Up-regulation of Cyclooxygenase-2 and Apoptosis Resistance by p38 MAPK in Hypericin-mediated Photodynamic Therapy of Human Cancer Cells

Received for publication, July 15, 2003, and in revised form, October 3, 2003
Published, JBC Papers in Press, October 13, 2003, DOI 10.1074/jbc.M307591200

Photodynamic Therapy (PDT) is an approved anticancer therapy that kills cancer cells by the photochemical generation of reactive oxygen species following absorption of visible light by a photosensitizer, which selectively accumulates in tumors. We report that hypericin-mediated PDT of human cancer cells leads to up-regulation of the inducible cyclooxygenase-2 (COX-2) enzyme and the subsequent release of PGE₂. Dissection of the signaling pathways involved revealed that the selective activation of p38 MAPK α and β mediate COX-2 up-regulation at the protein and messenger levels. The p38 MAPK inhibitor, PD169316, abrogated COX-2 expression in PDT-treated cells, whereas overexpression of the drug-resistant PD169316-insensitive p38 MAPK α and β isoforms restored COX-2 levels in the presence of the kinase inhibitor. Transcriptional regulation by nuclear factor-κB was not involved in COX-2 up-regulation by PDT. The half-life of the COX-2 messenger was drastically shortened by p38 MAPK inhibition in transcriptionally arrested cells, suggesting that p38 MAPK mainly acts by stabilizing the COX-2 transcript. Overexpression of WT-p38 MAPK increased cellular resistance to PDT-induced apoptosis, and inhibiting this pathway exacerbated cell death and prevented PGE₂ secretion. Hence, the combination of PDT with pyridinyl imidazole inhibitors of p38 MAPK may improve the therapeutic efficacy of PDT by blocking COX-2 up-regulation, which contributes to tumor growth by the release of growth- and pro-angiogenic factors, as well as by sensitizing cancer cells to apoptosis.

Photodynamic therapy (PDT) is an accepted therapeutic procedure suitable for the treatment of a variety of tumors and non-malignant disorders. PDT involves the administration and subsequent light activation (600–850 nm) of a photosensitizing compound (photosensitizer), which specifically accumulates in tumors (1, 2). This leads, in the presence of molecular oxygen, to the photochemical generation of reactive oxygen species, which ultimately kills the target cells (1, 2).

Hypericin is a naturally occurring photosensitizer synthesized by Hypericum plants (St. John’s wort). The interesting photosensitizing properties of hypericin together with its selective uptake in tumor tissues and its minimal dark cytotoxicity, have encouraged the evaluation of this hydroxylated phenanthroperylenequinone in the photodynamic treatment of cancers (3).

Although hypericin does not preferentially accumulate in mitochondria but localizes mainly to the membranes of the endoplasmic reticulum and Golgi apparatus, a rapid release of cytochrome c into the cytosol and the subsequent activation of the apoptosome, are the signaling events precipitating apoptotic cell death by the photodynamic stress (4–7). Since apoptosis is a major in vivo response to hypericin-mediated PDT (8), modulation of apoptotic vulnerability is critical for the therapeutic efficacy of this drug.

Cellular commitment to apoptosis, or the ability to evade apoptosis in response to cell damage, involves the integration of a complex network of both survival and death pathways. The MAPK family a central mediator of survival and cell death pathways, and MAPKs have been implicated in the response of tumor cells to very diverse antitumor signals, including PDT (2).

The three major MAPK pathways in mammalian cells include the extracellular signal-regulated kinases (ERKs), the c-Jun NH₂-terminal protein kinases (JNKS), and the p38 MAPKs (9). ERKs are acutely stimulated by growth and differentiation events through activated receptor tyrosine kinases, heterotrimeric G protein-coupled receptors, or cytokine receptors (9). The JNKS and p38 MAPKs are activated in response to a variety of stress signals including UV irradiation, chemotherapy, osmotic stress, hypoxia/anoxia, hyperthermia, and they have been involved in the regulation of apoptosis (9).

In a previous study we have shown that, parallel to the...
capase-activation cascade, hypericin-mediated PDT leads to inhibition of ERK2 (6), and to a sustained activation of the stress-activated protein kinases, JNK1 and p38 MAPK. Activation of JNK1 and p38 MAPK was suggested to have a protective role against PDT-induced apoptosis (6). However, downstream targets of these signaling cascades still need to be identified.

Some recent reports have implicated p38 MAPK in the up-regulation of the inducible cyclooxygenase-2 (cox-2) gene (10–14). COX catalyzes the conversion of arachidonic acid to prostaglandin (PG) H₂, the immediate substrate for a number of cell specific prostaglandin and tromboxane synthases, leading to the production and release of PGE₂ and other prostanoids, that contribute to the development of important immunomodulatory responses (15–17). Two isoforms, COX-1 and COX-2, have been identified and their expression is differently regulated. COX-1 is constitutively expressed in most cell types and may be responsible for housekeeping functions. By contrast, the expression of COX-2, which is regulated both at the transcriptional and post-transcriptional level, is barely detectable in normal tissues but is rapidly induced in response to tumor promoters, oncogenes, cytokines, and mitogens (15–17).

In a number of cellular and animal models, COX-2 has been shown to promote cell growth, to inhibit apoptosis, and to enhance cell motility and adhesion, and COX-2 overexpression is sufficient to cause tumorigenesis (reviewed in Refs. 16 and 17). In agreement with these observations, COX-2 is up-regulated in many tumors, and this up-regulation has been shown to promote cancer progression, recurrence, and invasiveness, as well as angiogenesis (16, 17). These studies suggest that the use of specific COX-2 inhibitors may be beneficial in the management of these neoplasms.

Interestingly, the combination of porphyrin- and chlorin-based PDT with a specific COX-2 inhibitor, was recently reported to increase the tumoricidal potential of PDT in vivo (18). However, the mechanisms underlying PDT-mediated COX-2 up-regulation and its protective effect were not further explored.

In the present study, we show that hypericin-mediated PDT leads to COX-2 up-regulation and secretion of PGE₂, which are completely dependent on the specific activation of p38 MAPK. We also provide evidence in support for a post-transcriptional role of p38 MAPK in stabilizing COX-2 mRNA and for a functional involvement of p38 MAPK in counteracting PDT-induced apoptosis. Our results suggest that inhibition of the p38 MAPK signaling cascade, which results in the repression of COX-2 up-regulation and concomitant increase in the susceptibility of the cells to undergo apoptosis, could be of clinical relevance to improve the efficacy of hypericin-mediated PDT of cancers.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hypericin was synthesized and purified as described in Ref. 8. All cell culture products were obtained from Bio-Whittaker (Verviers, Belgium). The p38 MAPK inhibitor PD98059, the selective COX-2 inhibitor NS-398 were purchased from Calbiochem (San Diego, CA), whereas the inhibitor of IκB kinase BAY117085 and the selective inhibitor of PI3-kinase LY294002 were from BIOMOL Research Laboratories (Plymouth, PA). Sytox Green was purchased from Molecular Probes (Leiden, The Netherlands). Actinomycin D was from Sigma. The fluorogenic caspase substrates Ac-DEVD-amc and Ac-IETD-amc were obtained from Peptide Institute, Inc. (Osaka, Japan). PARP antibody was from BIOMOL Research Laboratories (Plymouth, PA), anti-cytochrome c was obtained from Pharmingen (San Diego, CA), and anti-caspase-3 antibody and anti-COX-2 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p38 MAPK (Thr180/Tyr182) monoclonal antibody, which specifically recognizes the phosphorylated form of the kinase and anti-p38 MAPK antibody were purchased from New England Biolabs, Inc. (Beverly, MA). Monoclonal anti-α-tubulin antibody was from Sigma. Horseradish peroxidase-conjugated secondary antibodies were from DAKO (Denmark). GST-Jun-(1–223) and polyclonal antibodies to the human JNK1 and ERK2 were prepared as described elsewhere (6).

**Cell Culture**—HeLa cells (human cervix carcinoma cells) and T24 (human transitional cell carcinoma of the urinary bladder) were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle’s medium containing l-glutamine (2 mm), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal calf serum. Stable HeLa transfectants overexpressing mutated HA-tagged IκBα, were obtained as described in Ref. 19.

**Cell Photosensitization**—The cells were preincubated with hypericin for 16 h in subdued light conditions (< 1μW/cm²) and subsequently irradiated in Dulbecco’s modified Eagle’s medium by placing the sample, 5 cm above a set of seven L18W30 fluorescent lamps (Osrana) as described in Vantieghem et al. (4). The maximal emission of the fluorescent lamps was between 530 and 620 nm, which coincides with the major absorption peaks of hypericin (545 and 595 nm). At the surface of the diffuser, the uniform fluence rate was 4.5 mW/cm², as measured with an IL 1400 radiometer (International Light, Newport, MA). All inhibitors used were added to the cell culture medium 1 h prior to photosensitization at concentrations indicated in figure legends.

**Preparation of Cell Extracts and Western Blotting**—Cell extracts were prepared at indicated time points following photosensitization, and analysis of cytochrome c release was performed as described in Refs. 6 and 7. Protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce). Samples (30–80 μg of protein) were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose (Protran®), and processed as in Refs. 6 and 7.

**Caspase Assay**—Caspase activity was measured using the fluorogenic substrate Ac-DEVD-amc as described in Ref. 6.

**Immunoprecipitation and Protein Kinase Assay**—JNK1, ERK2, and p38 MAPK activities were measured as previously described in Refs. 6 and 7.

**Assessment of Cell Viability**—Cell viability was estimated by 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism as previously described (7).

**Transient Transfections**—The drug-resistant (DR) p38α was produced by mutating Thr106, His107, and Leu108 to Met, Pro, and Phe, respectively. In the case of the DR p38β, Thr50, Thr107, and Leu108 were changed to Met, Met, and Phe, respectively. Mutagenesis was performed with the QuikChange mutagenesis kit (Stratagene). DR p38α and p38β mutants were generated using complementary primers (only the forward primer is shown) 5'-caggtctacctgctgtttatgggcgccgacctgaacaa-3' and 5'-gaattctgtttagtggatatgctgagcgacgatcggacgctgaccaacatcg-3', respectively. FLAG-tagged p38α and FLAG-tagged p38β were kindly provided by Dr. J. Han (Scripps Institute, CA). For transient transfection cells were seeded at 1.0 × 10⁵ (T24) or 7.0 × 10⁴ (HeLa) cells/cm² Petri dish. After an overnight culture, pcDNA3, WT p38, MKK3, DR p38α, and pEGFP-C1 were transfected in 6-well plates with the FuGENE 6 transfection reagent (Roche Applied Science, Vilvoorde, Belgium) (HeLa cells) according to the manufacturer’s protocol. After 7 h of transfection the medium was changed with complete Dulbecco’s modified Eagle’s medium. Twenty-four hours after transient transfection, cells were incubated with or without hypericin for 16 h at 37 °C in dark. Cells were then irradiated as described above. Transfection efficiency was determined by calculating the percentage of fluorescent green cells over the total number of cells under a fluorescence microscope. Total lysates were prepared and analyzed by Western blotting.

**Cell Cycle Analysis**—Cell cycle distribution was determined by flow cytometry after staining of the DNA with Sytox Green according to Vantieghem et al. (7).

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were obtained, and EMSA was performed as described by Volanti et al. (20). DNA-protein complexes were resolved by nondenaturing PAGE. Gels were stained with the Fuji x-ray films. The sequence of the probes (Eugentech, Liége, Belgium) used was: NF-κB probe, 5'-GGTTGAGGAGATTTCCGCAGG-3'.

**PGE₂ Measurement**—Cells were photosensitized as described before. After the indicated time points, the medium was collected, dead cells were removed by centrifugation, and PGE₂ concentrations were measured using PGE₂, ELISA Kit (R&D Systems, Abingdon, UK) according to manufacturer’s instructions.

**RT-PCR**—After indicated time points, total RNA was isolated with the RNeasy Mini Kit (Qiagen, West Sussex, UK) and spectrophotometrically quantified, and the RT-PCR reaction was performed with the
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Fig. 1. Hypericin-mediated PDT induces apoptotic cell death in T24 cells via the intrinsic pathway of caspase activation. T24 cells were incubated for 16 h with 150 nM hypericin and then irradiated with a light dose of 4 J/cm² as described under “Experimental Procedures.” A, phase contrast microscopic analysis of untreated T24 cells (ctr) and PDT-treated cells at different time points after irradiation as indicated. B, at the indicated time points after irradiation, cells were harvested as described under “Experimental Procedures” and analyzed for the presence of cytosolic cytochrome c, caspase-3, and PARP cleavage by Western blot using specific antibodies. C, left panel shows the DEVD-directed caspase-3 and IETD-directed caspase-8 activity at different time points post-irradiation; right panel shows the hypericin dose-dependent loss of cell viability 24 h after irradiation as determined by MTT metabolism. Error bars represent S.D. of at least three independent experiments.

ThermoScript™ RT-PCR System (Invitrogen) according to manufacturer’s instructions. The reactions were heated at 94 °C for 3 min and then immediately cycled 26 times through a 30 s denaturing step at 94 °C, a 30 s annealing step at 55 °C and a 30 s extension step at 72 °C. After the cycling procedure, a final 10 min elongation step at 72 °C was performed. The following primers were used: COX-2, (F) 5'-TTCAATGAGATTCTGCGAAAAT-3' and (R) 5'-AGATCATCTCTGCCTGAGT-3'; GADPH, (F) 5'-GGCTACAGCACGCCCTTCATTGACC-3' and (R) 5'-GAAGGGCATGGCAGCTTCC-3'.

RNA Stability Assay—The cells were treated as described before. 5-h post-irradiation, the medium was changed to fresh medium containing actinomycin D (3 μg/ml) with or without the p38 MAPK inhibitor PD169316. At different time intervals, cells were collected for RNA preparation. RNA was examined by RT-PCR analysis.

RESULTS

PDT with Hypericin Induces Apoptosis in Cancer Cells via the Intrinsic Pathway of Caspase Activation—Dose- and time-dependent studies of the cell death response in the human TCC cell line T24, showed an accumulation of apoptotic cells starting at 3 h post-irradiation, using 150 nM as the optimal lethal hypericin concentration in combination with 4 J/cm² light dose (Fig. 1, A and C). T24 cells treated with 150 nM hypericin and irradiated, showed a time-dependent increase in the amount of cells with apoptotic morphology, which was paralleled by the increase in cytosolic cytochrome c and cleavage of procaspase-3 and its downstream substrate PARP (Fig. 1B). Apoptotic cell death could be blocked by the cell-permeable caspase inhibitors z-VAD-fmk and z-DEVD-fmk (data not shown), confirming that PDT-induced apoptosis in T24 cells is a caspase-dependent process. As shown in Fig. 1C, activation of procaspase-3 was clearly detectable 7 h after irradiation whereas a negligible increase in caspase-8 activity could be measured 24 h after PDT, likely as a result of a caspase-3 feedback activation loop (21). Similar kinetics of cytochrome c release and dependence on procaspase-3 activation for the progression of apoptosis, have been shown in HeLa cells (4, 6), indicating that the main mechanism triggering apoptotic cell death in response of hypericin-mediated PDT is conserved in a variety of human cancer cells.

Hypericin-mediated PDT of Cancer Cells Leads to Up-regulation of COX-2—Cyclooxygenase-2 is an inducible enzyme whose up-regulation has been linked to tumor recurrence and progression in different cancers, including TCC of the human urinary bladder (22). Since in an experimental animal system hypericin-based PDT of bladder TCC has been shown to be only partially effective because of recurrence (23), we set out to investigate whether photosensitization of T24 and HeLa cells with hypericin could cause COX-2 up-regulation.

Fig. 2A shows that while the basal level of COX-2 in the untreated T24 and HeLa cells was hardly detectable, PDT promoted a time-dependent and sustained increase in COX-2 protein levels. In both cancer cell lines the induction of COX-2 protein was detectable within 3 h after irradiation, attained a steady state at 7–16-h post-irradiation, reaching more than a 12-fold induction, and declined very gradually thereafter (Fig. 2A). Dose response experiments (data not shown) revealed COX-2 induction by hypericin concentrations starting from sublethal doses of 25 nM, corresponding to 80% cell survival
Fig. 1C), and increasing up to 150–200 nM, which corresponds to ~20% cell survival. In all cases this response was totally light-dependent.

Since COX-2 is the rate-limiting enzyme in the biosynthesis of PGE$_2$, we also evaluated the release of this prostaglandin into the medium at the post-irradiation time corresponding to maximal COX-2 induction. As compared with control cells, the levels of secreted PGE$_2$ increased severalfold following exposure of the cells to PDT (Fig. 2B). In the presence of the methyl sulfoxide NS-398, a selective COX-2 inhibitor (24), the release of PGE$_2$ was totally blocked (Fig. 2B), demonstrating that the induction of PGE$_2$ synthesis coincides with up-regulation of COX-2 protein levels in response to the photodynamic stress.

**Cyclooxygenase-2 Is Selectively Up-regulated by the PDT-induced p38 MAPK Signaling Pathway**—It has been reported that in T24 cells, which bear an oncogenic H-ras mutation, the MEKK1-p38 MAPK and the Raf-ERK cascades are constitutively activated (25). In agreement with this, we found that in untreated T24 cells the activity state of p38 MAPK and ERKs (Fig. 3) was elevated. However, whereas the activity level of ERKs was rapidly reduced by PDT, the basal phosphorylation level of p38 MAPK dropped drastically to an undetectable level, to be followed by a de novo p38 MAPK activation, which was specifically mediated by PDT stress (Fig. 3). Interestingly, PDT of HeLa cells, with either membrane- or mitochondria-targeted photosensitizers, has been shown to cause an immediate dose-dependent attenuation of the EGF- or inflammatory cytokine-mediated signal through rapid degradation of the EGF receptor or down-modulation of cytokine-responsive signaling pathways (26). These PDT-mediated early effects could indeed explain
why in HeLa (6) and T24 cells (Fig. 3) we observed a down-regulation of the basal activity levels of ERK and p38 MAPK immediately after irradiation.

No difference in the protein levels was observed as shown for p38 MAPK (Fig. 3), indicating that PDT effects are due to changes in the phosphorylation/activity state of these signaling enzymes exclusively (data not shown).

To delineate the contribution of these protein kinases to the PDT-mediated up-regulation of the COX-2 protein levels, we used as a first approach cell-permeable chemical inhibitors of each protein kinase cascade. Western blot analysis (Fig. 4A) showed no substantial difference in the COX-2 level in PDT-treated cells in the presence of the MEK-1 inhibitor, PD98059. The JNK inhibitor SP600125 did not affect the level of COX-2 by PDT either and neither did the PI3K inhibitor LY294002, although PDT treatment did reduce the PI3K-mediated activation of AKT (data not shown).

Preincubation of the cells with 1 μM PD169316, a specific p38 MAPK inhibitor, prior to irradiation, significantly blocked COX-2 up-regulation in both cell lines (Fig. 4A), suggesting an important and universal role for this signaling pathway in this PDT-mediated cellular response. It should be mentioned that at the concentration used in this study (e.g. 1 μM), the PD169316 p38 MAPK inhibitor did not show any nonspecific effects against JNK activity, whereas it clearly blocked p38 MAPK as measured by an in vitro kinase assay (data not shown).

The effect of PD169316 on the COX-2 mRNA level was further investigated by RT-PCR analysis. As shown in Fig. 4B, PD169316 blocked PDT induction of steady-state levels of COX-2 mRNA, mirroring that of protein levels.

Intriguingly, while the basal phosphorylation/activation state of p38 MAPK in the untreated T24 cells was not sufficient per se to cause COX-2 expression, the superimposed p38 MAPK activation induced by hypericin-based PDT led to a dramatic increase in the COX-2 protein levels. Although the molecular mechanism of such an observation is still elusive, it may suggest the existence of a negative feedback mechanism controlling COX-2 levels in the T24 cells in the absence of stress.

In order to strengthen the involvement of the p38 MAPK as an important upstream regulator of the signaling cascade leading to COX-2 up-regulation, we examined PGE$_2$ secretion in cells pretreated with either NS-398 or PD169316. Fig. 4C shows that inhibition of p38 MAPK drastically impaired the release of PGE$_2$, providing a further link between p38 MAPK activation and COX-2 induction.

Given the apparent role of p38 MAPK in the up-regulation of COX-2 by PDT, we endeavored to confirm the specificity of the inhibitory effects of PD169316 by transfecting cells with con-
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NF-κB, which is a well known oxidative stress-induced transcription factor (29). This transcription factor is a good candidate since the cox-2 promoter contains a NF-κB enhancer sequence (15), NF-κB can be activated by PDT (19), and p38 MAPK can induce the transcriptional function of NF-κB by stimulating the RelA/p65 transactivation subunit of NF-κB (30, 31). To this end, nuclear extracts of PDT-treated cells were prepared at different post-irradiation time points and analyzed by EMSA. TNF-α, a powerful activator of NF-κB, was used as a positive control.

Whereas treatment of cells with TNF-α rapidly elicited binding of nuclear proteins to the NF-κB site of the cox-2 promoter in both cell lines, PDT caused significant retardation of the NF-κB probe only 24 h after irradiation (Fig. 6A). Competition experiments carried out with a wild-type or a mutated unlabeled NF-κB probe (data not shown) proved that the upper retarded band was specific whereas the lower one (indicated as n.s. in Fig. 6A) was not. Consistent with results obtained by EMSA, degradation of the cytoplasmic NF-κB inhibitor IκBα occurred only at late time points, starting 16 h after PDT (Fig. 6B).

The late kinetics of the PDT-mediated activation of NF-κB do not correlate with the earlier onset of the COX-2 induction (Fig. 4), which was already detected 3 h after irradiation, suggesting that the latter cellular response is not regulated by a NF-κB-dependent pathway.

To further corroborate these observations, we monitored PDT-induced COX-2 levels in HeLa cell transfectants, which stably overexpresses the NF-κB inhibitor IκBα (19) and are devoid of NF-κB DNA binding activity (data not shown). In addition, we used the IκB-kinase chemical inhibitor BAY117085. Results clearly show that negative regulation of the NF-κB pathway, by either genetic or pharmacological means, did not alter the kinetics or the extent of COX-2 expression in cells exposed to photodynamic stress (Fig. 7). Collec-
tively taken, these observations support the concept that NF-κB is not involved in the up-regulation of COX-2 by PDT.

In addition, p38 MAPK inhibition by PD169316, had no effect on PDT-induced NF-κB activation (data not shown), further separating the mechanism of p38 MAPK-mediated COX-2 expression from the activation of this transcription factor in our cellular systems.

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**Inhibition of p38 MAPK Increases the Susceptibility of Cancer Cells to PDT-induced Apoptosis**—We further investigated the possible relevance of the p38 MAPK-COX-2 cascade in the regulation of the PDT-induced apoptosis. Increasing the expression levels of WT-p38 MAPK α, substantially lowered the efficacy of PDT-mediated apoptosis, as shown in Fig. 9 by the reduced number of transfected cells showing morphological features of apoptotic cell death.

One way by which PDT-induced p38 MAPK activation could protect cells against apoptosis, would be by enhancing the cellular levels of COX-2. FACS analysis showed a substantial increase in the amount of PDT-treated cells with a sub-G1 content of DNA, characteristic of apoptotic cells, 24 h after irradiation (Fig. 10). This sub-G1 region was considerably bigger in PDT-treated cells where the p38 MAPK was blocked by PD169316 (Fig. 10), thus confirming by a different approach the results shown in Fig. 9. As already shown in Fig. 4, inhibition of p38 MAPK in these cells also reduced the up-regulation of COX-2 expression and PGE2 secretion. However, blocking COX-2 activity by NS-398 did not result in a significant increase in cells with apoptotic DNA (Fig. 10). We also failed to see a significant anti-apoptotic effect of exogenously added PGE2, prior to PDT (data not shown).

In conclusion, our observations suggest that although p38 MAPK activation by PDT is required to up-regulate COX-2, this enzyme is not likely the direct downstream mediator of the p38 MAPK anti-apoptotic response.

**DISCUSSION**

The elucidation of the mechanism by which PDT kills cancer cells requires a deeper understanding of the signaling pathways activated by this antitumoral therapy in cancer cells. It has become increasingly clear that these cellular pathways involve not only signals precipitating cell death but also pathways that act in rescuing the photodamaged cells from apoptosis.

The TCC bladder cancer cell line T24 provides an interesting in vitro cellular model to explore at the molecular level the photodynamic actions of hypericin, because this sensitizer specifically localizes to this cancer lesion (36). Therefore hypericin-mediated PDT of bladder tumors may be a good therapeutic prospect. In an experimental animal model, hypericin-mediated PDT of urinary bladder TCC resulted in a dramatic loss of tumor cells clonogenic survival, but recurrence was found to occur (23). Tumor re-growth after PDT is suggested for the activation of cancer-rescuing responses allowing survival and further expansion of a clone of TCC cells. The primary goal of this study was to identify, at the molecular level, potential targets mediating the cancer cell adaptive responses to hypericin-mediated PDT.

An increasing number of studies suggest the involvement of COX-2 in tumor progression, and carcinogenesis and different protein kinase-mediated pathways have been shown to regulate COX-2 expression in response to different cellular stress signals (reviewed in Refs. 15–17). COX-2 protein levels are up-regulated in cancer cells by hypericin-mediated PDT in a time- and dose-dependent fashion. This COX-2 expression is remarkably dependent on the PDT stress since the basal levels of COX-2 in HeLa and T24 cells are very low or undetectable.

We have used different strategies to unambiguously show that PDT-induced up-regulation of COX-2 in cancer cells requires the activation of the p38 MAPK pathway. This conclusion was first documented by the use of pharmacological inhibitors of the p38 MAPK (PD169316), ERKs (PD98059), PI3K (LY294002), and JNK (SP600125) pathways. Only pretreatment of the cancer cells with 1 μM PD169316, a specific p38 MAPK inhibitor, blocked COX-2 up-regulation at the protein and messenger levels (Fig. 4) in both cell types. This indicates that p38 MAPK is a common target of hypericin-based PDT and a general mediator of COX-2 up-regulation in photodamaged cancer cells.

In this study we challenged the effect of the drug PD169316 as a specific inhibitor of the p38 MAPK-mediated signals by overexpressing DR mutants of the p38 MAPK α and β isoforms (27). With this strategy we sought to determine whether the...
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**Fig. 10. Effect of PD169316 and NS-398 on PDT-induced apoptosis.** Untreated and hypericin-PDT treated HeLa cells, in the presence or absence of 1 μM PD169316 or 50 μM NS-398, were analyzed by FACS for DNA content after staining with Sytox Green, 24 h after irradiation (4 J/cm²). The data were analyzed by the SYSTEM II™ software. Histograms are typical of three independent experiments, and the graph summarizes the results obtained in which error bars indicating S.D. are shown.

Inhibitory effect of PD169316 on the COX-2 expression induced by PDT could be specifically overcome by the expression of the DR p38 MAPK α and β isoforms. In cells where the endogenous p38 MAPKs were kept inhibited by the presence of the PD169316, and where COX-2 levels did not rise in response to PDT, overexpression of DR-p38 MAPK mutants re-established similar levels of COX-2 as found in PDT-treated cells in the absence of the inhibitor (Fig. 5). This clearly demonstrates that the observed effect of PD169316 is due to p38 MAPK inhibition. In addition, overexpression of the MKK3/p38 MAPK signaling module largely increased the expression of COX-2 with or without PDT treatment, further supporting the specific involvement of this signaling cascade in the up-regulation of COX-2. An important question in defining a role for the p38 MAPK signal in the PDT-mediated cellular response is clarifying how it leads to COX-2 induction.

The promoter region of the human cox-2 gene contains consensus sequences for NF-κB, NF-IL6 (C/EBPβ), and CRE sites (15). NF-κB has been reported to be important in the transcriptional activation of COX-2 in many inflammatory responses (15). NF-κB activation is initiated by inflammatory stimuli including IL-1β, TNFα, LPS (37), as well as by PDT with different photosensitizers (19, 20, 38). Since p38 MAPK has been implicated in the regulation of the NF-κB transcriptional activity (30, 31), we have evaluated the possible functional link between p38 MAPK-NF-κB activation and COX-2 expression using several different approaches.

All our results argue against the involvement of this particular transcription factor in the signaling cascade leading to COX-2 expression in our system. In cells photosensitized with hypericin, binding of nuclear proteins to the NF-κB site of the cox-2 promoter occurs with slow kinetics (16–24 h post-irradiation) whereas COX-2 protein levels are readily detectable 3 h following PDT. Moreover, in cells where the nuclear translocation of NF-κB is counteracted by overexpressing IκBα, or in cells treated with the chemical inhibitor of the IκB kinase, which prevents the phosphorylation-mediated degradation of IκBα, no alterations in COX-2 expression by PDT could be found (Figs. 6 and 7). Furthermore, inhibition of p38 MAPK did not affect NF-κB activation by PDT.

COX-2 expression can be regulated at the post-transcriptional level by signals stabilizing its rapidly degraded mRNA (10, 11, 14, 34, 35). The 3'-UTR of the COX-2 transcript contains 22 copies of an AU-rich element (ARE), consisting of the destabilizing AUUUA motif (32, 33). This motif is also found in a number of mRNAs of inflammatory mediators and immediate early genes, and has been shown to bind proteins involved in their post-transcriptional regulation. Some recent reports have implicated the p38 MAPK-MAPKAPK-2 signaling module in the stabilization of COX-2 mRNA (10, 12, 14).

Using a classical RT-PCR approach, we found that when transcription was blocked by the addition of actinomycin D, the stability of the PDT-induced COX-2 mRNAs rapidly declined when the p38 MAPK-dependent signal was inhibited by PD169316 (Fig. 8). Although the contribution of other possible p38 MAPK-regulated transcription factors that bind to the ATF/CRE responsive element of the cox-2 promoter was not explored in this study, our results unambiguously show that p38 MAPK is required to extend the half-life of the COX-2 transcript in cells exposed to PDT.

Given the prevalent role of p38 MAPK in the PDT-mediated COX-2 up-regulation, we also wanted to find out whether the p38 MAPK and COX-2 signaling cascades could be functionally linked. Blocking p38 MAPK activity in PDT-treated cells obliterated not only the induction of COX-2 at the protein and messenger levels, but also the subsequent secretion of PGE₂ (Fig. 4). Besides playing an important role in inflammatory responses, PGE₂, the major metabolite of the cyclooxygenation reaction, is also an important molecule in the control of cell growth, the prevention of apoptosis and angiogenesis, and the promotion of cell motility and adhesion (15–17). Consistent with data supporting a role of COX-2 and PGs in carcinogenesis (17), a functional link between increased levels of COX-2 and cellular resistance against apoptosis in response to a variety of extracellular and intracellular stresses has recently been established (39–42).

Hence, we explored the possibility that the p38 MAPK cascade leading to COX-2 expression was protecting cancer cells against the PDT-induced apoptosis. Increased expression of WT-p38 MAPK (Fig. 9) or blocking its activity by PD169316 (Fig. 10) resulted in a significant reduction or enhancement of the degree of PDT-induced apoptosis, respectively, supporting the involvement of this signal in a cellular response that counteracts PDT-induced apoptosis. However, blocking COX-2 ac-
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