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Pro-Survival and Pro-Growth Effects of Stress-Induced Nitric Oxide in a Prostate Cancer Photodynamic Therapy Model

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Abstract

We discovered recently that human breast cancer cells subjected to photodynamic therapy (PDT)-like oxidative stress localized in mitochondria rapidly upregulated nitric oxide synthase-2 (NOS2) and nitric oxide (NO), which increased resistance to apoptotic photokilling. In this study, we asked whether human prostate cancer PC-3 cells would exploit NOS2/NO similarly and, if so, how proliferation of surviving cells might be affected. Irradiation of photosensitized PC-3 cells resulted in a rapid (<1 h), robust (~12-fold), and prolonged (~20 h) post-irradiation upregulation of NOS2. Caspase-3/7 activation and apoptosis were stimulated by NOS2 inhibitors and a NO scavenger, implying that induced NO was acting cytoprotectively. Cyclic GMP involvement was ruled out, whereas suppression of pro-apoptotic JNK and p38 MAPK activation was clearly implicated. Cells surviving photostress grew back ~2-times faster than controls. NOS2 inhibition prevented this and the large increase in cell cycle S-phase occupancy observed after irradiation. Thus, photostress upregulation of NOS/NO elicited both a prosurvival and pro-growth response, both of which could compromise clinical PDT efficacy unless suppressed, e.g. by pharmacological intervention with a NOS2 inhibitor.

Keywords

photodynamic therapy; pancreatic cancer; nitric oxide synthase; nitric oxide

1. Introduction

Nitric oxide (NO), a free radical molecule produced naturally by enzymes of the nitric oxide synthase (NOS) family, plays an important role in many different biological processes [1]. When generated at high rates, e.g. by activated neutrophils, NO can have pro-oxidant cytotoxic effects, whereas at low rates, it may act as a cytoprotective antioxidant, fostering cell survival under an oxidative challenge [2,3]. Numerous studies have shown that low flux NO in tumors can signal for promotion of cell growth, angiogenesis, and inhibition of apoptosis [4–6]. We showed previously [7] that breast tumor COH-BR1 cells sensitized with disseminated 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) were protected against necrotic photokilling by NO from exogenous donors and attributed this to

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Conflict of interest statement

The authors declare that they have no conflicts of interest in the studies described.

free radical scavenging. Subsequent work revealed that these cells died by apoptosis when PpIX was restricted to mitochondria and that exogenous NO also inhibited this [8]. However, it did so to a significant extent by interfering with stress activation/deactivation of mitogen-activated protein kinases (MAPKs), i.e. activation of pro-apoptotic JNK and p38 α , and deactivation of anti-apoptotic ERK1/2 and p38 α [8]. More recently, we discovered that irradiation of ALA-treated COH-BR1 cells caused a rapid and prolonged upregulation of inducible NOS (NOS2) along with NO [9,10]. Apoptotic photokilling of these cells was markedly enhanced by NOS competitive inhibitors, a NO scavenger, and shRNA-based NOS2 knockdown [9,10], implying that stress-induced NOS2/NO played an important role in photoresistance. Similar findings had been made by others using different cells and stress systems [11]. However, ours (9) was the first evidence for such an effect in the context of anti-tumor photodynamic therapy (PDT), albeit for an *in vitro* model system [12,13].

We have now extended our studies to another cancer line, human prostate PC-3 cells, and have shown that photostress-induced NOS2/NO not only provides protection against apoptosis, but elicits a striking post-irradiation growth spurt in surviving cells which lasts for at least 72 h. This is the first reported example of NO-dependent growth stimulation in cancer cells exposed to a PDT-like oxidative stress. The pro-survival/pro-growth response that we describe could be a general phenomenon in NOS-expressing tumors subjected to PDT, and one that might seriously compromise treatment effectiveness unless counteracted in some way. One of the NOS2 inhibitors used in this study, GW274150, provides some promise along these lines, given that it has already been tested in asthmatic humans as an anti-inflammatory agent [14].

2. Materials and methods

2.1. General materials

Cayman Chemicals (Ann Arbor, MI) supplied the non-specific NOS inhibitor L-*N*^G-nitroarginine methyl ester (L-NAME), the NOS2-specific inhibitor N-[3-(aminomethyl)benzyl]acetamide (1400W), the NO trap 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), and the Annexin V-FITC plus PI kit for detecting apoptosis vs. necrosis. The NOS2 inhibitor GW274150 was kindly supplied by GlaxoSmithKline, LLC (Research Triangle Park, NC) via a material transfer agreement. The NO probe 4,5-diaminofluorescein diacetate (DAF-2DA) was obtained from EMD Biosciences (San Diego, CA). Calbiochem (Gibbstown, NJ) supplied the N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (Ac-DEVD-AMC) and 1 *H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ). Most of the other reagents and cell culture materials were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

Human prostate cancer PC-3 cells obtained from the ATCC repository (Manassas, VA) were grown under standard culture conditions, using DME/F12K medium containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μ g/ml). All experiments were carried out on cells that had been passaged fewer than 10 times. Other details were as described previously [9,10].

2.3. Cell sensitization and treatment with NOS inhibitors or NO scavenger

For evaluating sensitivity to photokilling, PC-3 cells were plated in 35-mm culture dishes for determining caspase-3/7 activity or viability by MTT (thiazolyl blue) assay, and on coverslips in 35 mm dishes for determining apoptosis by Annexin V-FITC staining. At 50–60% confluency, the cells in phenol red- and serum-free RPMI medium were metabolically sensitized with PpIX by treating with 1 mM ALA for 30 min in the dark at 37 °C. When a

NOS inhibitor (L-NAME, 1400W, or GW274150) or a NO scavenger (cPTIO) was used, it was introduced 30 min before ALA and maintained at its initial concentration throughout irradiation and post-irradiation incubation. Prior to irradiation, the subcellular location of PpIX in ALA-treated cells was determined by confocal fluorescence microscopy, using MitoTracker Green as a mitochondria marker and the excitation and emission wavelengths described previously for another cell line [8].

2.4. Cell irradiation

Immediately after ALA treatment, cells were switched to fresh RPMI medium without ALA, which either lacked or contained a NOS inhibitor or cPTIO, and irradiated at room temperature on a translucent plastic platform over a bank of four 40-W cool-white fluorescent tubes [15]. The fluence rate of incident light, measured with a YSI radiometer (Yellow Springs, OH), was $\sim 1.1 \text{ mW/cm}^2$. After a given irradiation period, the medium was replaced with 1% serum-containing DME/F12K (without or with a NOS inhibitor or cPTIO) and cells were returned to the incubator. At various times, cells were recovered for determination of total protein, Western analysis, overall viability, caspase-3/7 activity, or apoptotic vs. necrotic cell death.

2.5. Post-irradiation determination of cell viability and apoptotic death

The effects of increasing photodynamic stress on overall cell viability were determined by MTT assay [15], typically carried out 20 h after irradiation. Photostress-induced apoptosis/necrosis was assessed using the nuclear fluorophores Hoechst 33258 (Ho) and propidium iodide (PI), the former to detect sustained apoptosis with chromatin condensation, and the latter to detect any concurrent necrosis [8,15]. Early stage apoptosis, as indicated by externalization of plasma membrane phosphatidylserine, was determined by Annexin V-FITC staining with fluorescence microscopy, following instructions provided by the reagent supplier. Photostress activation of caspase-3/7 was monitored as described previously (9), using the fluorogenic substrate Ac-DEVD-AMC.

2.6. Western blot analyses

The level of NOS2 in PC-3 cells that had been exposed to a photodynamic stress was determined by Western blot analysis. Lysates of ALA/light-treated cells, along with ALA-only dark controls, were prepared as described [8–10], analyzed for total protein, and subjected to SDS-PAGE. Separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane and analyzed, using primary antibodies against NOS2 (Santa Cruz Biotechnology, Santa Cruz, CA) and β -actin (employed as a loading standard). For protein detection, a peroxidase-conjugated secondary antibody and a SuperSignal West Pico chemiluminescence kit (Thermo Scientific, Rockford, IL) were used. Other details, including determination of band intensities relative to β -actin as a loading standard, were as described previously [8,9].

2.7. Evaluation of surviving cell proliferation after exposure to photostress

The number of viable cells at various post-irradiation times after ALA/light or ALA/1400W/light exposure was determined by trypan blue dye exclusion assay. PC-3 cells at 40–45% confluency were switched to serum-free medium, dark-incubated with 1 mM ALA for 30 min, and exposed to a 0.7 J/cm^2 light fluence. Immediately thereafter, the medium was replaced with DME/F12K plus 10% FBS and cells were returned to the incubator. At increasing intervals up to 48 h, cells were recovered by trypsinization, centrifuged, and washed with ice-cold PBS. After staining with 0.4% trypan blue, the titer of live (dye-excluding) cells was determined with a hemocytometer. Extent of viable cell grow-back after photostress was also determined by MTT assay [10,15]. After sensitization and

irradiation, cells were switched to DME/F12K medium plus 10% FBS and placed in the incubator. At each of three 24 h intervals up to 72 h, the medium was removed and replaced with 1.0 ml of RPMI containing MTT (0.5 mg/ml). After 4 h of incubation, cells were solubilized in 1.0 ml of isopropanol and the viable fraction based on formazan absorbance at 563 nm was determined.

2.8. Post-irradiation cell cycle analysis

For analyzing the effects of photostress on cell cycle distribution, ALA-treated PC-3 cells were irradiated, dark-incubated for various periods up to 36 h, then trypsinized as described in the preceding section and suspended in ~5 ml of 70% ethanol. After standing for 1 h on ice, the cells were centrifuged and resuspended in 1.0 ml of cell cycle assay solution containing 0.38 mM citrate, ribonuclease A (0.5 mg/ml), and PI (10 µg/ml). Final cell concentration was $\sim 5 \times 10^5$ /ml. Other details were as described elsewhere [16]. An Accuri-C6 Flow Cytometer with FlowJo software (Ashland, OR) was used for assessing cell cycle distribution based on PI-labeled DNA.

2.9. Data analysis

The two-tailed Student's *t*-test was used for assessing the significance of perceived differences between experimental values, $p < 0.05$ being considered statistically significant.

3. Results

3.1. Effects of NOS inhibitors on PC-3 cell photokilling

Subconfluent PC-3 cells were routinely sensitized with PpIX by incubating with 1 mM ALA in serum-free medium for 30 min in the dark. Before irradiation was carried out, we examined the subcellular location of PpIX by fluorescence confocal microscopy, using MitoTracker Green to mark mitochondria. As shown in Fig. 1A, the perinuclear zone of PpIX fluorescence completely overlapped that of MitoTracker fluorescence. Thus, under the conditions used, PpIX was localized in mitochondria, where it is known to arise via the heme biosynthetic pathway [13].

In an initial experiment, we asked how photoinactivation of ALA-treated cells would be affected by different NOS inhibitors (L-arginine competitors). As shown in Fig. 1B, cells irradiated in the absence of an inhibitor underwent a light fluence-dependent loss of MTT-assessed Mito dehydrogenase activity (measured 20 h after irradiation), the value for 50% loss (LD_{50}) being ~ 1.5 J/cm². ALA treatment without irradiation was innocuous (results not shown), as was irradiation without ALA treatment (Fig. 1B). Phototoxicity was significantly enhanced by NOS inhibitors, the LD_{50} values for 1 mM L-NAME, 50 µM GW274150, and 20 µM 1400W being ~ 1.3 , ~ 1.0 , and ~ 0.8 J/cm², respectively (Fig. 1B). L-NAME is a low affinity non-selective inhibitor which can act on NOS1 (neuronal) and NOS3 (endothelial) as well as NOS2, whereas GW274150 and 1400W are relatively high affinity, NOS2-selective inhibitors [17]. The observed LD_{50} order for these inhibitors (1400W < GW274150 < L-NAME) is consistent with their known 50% inhibitory concentration (IC_{50}) values for pure NOS2 [17], despite the fact that the concentrations we used varied in the same order. These findings are similar to those previously reported for COH-BR-1 breast tumor cells [9,10], and suggest that exploiting NOS2 for cytoprotection may be a general strategy of cancer cells for coping with photooxidative stress.

3.2. NOS2 and NO upregulation in photostressed PC-3 cells

We subjected ALA/light-treated cells to Western analysis to determine whether NOS2 protein might be upregulated in response to photodynamic stress. As shown in Fig. 1C, PC-3

cells expressed a very low constitutive level of immunodetectable NOS2, which did not change significantly after treating with ALA in the dark for 30 min or up to 20 h. However, there was a substantial elevation in NOS2 after irradiating ALA-treated cells for 10 min (light fluence $\sim 0.7 \text{ J/cm}^2$), beginning ~ 30 min after irradiation and persisting for at least 20 h (Fig. 1C). The densitometrically-determined protein level, normalized to β -actin, was ~ 12 -times greater than that of the dark control 1 h after irradiation and remained there for at least another 19 h. Whether other NOS isoforms (NOS1, NOS3) are expressed in these cells and, if so, whether they might be overexpressed in response to photostress was not determined. However, in a previous study [9], we probed for all three isoforms in ALA/light-treated COH-BR1 cells and found that only NOS2 was upregulated. From the results in Fig. 1C, we predicted that NO produced by stress-induced NOS2 played a key role in photokilling resistance. To examine this, we monitored NO production in photostressed PC-3 cells using the fluorophore DAF-2DA. Upon internalization, DAF-2DA is hydrolyzed to DAF-2, which can be nitrosated by the NO by-product, N_2O_3 , to give DAF-2 triazole, the fluorescent reporter that is detected [18]. As shown in Fig. 1D, control cells exposed to ALA alone exhibited relatively little fluorescence 2 h or 22 h later. However, ALA/light-treated cells gave a strong signal 2 h after irradiation and it was much stronger after an additional 20 h, the integrated fluorescence intensity being ~ 3 -times and ~ 50 -times, respectively, that of the control. The signals at both time points were strongly suppressed by 1400W (Fig. 1D), supporting the idea that increased NO output as a result of robust and prolonged NOS2 upregulation (Fig. 1C) played a crucial role in the observed stress-induced resistance.

3.3. Effects of NOS2 inhibitors and a NO scavenger on apoptotic photokilling

As most of the newly synthesized PpIX in ALA-treated PC-3 cells was in mitochondria (Fig. 1A), we predicted that photokilling would mainly occur via intrinsic apoptosis [19] due to PpIX-sensitized oxidative damage in that compartment. In support of this, we found that caspase-3/7 activity was elevated by ALA/light treatment, the level measured 4 h after irradiation (0.7 J/cm^2) being nearly twice that of a dark control (Fig. 2A). Irradiation in the presence of a NOS2 inhibitor resulted in an additional increase in caspase-3/7 activity, $50 \mu\text{M}$ GW274150 raising it to ~ 3 -times the control level and $20 \mu\text{M}$ 1400W to ~ 4 -times the control level (Fig. 2A). To learn whether NO itself (presumably upregulated in parallel with NOS2 [9,10]) was involved in photokilling resistance, we used cPTIO, a nitronyl nitroxide known to be a highly specific NO scavenger [20]. As shown in Fig. 2A, $10 \mu\text{M}$ cPTIO also caused a large additional increase in photostress-induced caspase-3/7 activation, raising it more than 5-times over the control. At the concentrations used, 1400W, GW274150, and cPTIO by themselves were completely non-toxic to cells in the dark or light [9,10]. Consistent stimulatory effects of the NOS2 inhibitors and cPTIO were observed when apoptotic cell count was assessed using Annexin V-FITC staining with fluorescence microscopy. As shown by the fluorescence micrographs in Fig 2B, non-irradiated ALA-treated cells were essentially negative to Annexin V, but gave strong signals after being exposed to a 0.7 J/cm^2 light fluence and standing in the dark for 4 h. The number of apoptotic cells rose even higher when 1400W was present during and after irradiation (Fig. 2B), consistent this inhibitor's effect on caspase-3/7 activation. Little, if any, PI fluorescence was detected, ruling out any significant cell death via necrosis. Quantification of the Fig. 2B imaging results, along with those obtained when GW274150 or cPTIO was present, is shown in Fig. 3C. As can be seen, the iNOS inhibitors and NO trap produced dramatic increases in apoptotic cell count over that observed with ALA/light alone, and were consistent with the observed increases in caspase-3/7 activation. Taken together, the data in Figs. 1 and 2 clearly indicate that ALA/light-stressed PC-3 cells are capable of rapidly and robustly inducing NOS2 and NO as a cytoprotective tactic.

3.4. Effect of inhibiting soluble guanylyl cyclase on apoptotic photokilling

We asked whether NO-activatable soluble guanylyl cyclase (sGC), known to be expressed in PC-3 cells [21], might play a role in photostress-induced resistance. Cyclic-GMP (cGMP) generated by sGC has a variety of biological effects, including activation of protein kinase G (PKG), which is known to play an anti-apoptotic, pro-survival role in certain cancer cells [22]. A previous PDT-related study [23] showed that apoptotic photokilling of lymphoblastoid cells was suppressed by NO from a chemical donor and that this could be antagonized by inhibitors of sGC and PKG, suggesting involvement of cGMP-dependent PKG in the NO effect. In contrast to this, we found that the sGC inhibitor ODQ, in increasing concentration up to 10 μ M (which was maximally active in the cited study [23]) had no significant effect on caspase-3/7 activation in photostressed PC-3 cells. Actual activation levels at 0, 0.1, 1.0, and 10.0 μ M ODQ were 2.23 ± 0.03 , 2.22 ± 0.11 , 2.17 ± 0.16 , and 2.23 ± 0.18 , respectively, relative to a dark control. Consistently, the cell-permeating cGMP analogue 8-Br-cGMP showed no significant inhibitory effect on photoinduced apoptosis (results not shown). Collectively, these results suggest that the protective effects of stress-induced NO that we observed (Figs. 1 and 2) were not mediated by sGC-generated cGMP.

3.5. Influence of photostress-induced NOS2/NO on MAPK activation status

In previous work, we examined the effects of exogenous NO (from spermine NONOate) on MAPK status in ALA/light-stressed COH-BR1 cells, showing that NO suppressed the phosphorylation-activation of pro-apoptotic JNK and p38 α as well as deactivation of pro-survival ERK1/2 and p38 α (8). We showed recently that endogenous, photostress-induced NOS2/NO in COH-BR1 cells had similar effects on these MAPKs [24]. It was of interest in the present study to determine whether comparable MAPK responses would be seen in PC-3 cells. After ALA treatment and exposure to a 0.7 J/cm² fluence, PC-3 cells were subjected to MAPK Western blot analysis at various times of post-irradiation dark incubation. As shown in Fig. 3, JNK was inactive in a dark control, but became transiently activated after photostress. Phosphorylation of its two major isoform subgroups, JNK1 (55 kDa) and JNK2 (46 kDa), was barely evident immediately after irradiation, but increased rapidly thereafter, maximizing after ~30 min, and then declining until it was almost undetectable after 20 h. When cell sensitization, irradiation, and subsequent dark incubation were carried out in the presence of 1400W, there was an intensification and prolongation of JNK phosphorylation, which could now still be detected after as long as 20 h (Fig. 3). A similar response to photostress was observed for p38, i.e. a transient activation that peaked ~30 min after irradiation and then declined (Fig. 3); however, the activation was more prolonged when 1400W was present throughout. The phosphorylation pattern of the ERK1/2 isoforms (46 kDa ERK1 and 44 kDa ERK2) was strikingly different from that of JNK and p38. As shown in Fig. 3, ERK1/2 was strongly expressed in PC-3 cells, but little (if any) phosphorylated ERK1/2 was observed in a dark control or during dark incubation for as long as 2 h after ALA/light treatment. However, both phosphorylated isoforms were detected after 20 h, p-ERK2 appearing to be more abundant than p-ERK1. In striking contrast to JNK and p38 phosphorylation, delayed ERK1/2 phosphorylation was abolished by 1400W (Fig. 3). These findings support the notion that stress-upregulated NOS2/NO functioned cytoprotectively by inhibiting the activation of pro-apoptotic JNK and p38 while subsequently stimulating the activation of pro-survival/pro-growth ERK1/2.

3.6. NOS2/NO-dependent proliferation enhancement in cells surviving a photostress challenge

ERK1/2 activation is typically associated with cell survival and proliferation [25]. The observation that delayed ERK1/2 activation by photostress could be prevented by 1400W (Fig. 3) prompted us to look more directly at whether iNOS-derived NO could, in fact,

stimulate cell growth after photostress. Two different approaches were used: (i) assessing the titer of live (trypan blue-excluding) cells at various post-irradiation times, and (ii) measuring the viable (MTT-active) cell fraction at various post-irradiation times. As shown in Fig. 4A, the live cell count in a dark control (ALA alone) increased by ~30% from 24 h to 48 h, whereas in ALA/light-stressed cells, it increased by more than 90%. When 1400W was present during and after photostress, the live cell count was much lower at 24 h (as expected), but it now increased by only ~25% over the next 24 h (Fig. 4A).

We obtained similar results when the viable fraction was tracked over a 3-day post-irradiation period. For example, after ALA/light treatment, the fraction increased by ~170% from 24 h to 72 h, whereas in an ALA-only dark control, it increased by only ~30% over the same interval. With 1400W present in the ALA/light system, the viable fraction started much lower at 24 h and increased ~90% by 72 h, i.e. significantly less than when 1400W was absent (Fig. 4B). Two light-only controls, one with 1400W present, showed essentially the same growth rates from 24 h to 72 h as the dark control. Collectively, the data in Fig. 5 demonstrate that photostress-induced NOS2/NO not only protected PC-3 cells against apoptosis, but stimulated surviving cells to grow back more rapidly. It is important to point out that 1400W did not reduce the growth rate of non-stressed cells (Fig. 4B), suggesting that their low constitutive NOS2 level (Fig. 1C) had little effect on proliferation. However, this situation changed dramatically after robust upregulation of the enzyme in response to photostress.

Additional evidence for a pro-growth response was sought by examining cell cycle distribution at various times after photodynamic challenge. Fig. 5A shows phase distribution histograms at 36 h after ALA, ALA/light, and ALA/1400W/light treatment, while Supplementary Table 1 shows actual percentages of different phases at 0, 24, and 36 h after treatment. Note that the most dramatic shifts in phase distribution as a function of post-irradiation time occurred in the ALA/light system, which revealed a progressive increase in S-phase occupancy from 24 h to 36 h (Fig. 5B), decrease in G₀/G₁ occupancy, and slight increase in Sub-G₀/G₁ and G₂/M. Especially noteworthy was the fact that the large increase in S-phase from 24 h to 36 h was abolished by 1400W, which also caused a significant increase in Sub-G₀/G₁ occupancy. In specific terms, ALA/light caused an S-phase increase of ~70% after 24 h and ~140% after 36 h relative to the level with ALA-only or ALA/light measured immediately after irradiation (Fig. 5B). Inhibitor 1400W not only negated these effects, but actually caused a slight decrease in S-phase level (~25% after 36 h). Because S-phase represents cells that have effectively doubled their DNA content in preparation for division, these findings help to explain why photostress-surviving cells exhibited a strong NO-dependent growth spurt.

4. Discussion

That NO plays an important pro-survival/pro-growth role in various tumors has been known for many years [4]. This role is often linked to constitutive expression of NOS2 because inhibition or knockdown of this enzyme typically has a negative impact on tumor cell survival and proliferation *in vitro* [26,27] as well as on angiogenesis and tumor progression *in vivo* [28,29]. It is also clear that endogenous NOS2/NO, acting cytoprotectively, can antagonize the antitumor effects of ionizing radiation and chemotherapeutic agents. For example, the NOS2 inhibitor L-nil was recently shown to act synergistically with cisplatin in reducing the proliferation rate of a human melanoma cell line both *in vitro* and *in vivo* [30]. Another recent study revealed that the sensitivity of hypoxic non-small cell lung cancer cells to radiation-induced apoptosis was strongly enhanced by the NOS inhibitor L-NMMA, an effect attributed primarily to NOS2 inhibition [31].

How NO produced by tumor cells themselves or surrounding vascular cells might influence PDT effectiveness has received surprisingly little attention. An early study using isolated rat aorta as a model suggested that NO generation in tumor vasculatures is impaired by PDT, resulting in tumor suppression due to vasoconstriction [32]. Later work revealed that NOS1 in epidermoid cancer cells was rapidly upregulated by photodynamic stress [33]. The resulting NO was assumed to play a toxic, pro-apoptotic role, but the more likely possibility of it being involved in pro-survival signaling was not considered. Two subsequent studies have provided strong support for the latter idea by showing that the Photofrin-PDT cure rate for various mouse-borne tumors was significantly increased by NOS inhibitors, especially tumors with relatively high constitutive NO output [34,35]. Similar results were obtained in more recent studies using an ALA-PDT mouse model [36]. The observed NOS inhibitor effects [34–36] were attributed mainly to NO's well known vasodilatory effects acting in opposition to PDT's tumor-suppressing vasoconstrictive effects. Little else was done in an effort to better understand NO-dependent PDT resistance, particularly at the cellular and molecular level. In addressing this issue, we recently showed that breast COH-BR1 cells could mount an endogenous resistance to mitochondria-targeted ALA/light-stress by rapidly upregulating NOS2 and NO [9,10,24].

A similar response was observed for prostate PC-3 cells in the present study. We saw a striking 10–12-fold induction of NOS2 protein within 1 h after exposing ALA-primed PC-3 cells to a 0.7 J/cm² light fluence. This persisted for at least another 20 h and was accompanied by a dramatic increase in NO output. The background NOS2 level in these cells was much lower than that observed in COH-BR1 cells [10,24]. However, the extent of NOS2 upregulation (even with a smaller light fluence) was much greater, suggesting that PC-3 cells are more sensitive to this type of photostress response. NOS2 mRNA level was not determined, but it was probably also elevated by photostress, considering the nearly 2-fold elevation of NOS2 message observed in COH-BR1 cells [9]. The functional significance of NOS2 induction in ALA/light-treated PC-3 cells was demonstrated by showing that cell sensitivity to caspase-3/7 activation and apoptotic death was strongly enhanced by NOS inhibitors (L-NAME, 1400W, GW274150) and also by a NO trap (cPTIO), consistent with stress-induced NOS2/NO playing a major role in cellular resistance to photokilling. The apoptosis-promoting effects of GW274150 were consistently smaller than those of 1400W, even at higher concentrations of the former. This was not surprising, however, because the IC₅₀ value of GW274150 for isolated pure NOS2 is ~6-times greater than that of 1400W [17].

We have examined the activation status of some key pro-apoptotic vs. pro-survival signaling MAPKs in photostressed PC-3 cells and how this is affected by NOS2-derived NO. Continuous inhibition of NOS2 with 1400W resulted in a remarkable increase in extent and duration of JNK and p38 activation, whereas the exceedingly slow activation of ERK1/2 was abolished. These findings suggest that stress-upregulated NO acted cytoprotectively by inhibiting any sustained pro-apoptotic activation of JNK and p38 while stimulating pro-survival activation of ERK1/2. Similar effects of NO on these MAPKs have been reported for other oxidative stress systems [37]. Whether sGC/cGMP signaling played a role in cytoprotection was also examined. Activation of sGC by NO results in formation of cGMP, a known activator of PKG, which has been reported to promote survival and/or inhibit apoptosis of tumor cells [23]. In contrast to results with another cell line [23], we found that ODC over the concentration range 0.1 to 10 μM, which avoids non-specific effects [21], had no effect on ALA/light-induced caspase-3/7 activation. Thus, any cGMP involvement in apoptotic resistance was ruled out in our system. Similar non-effects of ODC have been observed for COH-BR1 cell photokilling [24], implying that both cell types rely on some other NO-dependent pro-survival signaling pathway(s). Among the possibilities worth investigating are: (i) inhibition of caspase-9 and/or caspase-3 activation via NO-mediated S-

nitrosation [37], (ii) S-nitrosation inhibition of JNK and/or its upstream activator, ASK1 [38,39], and (iii) S-nitrosation-activation of MAPK phosphatase-1 [40].

In addition to mounting a NO-dependent resistance to photokilling like COH-BR1 cells [9,10,24], PC-3 cells exhibited a remarkable growth spurt in their surviving fraction, and NO also played a role in this. Although post-PDT stimulation of residual cell proliferation can be inferred from studies by others, where increased VEGF expression and angiogenesis was observed [41], our findings are the first to implicate NOS2/NO in this response. Using two complementary approaches, one tracking number of Trypan blue-excluding live cells and the other, MTT-assessed metabolically active cells, we showed that remaining viable PC-3 cells after a photostress grew back much faster than controls, and in 1400W-inhibitable fashion. Cell cycle analyses provided strong confirmatory evidence for this accelerated proliferation. One of the most striking observations was the increasingly greater occupancy of S-phase over a 36 h post-irradiation period and the prevention of this by 1400W, again indicating that stress-induced NOS2/NO played a key role. Similar results have been reported by others, using various other cell lines [42,43]. However, in those studies NO was introduced exogenously, either in gaseous form [42] or indirectly as nitrite [43]. Therefore, in the context of NO-stimulated cell growth, our findings are unique because they demonstrate the involvement of endogenous stress-induced NOS2/NO. We are in the process of probing more deeply into the mechanism of NO-enhanced PC-3 proliferation by examining the post-photostress status of cell cycle proteins such as cyclin-dependent kinases, kinase inhibitors, and retinoblastoma protein [42,43].

In summary, we have shown for the first time that prostate PC-3 cancer cells respond promptly to PDT-like stress by upregulating NOS2/NO, which enhances apoptotic resistance and stimulates proliferation. Yet to be investigated is whether stressed PC-3 cells use additional cytoprotective strategies, e.g. induction of cyclooxygenase-2 [44], and if so, their importance relative to NOS2/NO. Our finding that photostress-surviving cells used NO to proliferate more rapidly than controls is entirely novel in the context of PDT. If occurring in cancer patients, this response, along with hyperresistance to apoptosis, could seriously undermine PDT effectiveness. One possible answer is rational pharmacologic intervention with a NOS2 inhibitor [45]. Tested for the first time in this study involving a prostate cancer-PDT model, GW274150 would be an excellent candidate, having already been tested in humans as an anti-inflammatory agent [14].

Supplementary Material

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Figure 1. PC-3 cell sensitization, photodynamic challenge, and induction of NOS2/NO

(A) Subcellular localization of PpIX. PC-3 cells grown on coverslips to ~60% confluency were switched to serum-free RPMI medium and incubated with 1 mM ALA for 30 min in the dark. The cells were then switched to ALA-free medium, treated with 100 nM MitoTracker Green for 10 min, and examined by confocal fluorescence microscopy, using 488 nm excitation with 620–650 nm emission for PpIX and 490 nm excitation with 516 nm emission for MitoTracker Green. Scale bar: 25 μm . (B) Phototoxicity. Cells were treated with ALA in the absence or presence of L-NAME (1 mM), GW274150 (50 μM), or 1400W (10 μM), each introduced 15 min before ALA. After exposure to increasing light fluences (J/cm^2), cells were switched to 1% serum-containing RPMI medium with or without L-NAME, GW274150, or 1400W at the indicated concentration. Extent of phototoxicity was assessed 20 h later by MTT assay. A light-only control (no ALA) was analyzed alongside. Plotted data are relative to non-treated controls (no ALA, light, or inhibitor); means $\pm$ SD of values from three separate experiments are shown. (+) light only; (x) ALA/light; (O) ALA/L-NAME/light; () ALA/GW274150/light; (∇) ALA/1400W/light. (C) NOS2 upregulation. ALA-treated cells were either kept in the dark for 20 h (ALA) or exposed to a $0.7 \text{ J}/\text{cm}^2$ light fluence (ALA/hv). After 0 h to 20 h of post-irradiation dark incubation, lysates were prepared and analyzed for NOS2 by Western blotting, using β -actin as a loading standard (total protein: 125 μg per lane). Band intensities normalized to β -actin and relative to the dark control (ALA) are indicated below the various time points. Data are from one experiment representative of two with identical results. (D) NO generation. ALA-treated cells were either not irradiated (ALA) or irradiated in the absence (ALA/hv) or presence of 20 μM 1400W (ALA/W/hv). After 2 h and 20 h in the dark, cells were treated with 20 μM DAF-2DA for 30 min, and then examined by fluorescence microscopy. Number below each

image panel represents integrated fluorescence intensity; means $\pm$ SD of values from three different viewing fields are shown. Bar: 200 μ m.

Figure 2. Effects of NOS2 inhibitors and a NO scavenger on extent of caspase-3/7 activation and apoptosis of photostressed PC-3 cells

ALA-treated cells were either not irradiated (ALA) or irradiated for 10 min (0.7 J/cm^2) in the absence (ALA/hv) or presence of $20 \mu\text{M}$ 1400W (ALA/W/hv), $50 \mu\text{M}$ GW274150 (ALA/GW/hv), or $10 \mu\text{M}$ cPTIO (ALA/cP/hv). (A) Caspase-3/7 activity relative to the dark control (ALA) measured 4 h after irradiation; mean values $\pm$ SD ($n = 3$). (B) Representative fluorescence micrographs of Annexin V-FITC-stained cells 8 h after irradiation; a dark control (ALA-only) is also represented. Scale bar: $200 \mu\text{m}$. (C) Annexin V-positive apoptotic cells as a percentage of total cells for the various reaction conditions; mean values $\pm$ SD ($n = 3$).

Figure 3. Photostress activation of MAPKs in the absence *versus* presence of a NOS2 inhibitor ALA-treated PC-3 cells were irradiated (0.7 J/cm^2) in the absence (ALA/hv) or presence (ALA/W/hv) of $10 \mu\text{M}$ 1400W. Immediately thereafter (0 min) or after increasing periods of dark incubation from 15 min to 20 h, cells were recovered, lysed, and homogenized. After protein determination, samples were subjected to Western blot analysis, using antibodies against p-JNK, p-p38, p-ERK1/2, and overall JNK, p38, and ERK1/2. Dark controls (DC), i.e. ALA-treated cells kept in the dark for 20 h are also represented. Total protein per lane: $125 \mu\text{g}$. Number below each lane for the phosphorylated MAPKs represents band intensity normalized to total MAPK and relative to DC. Blots are from one experiment representative of three for each condition.

Figure 4. Effect of photostress-induced NOS2/NO on PC-3 cell proliferation

Cells at ~45% confluency in 35-mm dishes were dark-incubated with 1 mM ALA for 30 min in the absence or presence of 1400W (10 μ M) and then irradiated (0.7 J/cm²). Cells not irradiated after ALA treatment or irradiated after 1400W treatment were used as controls. (A) After the indicated intervals of post-irradiation dark incubation, live cell counts based on trypan blue dye exclusion were determined. (B) Cells were recovered after the indicated times of post-irradiation incubation and the viable fractions were determined by MTT assay. Annotations are as follows: ALA, dark control; ALA/hv, irradiation after ALA treatment; ALA/W/hv, irradiation after ALA and 1400W treatment; hv, irradiation without ALA or 1400W treatment; W/hv, irradiation after 1400W treatment. Means $\pm$ SD of values from

three separate experiments are plotted in (A) and (B). * $P < 0.005$ (A) or < 0.001 (B) compared with ALA/hv at the same given post-irradiation time.

Figure 5. Effect of photostress-induced NOS2/NO on cell cycle progression

(A) Cell cycle phase distribution. Histograms show occupancy of the sub-G₀/G₁, G₁, S, and G₂/M phases 36 h after exposing cells preincubated with 1 mM ALA or 1 mM ALA plus 10 μ M 1400W to a 0.7 J/cm² light fluence. An ALA-only dark control is also represented. (B) Changes in S-phase occupancy at various times after treating cells as described in (A). Means $\pm$ SD of values from three independent experiments are plotted. *P<0.001 compared with ALA/hv at the same post-irradiation time.