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Original Contribution

# Cytoprotective Signaling Associated with Nitric Oxide Upregulation in Tumor Cells Subjected to Photodynamic Therapy-like Oxidative Stress ☆

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ABSTRACT

Photodynamic therapy (PDT) employs photoexcitation of a sensitizer to generate tumor-eradicating reactive oxygen species. We recently showed that irradiating breast cancer COH-BR1 cells after treating with 5-aminolevulinic acid (ALA, a pro-sensitizer) resulted in rapid upregulation of inducible nitric oxide (NO) synthase (iNOS). Apoptotic cell killing was strongly enhanced by an iNOS inhibitor (1400 W), iNOS knockdown (kd), or a NO scavenger, suggesting that NO was acting cytoprotectively. Stress signaling associated with these effects was examined in this study. ALA/light-stressed COH-BR1 cells, and also breast adenocarcinoma MDA-MB-231 cells, mounted an iNOS/NO-dependent resistance to apoptosis that proved to be cGMP-independent. Immunocytochemistry and subcellular Western analysis of photostressed COH-BR1 cells revealed a cytosol-to-nucleus translocation of NF-κB which was negated by the NF-κB activation inhibitor Bay11. Bay11 also enhanced apoptosis and prevented iNOS induction, consistent with NF-κB involvement in the latter. JNK and p38 MAP kinase inhibitors suppressed apoptosis, implicating these kinases in death signaling. Post-irradiation extent and duration of JNK and p38 phosphorylation were dramatically elevated by 1400 W or iNOS-kd, suggesting that these activations were suppressed by NO. Regarding pro-survival stress signaling, rapid activation of Akt was unaffected by 1400 W, but prevented by Wortmannin, which also enhanced apoptosis. Thus, a link between upstream Akt activation and iNOS induction was apparent. Furthermore, p53 protein expression under photostress was elevated by iNOS-kd, whereas robust Survivin induction was abolished, consistent with p53 and Survivin being negatively and positively regulated by NO, respectively. Collectively, these findings enhance our understanding of cytoprotective signaling associated with photostress-induced NO and suggest iNOS inhibitor-based approaches for improving PDT efficacy.



## Introduction

Produced naturally by nitric oxide synthase (NOS) enzymes, nitric oxide (NO) has numerous biological roles, including involvement in vasorelaxation, neurotransmission, and eradication of pathogenic microorganisms [1]. When generated at high rates (e.g. by activated macrophages and neurtophils), NO can have prooxidant cytotoxic effects, whereas at low rates, it may act cytoprotectively and promote cell growth/survival [2,3]. There are numerous examples of endogenous (NOS-derived) or exogenous (chemical donor-derived) NO acting in a salutary fashion on normal (non-transformed) cells. Studies with primary hepatocytes [4,5] and differentiated (neuron-like) PC12 cells [6] showed that apoptosis induced by growth factor or serum deprivation was inhibited by exogenous NO through activation of soluble guanylyl cyclase (sGC) and thence cGMP activation of protein kinase G (PKG). A more recent study [7] revealed that human keratinocyte apoptosis provoked by ultraviolet-B radiation was strongly enhanced by NOS and sGC inhibitors, but attenuated by overexpression of inducible NOS (iNOS). These findings [7] demonstrated an antiapoptotic role for endogenous NO that was mediated at least in part by cyclic GMP. However, whether this NO derived solely from constitutive NOS or whether any stress-induced NOS might have been involved was not determined. There is increasing evidence that tumor cells can also exploit NO

*Abbreviations:* ALA, 5-aminolevulinic acid; Akt, protein kinase-B; 8-Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; Ho, Hoechst-33258; iNOS, inducible nitric oxide synthase; IκB, inhibitor of NF-κB; Iκκ, IκB kinase; JNK, c-Jun-N-terminal kinase; 1400W, N-[3-(aminomethyl)benzyl]acetamidine; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazolyl-2-yl)-2–5-diphenyltetrazolium bromide; NF-κB, nuclear factor-kappa B; ODQ, 1H-[1,2,4]oxadiazolo[4,3–a]quinoxalin-1-one; PI, propidium iodide; PDPK1, 3-phosphoinositide-dependent protein kinase-1; PDT, photodynamic therapy; PI3K, phosphoinositide-3-kinase; PpIX, protoporphyrin IX; p38, p38 mitogen-activated protein kinase; sGC, soluble guanylyl cyclase; SPNO, spermine-NONOate Suvsurvivin; Wo, Wortmannin

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as an anti-apoptotic/pro-survival signaling molecule [8,9]. This NO may derive from the tumor cells themselves as well as from macrophages and endothelial cells in the tumor vasculature. In contrast to normal cells [4–7], relatively little is known about the NOS/NO status of tumor cells subjected to oxidative challenges, including therapeutic challenges, or whether NO might play a role in cellular resistance to the lethal effects of these conditions. Of special interest along these lines are earlier studies [10–12] showing that administration of non-specific NOS inhibitors markedly improved the responses of various mouse-borne tumors to Photofrin-sensitized photodynamic therapy (PDT). The results were mainly attributed to diminished relaxation of tumor blood vessels by NO acting in opposition to PDT's known vasoconstrictive effects [10,11]. Surprisingly little else has been done to further characterize NO's anti-PDT activity in terms of (a) whether the NO derives from tumor cells *per se*, tumor vasculature cells, or both; (b) which of the three NOS isoforms is most important in supplying the NO; and (c) whether the NOS/NO involved is constitutive or possibly stress-upregulated. Of added importance, but not investigated up to now is how endogenous tumor NO might modulate the stress signaling events that underlie PDT. Using a PDT model comprised of breast tumor COH-BR1 cells sensitized by 5-aminolevulinic acid (ALA)-generated protoporphyrin IX (PpIX), we showed recently that apoptotic cell photokilling was markedly enhanced by iNOS inhibitors or iNOS knockdown [13,14]. We found, moreover, that both iNOS and NO were rapidly upregulated in photostressed cells and that scavenging NO with a chemical trap markedly increased the apoptotic count. The implication of these findings is that if, in a clinical PDT setting, tumor cells upregulate iNOS/NO as a cytoprotective strategy, this could diminish treatment effectiveness. In order to better understand NO-mediated resistance to photokilling from a mechanistic standpoint, we have begun to investigate the pro-survival *vs.* pro-death signaling events associated with iNOS and NO upregulation in ALA/light-stressed COH-BR1 cells. Recent findings along these lines are described.

## Materials and methods

### *General materials*

5-Aminolevulinic acid (ALA), Hoechst-33258 (Ho), propidium iodide (PI), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), Wortmannin (Wo), sodium orthovanadate, $\beta$-glycerophosphate, and a primary monoclonal anti-$\beta$-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO). A 10 μM stock solution of Wo in DMSO was prepared immediately before adding to cells. Invitogen Life Technologies (Grand Island, NY) supplied the Dulbecco's modified Eagles's/Kaighn's-modified Ham's nutrient F12K (DME/F12K) growth medium, fetal bovine serum, antibiotics, and geneticin. Spermine NONOate (SPNO), N-[3-(aminomethyl)benzyl]acetamidine (1400 W), 1 H-[1,2,4]oxadiazolo[4,3–a]quinoxalin-1-one (ODQ), and Bay11-7082 (Bay11) were from Cayman Chemicals (Ann Arbor, MI). Immediately before experimental use, stock solutions of 25 mM SPNO, 1 mM 1400 W, 1 mM Bay11, and 50 mM ODQ were prepared in 10 mM NaOH, pH 7.4 phosphate buffer, ethanol, and DMSO, respectively. The p38 inhibitor SB202190 and JNK inhibitor SP600125 were from Calbiochem (Gibbstown, NJ). Freshly prepared stock solutions of 1 mM SB202190 in water and 5 mM SP600125 in ethanol were used for experiments. An Annexin V-FITC cell staining kit was from Roche Applied Sciences (Indianapolis, IN). Santa Cruz Biotechnology (Santa Cruz, CA) supplied the polyclonal antibody against human iNOS, the monoclonal antibody against $\beta$-actin, and monoclonal antibody against p53. Monoclonal antibodies against human JNK, phospho-JNK, p38 (all forms), phospho-p38 (all forms), p38α, p38$\beta$, NF-κB (p65 subunit), histone H3, and α-tubulin, along with horseradish peroxidase-conjugated IgG secondary antibodies, were from Cell Signaling Technology (Danvers, MA). The antibody against human Survivin was from AbCam (Cambridge, MA), that against human phospho-Survivin was from Novus Biologicals (Littleton, CO), and the Alexa 488-conjugated secondary antibody was from Invitrogen. Pierce Chemical Co. (Rockford, IL) supplied reagents for the bicinchoninic (BCA) protein assay and for the SuperSignal West Pico chemiluminescence detection of proteins on immunoblots. The iNOS inhibitor GW274150 was kindly supplied by GlaxoSmithKline, LLC (Research Triangle Park, NC) via a material transfer agreement.

### *Cell culture*

Wild-type (WT) COH-BR1 cells, an epithelial line derived from pleural effusion of a patient diagnosed with breast cancer [15], were obtained as a research gift from Dr. James Doroshow, City of Hope Cancer Center. Cytogenetic analysis (R.S. Esworthy, personal communication; Ref 15) confirmed that the cells were of human origin and consisted of two subpopulations, one being near diploid (56 chromosomes) and one near haploid [27,28chromosomes]. These cells lack selenoperoxidase GPx4, which detoxifies complex lipid hydroperoxides [16] and are quite sensitive to singlet oxygen-mediated photooxidative stress such as used in this study. Also used was a previously generated shRNA-based iNOS-knockdown (kd) clone of COH-BR1 cells [14]. This clone was one of four generated with plasmids containing iNOS-shRNA inserts (SABiosciences, Frederick, MD). The selected clone expressed the lowest constitutive level of iNOS relative to WT cells ($<20\%$), as determined by Western analysis [14]. Human breast adenocarcinoma MDA-MB-231 cells were obtained from the ATCC repository, which provided the necessary authentication details. All cells were grown under standard culture conditions, using DME/F12K medium supplemented with 10% fetal bovine serum and antibiotics [13,14,16]. Growth media for iNOS-kd cells contained geneticin (0.1 mg/ml), which was omitted 24 h before an experiment.

### *Cell sensitization and irradiation*

Cells at 60–65% confluence were metabolically sensitized with PpIX by incubating with 1.0 mM ALA in serum-free DME/F12K medium for 45 min in the dark at 37 °C. Most of the PpIX at this point was localized in mitochondria, where it originates [14,17]. Where indicated, an iNOS inhibitor (1400 W or GW274150), JNK inhibitor (SP600125), p38 inhibitor (SB202190), NF-κB activation inhibitor (Bay11-7082), PI3K inhibitor (Wo), soluble guanylyl cyclase inhibitor (ODQ), or 8-Br-cGMP was added 15 min before ALA and maintained at a given concentration during all subsequent steps. The exogenous NO donor, spermine NONOate (SPNO), was used in one experiment. Immediately after sensitization, cells were switched to ALA- and serum-free medium either lacking or containing one of the above agents, as indicated. The cell dishes were irradiated at room temperature as described [14]; light fluence rate was $\sim$1.1 mW/cm$^2$, corresponding to a delivered fluence of $\sim$1 J/cm$^2$ after 15 min of irradiation.

### *Evaluation of cell death*

ALA-treated cells on coverslips were irradiated in the absence or presence of a given inhibitor (1400 W, ODQ, Bay11, Wo) or NO donor (SPNO) and after 20 h of dark incubation analyzed for extent of apoptotic cell death. Cell coverslips were treated with an