Cyclooxygenase-2 Inhibitor Treatment Enhances Photodynamic Therapy-mediated Tumor Response

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Abstract

Photodynamic therapy (PDT) continues to be used in the treatment of solid tumors. Clinical results are promising, but the therapy has not been thoroughly optimized, and tumor recurrences can occur. Recently, it has been shown that inhibitors of cyclooxygenase (COX)-2 can be effective in combination with conventional chemotherapy and radiation therapy. In the current study, we examined the parameters of PDT-mediated activation of COX-2 expression. We also examined the tumoricidal effectiveness of combining PDT with the selective COX-2 inhibitor NS-398. PDT induced the transcriptional activation of COX-2. Prolonged expression of COX-2 protein was observed in PDT-treated mouse sarcoma and carcinoma cell lines, whereas COX-1 was not inducible by PDT. Prostaglandin (PG) E2 synthesis was also increased in PDT-treated cells, and PGE2 levels were attenuated in cells coincubated with NS-398, indicating that PDT induced the expression of biologically active COX-2. Both porphyrin- and chlorin-based photosensitizers were able to elicit PDT-mediated COX-2 expression. COX-2 was also elevated in radiation-induced fibrosarcoma (RIF) tumors after treatment with PDT. We also observed that systemic administration of NS-398 decreased PDT induction of both PGE2 and vascular endothelial growth factor in treated RIF tumors. Additionally, we demonstrated that NS-398 enhanced PDT responsiveness in RIF tumors without increasing toxicity to normal tissue. These results provide strong evidence that combination procedures involving selective COX-2 inhibitors may improve the therapeutic effectiveness of PDT.

Introduction

PDT involves the systemic administration of a tumor-localizing photosensitizer followed by focal light activation (1). This procedure results in the photochemical generation of cytotoxic reactive oxygen species such as singlet oxygen within the target tissue (2). PDT clinical trials using the photosensitizer PH, as well as a variety of second-generation photosensitizers, show promise in treating malignancies of the esophagus, bronchi, brain, peritoneal cavity, skin, bladder, and head and neck, as well as in treating nononcologic disorders such as age-related macular degeneration and psoriasis (2, 3). Initial treatment responses after PDT are normally positive; however, recurrences can occur, and therefore methods to improve PDT responsiveness are needed (2).

As part of ongoing mechanistic studies on oxidative stress, we examined gene expression using microarray analysis in mouse RIF cells treated with PH-mediated PDT. One of the genes consistently overexpressed after PDT was COX-2. In retrospect, this observation seems logical because earlier work (4) indicates that PDT stimulates the release of PGE2 from macrophages and RIF cells grown in culture, and this release is blocked by indomethacin. Studies also demonstrated that the decrease in arteriolar blood flow after PDT is inhibited by pretreatment with indomethacin, suggesting a possible COX pathway in modulating the PDT microvascular response (5). Previous work from our laboratory identified endogenous PG release in mice exhibiting a systemic shock reaction after localized PDT, which could be attenuated with NSAIDs (6). PDT also causes rapid release of the PG precursor arachidonic acid in treated mouse lymphoma cells, which was assumed to occur via phospholipase A2-dependent breakdown of membrane phospholipids (7).

COXs are rate-limiting enzymes in the metabolism of arachidonic acid to PGs (8). Different genes with unique expression properties and physiological functions encode the two isoforms of COX, COX-1 and COX-2. COX-1 is constitutively expressed in many tissues. This enzyme is involved in the production of PGs important in normal homeostatic functions including protection of the gastric mucosa (8). COX-2 is an inducible form of COX and is considered an early response gene involved in inflammation and mitogenesis (8, 9). NSAIDs decrease PG production by inhibiting the activity of both COX isoforms. A variety of PGs are elevated at sites of inflammation, and NSAIDs prevent the pathological increase of PGs (10). The initial application for COX-2-specific inhibitors was blocking PG-mediated inflammation and pain associated with arthritis (11). Interestingly, COX-2 inhibitors are now being examined for the chemoprevention and/or treatment of various cancers (9, 12–15). Evidence is emerging that COX-2-specific inhibitors may play an important role in regulating tumor tissue angiogenesis and/or apoptosis (10, 16–19). Recent studies indicate that combination treatments with COX-2-specific inhibitors and either chemotherapy or radiation therapy further enhance antitumor activity in established tumors growing in mice (20–22). It is relevant to note that this procedure does not appear to adversely affect normal tissue responses.

In the current study, we examined in vitro and in vivo expression profiles and biological activity of COX-2 after PDT. In addition, we evaluated the effectiveness of a combined modality approach using the COX-2-selective inhibitor NS-398 and PDT in treating RIF tumors.

Materials and Methods

Drugs and Reagents. The photosensitizer PH was a gift from Axcane Scandipharm Inc. (Birmingham, AL) and was dissolved in 5% dextrose in water to make a 2.5 mg/ml stock solution. NP6 was a gift from Porphyrin Products Inc. (Logan, UT) and was dissolved in saline to make a 2.5 mg/ml stock solution. The COX-2-selective inhibitor NS-398 [N-(2-cyclohexylxoy-4-nitrophenyl)-methanesulfonamide] was purchased from Cayman Chemical Co. (Ann Arbor, MI) and dissolved in DMSO to make stock solutions of 8.0
and 3.1 mg/ml for in vivo and in vitro experiments. Final working solutions of NS-398 were obtained by diluting the stock solutions either in saline for in vivo experiments or in RPMI 1640 for in vitro experiments.

**Cells and Tumor Model.** Mouse RIF, BA (mouse mammary carcinoma), and LLC cells were used for in vitro experiments (23–25). Cells were grown as monolayers in RPMI 1640 supplemented with either 15% FCS (RIF cells) or 10% FCS (BA and LLC cells) and antibiotics. For in vivo experiments, RIF tumors were generated by trocar injection of 1-mm3 pieces of tumor into the right flank of 8–12-week-old female C3H/HeJ mice.

**In Vitro and in Vivo Treatment Protocols.** Tumor cells were seeded in plastic Petri dishes and incubated overnight in complete growth media for in vitro PDT treatments. Cells were then incubated in the dark with either PH or NPe6 (25 µg/ml) for 16 h at 37°C in media supplemented with 5% FCS, incubated for 30 min in fresh growth media, and exposed to red light. Cells incubated with PH were exposed to broad-spectrum red light (570–650 nm, 0.35 mW/cm²) generated by a parallel series of 30-W fluorescent bulbs. NPe6-treated cells were exposed to 665 nm light (2 mW/cm²) generated by an argon-pumped dye laser. Treated cells were refed with either complete growth media to determine clonogenic survival and COX-2 expression or serum-free media for analysis of PGE2 secretion. Cells were incubated with NS-398 (100 µM) immediately after light treatment in selected experiments. In vivo PDT tumor treatments were performed as reported previously on mice with s.c. tumors measuring 6–7 mm in the largest diameter. Briefly, mice received an i.v. injection of PH (5 mg/kg) followed 24 h later with nonthermal laser tumor irradiation using an argon-pumped dye laser emitting red light at 630 nm (23, 24). Light was delivered via a quartz fiber micro lens delivery system, and outputs were measured with a power meter. A light dose rate of 75 mW/cm² and total light doses of 200 and 300 J/cm² were used. Tumors were treated with PDT and monitored 3 times/week for detection of tumor recurrence. Cure was defined as being disease free for up to 90 days after PDT. COX-2 inhibitor treatment was performed using NS-398. The COX-2 inhibitor was administered by multiple i.p. injections at a dose of 10 mg/kg immediately after light exposure (time 0); at 4, 24, and 48 h after light exposure; and every other day for 20 days after treatment.

**PDT-mediated Normal Skin Response.** Normal skin response to PDT was evaluated in albino Swiss Webster mice as described previously (26). The right hind limb of each mouse was treated with PDT in a manner identical to that described above in the tumor response protocol. A quantitative skin scoring system monitoring the appearance and decline of edema, erythema, and desquamation was used to determine the degree of normal tissue damage induced by each treatment.

**Nuclear Run-off.** Transcription rate was measured by nuclear run-off as described previously (27). Briefly, cells were collected in cold PBS, pelleted and resuspended in reticulocyte standard buffer [10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.1 mM CaCl₂] with 5% NP40. Nuclei were collected and stored in nuclei storage buffer [40% glycerol, 50 mM Tris (pH 8.3), 5 mM MgCl₂, and 0.1 mM EDTA]. Nascent RNA was labeled with [α-³²P]UTP. Nitrocellulose filters were blotted with 10 µg of COX-2 and glyceraldehyde-3-phosphate dehydrogenase cDNA, and equal amounts of labeled RNA (5 × 10⁵ cpm) were used for overnight hybridization.

**Western Immunoblot Analysis.** In vitro and in vivo COX-2 expression was documented by Western immunoblot analysis. RIF cells were collected at various times after treatments in a SDS lysing buffer [4% SDS, 0.125 M Tris base, 10% glycerol, 4% 2-mercaptoethanol, and 0.02% bromphenol blue (pH 6.8)]. RIF tumors were homogenized with a Polytron in 1× reporter lysis buffer (Promega, Madison, WI), and all samples were evaluated for protein expression as described previously (23, 24). Protein samples were size-separated on 10% discontinuous polyacrylamide gels and transferred overnight to nitrocellulose membranes. Filters were blocked for 2 h with 5% nonfat milk and then incubated for 3 h with either

![Fig. 1. Nuclear run-off and Western analysis demonstrating transcriptional activation of cox-2 and inducible COX-2 expression in cancer cell lines treated with PDT. A. RIF cells were incubated with PH (25 µg/ml) for 16 h and then exposed to 315 J/m² red light. Cell nuclei were collected 1.5 or 3 h after treatment, and newly synthesized [³²P]-32 P]UTP.](cancerres.aacrjournals.org)
Results

PDT Induces Strong and Prolonged COX-2 Expression in Cancer Cells. Numerous physiological stimuli induce COX-2 expression. We previously performed a gene expression analysis of PDT-treated RIF cells and observed a 2.5-fold increase in COX-2 mRNA expression. To determine whether PDT activates COX-2 transcription, we performed a nuclear run-off experiment. Fig. 1A shows enhanced COX-2 transcription in RIF cells treated with a PDT dose resulting in 30% clonogenic survival. We also performed Western immunoblot analysis on RIF cells treated with PDT to evaluate protein expression patterns. Fig. 1B shows COX-2 expression as a function of PDT dose for cells collected 24 h after light treatment. COX-2 expression increased with PDT for treatment doses corresponding from 95% survival (105 J/m²) to 5% survival (420 J/m²). Control conditions such as photosensitizer alone or light alone (data not shown) did not induce COX-2 expression above basal levels. Fig. 1C shows kinetic data for COX-2 expression in RIF cells after PDT using a 315 J/m² light dose. Increased COX-2 levels were still observed 192 h (8 days) after light treatment and assayed for COX-1, COX-2, and actin levels by Western immunoblot.

a mouse monoclonal anti-COX-2 (clone 33; BD Transduction Laboratories, San Diego, CA) or a rabbit polyclonal anti-COX-1 (1601009; Cayman Chemical Co.) antibody to document COX-2 and COX-1 expression in RIF cells. A goat polyclonal anti-COX-2 antibody (sc-1745; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to document COX-2 expression in RIF tumors. Filters were then incubated with an antistreptavidin, antirabbit, or goat peroxidase conjugate (Sigma, St. Louis, MO), and the resulting complexes were visualized by enhanced chemiluminescence autoradiography (Amersham Life Science, Chicago, IL). Protein loading was evaluated by incubating the filters with a mouse monoclonal anti-actin antibody (clone C-4; ICN, Aurora, OH).

ELISAs. A PGE₂ enzyme immunoassay kit (Cayman Chemical Co.) was used to quantify PGE₂ concentrations in culture media and in tumor extracts. A Quantikine M mouse VEGF ELISA kit (R&D System, Minneapolis, MN) was used to quantify VEGF levels in tumor extracts from control and treated mice. Results were normalized to protein concentrations.

Statistics. Statistical analysis of tumor cure rates was based on the log-rank test. Statistical significance of the normal skin response, PGE₂ levels, and VEGF levels were obtained by two-way ANOVA followed by an unpaired Bonferroni multiple comparison t test for analysis of differences between two groups. Results with P < 0.05 were considered significant.

Statistical significance of the normal skin response, PGE₂ levels, and VEGF levels was used to quantify VEGF levels in tumor extracts from control and treated mice. Results were normalized to protein concentrations.
ENHANCING PDT WITH COX-2 INHIBITION

**Fig. 1.** Inducible COX-2 expression in tumors treated with PDT using the chlorin-based photosensitizer NPe6 could also induce COX-2 expression. As shown in Fig. 1E, inducible COX-2 expression was also observed after PDT with this second-generation photosensitizer. The decrease in COX-2 expression at the highest NPe6 PDT dose is likely due to excessive toxicity and a concomitant decrease in inducible protein expression. These data indicate that our initial findings can be extended to multiple cell lines and photosensitizers with differing subcellular targets and confirm that PDT can effectively activate COX-2 expression.

**PDT Induces Biologically Active COX-2 but not COX-1.** COX-2 is a rate-limiting enzyme in the biosynthesis of PGs such as PGE2. To evaluate the biological functionality of the COX-2 expressed after PDT, we assayed for PGE2 levels released into the supernatant of control and treated cells. Fig. 2A shows that PGE2 levels were significantly increased after PDT, and this increase followed a PDT dose-dependent pattern. Basal PGE2 levels were detected in growth medium collected from untreated cells and cells treated with PH alone, and levels increased more than 3-fold with PDT dose. To confirm that the increase in PGE2 was related to COX-2, we included the selective COX-2 inhibitor in the experimental protocol. Selective COX-2 inhibitors such as NS-398 function by binding to a hydrophilic pocket of the protein and blocking enzymatic function. This enzymatic blockade leads to a reduction in the biosynthesis of PGs. Fig. 2A also shows that incubation of PDT-treated cells with NS-398 (100 μM) blocked PGE2 secretion in both treated and untreated cells. This indicates that COX-2 was directly involved in the PGE2 released by RIF cells. Interestingly, PDT-mediated COX-2 protein expression was also attenuated by NS-398 as shown in Fig. 2B. NS-398 does not function at the level of gene transcription or translation. Our observation is therefore consistent with previous reports indicating that the COX-2 product PGE2 is involved in a positive feedback loop in activating COX-2 transcription and/or stabilizing COX-2 mRNA (28). We also examined whether PDT affected expression of the COX-1 isoform. COX-1 is constitutively expressed at low levels in most cells. Fig. 2C shows Western immunoblots of both COX isoforms in control and PDT-treated RIF cells in either the presence or absence of NS-398. Basal COX-1 expression was observed in RIF cells, but this expression was not affected by PDT, nor was it inhibited by NS-398. These results indicate that PDT induces only the COX-2 isoform of COX.

**In Vivo PDT Induces Biologically Active COX-2 Expression in Treated RIF Tumors.** We next examined whether COX-2 expression and function were modulated in solid tumors after PDT. Western immunoblot analysis was used to measure COX-2 expression in control and PDT-treated RIF tumors growing in C3H mice. Fig. 3A shows that tumors treated with PDT and collected 24 h after light exposure exhibited elevated COX-2 levels. The biological activity of the expressed COX-2 was determined by assaying for PGE2 levels in tumor lysates. The COX-2-selective inhibitor NS-398 was also included in these experiments. Fig. 3B shows that increased PGE2 levels were observed in tumor lysates 24 h after PDT, and this agrees with our in vitro data. Likewise, the COX-2-selective inhibitor NS-398 was again effective at reducing the PDT-mediated increase of PGE2 in treated tumors. These results indicate that in vivo PDT induces biologically active COX-2. COX-2 expression has been implicated in various physiological activities including tumor angiogenesis. Induction of COX-2 and PGE2 is often associated with increased expression of angiogenic factors, and inhibition of COX-2 can induce multiple physiological responses including an antiangiogenic reaction. Fig. 3C shows that PDT treatment induced increased expression of VEGF in RIF tumors and that VEGF levels were attenuated by administration of NS-398 to treated mice. These results indicate that COX-2 inhibi-
tion in PDT-treated tumors may involve modulating expression of angiogenic biomolecules.

**COX-2 Inhibition Enhances PDT Responsiveness in RIF Tumor but not in Normal Tissue.** Inhibitors of COX-2 are currently being examined in a combined modality approach with chemotherapy or radiation therapy to improve antitumor responses in established tumors. We observed that PDT activated biologically functional COX-2 in treated tumors, and this response could be blocked with the selective COX-2 inhibitor NS-398. We next examined the tumoricidal effect of combining PDT with NS-398 in a mouse tumor model. RIF tumors measuring 5–7 mm in diameter were treated with PH PDT using light doses of either 200 or 300 J/cm². In some mice, NS-398 was administered as multiple i.p. injections (10 mg/kg/injection) starting immediately after PDT; 4, 24, and 48 h after PDT; and then once every other day up to day 20. Additional mice were treated with NS-398 alone. All treated mice were monitored for tumor growth during a 90-day evaluation period. Results of these experiments are shown in Fig. 4, A and B, and depict tumor-free mice as a function of days after treatment. PDT alone using the 200 J/cm² light dose did not produce any long-term cures, whereas PDT delivered at 300 J/cm² resulted in a 25% cure rate. Interestingly, the combination of PDT plus NS-398 resulted in statistically significant increases in tumor cures for both PDT doses. Administration of NS-398 alone did not affect tumor response (data not shown). These results demonstrate that selective COX-2 inhibition can enhance PDT-mediated tumor response. However, in order for a combination therapy approach to be beneficial, the enhanced response in tumor toxicity must be greater than any potential enhanced response observed in dose-limiting normal tissue. We next evaluated the effect of NS-398 on PDT-mediated normal skin damage. Fig. 3C shows the average skin response as function of time after either PDT or PDT plus NS-398 applied the same dose and multidelivery schedule used for tumor treatments. The expected skin photosensitization (edema, erythema, and desquamation) was observed after PDT. However, no significant differences in the magnitude or repair of skin damage were observed between the two treatment groups. These results demonstrate that a selective COX-2 inhibitor does not alter PDT-associated normal skin photosensitization response while enhancing the therapeutic effectiveness of PDT in treating solid tumors.

**Discussion**

The main finding of this study is that a combination therapy approach using PDT and a selective COX-2 inhibitor was more effective than either procedure alone at producing long-term tumor cures. Our results also indicate that this combination protocol does not adversely affect normal skin photosensitization. These observations may have significant translational importance for the development of improved clinical treatment regimens. PDT is currently used as a stand-alone procedure in the clinical treatment of a variety of solid tumors, but recurrences can still arise (1, 2). Likewise, COX-2 inhibitor procedures are now being examined for the clinical prevention and treatment of tumors (14–17). Inhibitors of COX-2 are being shown to be most effective in improving tumor responsiveness when combined with conventional therapies such as chemotherapy and/or radiation therapy (20–22). Our results strongly support the consideration and development of protocols to evaluate the clinical efficacy of combining PDT with COX-2 inhibitor therapy.

Our study also demonstrated that PDT is an efficient inducer of biologically active COX-2 expression. These results were obtained using multiple cell lines and two photosensitizers with differing subcellular localization sites and targets. PH localizes primarily in the mitochondria, whereas NPe6 localizes in the lysosomes (2). It appears that PDT can therefore be added to the growing list of therapeutic, physiological, and environmental stimuli that activate COX-2 expression. Our nuclear run-off data indicate that PDT-mediated COX-2 expression is due in part to transcriptional activation. The 5′-flanking region of the COX-2 gene contains regulatory elements including a TATA box, E box, CRE motifs, AP-1 site, C/EBP (NF-IL-6) motifs, and NF-kB sites (8, 9). PDT induces both oxidative stress and tumor tissue hypoxia, which can activate a number of transcription factors and signaling pathways involving these regulatory elements (2). Studies are currently under way to identify the specific transcriptional regulatory elements, transcription factors, and signaling pathways responsible for PDT-induced COX-2 expression. However, PDT may be involved in both transcriptional and posttranscriptional pathways of COX-2 activation. Our results show extended expression of COX-2 after PDT, which may suggest mRNA stabilization. Recently, it has been shown that PGE₂ is involved in stabilization of COX-2 mRNA (28). It is therefore feasible that PDT-mediated PGE₂ synthesis is involved in a positive feedback loop resulting in COX-2 mRNA stabilization. Studies are also under way to define the role that mRNA stabilization plays in PDT-mediated COX-2 expression.

The mechanism(s) by which COX-2 inhibition enhances PDT responses is currently unclear. Numerous studies indicate that COX-2 expression modulates a variety of biological actions in tumor tissue including angiogenesis and apoptosis (16–19). There is growing evidence that COX-2 expression is associated with increased activation of multiple angiogenic factors and that COX-2-selective inhibitors can decrease expression profiles of angiogenic peptides (16). In the current study, we show that PDT induces VEGF expression in treated tumors and that NS-398 attenuated the VEGF expression. This finding is in agreement with other studies that have shown that COX-2 expression leads to increased VEGF synthesis and that inhibition of COX-2 reverses this response. Therefore, COX-2 inhibitor treatment may be enhancing PDT in part by an antiangiogenic pathway. We have previously shown that selective inhibitors of VEGF can improve PDT-mediated tumoricidal response, and therefore an antiangiogenic role of COX-2 inhibition is likely to be involved in the observed improvement in PDT (24). Whereas COX-2 inhibitors can decrease angiogenic pathways, it is also likely that these inhibitors enhance tumor response by increasing apoptosis in target tissue. Additional studies are needed to determine the actual cytotoxic mechanisms of action of combining PDT with COX-2 inhibitors.

In summary, this study demonstrated that PDT was a strong inducer of only the COX-2 isoform of COX and that PDT-mediated COX-2 expression was due in part to transcriptional activation. PDT-induced COX-2 was biologically active, as shown by the increased synthesis and release of PGE₂. COX-2 is also a therapeutically relevant enzyme for evaluation with PDT. The selective COX-2 inhibitor, NS-398, decreases PDT induction of PGE₂ and VEGF, and NS-398 treatments enhance the tumoricidal action of PDT without affecting normal tissue repair. These results strongly support the initiation of clinical studies combining PDT with selective COX-2 inhibitor therapy.

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**References**

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