamin D, which enhanced PpIX formation [33].

It is now well known that PDT generates tumor antigens that serve as 'danger signals' to activate the innate immune system [2,34,35]. Activation of this system requires priming of tumor-specific T-lymphocytes, which assist in recognition and elimination of tumor cell targets. PDT can also elicit an 'immune memory', which can activate eradication if the tumor redevelops at some later time [34,35]. Stimulation of anti-tumor immunity can be thought of as a back-up or indirect effect of PDT which can complement the directly damaging effects of this approach on tumor cells per se or cells in the proximal tumor vasculature. Since SCID mice are deficient in functional B- and T-lymphocytes [36], they would be impaired in ability to mount a post-PDT immune response. The latter could involve phagocytic cells that

produce NO and ROS at cytotoxic levels [37]. It is also important to point out that NO is known to be capable of immunosuppression and that this can promote survival and progression of many different tumor types [37,38]. This aspect, though also applicable to syngeneic models, is irrelevant for the SCID model that we used. Based on these various considerations, we believe that this model provides the most accurate *in vivo* representation of how NO produced by PDT-induced iNOS in tumor cells per se can promote resistance and even tumor progression. Thus, as mentioned above, we believe that our *in vivo* findings regarding NO-mediated resistance to PDT closely recapitulate those obtained with a simple *in vitro* model. This is the first such recognition to be reported in the context of PDT.

The MDA-MB-231 cells used in this study express a mutated form of p53 that lacks wild type tumor suppressor activity, thus providing a significant anti-apoptotic/pro-survival advantage [39,40]. Studies have shown that iNOS-overexpressing colon cancer cells harboring mutant p53 grew faster than controls, whereas iNOS-overexpressing counterparts equipped wild type p53 grew slower [41]. Based on this and related evidence, it has been proposed that tumor p53 and iNOS act in opposing regulatory fashion. Wild type p53 is known to suppress iNOS transcription and also to interact with the enzyme and reduce its activity [42–44]. On the other hand, NO has been shown to modify wild type p53 in breast cancer cells, reducing its pro-apoptotic activity [45]. In subsequent studies, it will be of interest to learn how cells with wild type p53, e.g. human breast carcinoma MCF-7 cells [39,40], respond to ALA-PDT *in vitro* and *in vivo*, i.e. whether any iNOS-mediated resistance is repressed compared with that observed with MDA-MB-231 cells.

Many investigators have advocated pharmacologic use of iNOS inhibitors to retard growth, migration, and invasion of malignant tumors known to exploit iNOS/NO for these purposes [28–30,46]. Such inhibitors might be used alone or in combination with existing chemotherapeutic or radiotherapeutic approaches in order to improve treatment outcomes. Based on our findings in this study, such ideas could also apply to PDT, including ALA-based PDT. Thus, there is good reason to believe that PDT outcomes for breast carcinomas and a variety of other solid malignancies could be improved by administration of select iNOS inhibitors as pharmacologic adjuvants. Although inhibitors such as L-NAME and L-NNA have already been tested in animal models [21–25], these are not specific for iNOS, so their effects could be far from optimal if iNOS is the primary source of NO after PDT. We found that iNOS-specific GW274150 [47,48] significantly increased MDA-MB-231 cell photokilling *in vitro* and tumor regression *in vivo* after ALA-PDT. Since GW274150 has already been used in a human clinical trial unrelated to cancer or PDT, and with no unfavorable side effects [49], it should be a good candidate for future testing in a clinical PDT setting. The same should apply to the iNOS inhibitor L-NIL, which has tested favorably in a clinical trial dealing with suppression of asthmatic inflammation [50].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlight

- Human breast cancer MDA-MB-231 cells *in vitro* overexpress iNOS after a 5-aminolevulinic acid (ALA)-photodynamic therapy (PDT)-like challenge.
- iNOS-derived NO acts cytoprotectively and stimulates surviving cell growth and invasion.
- ALA-PDT activates/upregulates pro-survival/progression effector proteins *in vitro*.
- ALA-PDT suppresses SCID mouse-borne MDA-MB-231 tumor xenografts and iNOS inhibition enhances this suppression.
- ALA-PDT upregulates iNOS protein and NO-derived nitrite in MDA-MB-231 tumors.
- This is the first report of iNOS/NO-enhanced resistance to PDT in a human tumor model.

Fig. 1.

Western blot depicting iNOS upregulation in ALA/light-challenged MDA-MB-232 cells. Cells in serum-free medium were dark-incubated with 1 mM ALA for 30 min, switched to ALA-free medium, and exposed to a 1 J/cm² fluence of broad-band visible light. Immediately after irradiation (0 h) and after increasing periods of subsequent dark incubation up to 24 h, cells were retrieved for Western analysis of iNOS and β -actin. A dark control (DC: ALA alone for 24 h) was also analyzed. Number below each iNOS band indicates band intensity relative to β -actin and normalized to DC. Total protein load per lane: 100 μ g.

Fig. 2. iNOS/NO-induced resistance of MDA-MB-231 cells to photosensitized toxicity. ALA-treated cells were either not irradiated or irradiated in the absence or presence of 25 μ M 1400W (W), using the indicated light fluences. Non-ALA-treated cells were also irradiated as controls. Following treatment, cells were switched to 10% FBS-medium after removal of any detached cells. (A) MTT-assessed viable fraction after 20 h of dark incubation; (□) W absent, (△) W present, (○) light-only control. (B) AnnexinV-FITC-assessed apoptosis after 4 h of dark incubation; 25 μ M camptothecin (CPT) served as a reference for induction of complete apoptosis. Data in panels (A) and (B) are means $\pm$ SEM of values from three separate experiments. * $P < 0.02$ compared with ALA/hv.

Fig. 3.

Accelerated proliferation and invasion of MDA-MB-231 cells surviving an ALA/light challenge: role of iNOS/NO. ALA-treated cells were exposed to a 1 J/cm² light fluence in the absence *vs.* presence of 25 μM 1400W or 25 μM cPTIO. Cells treated with ALA alone or 1400W alone served as controls. Twenty-four hours after treatment, cells were switched to fresh FBS-medium. (A) Proliferation assessed by MTT-detectable viable cell number. (B) Invasion assessed by trans-well assay. Cells traversing the trans-well filter and collecting on its lower surface were photographed (upper images), then removed by centrifugation and quantified by MTT assay (lower image). Plotted data in panels (A) and (B) are means ± SEM of values from three replicate experiments. *P<0.01 compared with ALA/hv.

Fig. 4.

Altered expression or activity of effector proteins in ALA/light-treated cells: effects of iNOS inhibition. After pre-incubation with ALA, MDA-MB-231 cells were exposed to a 1 J/cm^2 light fluence in the absence (ALA/hv) or presence of $25 \mu\text{M}$ GW274150 (ALA/GW/hv). ALA-only dark controls (DC) were run alongside. At the indicated post-irradiation times, cells were retrieved for Western analysis of Bcl-xL, Bax, Survivin, S100A4, phosphorylation-activated Akt (p-Akt), total Akt, and β -actin. Number below each effector protein band represents band intensity relative to β -actin and normalized to DC. Total protein load: $50 \mu\text{g}$ per lane.

Fig. 5. View of the animal and light source arrangement for ALA-PDT. (A) Typical positioning of a mildly anesthetized mouse in an opaque conical restraint on the Omnilux-reviveTM illuminator. Arrow points to the tumor mass, which has been exposed by a cut-out in the restraint. (B) View of a restrained mouse during 633 nm irradiation. Light fluence rate at the illuminator surface was 1050 W/m². Note that the animal's nose at the restrainer tip opening projects off the light field.

Fig. 6.

ALA-PDT suppression of mouse-borne tumor xenografts: improved response after multiple administrations of iNOS inhibitor 1400W (W) or GW274150 (GW). (A) Effects of iNOS inhibition on light-only controls. Female SCID mice bearing MDA-MB-231 tumors were injected i.p. with PBS vehicle or 1400W (10 mg/kg) in PBS. After 4 h, each animal was mildly anesthetized with isoflurane and placed into an opaque restraining cone with a nose opening and a cutout for tumor exposure to irradiation. Omnilux-revive™ irradiation was carried out for 15 min, corresponding to a delivered light fluence of ~95 J/cm². After irradiation, animals were re-injected with 1400W every day until termination, tumor volumes being measured every third day. The mean $\pm$ SEM of values for 3 animals is plotted for each time point. (B) Effects of iNOS inhibition on ALA-PDT. MDA-MB-231 tumor-bearing SCID mice were injected i.p. with ALA (100 mg/kg) or PBS vehicle (light-only controls), followed by 1400W (10 mg/kg) or GW274150 (25 mg/kg). After 4 h in the dark, each animal was mildly anesthetized, placed in a restraining cone, and irradiated as described in (A). After PDT, animals were caged under subdued room light and re-injected with 1400W or GW274150 every day and tumor volumes were measured every third day. For each time point, the mean $\pm$ SEM of values for the following animal numbers are plotted: 6 (hv); 6 (ALA/hv); 3 (ALA/W/hv); 3 (ALA/GW/hv). $P < 0.05$ for ALA/W/hv vs. ALA/hv at 6, 9, and 12 days; $P < 0.05$ for ALA/GW/hv vs. ALA/hv at 9 and 12 days.

Fig. 7. iNOS upregulation in MDA-MB-231 tumor tissue after ALA-PDT. Tumor-bearing SCID mice were irradiated after prior administration of ALA in PBS (ALA/hv) or PBS alone (hv, a light-only control). After irradiation as described in Fig. 6, tumors were either harvested immediately (day-0) or after a 2 or 6 days delay, during which animals were kept away from any bright room lighting. Tumors were homogenized, homogenates centrifuged, and supernatants recovered for Western blot analysis of iNOS as described in Sect. 2.9. Each lane in the blots shown represents material from pooled homogenates of duplicate experimental tumors. Numbers below iNOS bands indicate band intensities relative to β-actin and normalized to day zero. Numbers below β-actin bands indicate average size of tumors used for preparing these Western blots. Total tumor protein applied per lane: 40 μg.

Fig. 8.

Nitrite/nitrate levels in tumor tissues after ALA-PDT. Homogenates of tumors from light-control animals and animals subjected to ALA-PDT in the absence *vs.* presence of 1400W were first treated with nitrate reductase and then analyzed for total nitrite via the Griess assay, as described in Sect. 2.8. Tumor samples were analyzed immediately after irradiation (day-0) and after animals had been caged for 3 and 6 days after irradiation. Plotted data are means $\pm$ SEM of values obtained for three replicate tumors at each post-irradiation time.

* $P < 0.01$ *vs.* ALA/hv.

Fig. 9.

Effects of ALA-PDT on expression of key pro- vs. anti-survival effectors in tumor samples after ALA-PDT: effects of an iNOS inhibitor. At the indicated times (days) after subjecting MDA-MB-231 tumor-bearing SCID mice to irradiation alone (hv), irradiation after ALA administration (ALA/hv), or irradiation after ALA and GW274150 administration (ALA/GW/hv), tumors were isolated and homogenates prepared. Samples were used for Western blot analysis of Bcl-xL, Bax, Survivin, S100A4, and β -actin levels. Numbers below effector protein bands indicate band intensity relative to β -actin and normalized to day zero, i.e. immediately after PDT. Total tumor protein loaded: 40 μ g per lane.