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SIGNALING EVENTS IN APOPTOTIC PHOTOKILLING OF 5-AMINOLEVULINIC ACID-TREATED TUMOR CELLS:

INHIBITORY EFFECTS OF NITRIC OXIDE

Reshma Bhowmick and Albert W. Girotti*

Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA

Abstract

Antitumor photodynamic therapy (PDT) employs a photosensitizing agent, molecular oxygen, and visible light to produce reactive oxygen species that can destroy tumor and tumor vasculature cells. NO produced by these cells could be pro-carcinogenic by inhibiting apoptosis and promoting angiogenesis and tumor growth. We recently showed that NO from a chemical donor or activated macrophages makes COH-BR1 breast tumor cells more resistant to photokilling sensitized by 5-aminolevulinic acid (ALA)-generated protoporphyrin IX (PpIX). Signaling events associated with this hyperresistance have now been examined. ALA-treated COH-BR1 cells containing mitochondria-localized PpIX died mainly by apoptosis after being irradiated. Underlying redox signaling associated with MAP kinase (ERK1/2, p38, JUN) phosphorylation-activation and heme oxygenase-1 (HO-1) upregulation was studied using immunoprecipitation and Western blot methodology. ALA/light treatment resulted in activation of pro-apoptotic JNK and p38 α , and deactivation of pro-survival p38 β and ERK1/2. Involvement of both JNK and p38 in apoptosis was established by using a specific inhibitor for each. Spermine NONOate-derived NO, introduced immediately before irradiation, provided substantial protection against apoptosis. This was accompanied by greater HO-1 induction and strong inhibition of each MAP kinase effect seen in the absence of NO. Downstream of JNK and p38 α activation, a marked upregulation/activation of pro-apoptotic Bax and Bid was observed along with down-regulation of anti-apoptotic Bcl-xL, each response being reversed by NO. These findings provide new insights into signaling activity associated with the intrinsic apoptotic pathway in ALA-PDT and how this activity can be modulated by NO.

Keywords

Nitric Oxide; 5-Aminolevulinic Acid; Tumor Cells; Photodynamic Therapy; Redox Signaling; Mitogen-Activated Protein Kinase (MAPK); Apoptosis

Introduction

Photodynamic therapy (PDT)¹ was introduced about 35 years ago as a novel means of selectively eradicating a variety of solid tumors (1,2). PDT is a three-component treatment

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*To whom correspondence should be addressed: Albert W. Girotti, Ph.D., Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, 53226, USA, Tel: 414-456-8432, Fax: 414-456-6510, Email: agirotti@mcw.edu

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modality involving (a) systemic or topical application of a sensitizing agent or metabolic precursor; (b) photoexcitation of the sensitizer, typically by light in the visible wavelength range (400-750 nm); and (c) molecular oxygen. Photoexcited triplet state sensitizers can give rise to cell-modifying reactive oxygen species (ROS), either free radicals such as superoxide and hydroxyl radical via the Type I (electron or hydrogen transfer) mechanism or non-radical singlet molecular oxygen ($^1\text{O}_2$) via the Type II (energy transfer) mechanism (3). Most PDT sensitizers are believed to mediate Type II reactions in which $^1\text{O}_2$ may trigger a variety of responses ranging from antioxidant induction to apoptotic death, or in some cases autophagic death (4-6). There is considerable interest in elucidating the signaling pathways leading to these responses in order to better understand how PDT operates at the molecular and cellular level (4,5). Whether PDT-stressed cells die via apoptosis (programmed dismantling through signaling pathways) or necrosis (non-programmed dismantling) depends on many different factors, including cell type, light dose, and subcellular location of the sensitizer (4). Two major mechanisms of apoptosis exist: the extrinsic (death receptor) pathway activated at the plasma membrane and the intrinsic pathway activated in mitochondria (7). The importance of sensitizer localization on mode of photokilling was demonstrated in our previous studies with COH-BR1 tumor cells sensitized with 5-aminolevulinic acid (ALA)-induced protoporphyrin IX (PpIX) (8,9). We found that photokilling occurred by intrinsic apoptosis when PpIX was restricted to mitochondria (where it originates), but switched to necrosis when PpIX was allowed to diffuse away to peripheral sites, including plasma membrane. Apoptosis was preceded by two crucial events: (i) peroxidation of cardiolipin, which tethers cytochrome c to the mitochondrial inner membrane, and (ii) release of cytochrome c into the cytosol (9). In the present study, we have probed more extensively into the oxidative signaling events associated with ALA/light-induced cell killing, particularly those mediated by the mitogen-activated protein kinases (MAPKs) JNK and p38 (typically associated with apoptosis) and ERK1/2 (typically associated with cell survival and proliferation). These kinase mediators have been studied relatively little in the context of PDT stress, especially ALA-PDT stress.

Nitric oxide (NO), a multifunctional free radical gas produced naturally by nitric oxide synthases, is known to have cytotoxic (prooxidant) as well as cytoprotective (antioxidant) effects on mammalian cells, the former typically associated with high rates of NO generation/delivery and the latter with relatively low rates (10). NO can react directly with biological targets or indirectly in the form of strongly oxidizing NO-derived species such as peroxynitrite and nitrogen dioxide (10,11). Direct reactions may be regulatory, cytotoxic, or protective, depending on the circumstances, whereas indirect reactions tend to be mainly cytotoxic. An antiapoptotic cytoprotective role for NO has been described for a variety of stress-inducing systems, including TNF α , Fas ligand, and serum- or glucose-deprivation (12-14). Reported mechanisms include (i) caspase inactivation via S-nitrosation of catalytic cysteine residues (15), (ii) inhibition of membrane lipid peroxidation via oxyl and peroxy radical scavenging (16), (iii) inhibition of the proapoptotic cardiolipin peroxidase activity of cytochrome c (17), and (iv) upregulation of cytoprotective proteins such as heme oxygenase-1 (HO-1) and heat shock protein-70 (18,19). In previous work we showed that COH-BR1 cells containing disseminated ALA-induced PpIX were strongly protected against necrotic photokilling when exposed to different sources of NO during irradiation, an effect attributed to interception of plasma membrane lipid-derived radicals (20). We discovered subsequently that resistance against such killing can also be preconditioned, i.e. elicited by NO which is no longer present when the photochallenge is applied (21). Proapoptotic signaling triggered by photoexcitation of ALA-elevated, mitochondrion-localized PpIX was examined in the present study, with special attention given to the MAPK pathways and how their activation is affected by exogenous NO.

Materials and methods

General materials

Sigma Chemical Co. (St. Louis, MO) supplied the 5-aminolevulinic acid (ALA), hemin (ferriprotoporphyrin IX), bovine trypsin, penicillin, streptomycin, Hoechst 33258 (Ho), propidium iodide (PI), rhodamine 123 (Rh123), NP-40 detergent, Dulbecco's Modified Eagle's/Ham's Nutrient F12 (DME/F12) medium, and fetal bovine serum. Boehringer Mannheim (Indianapolis, IN) supplied the Complete Mini-mixture of protease inhibitors. Primary monoclonal antibodies against human p38 (all forms), phospho-p38 (all forms), p38 α , p38 β , JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, and β -actin, along with horseradish peroxidase-conjugated IgG secondary antibodies, were obtained from Cell Signaling Technology (Danvers, MA). Monoclonal antibodies against human heme oxygenase-1 (HO-1) and β -actin were from Alpha Diagnostics (San Antonio, TX). Calbiochem (Gibbstown, NJ) supplied the SB202190 (a p38 inhibitor) and the SP600125 (a JNK inhibitor). Stock solutions of 1.0 mM SB202190 in water and 5.0 mM SP600125 in ethanol were prepared immediately before adding to cells. Spermine NONOate (SPNO) was obtained from Cayman Chemical (Ann Arbor, MI). Stock solutions of 25 mM SPNO in 10 mM NaOH were stable indefinitely when stored at -80 °C. Active SPNO decomposes with a half-life of ~39 min at pH 7.4 and 37 °C, releasing two equivalents of NO in the process (22). Decomposed SPNO (dSPNO) for control incubations was prepared by acidifying a stock sample to pH ~3.

Cell culture conditions

COH-BR1 cells, a selenoperoxidase GPx4-null epithelial subline derived from a human breast tumor (23) were originally obtained from Dr. James Doroshow, City of Hope Cancer Center (Duarte, CA). The cells were grown and maintained in humidified 5% CO₂/95% air at 37 °C, using DME/F12 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). Proliferating cells received fresh medium every third day. All experiments were carried out on cells at fewer than ten passages.

Cell sensitization and irradiation

COH-BR1 cells at ~60% confluency (either on cover slips in 35-mm culture dishes or in 90-mm dishes without slips) were washed once with DME/F12 medium lacking phenol red, serum and antibiotics, and incubated with 1.0 mM ALA in DMEM/F12 for 45 minutes in the dark at 37°C. For some experiments, an inhibitor of p38 (SB202190) or JNK (SP600125) was added to cells 1 h before irradiation, i.e. 15 min before ALA. In the case of SP600125, an ethanol-containing (vehicle) control was also prepared. Immediately before irradiation, the medium was aspirated and quickly replaced with fresh non-supplemented DMEM/F12. Cells were irradiated on a translucent plastic platform over a bank of four 40-W cool white fluorescent tubes (8,9). The light fluence rate at the platform surface was ~1.1 mW/cm², as determined with a YSI radiometer (Yellow Springs, OH). Cells were exposed to fluences ranging from 2 J/cm² to 6 J/cm². Temperature at the platform surface was maintained at 26-28 °C. Immediately after light exposure, medium was removed, replaced with DMEM/F12 containing 1% FBS plus antibiotics, and cells were returned to the incubator for various time intervals ranging from 5 min to 20 h, after which they were recovered for immunoblot, immunoprecipitation with immunoblot, or viability analyses. Dark controls (non-irradiated, ALA-treated cells) and light controls (irradiated, non-ALA-treated cells) were run alongside.

Cell treatment with SPNO or hemin

Where indicated, cells were treated with the active NO donor SPNO (0.2 mM) 10 min before being irradiated. At this concentration, SPNO was innocuous to the cells. Decomposed SPNO, fully depleted of NO, was used as a control. Cells were also treated with 5 μ M hemin 10 min

before irradiation, a freshly prepared stock solution of 2.0 mM hemin in 10 mM KOH being used.

Confocal Microscopy

COH-BR1 cells grown on a cover slip in DMEM/F12 medium were incubated with 1.0 mM ALA for 45 min and then with the mitochondrial probe Rh123 (~1 µg/ml) for 10 min. After extensive washing with PBS, the cells were overlaid with a dilute solution of sodium hydrosulfite in nitrogen-sparged PBS (to prevent PpIX photodegradation during viewing) and analyzed by confocal microscopy for subcellular distribution of PpIX, using a Leica TCS/SCP2 instrument (Leica Microsystems, Knowlhill, UK). Rh123 and PpIX were photoexcited at 488 nm, the former being detected by its fluorescence emission in the 520-580 nm range, and the latter by its fluorescence emission in the 620-650 nm range. Other details were as described previously (8).

Evaluation of cell death

Extent and mechanism of cell death in ALA/light- or ALA/SPNO/light-treated cells on cover slips was assessed by fluorescence microscopy using Ho and PI as indicators of apoptosis and necrosis, respectively. Membrane-traversing HO stains normal, necrotic, and apoptotic nuclei faintly, more intensely, and highly intensely at condensed chromatin, respectively (24). PI, which only traverses damaged or “leaky” membranes, does not reveal normal nuclei, but stains necrotic (including aborted apoptotic) nuclei intensely (24). Ho and PI were used simultaneously, the former to detect genuine sustained apoptosis and the latter to confirm necrosis (typically not discernable with Ho alone). Cells were treated with 5 µM Ho and 50 µM PI, returned to the incubator for 30 min, then washed 3-times with PBS, waiting 10 min between aspirations. The cover slips were mounted on slides with one drop of fluomount G and examined by fluorescence microscopy. For each specimen, approximately 100 cells in 3-4 viewing fields were evaluated.

Immunoblot analysis

Cell extracts were prepared as described (25) with some modifications. At the indicated times of dark incubation following exposure to a given light fluence, cells were collected by trypsinization, centrifuged, and washed with ice-cold PBS. They were then suspended in cold pH 7.5 lysis buffer (25 mM HEPES, 1% Triton X-100, 10% glycerol, 0.3 M NaCl, 1.5 mM MgCl₂, 1 mM DTT, 2 mM EDTA, 2 mM EGTA, 20 mM β-glycerophosphate, 1 mM Na₃VO₄ plus Complete-mini mixture of protease inhibitors) and homogenized by 5-6 passages through a 22-gauge needle. The resulting lysate was centrifuged at 8,000 xg for 10 min at 4 °C. The supernatant fraction was recovered and its protein concentration determined by bicinchoninic acid (BCA) assay, using reagents from Pierce Chemical Co. (Rockford, IL). For Western blotting, equal amounts of lysate protein were subjected to Laemmli SDS-PAGE, using 10% acrylamide/bisacrylamide for MAPKs and 12.5% acrylamide/bisacrylamide for Bcl-2 family proteins. The separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane, using a Genie blotter (Idea Scientific, Minneapolis, MN). Membranes were incubated with primary and secondary antibodies diluted according to supplier recommendations. Protein visualization, including β-actin as a loading standard, was accomplished using the Supersignal West Pico chemiluminescence detection kit (Thermo Scientific, Rockford, IL). Quantitation of immunoblot bands was carried out using LabWorks image acquisition and analysis software from UVP (LLC Upland, CA). Additional details were as described (25).

Immunoprecipitation procedure

The extent of p38 α or p38 β activation via phosphorylation was assessed by immunoprecipitation assay using a published procedure (26) with certain modifications. Briefly, photostressed cells, along with controls, were recovered and washed with chilled PBS. The cells were suspended in ice-cold pH 7.5 homogenization buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM Na₃VO₄, 1 mM β -glycerophosphate, 2.5 mM sodium pyrophosphate, 1% NP-40 plus protease inhibitors) and lysed by repeated passage through a 22-gauge needle. After centrifugation at 10,000 $\times$ g for 10 min, the supernatant fraction was recovered for determination of p38 α and p38 β phosphorylation (activation) status. For immunoprecipitation assay, anti-p38 α or anti-p38 β antibody was incubated with protein G-conjugated beads in PBS (~1 μ g antibody/40 μ l) for 45 min at room temperature. After centrifugation and removal of antibody solution, the beads were washed once with homogenization buffer, and mixed with cell lysate. After a 2 h incubation at 4 $^{\circ}$ C, the immunocomplex-containing beads were washed 4-times with pH 7.4 washing buffer (10 mM Tris-HCl, 150 mM NaCl, 0.2 mM Na₃VO₄, 1% Triton X-100, and protease inhibitor cocktail) at 4 $^{\circ}$ C. The immunocomplex was then eluted with SDS-PAGE loading buffer (62.5 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, pH 6.8) and subjected to SDS-PAGE, followed by Western blot analysis as described in the preceding paragraph. Other details were as described previously (26).

Statistics

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

Results

Localization and content of PpIX in ALA-treated cells

COH-BR1 cells were incubated in the presence of ALA (1.0 mM) and the mitochondrial marker Rh123 (~1 μ g/ml) in serum-free DME/F12 medium for 45 min in the dark. After removal of the medium and replacement with DME/F12 without ALA and Rh123, the cells were examined by confocal fluorescence microscopy, using appropriate excitation and emission wavelengths for PpIX and Rh123. As shown in Fig. 1A, a strong perinuclear zone of PpIX fluorescence was observed, which completely overlapped the zone of Rh123 fluorescence. Thus, most of the ALA-generated PpIX under the incubation conditions used was restricted to mitochondria. The pre-irradiation intracellular content of PpIX was found to be 0.44 ± 0.05 nmol/mg of protein (mean $\pm$ SD; $n = 3$), as determined spectrofluorimetrically (λ_{ex} 405 nm; λ_{em} 630 nm) using a PpIX standard (27). This value was reproducible within 10% for routine cell incubations with ALA as described, and SPNO had no significant effect on it.

Apoptosis-dominated photokilling: effects of exogenous NO

Cells sensitized with ALA-enhanced PpIX as described were irradiated at room temperature with broad-band visible light for different periods and checked for apoptotic vs. necrotic death after a subsequent 20 h incubation period in the dark. As shown by the fluorescent micrographs in Fig. 1B, irradiation for 30 min (delivered fluence ~2 J/cm²) resulted in extensive cell death, as assessed 20 h later by comparing degrees of nuclear staining with Ho and PI. Ho staining was much more intense and condensed than PI, indicating that more cells had died via apoptosis than necrosis. This was confirmed by cell counting, which showed that ~55% of the population was apoptotic and ~10% necrotic compared with <5% in each category for an ALA-only dark control (Fig. 1B). Increasing the light fluence to 4 J/cm² resulted in ~70% apoptotic and ~15% necrotic cells after 20 h, whereas a higher fluence, 6 J/cm², appeared to reduce apoptotic kill somewhat while increasing necrosis to nearly 30%. This suggests that the conditions favoring apoptosis (e.g. sufficient ATP, intact plasma membrane) were being compromised at the

highest fluence used. When ALA-treated cells were irradiated in the presence of active SPNO (0.2 mM, introduced 10 min before light), the extent of photokilling was substantially reduced compared to that observed without SPNO, the apoptotic cell count being ~5% and ~22% at light fluences of 2 J/cm² and 4 J/cm², respectively (Fig. 1C). Cell kill was insignificant in non-irradiated ALA/SPNO-treated cells, indicating that at the fluxes used, NO was innocuous to the cells. Importantly, when decomposed SPNO was substituted (Fig. 1D), the extent of apoptosis at the different fluences was similar to that seen in the non-SPNO control (Fig. 1C), which confirms that NO was the cytoprotective agent released by SPNO. Under the conditions used, NO was released at an initial rate of ~1.4 μ M/min, as determined from the initial concentration of SPNO (0.2 mM) and its half-life at 25 °C (~200 min) (22). This is in the range of NO fluxes that are estimated to be produced by activated macrophages, e.g. at tumor inflammatory sites (10,11).

We also examined photokilling by thiazolyl blue (MTT) assay based on spectrophotometric determination of mitochondrial dehydrogenase-generated formazan (8,9). For COH-BR1 cells treated as described in Fig. 1, plots of MTT-assessed viability vs. light fluence revealed that loss of overall viability was markedly reduced when NO was supplied during irradiation (not shown). The fluence for 50% loss increased from ~1.7 J/cm² without NO to ~5.7 J/cm² with NO, in keeping with the results obtained by Ho/PI staining (Fig. 1). Apoptotic photokilling of two other ALA-treated cell lines, breast cancer MDA-MB-231 and cervical cancer HeLa, was also found to be strongly inhibited by SPNO (results not shown), indicating that effects such as shown in Fig. 1 are not restricted to COH-BR1 cells.

MAPK activation in ALA/light-treated cells: effects of NO

The first known study showing that the JNK and p38 MAPKs are activated in irradiated ALA-exposed cells was carried out using human keratinocytes *in vitro* (28). A long preincubation time with 1 mM ALA was used (24 h), which would have resulted in PpIX accumulation outside as well as inside mitochondria. Also, MAPK activation was assessed after only one relatively short post-irradiation time, 30 min (28). In the present study, stress signaling elicited by PpIX photoexcitation would have originated in mitochondria, given that most of the newly synthesized porphyrin was located there. Western immunoblot analysis was used to monitor three major MAPKs (p38, JNK, and ERK1/2) as a function of (i) a single post-irradiation time after exposing sensitized COH-BR1 cells to various light fluences, and (ii) various post-irradiation times after exposing the cells to a single light fluence. Using an antibody that does not distinguish between the various known isoforms (29,30) of total p38 or phosphorylation-activated p38 (p-p38), we found that this MAPK exhibited significant basal activation (Fig. 2). There was no obvious net change in p-p38 level 20 h after ALA-treated cells were exposed to light fluences up to 6 J/cm², either in the absence or presence of 0.2 mM SPNO (Fig. 2). Similarly, no change in p-p38 or total p38 was apparent when non-ALA-treated cells were irradiated, suggesting that any background photosensitization was insignificant. Though detected in our cells, the two isoforms of JNK (54 kDa and 46 kDa) exhibited no significant phosphorylation (p-JNK) 20 h after a light-only or ALA/light challenge, even at the highest applied fluence, 6 J/cm² (Fig. 2). In contrast, substantial phosphorylated 46 kDa ERK1 and 44 kDa ERK2 (p-ERK1/2), like p-p38, was detected prior to stressing cells. Although the p-ERK1/2 level remained unchanged in a light-only control, it decreased progressively with light fluence after a photodynamic (ALA/light) challenge, p-ERK1 becoming barely detectable at and above 4 J/cm² (Fig. 2). Importantly, SPNO-derived NO reversed this trend, slowing the decline of both p-ERKs. The immunoblots in Fig. 2 are from one experiment representative of two with similar results. Means $\pm$ deviation of relative band intensities for each phosphorylated MAPK in these experiments (except JNK) are shown in the Fig. 2 legend.

We also studied MAPK activation as a function of time after cell exposure to photodynamic action. As shown in Fig. 3, overall p-p38 was elevated over the dark control level 15-30 min after 4 J/cm² of irradiation and then gradually subsided over the next several hours. Phosphorylation of JNK, which was insignificant at the outset, also increased rapidly after irradiation, reaching a maximum after ~15 min and then declining steadily until it became barely detectable after 4 h (Fig. 3), consistent with the absence of a p-JNK signal at 20 h post-irradiation in the Fig 2 experiment. There was no apparent change from the pre-existing level of p-ERK1/2 up to 4 h after irradiation, but a significant drop was evident at 20 h (data not shown), in agreement with the Fig. 2 data. In this format (varying post-irradiation time), NO again appeared to act in opposition to p38 and JNK activation and ERK1/2 deactivation (Fig. 3). ERK1/2 even showed a sizeable increase in activation, at least up to 4 h after irradiation. The effect on JNK was most dramatic, i.e. the transient spike in its activation 15-30 min after irradiation was abolished by NO. As shown by the dark control (Fig. 3), NO on its own did not elicit any significant early MAPK activation (JNK, p38) or deactivation (ERK1/2). The immunoblots in Fig. 3 are from one experiment representative of three with similar results. Means $\pm$ standard deviation of relative band intensities for each phosphorylated MAPK in these experiments (except JNK in the ALA/SPNO/hv system) are shown in the Fig. 3 legend.

The alpha and beta isoforms of p38 have been reported to affect apoptosis in opposing ways, p38 α acting pro-apoptotically and p38 β anti-apoptotically (30-32). Whether this might apply under conditions of photooxidative challenge has not been described previously. To address this, we recovered cells at different post-irradiation times, separated p38 α and p38 β by immunoprecipitation with specific antibodies, and compared phosphorylation of the individual isoforms by Western blotting, using a phospho-specific antibody recognized by both. As shown in Fig. 4A, p38 α was strongly activated 15 min after a 4 J/cm² fluence, reaching ~30-times its control level. By contrast, p38 β remained at nearly the same level as the relatively high dark control. The contrasting effects of photodynamic stimulation on p-p38 α and p-p38 β were also evident 20 h after irradiation (Fig. 4B). Thus, p38 α exhibited progressively greater activation with increasing light fluence, maximizing at ~4-times the dark control level. Concomitantly, p38 β underwent progressive deactivation, reaching ~50% of the control phosphorylation level at the highest fluence used, 6 J/cm² (Fig. 5B). (Notice in Fig. 4A that the post-irradiation decline in p-p38 β was not yet apparent after 15 min.) The sharp difference in starting level phosphorylation between the two p38 isoforms is more obvious for the experiment represented in Fig. 4A than that in Fig. 4B. The observed reciprocity in p38 α and p38 β could explain our inability to detect any significant changes in the Fig. 2 experiment, where total p-p38 was monitored. As shown in Fig. 4C, NO produced a striking reversal of the observed 20 h post-irradiation trends in p-p38 α and p-p38 β , inhibiting elevation of the former and decay of the latter with increasing light dose. These reciprocal effects were most dramatic at the 4 J/cm² fluence level, subsiding somewhat at 6 J/cm². The latter effect correlates with our findings that NO markedly curbed apoptosis at both 2 J/cm² and 4 J/cm², but much less so at 6 J/cm² (Fig. 1 B,C).

Direct evidence for p38 and/or JNK involvement in apoptosis was sought by using ATP-competing inhibitors of these MAPKs, viz. SB202190 for p38 and SP600125 for JNK (33, 34). As shown in Fig. 5A, 20 μ M SB202190, introduced 1 h before a 4 J/cm² light dose (15 min before ALA), decreased apoptotic photokilling by ~55%, whereas 25 μ M SP600125 decreased it by ~50%. At the given concentration, neither compound on its own had any significant effect on cell viability (not shown). Also, the ethanol vehicle for SP600125 had no effect. To confirm that these inhibitors were acting as expected, i.e. by interfering with phosphorylation-activation (33), we determined the p-p38 and p-JNK levels by Western analysis. As shown in Fig. 5B, SB202190 dose-dependently suppressed p38 photoactivation, 20 μ M reducing it to ~50% of the control value without inhibitor. Likewise, SP600125 inhibited JNK photoactivation, 25 μ M reducing it to ~25% of the control value. Each of the inhibitors

exhibited ~15% cross-reactivity at the highest concentration used, indicating that specificity for a given MAPK, though high as observed previously (33,34), was not absolute. These findings confirm that each of the MAPK signaling pathways examined (JNK and p38) made a significant contribution to photosensitized apoptosis.

HO-1 upregulation in ALA/light-treated cells: effects of NO and hemin

Inducible heme oxygenase-1 (HO-1) has been reported to play an important role in low flux NO's cytoprotective effects against various types of oxidative challenge, including photodynamic challenge (18,19,21,35). We therefore, asked whether HO-1 might be involved in the effects we describe for ALA/SPNO/light-treated COH-BR1 cells. As shown by the immunoblots in Fig. 6A, which represent one of two independent experiments with similar results, HO-1 was undetectable in a light-only control 20 h after irradiation, but was progressively elevated with increasing fluence in ALA/light-treated cells. Note that exposure to ALA alone (0 J/cm²) appeared to cause in a slight induction of HO-1, which was further enhanced by irradiation. An ALA/SPNO dark control (0 J/cm²) showed elevated HO-1 relative to the ALA-only dark control; however, irradiation of ALA/SPNO-treated cells resulted in a strong additional elevation of HO-1 (Fig. 6A). A similar response to the latter was seen when hemin, a well-recognized HO-1 inducer (36), was introduced before irradiation instead of SPNO (Fig. 6A). As shown in Fig. 6B, photodynamic apoptosis in ALA/light-stressed cells was strongly inhibited by SPNO (*cf.* Fig. 1C) as well as by hemin. Importantly, the protective effects of SPNO and hemin were found to be substantially diminished when cells were pre-incubated with ZnPPIX, a non-photoactivatable inhibitor of heme oxygenase (37) (data not shown). Thus, HO-1 upregulation, which was strongly enhanced by NO delivered concurrently with photodynamic stress, clearly appeared to be involved in NO's observed cytoprotective effects. However, the actual mechanism by which NO could have been cytoprotective via HO-1 induction remains to be investigated.

Expression of Bcl-2 family proteins in ALA/light- and ALA/NO/light-treated cells

MAPK activation could play a role in the expression of downstream Bcl-2 family proteins that either promote or suppress apoptosis (4,5). To begin studying this in our system, we used immunoblot analysis to examine the status of pro-apoptotic Bax and anti-apoptotic Bcl-xL in ALA/light-stressed cells. As shown in Fig. 7A, the amount of Bax measured 20 h after irradiation increased progressively with light fluence, reaching at 4 J/cm² ~3-times the level in a non-irradiated control. This upregulation was strongly suppressed by SPNO-derived NO, the increase being reduced to only ~20% (Fig. 7B). In striking contrast to Bax, immunodetectable Bcl-xL decreased progressively with increasing fluence, reaching ~10% of the control level by 4 J/cm² (Fig. 7A). As in the case of Bax, this effect was strongly attenuated by NO, which, at 4 J/cm², left Bcl-xL at ~90% of its control level (Fig. 7B). Thus, the expression of two proteins which act in opposing fashion on apoptosis was modulated consistently by photooxidative stress originating in mitochondria, and NO reversed each response, in keeping with its observed cytoprotective action. As shown in Fig. 7C, the level of pro-apoptotic full-length Bid was markedly reduced 20 h after ALA/light treatment, presumably because proteolytic truncation activated it for mitochondrion-elicited apoptosis (38). However, we were not able to detect C-terminal truncated Bid, the active fragment, possibly because of degradation once apoptosis was initiated. Importantly, Bid loss was strongly inhibited by NO, which is again consistent with the cytoprotective (anti-apoptotic) effects of NO which we observed.

Discussion

The role of MAPK activation in PDT-related oxidative cell killing has been examined in a number of recent studies using different photosensitizing agents and tumor cell types (5,

39-41). However, in contrast to other oxidative challenges (e.g. UV radiation, H₂O₂ exposure), relatively little is known about PDT-elicited stress signaling in terms of MAPK activation mechanisms and possible cross-talk among different pathways. This is particularly true for ALA-based PDT, a variant of classical PDT in which ALA or an ester thereof is used as a “pro-drug”. Upon entering cells, the pro-drug is metabolized via the heme biosynthetic pathway to PpIX, the active amphiphilic photosensitizing agent (42). ALA-PDT has been used for both systemic and dermal cancers (e.g. squamous and basal cell carcinomas), the pro-drug usually being applied topically in the latter case (42). The first known study dealing with stress signaling in this modality showed rapid activation of p38 and JNK, but not ERK1/2, in ALA/light-treated keratinocytes and melanoma cells *in vitro* (28). In this study, however, cells were pre-incubated with ALA for 24 h in serum-containing medium, which would have allowed widespread redistribution of PpIX beyond mitochondria, where it originates. Thus, stress signaling in this case (28) may have originated in more than one cellular compartment, depending on the location of redox-activatable sensor molecules in relation to PpIX. In a more recent example (43), ALA/light-stressed HL-60 cells exhibited activation of both mitochondrion- and endoplasmic reticulum-induced apoptosis, the latter reflecting Ca²⁺ delocalization. Photodamage at more than one subcellular site in this case might again be explained by use of a relatively long pre-incubation time with ALA, allowing nascent PpIX to diffuse away from mitochondria.

One of the highlights of the present study, which distinguishes it from many preceding PDT-related studies (28,39-43) or even those involving simple addition of an oxidant such as H₂O₂ to cells (44-46), is that initial oxidative pressure was applied site-specifically, i.e. predominantly in mitochondria. This follows from our observation that in the sensitization procedure used, ALA-induced PpIX was restricted to the mitochondrial compartment, as visualized by confocal microscopy with a mitochondrial marker for verification. Singlet oxygen (¹O₂), the major ROS generated by photoexcitation of PpIX, can react with unsaturated lipids and other biomolecules, typically giving hydroperoxide derivatives (47,48). The mean lifetime of ¹O₂ is expected to be much shorter in mitochondria than in water (~2 μs), given the abundance of molecular targets in the former and their high reaction rate constants (49). Thus, the probability of ¹O₂ escape into cytosol would have been low in our system, assuming that most of the PpIX was located in the mitochondrial matrix and inner membrane. This suggests that oxidative events occurring in mitochondria were responsible for the observed activation of MAPK signaling pathways associated with apoptotic cell death.

Consistent with other studies using different cell lines and oxidative challenges (44,46), we observed a rapid, albeit transient, appearance of activated JNK isoforms in photodynamically-stressed COH-BR1 cells. Concomitantly, there was a striking light dose-dependent redistribution in the contents of two activated p38 isoforms, p38α and p38β, the former showing a large increase over its low preexisting level, and the latter a large decrease from its relatively high preexisting level. Like p38β, ERK1/2 was de-activated by photostress. The indicated p38 trends are compatible with recent evidence that p38α and p38β have opposing effects on apoptosis, the α-isoform promoting it and the β-isoform suppressing it (31,32). We found that stress-induced apoptosis and p38α or JNK activation were both attenuated by a p38 or JNK inhibitor, suggesting at least partial involvement of each MAPK in death signaling. JNK and p38 are downstream members of two 3-tiered stress-signaling cascades (30,44), activation of which begins with auto-phosphorylation of a primary kinase (MAP3K), which acts on an intermediate kinase (MAP2K: M2K3/4/6 for p38; M2K4/7 for JNK), which in turn acts on the terminal MAPK. Of the several known stress-activated MAP3Ks, apoptosis signal regulating kinase 1 (ASK1) is the best characterized in terms of regulatory mechanism (46). ASK1 is inactive when associated with reduced thioredoxin (Trx) or glutaredoxin (Grx), which acts as a redox sensor. Upon oxidative conversion from the dithiol to disulfide form, Trx or Grx dissociates from ASK1, thus permitting its activation and thence activation of downstream

MAPKs (46,50). Triggering ASK1 by H_2O_2 or UV oxidative stress has been well documented for various cell types (46,51). However, whether it responds to $^1\text{O}_2$ -mediated photodynamic stress has not been determined, and we are currently exploring this for the ALA/light system described. ASK1 and downstream MAPKs are typically found in the cytosolic compartment of mammalian cells, but there is recent evidence that ASK1 also exists in mitochondria (52). Consequently, if stress signaling had been triggered by $^1\text{O}_2$ attack on ASK1 in our system, this would likely have occurred in the mitochondrial compartment rather than the cytosolic due to $^1\text{O}_2$'s short lifetime (see above). On the other hand, $^1\text{O}_2$ adducts such as relatively long-lived and mobile lipid hydroperoxides (53) might have migrated outside mitochondria and activated MAPK cascades there. We can only speculate on where activation took place in our system until mechanistic evidence along these lines is forthcoming.

JNK and p38 have been reported to activate apoptotic signaling pathways via transcriptional upregulation of various pro-apoptotic proteins or modulation of pro- and anti-apoptotic protein activities through phosphorylation (30,44). For example, p38-mediated down-regulation of Bcl-xL and up-regulation of p53 were found to play a crucial role in oxidant-induced apoptosis of endothelial cells (54). In the case of JNK, phosphorylation-activation of pro-apoptotic Bax has been reported (55), as well as activation via release from sequestering 14-3-3 proteins upon their phosphorylation (56). Suppression of the anti-apoptotic activities of Bcl-2, Bcl-xL, and Mcl-1 by JNK-mediated phosphorylation has also been described (57). In this study we have found that Bax and Bcl-xL protein levels were reciprocally altered in photodynamically-stressed COH-BR1 cells, the former increasing markedly and the latter decreasing, both effects being consistent with the observed apoptotic end-point. Whether Bax was phosphorylated in addition to being upregulated in stressed cells was not assessed, nor was targeting to the outer mitochondrial membrane for release of apoptogenic proteins, but this probably occurred, based on considerable evidence from other stress systems (55,58).

In addition to providing new information about MAPK-mediated stress signaling in an ALA-PDT model, this study describes for the first time the effects of NO on this signaling. In the low fluxes used, NO did not activate apoptotic signaling on its own, but instead strongly inhibited ALA/light-induced JNK and p38 α activation, ERK1/2 and p38 β deactivation, and apoptotic cell death. Consistent with these antiapoptotic/pro-survival effects was NO's striking inhibition of Bax upregulation, Bcl-xL down-regulation, and Bid activation in stressed cells, along with its ability to induce HO-1 to higher levels than attained with ALA/light alone. How might these findings be explained? NO does not deactivate $^1\text{O}_2$ (20), so this can be ruled out as a protective mechanism. However, as shown previously (16,20), NO can diffuse into membrane bilayers and scavenge chain-propagating lipid-derived radicals, which would otherwise amplify production of relatively mobile electrophiles and lipid hydroperoxides. As indicated, these species could potentially activate stress proteins such as the MAP3K ASK1. In addition to interfering with MAPK-mediated signaling by acting as a chain-breaking antioxidant, NO can modify proteins via S-nitrosation of specialized cysteine residues, either by reaction of its oxidation product N_2O_5 with a thiolate group or direct reaction of NO with an oxidized thiolate in the form of a thiyl radical (59). In the case of cellular ASK1, it has been reported that inducible NO synthase-dependent S-nitrosation of a specific cysteine residue in the kinase's catalytic domain prevents autophosphorylation-activation in response to H_2O_2 exposure (60). Other cellular studies have shown that activation of downstream JNK1 can also be inhibited by S-nitrosation, in this case induced by either exogenous or endogenous NO (61). Similar derivatization of the antiapoptotic protein Bcl-2 is reported to prolong its lifetime by inhibiting ubiquitination and proteosomal degradation (62). If occurring in our system, S-nitrosation of any of these target proteins could explain, at least in part, the antiapoptotic effects of NO that we observed. As with these possibilities, the mechanism of HO-1 hyperinduction in ALA/SPNO/light-treated cells is yet to be investigated. However, based on recent findings (63), activation of the Nrf2 transcription factor upon S-nitrosation and release of Keap1, its

associated regulatory protein, is a distinct possibility. Upregulated HO-1 could have suppressed photooxidative apoptosis via secondary upregulation of ferritin with sequestration of redox iron, as we reported previously (21). Alternatively, as suggested by recent evidence (32), HO-1's antiapoptotic/pro-survival effects might have been due to enhanced proteosomal degradation of p38 α with sparing of p38 β . Clearly much remains to be learned about NO's mechanism of action in the ALA-PDT model that we have described.

In summary, this is the first in-depth investigation of the stress signaling that underlies antitumor ALA-PDT and how it can be modulated by NO to potentially compromise therapeutic outcome. Although we used an ALA-PDT model in this work, our findings should prove important to the understanding of PDT stress signaling in general and how NO generated by tumor cells or nearby vascular cells can affect it. With regard to the latter point, we recently discovered that ALA/light treatment caused a rapid and prolonged upregulation of inducible nitric oxide synthase (iNOS) in COH-BR1 cells and that apoptotic cell death was markedly enhanced by addition of the NOS inhibitor L-NAME before irradiation (results not shown). These findings suggest that stress-elevated endogenous NO could act cytoprotectively and that this could be augmented when we used an exogenous NO donor. One of the highlights of the present study which distinguishes it from earlier ALA-PDT-related studies is that oxidative pressure in the form of ¹O₂ from photoactivated PpIX was applied in a sub-cellular site-specific manner, i.e. in mitochondria, where PpIX was localized. Thus, stress signaling emanated from this organelle rather than from multiple points in the cell and this should facilitate mechanistic deductions in ongoing studies.

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Abbreviations

PDT, photodynamic therapy
 ALA, 5-aminolevulinic acid
 DME/F12, Dulbecco's modified Eagle's/Ham's nutrient F12
 DTT, dithiothreitol
 Ho, Hoechst 33258
 HEPES, *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid
 HO-1, heme oxygenase-1
 PBS, phosphate-buffered saline (25 mM sodium phosphate/125 mM NaCl, pH 7.4)
 PI, propidium iodide
 PpIX, protoporphyrin IX
 Rh123, rhodamine 123
 SPNO, spermine NONOate
 dSPNO, decomposed spermine NONOate
 MAPK(s), mitogen-activated protein kinase(s)
 JNK, *c*-Jun N-terminal kinase
 ERK1/2, extracellular signal-regulated kinase-1/2
 p38, p38 mitogen-activated protein kinase
 SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Figure 1.

Subcellular localization of PpIX in ALA-treated cells: susceptibility to PpIX-sensitized photokilling in the absence vs. presence of SPNO. COH-BR1 cells at ~60% confluency in serum-free DME/F12 medium were incubated with 1.0 mM ALA for 45 min, washed, and then incubated with Rh123 (1 μ g/ml) for 10 min, all in the dark at 37 °C. (A) Confocal images of cells showing sites of major PpIX fluorescence, Rh123 fluorescence, and overlaying of the two. Each bar represents 75 μ m. (B) Apoptotic vs. necrotic photokilling of cells preincubated with 1.0 mM ALA for 45 min. Twenty hours after exposure to a light fluence of 2 J/cm^2 (Irrad), cells were stained with Ho and PI, and examined for numbers of apoptotic and necrotic nuclei by fluorescence microscopy. ALA-treated cells kept in the dark were analyzed alongside as a control (Dark). Approximately 100 cells in 4 viewing fields were scored for each sample. Plot shows extent of apoptosis (black bars) and necrosis (gray bars) after cell exposure to the following fluences: 0, 2, 4, and 6 J/cm^2 . (C) and (D) Effect of active vs. decomposed SPNO on photokilling. ALA-treated cells were irradiated in the presence of active donor (SPNO) or decomposed donor (dSPNO); 20 h later they were stained with Ho and PI and scored for apoptosis and necrosis. Plots in (B), (C), and (D) show means $\pm$ SD of values from three separate experiments.

Figure 2.

MAPK status in photodynamically challenged cells: increasing light fluence/single post-fluence time format. COH-BR1 cells were dark-incubated with 1.0 mM ALA for 45 min and then exposed to the indicated fluences (J/cm^2), either directly (ALA/hv) or after addition of 0.2 mM SPNO (ALA/SPNO/hv). A control not treated with ALA or SPNO was irradiated alongside (hv). Twenty hours after each irradiation, the cells were recovered by trypsinization, washed, and lysed in preparation for Western blot analyses. Samples of equivalent total cellular protein (120 μg per lane) were subjected to SDS-PAGE using 10% acrylamide/bis-acrylamide, followed by transfer to a PVDF membrane. After blocking, the membrane was treated sequentially with primary antibodies against p-p38, p38, p-ERK1/2, ERK1/2, p-JNK, and JNK, followed in each case by reporter secondary antibody and chemiluminescence measurement, with antibody stripping between each of the six determinations. β -Actin was also monitored

as a loading reference. For p38 and ERK1/2 (but not JNK because p-JNK was undetectable), densitometrically-determined intensity of phosphorylated enzyme relative to overall intensity (e.g. p-p38/p38) was as follows: **p38**: hv [1.0 ± 0.1 (2), 1.1 ± 0.0 (4), 1.0 ± 0.2 (6)]; ALA/hv [1.1 ± 0.1 (2), 1.0 ± 0.2 (4), 1.1 ± 0.0 (6)]; ALA/SPNO/hv [0.9 ± 0.1 (2), 0.8 ± 0.1 (4), 0.9 ± 0.0 (6)]. **ERK1/2**: hv [0.9 ± 0.0 (2), 0.8 ± 0.1 (4), 0.8 ± 0.1 (6)]; ALA/hv [0.4 ± 0.0 (2), 0.2 ± 0.0 (4), 0.3 ± 0.0 (6)]; ALA/SPNO/hv [1.2 ± 0.1 (2), 0.8 ± 0.1 (4), 0.9 ± 0.0 (6)]. Numbers in brackets are means $\pm$ deviation of values from two separate experiments for each reaction condition, light fluences being indicated in parentheses. The immunoblots shown are from one of these experiments. Values are relative to the dark control (0-fluence) in each case.

Figure 3.

MAPK status in photodynamically challenged cells: single light fluence/increasing post-fluence time format. COH-BR1 cells sensitized with ALA-generated PpIX as described in Figs. 1 and 2, were exposed to a 4 J/cm² light fluence in the absence (ALA/hv) or presence (ALA/SPNO/hv) of 0.2 mM SPNO, then trypsinized and recovered, either immediately (0 min) or after increasing periods of dark incubation ranging from 15 min to 4 h. For each condition, a non-irradiated dark control (DC) was run alongside and cells were recovered at the 15 min time point. Samples with the same amount of total cell protein (120 µg per lane) were subjected to SDS-PAGE and Western blotting, using primary antibodies against p-p38, p38, p-ERK1/2, ERK1/2, p-JNK, and JNK. For each MAPK (except JNK in ALA/SPNO/hv system), densitometrically-measured intensity of phosphorylated enzyme relative to overall intensity was as follows: **JNK**: ALA/hv [2.4±0.2 (0.25), 1.7±0.2 (0.5), 1.3±0.1 (1), 1.1±0.1 (2), 1.0±0.1 (4)]. **p38**: ALA/hv [3.2±0.2 (0.25), 3.4±0.3 (0.5), 1.5±0.1 (1), 1.0±0.1 (2), 0.9±0.1(4)]; ALA/SPNO/hv [1.0±0.1 (0), 1.1±0.1 (0.25), 1.0±0.0 (0.5), 0.9±0.2 (2), 0.9±0.1 (4)]. **ERK1/2**: ALA/hv [0.9±0.1 (0.25), 1.0±0.1 (0.5), 0.9±0.1 (1), 0.9±0.1 (2), 0.9±0.1 (4)]; ALA/SPNO/hv [1.4±0.1 (0), 4.5±0.1 (0.25), 4.3±0.0 (0.5), 3.8±0.1 (2), 4.8±0.1 (4)]. Numbers in brackets are means ± SD of values from three separate experiments for each reaction condition, cells being sampled at the post-irradiation times indicated in parentheses (hours). The immunoblots shown are from one of these experiments. Values are relative to the dark control (0-fluence) in each case.

Figure 4.

Phosphorylation state of p38 α and p38 β in photochallenged cells. ALA-treated cells were (A) exposed to a light fluence of 4 J/cm² and examined after 15 min in the dark, or (B) exposed to fluences of 2, 4, and 6 J/cm² and examined after 20 h in the dark. ALA-treated cells were also irradiated in the presence of 0.2 mM SPNO and examined 20 h later (C). Non-irradiated ALA-treated cells are also represented in each case (0 J/cm²). The p38 α and p38 β isoforms were immunoprecipitated using monoclonal antibodies specific for each and then analyzed by immunoblotting, using p-p38 antibody to probe for extent of phosphorylation and the individual antibodies to probe for total amount of each p38 isoform. The latter values were used for standardizing sample loads and normalizing the amounts of corresponding p-p38 in

the different sample lanes. Graphs beside the immunoblots show integrated levels of each p-p38 isoform: in (A) relative to p-p38 α at 0 J/cm²; in (B) and (C), each relative to its own level at 0 J/cm². Means $\pm$ deviation of values from two separate experiments are plotted in (B).

Figure 5.

Effects of MAPK inhibitors on extent of photosensitized MAPK phosphorylation and apoptotic cell killing. (A) Cell sensitization with ALA-derived PpIX was as described in Fig. 1. Where indicated, cells were pre-incubated with p38 or JNK inhibitors for 1 h (ALA being included during the last 45 min), after which irradiation was carried out (4 J/cm²) and apoptosis assessed 20 h later. DC, dark control without inhibitor; IC, irradiated control without inhibitor; SB, 20 μM SB202190 before irradiation; SP, 25 μM SP600125 before irradiation. Plotted data are means ± SD of values from three separate experiments; *significantly less than corresponding IC value, $p < 0.005$. (B) Phosphorylation status of p38 and JNK after irradiation of sensitized cells that had been treated with 0, 10, or 20 μM SB202190; *significantly lower than the value

at 0 μM inhibitor, $p < 0.005$. (C) Phosphorylation status of JNK and p38 after irradiation of sensitized cells that had been treated with 0, 10, or 25 μM SP600125; *significantly lower than the value at 0 μM inhibitor, $p < 0.001$. Western analyses in (B) and (C) were carried out 15 min after 4 J/cm^2 of light exposure. Plotted data in each case are means $\pm$ SD of values from three separate experiments, one of which is represented in the immunoblots (inset).

Figure 6.

HO-1 expression in relation to apoptosis in ALA/light-treated cells: effects of NO and hemin. ALA-treated cells were either kept in the dark (0 J/cm²) or exposed to light fluences of 2, 4, and 6 J/cm², either directly (ALA/hv) or after addition of 0.2 mM SPNO (ALA/SPNO/hv) or 5 μM hemin (ALA/hemin/hv). A control not treated with ALA or the other agents was irradiated alongside (hv). Twenty hours after irradiation, samples were recovered and analyzed for degree of HO-1 expression and apoptosis. (A) HO-1 protein level, as determined by Western analysis. Band intensities normalized to β-actin and relative to 0-fluence were as follows: ALA/hv [1.7±0.0 (2), 1.8±0.2 (4), 2.0±0.0 (6)]; ALA/SPNO/hv [3.4±0.2 (2), 2.7±0.2 (4), 3.9±0.3 (6)]; ALA/hemin/hv [0.9±0.1 (2), 1.0±0.2 (4), 1.3±0.1 (6)]. Numbers in brackets are means ±

deviation of values from two independent experiments at fluence levels indicated in parentheses; the blots shown are from one of these experiments. (B) apoptosis, as determined by fluorescence microscopy of Ho and PI-stained cells. IC, irradiated control without SPNO or hemin. Plotted data are means $\pm$ SD of values from three separate experiments; *significantly less than corresponding IC value, $p < 0.001$.

Figure 7.

Changes in Bcl-2 family protein levels in ALA/light- and ALA/SPNO/light-treated cells. Cells sensitized with ALA-induced PpIX were exposed to light fluences of 2 and 4/cm² in the absence (A) or presence (B) of SPNO (0.2 mM). A non-irradiated control (0 J/cm²) was run alongside. Twenty hours after each irradiation, samples were analyzed for Bax and Bcl-xL by Western blotting, β -actin serving as a loading standard. Plots in (A) and (B) show changes in Bax (●) and Bcl-xL (○) levels relative to the non-irradiated control for the respective blots. Samples were also analyzed for Bid by Western blotting (C); plots show changes in Bid level after irradiation in the absence (Δ) or presence (▲) of SPNO. Means $\pm$ deviation of immunoblot data from two separate experiments for each reaction condition are plotted in (A), (B), and (C). The blots shown are from one of these experiments.