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Cytoprotective Induction of Nitric Oxide Synthase in a Cellular Model of 5-Aminolevulinic Acid-Based Photodynamic Therapy

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Abstract

Photodynamic therapy (PDT) employs a photosensitizing agent, molecular oxygen, and visible light to generate reactive species that kill tumor and tumor vasculature cells. Nitric oxide produced by these cells could be pro-carcinogenic by inhibiting apoptosis or promoting angiogenesis and tumor growth. The purpose of this study was to determine whether tumor cells upregulate NO as a cytoprotective measure during PDT. Breast tumor COH-BR1 cells sensitized in mitochondria with 5-aminolevulinic acid (ALA)-derived protoporphyrin IX died apoptotically following irradiation, ALA- and light-only controls showing no effect. Western analysis revealed that inducible nitric oxide synthase (iNOS) was upregulated >3-fold within 4 h after ALA/light treatment, while other NOS isoforms were unaffected. Exposing cells to a NOS inhibitor (L-NAME or 1400W) during photochallenge enhanced caspase-3/7 activation and apoptotic killing up to 2–3-fold while substantially reducing chemiluminescence-assessed NO production, suggesting that this NO was cytoprotective. Consistently, the NO scavenger cPTIO enhanced ALA/light-induced caspase-3/7 activation and apoptotic kill by >2.5-fold. Of added significance, cells could be rescued from 1400W-exacerbated apoptosis by an exogenous NO donor, spermine-NONOate. This is the first reported evidence for increased tumor cell resistance due to iNOS upregulation in a PDT model. Our findings indicate that stress-elicited NO in PDT-treated tumors could compromise therapeutic efficacy, and suggest NOS-based pharmacologic interventions for preventing this.

Keywords

Nitric oxide; iNOS; 5-aminolevulinic acid; photodynamic therapy; apoptosis; 1400W; cPTIO

Introduction

Photodynamic therapy (PDT) is a relatively new antitumor treatment based on site-specific photogeneration of singlet oxygen ($^1\text{O}_2$) and other cytotoxic oxidants [1,2]. Like all photodynamic reactions, PDT requires (i) systemic or topical application of a tumor-localizing sensitizing agent or metabolic precursor thereof, (ii) sensitizer-exciting light, ideally in the long visible wavelength range, and (iii) molecular oxygen. Irradiation (e.g. from a laser source) is restricted to the tumor area for added selectivity and the sensitizer is usually innocuous unless photoactivated. 5-Aminolevulinic acid (ALA)-based PDT is a variant of classical PDT in which administered ALA itself or an ALA ester enters cancer

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cells and is metabolized to the active sensitizer, protoporphyrin IX (PpIX), via the heme synthetic pathway [3,4]. ALA-PDT is particularly effective for cutaneous carcinomas, in which case the pro-sensitizer can be conveniently applied topically, i.e. directly at the tumor site [4,5]. In tumor cells exposed to ALA, overproduced PpIX originates in mitochondria, but can diffuse to peripheral sites, including plasma membrane, after ALA is removed [4]. Using a breast tumor cell line (COH-BR1), we demonstrated previously that subcellular localization of PpIX has a strong impact on the mechanism of photokilling [6]. Thus, cells died via the intrinsic apoptotic pathway when PpIX was restricted to mitochondria, but via necrosis when it was allowed to disseminate. Our recent investigation of mitogen-activated protein kinase (MAPK) signaling in ALA/light-induced apoptosis [7] revealed that certain MAPKs (JNK and p38 α) were activated by photostress, whereas others (ERK1/2 and p38 β) were deactivated, in agreement with reported responses of these enzymes to other types of oxidative stress [8,9].

The multifaceted free radical, nitric oxide (NO), is known to have both cytotoxic and cytoprotective properties, depending on the biological conditions under which it is generated by nitric oxide synthase (NOS) enzymes. In the context of cancer, NO produced by tumor and tumor vasculature cells in relatively low fluxes could be pro-carcinogenic by inhibiting apoptosis on the one hand and promoting angiogenesis and tumor growth on the other [10,11]. We reported previously that NO from a chemical donor or activated macrophages made COH-BR1 cells more resistant to ALA/light-induced necrosis, an effect attributed to interception of plasma membrane lipid-derived radicals [12]. More recent work revealed that exogenous NO could inhibit ALA/light-induced apoptosis of these cells by antagonizing pro-apoptotic JNK and p38 α activation as well as anti-apoptotic ERK1/2 and p38 β deactivation [7].

We now report that ALA/light treatment of COH-BR1 cells and one other breast tumor line causes a rapid and prolonged upregulation of inducible NOS (iNOS) with accompanying overproduction of NO. That this NO was anti-apoptotic was demonstrated by the strong death-promoting effect of the iNOS inhibitor 1400W or the NO interceptor cPTIO introduced before irradiation. These findings indicate that photostress-elevated endogenous NO can act cytoprotectively on its own, i.e. in the absence of exogenous NO sources. This is the first reported evidence for cytoprotective iNOS and NO induction in a PDT stress model. On the basis of these findings, we propose that stress-elicited NO *in vivo* in tumors subjected to PDT can compromise therapeutic outcome, a prospect that has not been well recognized up to now.

Materials and Methods

General materials

5-Aminolevulinic acid (ALA), Hoechst 33258 (Ho), propidium iodide (PI), DME/F12 growth medium, fetal bovine serum, and antibiotics were from Sigma-Aldrich (St. Louis, MO). N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was from Calbiochem (Gibbstown, NJ). L-N^G-nitroarginine methyl ester (L-NAME), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), spermine NONOate (SPNO), and N-[3-(aminomethyl)benzyl]acetamide (1400W) were obtained from Cayman Chemicals (Ann Arbor, MI). Santa Cruz Biotechnology (Santa Cruz, CA) supplied the polyclonal antibodies against human iNOS and eNOS, and the monoclonal antibodies against human nNOS and β -actin.

Cell culture conditions

COH-BR1 cells, an epithelial subline derived from a human breast tumor [13], were originally obtained from Dr. James Doroshow, City of Hope Cancer Center (Duarte, CA). The cells were grown under standard incubation conditions, using phenol red-free DME/F12 medium (pH 7.4) supplemented with 10% fetal bovine serum and antibiotics [7]. MDA-MB-231 cells, a human breast adenocarcinoma line obtained from the American Type Culture Collection (Manassas, VA), were cultured under similar conditions. Proliferating cells received fresh medium every third day and were passaged fewer than ten times for all experiments.

Cell sensitization, irradiation, and death evaluation

Cells at ~60% confluency in 35-mm culture dishes were metabolically sensitized by incubating with 1.0 mM ALA in serum-free DME/F12 medium for 45 min in the dark, resulting in an abnormally high level of PpIX localized mainly in mitochondria [6,7]. Where indicated, L-NAME, 1400W, or cPTIO was added to cells in medium 15 min before ALA. Immediately after sensitization, cells were switched to fresh medium without ALA, which either lacked or contained L-NAME or 1400W, 1400W plus SPNO, or cPTIO at the indicated concentrations, and irradiated at room temperature on a translucent plastic platform, using broad-band visible light at a fluence rate of ~1.1 mW/cm². A 15-min exposure period corresponded to a delivered light fluence of ~1 J/cm². After irradiation, medium was removed, replaced with 1% serum-containing DME/F12 (without or with L-NAME, 1400W, or cPTIO, as required) and cells were returned to the incubator for various periods, after which the following were assessed: (i) apoptosis vs. necrosis using the nuclear fluorophores Ho and PI [7,12]; (ii) caspase-3/7 activity; (iii) immunodetectable NOS levels, and (iv) NO-derived nitrite levels.

Determination of caspase activity

Activation of caspase-3/7 in photodynamically stressed cells was monitored using the fluorogenic substrate Ac-DEVD-AMC. At the indicated post-irradiation time(s), cells were scraped from dishes into ice-cold PBS, pelleted, and resuspended in 50 mM HEPES/5 mM CHAPS/10 mM DTT/4 mM EDTA (pH 7.4) lysis buffer. Following protein determination, each lysate was incubated with 25 μ M Ac-DEVD-AMC for 30 min at 25 °C in the dark. Fluorescence of liberated AMC was measured with a PTI QM-7SE spectrofluorometer (London, Ontario, Canada), using 360 nm excitation and 460 nm emission.

Immunoblot analyses

For determination of NOS levels by Western blotting, lysates of ALA/light-treated cells and dark controls were prepared as described and analyzed for total protein [7]. Samples of equal protein content (typically 150 μ g) were subjected to Laemmli SDS-PAGE using 10 % acrylamide/bisacrylamide. Separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane, followed by incubation with primary antibodies against iNOS, nNOS, or eNOS according to supplier directions, and then with peroxidase-conjugated secondary antibodies. Protein anlayses, including β -actin as a loading standard, were visualized using a Supersignal West Pico chemiluminescence detection kit (Thermo Scientific, Rockford, IL). Other details were as described [7].

Nitrite determination

The NO₂⁻ level in cells and cell media at various post-irradiation times was determined by ozone-based chemiluminescence assay, using a Sievers NOA-280 analyzer (Sievers Instruments, Boulder, CO). Medium was collected from each culture well and cells were recovered by trypsinization and centrifugation, followed by lysis and protein determination.

Samples were added to a glacial acetic acid solution of 0.3 M KI in the analyzer reaction chamber to convert NO_2^- back to NO, which was sent in an argon stream through 1 N NaOH into the chemiluminescence reaction cell. NO_2^- content of an experimental sample was calculated from the integrated area of the readout peak, corrected for the background signal of a sample without cells, and based on a NO_2^- standard curve.

Results

Twenty hours after being exposed to a light fluence of $\sim 1 \text{ J/cm}^2$, a significant number of ALA-treated COH-BR1 cells with mitochondria-localized PpIX [7] were apoptotic, as revealed by bright, condensed Ho fluorescence (Fig. 1A), but no significant PI fluorescence, as reported previously (7). Light alone or ALA treatment alone had no effect on viability (not shown). When sensitized cells were irradiated in the presence of the NOS activity inhibitor L-NAME (1 mM) or 1400W (10 μM), a striking increase in apoptotic cell count was observed after 20 h (Fig. 1A). This suggests a rapidly mounted, stress-induced cytoprotective effect of NOS-generated NO, particularly iNOS NO, based on 1400W's high specificity for iNOS (13). Percent apoptotic cells as a function of L-NAME or 1400W concentration is shown in Fig. 1B. Without inhibitor, 20–25% and 45–50% of the cells were apoptotic at delivered light fluences of 1 J/cm^2 and 2 J/cm^2 , respectively. At 1 J/cm^2 , the count rose relatively rapidly with increasing [L-NAME], reaching $\sim 50\%$ at 4 mM inhibitor, whereas at 2 J/cm^2 the increase was more gradual, reaching $\sim 70\%$. 1400W produced more dramatic responses at both fluence levels and over a much lower concentration range than L-NAME (Fig. 1B). In this case, there was a much sharper rise in apoptotic count over a low range, i.e. up to $1 \mu\text{M}$, and a more gradual increase thereafter such that at $10 \mu\text{M}$ 1400W, nearly 70% of the cells were apoptotic at 1 J/cm^2 and 85% at 2 J/cm^2 (Fig. 1B). These findings are consistent with iNOS being a key stress responder in this system. As shown in Fig. 1C, caspase-3/7 activity of COH-BR1 cells measured 8 h after photochallenge (when apoptosis was not yet detectable) was 4-fold greater than that of a light-only or ALA-only control, consistent with caspase-3/7 involvement in apoptotic progression. The process presumably started in mitochondria (intrinsic pathway [15]), since photostress originated there (7). When included in the culture medium before and after irradiation, L-NAME (1 mM) or 1400W (10 μM) caused an additional increase in caspase-3/7 activity, the former bringing it to 5-times that of the control, and the latter to 7-times (Fig. 1C). No significant increase above background was observed in L-NAME- or 1400W-containing light controls, ruling out any possible phototoxicity of these compounds. These results further support our deduction that apoptosis was suppressed by iNOS-generated NO.

Apoptotic photokilling of another breast tumor line, MDA-MB-231 cells, was also enhanced by L-NAME in dose-dependent fashion, the level reached at 4 mM L-NAME being $\sim 50\%$ higher than that of the control (Fig. 2). Thus, the observed effects were not limited to one cell type.

We asked whether NOS inhibitors would also stimulate apoptosis if a different type of oxidative challenge were used, *viz.* exposure to H_2O_2 . After a 4-h incubation in the presence of 0.5 mM H_2O_2 , followed by 16 h in its absence, $\sim 30\%$ of the cells in a COH-BR1 population were apoptotic (Fig. 3A,B). L-NAME (Fig. 3A) and 1400W (Fig. 3B) in increasing concentrations up to 4 mM and 10 μM , respectively, increased the apoptotic count by $<10\%$. Thus, for H_2O_2 producing approximately the same level of basal apoptosis, any NOS-associated cytoprotection was negligible compared with that induced by ALA/light (Fig. 1B), suggesting that the latter stress response is highly specific. However, the reason for this is not clear at this point.

Protein expression of different NOS isoforms in ALA/light-treated COH-BR1 cells was analyzed by Western blotting. nNOS could not be detected, either in a dark control or in samples analyzed immediately after and up to 20 h after photostress using a light fluence of 2 J/cm² (Fig. 4). eNOS could be detected, but there was no significant change in its level after photostress. In contrast, a relatively high initial content of iNOS was observed and this was rapidly and persistently elevated by photostress, the levels at 2 h and 20 h post-irradiation being 1.9-fold and 3.2-fold greater than that of the dark control, respectively (Fig. 4). When a 4 J/cm² fluence was used, eNOS and nNOS still showed no response, whereas iNOS was upregulated more rapidly (Fig. S1), its level at 15 min post-irradiation being ~6-times the control and remaining approximately the same for up to 20 h.

We used chemiluminescence-based NO analysis to determine whether iNOS overexpression in photostressed cells resulted in an elevated NO steady state level. As shown in Fig. 5, the medium of ALA/light (4 J/cm²)-treated COH-BR1 cells at 4 h post-irradiation contained nearly 3-times as much nitrite as an ALA-only control. At 20 h post-irradiation, the nitrite level was nearly 8-times higher. A light-only control showed no significant nitrite elevation after 20 h (Fig. 5), confirming that the changes observed in the ALA/light system were dependent on PpIX sensitization. Post-irradiation nitrite levels in the cellular compartment were also determined and showed the same general trends as in the medium, although cellular level trended lower, e.g. it was 60% of the medium level at 20 h (Fig. 5). Thus, the extracellular compartment was favored at equilibrium, but whether this reflected only NO redistribution or some combination of NO and NO₂⁻ redistribution is not known. Importantly, 1400W at a concentration that markedly increased apoptotic photokilling of ALA-treated cells (Fig. 1B) decreased the measured NO₂⁻ in both cells and medium, the levels at 20 h post-irradiation being <50% of those without 1400W (Fig. 5).

To establish whether NO was in fact involved in the cytoprotective effects described, we used the nitronyl nitroxide cPTIO, known to be a highly specific NO scavenger [16]. When present during and after ALA/light treatment, cPTIO produced a substantial concentration-dependent elevation in caspase-3/7 activity measured 8 h after irradiation, 10 μM cPTIO increasing it to ~3.5-times the basal value, and 100 μM to >4-times (Fig. 6A). Likewise, apoptotic cell count 20 h after irradiation was dramatically elevated by cPTIO, 1 μM increasing it by 1.7-fold and 100 μM by 2.8-fold (Fig. 6B). Clearly, therefore, stress-induced NO played a prominent role in cellular resistance to photokilling.

We determined whether a source of exogenous NO could in effect “rescue” ALA/light-stressed cells from 1400W-stimulated apoptosis. As shown in Fig. 7A, 10 μM 1400W increased the level of caspase-3/7 activity ~6-fold over background (ALA/light alone) and this was nullified by inclusion of 0.1 mM SPNO, a diazeniumdiolate NO donor [17], at the start of irradiation. In fact, SPNO reduced activity to below background, evidently because its effective steady state concentration was higher than that of endogenously generated NO. Fully decomposed SPNO had no effect (not shown), confirming that NO was the active agent. Active SPNO acted similarly in reversing 1400W-enhanced apoptosis and even reducing it to below the background level (Fig. 7B).

Discussion

A site-specific cell sensitization procedure was used in this study whereby PpIX overproduced by high ALA pressure resides mainly in mitochondria, where it originates (3,4). We determined previously (6) that photoactivation of this PpIX, with accompanying generation of ¹O₂ and possibly other oxidants, results in mitochondrial damage that can trigger an intrinsic apoptotic cascade [15]. The unique mitochondrial inner membrane phospholipid, cardiolipin, was found to be a prominent target of this photodamage, its

peroxidation resulting in release of associated cytochrome c [18], known to be required for activation of the apoptosome [15]. Other studies with COH-BR1 cells revealed a switch from apoptotic to necrotic photokilling when PpIX was allowed to delocalize from mitochondria [6,12]. When cells in this condition were irradiated in the presence of low NO fluxes, e.g. from SPNO or activated macrophages [12,19], both necrosis and peroxidation of plasma membrane lipids were strongly inhibited, consistent with involvement of free radical peroxidative damage in necrosis and NO's known ability to intercept lipid-derived radicals [20]. More recent work revealed that SPNO-derived NO could also protect against ALA/light-provoked intrinsic apoptosis by inhibiting JNK- and p38 α -mediated redox signaling [7]. Whether NO scavenging of lipid-derived radicals played a role in this case was not investigated.

Not addressed previously [7] was the possibility that ALA/light-stressed cells might mount an endogenous NO-dependent cytoprotective response by activating/upregulating one or more NOS isoforms. The first known evidence for this, not only in the context of ALA-PDT but PDT in general, is provided in this report. A key initial observation was that the apoptotic fraction of ALA/light-exposed COH-BR1 cells increased substantially when irradiation was carried out in the presence L-NAME or 1400W, suggesting involvement of iNOS-generated NO. Interestingly, no such effect was observed when similarly lethal oxidative pressure in the form of H₂O₂ was used. In accordance with the 1400W results, we saw a large and sustained upregulation of immunodetectable iNOS, but not eNOS or nNOS, in photodynamically stressed COH-BR1 cells. Moreover, 1400W-inhibitable accumulation of NO₂⁻ was observed in both the cells and cell medium, consistent with iNOS-enhanced production of readily diffusible NO. We confirmed that stress-induced NO was the cytoprotective agent by showing (i) that 1400W-enhanced cell kill could be reversed by SPNO and (ii) that the kill could be enhanced by the NO scavenger cPTIO. cPTIO by itself was non-toxic to cells in the dark or light. However, its light absorption in the visible range (λ_{max} ~560 nm) might have partially competed with PpIX absorption, resulting in a lower kill stimulation than might have been seen otherwise. Upon reacting with NO, cPTIO generates an imino nitroxide and potentially cytotoxic $\cdot\text{NO}_2$. However, $\cdot\text{NO}_2$ is rapidly scavenged by excess cPTIO to give NO₂⁻ and cPTIO⁺ [21], thus reducing the risk of $\cdot\text{NO}_2$ toxicity. Since cell permeability of cPTIO is limited [22], most of its reduction of intracellular [NO] may have occurred indirectly, i.e. by shifting the diffusion equilibrium of NO in favor of the extracellular compartment.

Inducible NOS effects similar to those we describe have been reported for various other types of oxidative stress. For example, ultraviolet B-induced apoptosis of human keratinocytes was found to be enhanced by L-NAME, and addition of the NO donor SNAP after irradiation abolished this [23]. A soluble guanylate cyclase inhibitor also enhanced apoptosis, suggesting that the UVB-induced cytoprotective effect of NO was somehow mediated by cGMP. iNOS or eNOS knockout mice exhibited greater UVB-provoked skin cell apoptosis than wild type, implicating both enzymes in NO-mediated cytoprotection. A more recent study with lung carcinoma cells showed that iNOS-generated NO played a key role in induced resistance to ionizing radiation lethality, an effect associated with suppression of p53 accumulation and activation [24]. Several examples of "pre-conditioned" cytoprotection by endogenous or exogenous NO have also been reported [25], including our finding that exposure to SPNO made COH-BR1 cells more resistant to ALA/light killing 20 h later, i.e. when NO was no longer in the system [19].

The important question of how NO produced by tumor cells per se or surrounding vascular cells might influence tumor responsiveness to PDT has been addressed in only a few studies to date. An early report [26] suggested that hindered NO production by endothelium during PDT plays an important role in the antitumor vasoconstrictive effects of this approach;

however, no mechanistic evidence was provided. A later study [27] linked a rapid, transient upregulation of nNOS and NO in photodynamically stressed epidermoid cancer cells *in vitro* to apoptotic cell death, presumably via formation of peroxynitrite and/or other toxic derivatives. This was the first description of NOS induction in a PDT model, but the more plausible possibility of it being a cytoprotective response was not considered. Subsequent *in vivo* investigations have supported this concept. One study showed that PDT eradication of mouse fibrosarcoma tumors was enhanced by the NOS inhibitor L-NNA [28]. Another showed that PDT cure of various mouse tumors was dramatically improved by L-NNA or L-NAME, tumors exhibiting relatively high constitutive NO production responding best [29]. The likely explanation for these findings [28,29] is that NO, by promoting vasodilation in the tumor vasculature, counteracts the vasoconstrictive effects of PDT, thereby compromising treatment efficacy. Surprisingly little has been done to further characterize these *in vivo* effects, e.g. determining (i) whether NOS upregulation occurs in PDT-stressed tumors and, if so, which NOS isoform(s), and (ii) the relative importance of overexpressed *vs.* constitutive NOS in the pro-survival/proliferative response. The present study sets the stage for rationally addressing these questions in the *in vivo* setting.

In summary, we have demonstrated that NO is rapidly overproduced in photodynamically stressed breast tumor cells due to iNOS overexpression and that this contributes substantially to cell resistance to apoptotic photokilling. The molecular mechanism of iNOS induction was not examined, but presumably involved activation of transcription factor AP-1 or NF- κ B, the latter via phosphorylation and dissociation/degradation of inhibitory I κ B, as demonstrated for other stress systems [30]. Similarly, the mechanism by which iNOS-generated NO inhibits apoptosis is not yet clear, but plausible possibilities include (i) S-nitrosation-inhibition of caspase activation or activity [25], (ii) S-nitrosation of MAPKs such as ASK1 and JNK1 [25], or (iii) downregulation of Bax and/or upregulation of Bcl-xL, as we recently reported for exogenous NO (7). Moreover, NO-mediated induction of cytoprotective heme oxygenase-1 could also play a role [7]. The key revelation from this study is that NO produced not only by surrounding vascular cells, but also by tumor cells themselves under stress, can play a crucial role in tumor resistance to PDT. Accordingly, development of rational pharmacologic interventions based on NOS inhibition, is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ac-DEVD-AMC	N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin
ALA	5-aminolevulinic acid
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DME/F12	Dulbecco's modified Eagle's/Ham's nutrient F12
Ho	Hoechst 33258
L-NAME	L-N ^G -nitroarginine methyl ester
iNOS	inducible nitric oxide synthase

nNOS	neuronal nitric oxide synthase
eNOS	endothelial nitric oxide synthase
1400W	N-[3-(aminomethyl)benzyl]acetamidine
PDT	photodynamic therapy
PI	propidium iodide
PpIX	protoporphyrin IX
SPNO	spermine NONOate

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Figure 1.

Effects of NOS inhibitors on ALA/light-provoked caspase activation and apoptotic cell death. Subconfluent COH-BR1 cells in serum-free DME/F12 medium were sensitized with ALA-induced PpIX and irradiated in the absence or presence of L-NAME (1 mM) or 1400W (10 μM), introduced 1 h before light. (A) Fluorescence micrographs of cells that had been irradiated, dark-incubated for 20 h in 1% serum-containing DME/F12 medium, and then stained with Ho; scale bar: 100 μm. (B) Extent of ALA/light-induced apoptosis after 20 h as a function of L-NAME (left) and 1400W (right) concentration; light fluence: 1 J/cm² (●), 2 J/cm² (○). (C) Caspase-3/7 activity measured 8 h after the indicated cell treatments; 1 mM L-NAME; 10 μM 1400W. Values shown are relative to ALA- or light-only control. Light fluence was 1 J/cm² in (A) and 2 J/cm² in (C). Plotted values in (B) and (C) are means ± SD of data from three independent experiments; *P<0.0001 compared with ALA- or light-only; #P<0.0005 compared with ALA/hv.

Figure 2.

Effect of L-NAME on ALA/light-induced apoptotic killing of MDA-MB-231 cells. Subconfluent cells in serum-free DME/F12 medium were incubated with 1 mM ALA for 45 min in the dark, then switched to ALA-free medium. As indicated, L-NAME in increasing concentrations up to 4 mM was added to the cells 15 min before ALA. All sensitized cells were exposed to a light fluence of 0.5 J/cm². After 20 h of dark incubation in 1% serum-containing medium, extent of apoptosis was determined. Values are means $\pm$ SD of data from three independent experiments.

Figure 3.

Effects of NOS inhibitors on H_2O_2 -provoked apoptosis. COH-BR1 cells in serum-free DME/F12 medium either lacking or containing L-NAME (A) or 1400W (B) at the indicated concentrations were incubated for 4 h in the presence of 0.5 mM H_2O_2 . The cells were then switched to 1% serum-containing DME/F12 without H_2O_2 and incubated for an additional 16 h, after which extent of apoptosis was determined. Means $\pm$ SD of values from three separate experiments are plotted.

Figure 4.

Expression of different NOS isoforms in ALA/light-treated cells. ALA-treated COH-BR1 cells were exposed to a light fluence of 2 J/cm^2 . Immediately after irradiation (0 h) and after various periods of dark incubation up to 20 h, the cells were analyzed for iNOS, eNOS, and nNOS protein levels by Western blotting, using β -actin as a loading standard. An ALA-treated dark control was also examined after 20 h (DC). For iNOS and eNOS, densitometrically-measured band intensity relative to DC is indicated below each time point. Data shown are from one experiment representative of three.

Figure 5.

Photodynamic stimulation of NO generation in ALA-treated cells. Subconfluent ALA-treated COH-BR1 cells in 30-mm dishes were irradiated (4 J/cm^2) in the absence or presence of $10 \mu\text{M}$ 1400W, then dark-incubated for 4 h or 20 h, after which cells were separated from media and both were subjected to chemiluminescence-based NO determination, the cells after being lysed and analyzed for total protein. The inset shows NO signal (mV) in medium as a function of retention time for two selected systems: (a) ALA-only at 20 h, (b) ALA/light at 20 h. Net nitrite measured in cells is shown by black bars and in medium by gray bars. Notations under bars are defined as follows: ALA(20), ALA-only at 20 h; hv(20), light-only at 20 h; ALA/hv(4) and ALA/hv(20), ALA plus light at 4 h and 20 h, respectively; ALA/hv/W(4) and ALA/hv/W(20), ALA plus 1400W and light at 4 h and 20 h, respectively. Plotted values are means $\pm$ SD of data from three separate experiments; * $P < 0.0005$ compared with ALA(20) medium; ** $P < 0.0001$ compared with ALA(20) medium or cells; # $P < 0.0001$ compared with ALA/hv(20) medium or cells.

Figure 6.

Effect of cPTIO on ALA/light-induced caspase activation and apoptosis. ALA-treated COH-BR1 cells in 30-mm dishes were irradiated (1 J/cm²) in the absence or presence of cPTIO, introduced at the indicated concentrations 1 h before light. After 8 h of post-irradiation incubation, caspase-3/7 activity was determined (A) and after 20 h, the extent of apoptosis (B). Means $\pm$ SD of values from three independent experiments are plotted in (A) and (B). (A) [#]P<0.0001 compared to control without cPTIO. (B) [#]P<0.0005 and ^{##}P<0.0001 compared to control without cPTIO.

Figure 7.

Effect of an exogenous NO donor on caspase activation and apoptosis of 1400W-inhibited, ALA/light-stressed COH-BR1 cells. Sensitized cells were irradiated in the absence or presence of 10 μ M 1400W (added 1 h before light) or 10 μ M 1400W plus 100 μ M SPNO (added 10 min before light). Caspase-3/7 activity was measured after 8 h of post-irradiation incubation (A) and apoptosis after 20 h (B). Means $\pm$ SD of values from three replicate experiments are plotted in (A) and (B); * $P < 0.0001$.