The activation state of the members of the mitogen-activated protein kinase family following photodynamic therapy (PDT) with benzoporphyrin derivative monoacid ring A was investigated using a naturally transformed murine keratinocyte cell line, Pam 212. PDT involves the use of photosensitizer molecules and a specific wavelength of visible light. The process of PDT generates singlet oxygen and other reactive oxygen intermediates (ROIs), and the cytotoxic effect of these ROIs is the basis for the use of PDT to treat cancer and psoriasis. PDT caused a strong dose- and time-dependent activation of both stress-activated protein kinase (SAPK) and p38 HOG1. The maximum activation of SAPK and p38 HOG1 occurred between 20 and 30 min following PDT treatment with 200 ng/ml benzoporphyrin derivative monoacid ring A and 2 J/cm² of red light at 690 nm. In our system, PDT did not cause significant activation of extracellularly regulated kinase (ERK) 1 and ERK2. Under the same experimental conditions, ultraviolet light irradiation caused strong activation of SAPK and p38 HOG1 and minimum activation of ERK1 and ERK2 in Pam 212 cells. A number of ROI scavengers were tested for their effect on PDT-induced SAPK and p38 HOG1 activation. Both L-histidine and N-acetyl-L-cysteine showed a significant inhibitory effect on PDT-induced SAPK and p38 HOG1 activation. This indicated that PDT-induced SAPK and p38 HOG1 activation may be partially mediated by ROI.

The members of the mitogen-activated protein kinase (MAPK) family propagate signals originating from a large collection of different external stimuli including growth factors, hormones, cytokines, oxidative stresses, and other forms of physical and chemical stress stimuli (1). An end point of this complex signaling network is the regulation of gene expression in the nucleus. MAPKs constitute a family of multifunctional proline-directed serine/threonine kinases that phosphorylate many target proteins, including the nuclear transcription factors c-Jun and p62 TCF. MAPKs phosphorylate proteins at serine or threonine residues that are present in the context of a consensus sequence (amino acid (basic or neutral)-(serine or threonine)-proline). The activation of the MAPK requires phosphorylation of both threonine and tyrosine residues near the subdomain VIII region of its catalytic domain. This phosphorylation step is carried out by a MAPK kinase such as MEK1. The three known mammalian MAPK family members include the extracellularly regulated kinase (ERK), stress-activated protein kinase/c-Jun NH₂-terminal kinase (SAPK/JNK), and p38 high osmolarity glycerol protein kinase (p38 HOG1). ERK, SAPK, and p38 HOG1 are at the center of three distinct but closely related cytoplasmic phosphorylation cascades that link extracellular stimuli to the regulation of gene transcription (2).

The ERK pathway is considered to be involved mainly in the signal transduction of growth stimulatory signals, whereas SAPK and p38 HOG1 pathways are implicated in the transduction of stress signals and stimulation by inflammatory cytokines. Both the SAPK and p38 HOG1 pathways are responsive to stimuli such as tumor necrosis factor α (TNF-α), interleukin-1, UV irradiation, heat, change in osmolarity, and increase in intracellular reactive oxygen intermediates (ROIs) (3–7).

ROIs are partially reduced oxygen species formed during the reduction of molecular oxygen. ROIs react readily with biological macromolecules including proteins, lipids, and DNA (8). A variety of exogenous chemical and physical agents evoke oxidative stress. These include TNF-α treatment (9), UV irradiation (10), ionizing radiation (11), and photo sensitization (12). TNF-α treatment increases production of ROIs in mitochondria. The process of ionizing radiation generates highly toxic hydroxyl radicals, whereas exposure to UV causes lipid peroxidation and consequently decreases the level of intracellular antioxidants. UV-induced cellular damage is partly mediated by the production of ROIs. UV irradiation triggers a transcriptional induction response known as the “UV response” (10),

© 1996 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.
which includes the activation of transcription factors and an increased expression of the early-response genes such as c-jun, c-fos, and c-myc. The early-response genes encode the nuclear proteins that positively regulate the transcription of other effector genes encoding proteins such as antioxidant enzymes, heat shock proteins, growth regulatory proteins, major histocompatibility proteins, and proinflammatory cytokines (13). UV irradiation strongly activates both SAPK/JNK and p38 HOG1 (3, 7) and also activates Src tyrosine kinases and the Ras/Raf/MEK/ERK pathway (14, 15).

In this report, photodynamic therapy (PDT) was used as a new form of stress stimulus to activate MAPK. PDT is a new therapeutic procedure being used to treat human malignancies and psoriasis. It is based on the dye-sensitized photodestruction of biological macromolecules. This involves selective delivery of a photosensitizer dye to the targeted tissue, followed by the generation of toxic molecules upon light activation. The mechanisms of the PDT-mediated cytotoxic effect are not fully understood; however, in vitro studies support the assumption that singlet oxygen and other ROS generated during the excitation of the photosensitizer are responsible for tissue destruction (12).

Cellular responses to PDT include necrosis and programmed cell death (16), but under certain experimental conditions, PDT has been shown to regulate physiological processes. For example, PDT induces the release of soluble factors such as prostaglandin E2 and TNF-α (17, 18) and inhibits the surface expression of receptor molecules. Krummann et al. (19) reported a superoxide radical-mediated specific inhibition of the high affinity Fe receptor on human monocytes by PDT. PDT has also been shown to induce the expression of stress responsive proteins such as glucose-regulated proteins (20), heme oxygenase, and heat shock proteins (21–23). These findings indicated that, in addition to its cytotoxic effect, PDT can act as a form of exogenous stress signal to regulate cellular functions. This may have clinical implications. For instance, PDT may be used to regulate cytokine release or adhesion molecule expression by epidermal cells. This may lead to the modulation of local and systemic immune responses (24).

A number of recent publications provide further information on the molecular mechanism of the PDT effect. Luna et al. (25) reported induction of the early-response genes c-fos, c-jun, c-myc, and egr-1 in murine fibrosarcoma cells by PDT. This PDT-induced expression of early-response genes can be inhibited by the protein kinase inhibitors staurosporine and H-7, and the phospholipase A2 inhibitor, quinacrine. Legrand-Poels et al. (26) and Ryter and Gomer (27) reported activation of nuclear factor-xB following photosensitization. Agrawal et al. (28) reported a phospholipase-mediated induction of apoptosis in mouse lymphoma cells following PDT. A phospholipase C inhibitor, U73122, blocks the transient increase in both inositol-1,4,5-trisphosphate and the intracellular Ca2+ levels as well as the DNA fragmentation induced by PDT. These observations indicate that PDT-induced cellular responses involve modifications in the signal transduction pathways and gene transcription. Understanding these signaling events may provide means to modulate the PDT effects at the molecular level. It can also be used as a model system to study the signal transduction pathways mediating extracellular stress stimuli and transcriptional regulation.

We report here that PDT induced a time- and dose-dependent activation of SAPK and p38 HOG1 in a naturally transformed murine keratinocyte cell line, Pam212. Pretreating the cells with antioxidants caused a significant inhibition of PDT-induced SAPK and p38 HOG1 activation.
Stimulation of SAPK and p38 HOG1 by Photodynamic Therapy

Fig. 1. Induction of tyrosine phosphorylation of a 40-kDa protein following PDT treatment. Pam212 cells were treated with 100 ng/ml PMA, 4 min exposure to UV irradiation, 200 ng/ml BPD plus 2 J/cm² of light (PDT), 200 ng/ml BPD only (BPD), or 2 J/cm² light only (Light). After various treatments, total cell extracts were prepared, and an equivalent of 50 µg of protein of cell extracts were subjected to SDS-PAGE. This was followed by Western blot using antiphosphotyrosine antibody, 4G10 (A), and anti-HOG1-NT antibody (B).

Electrophoresis and Immunoblotting—The different proteins and cell extracts were resolved on 15-mm thick 11% SDS-polyacrylamide gels using the buffer system described by Laemmli. After electrophoresis, the proteins were transferred to nitrocellulose or Immobilon P transfer membranes in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). The membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 (TTBS) and 4% bovine serum albumin. The membrane was incubated with primary antibodies diluted in TTBS for 2 h at room temperature. Following extensive washing, the membrane was incubated with peroxidase-conjugated or alkaline phosphatase-conjugated secondary antibody at room temperature for 1 h, and the proteins were visualized by ECL or color development.

Immunoprecipitation—SAPK/JNK, HOG1, ERK1, and ERK2 were immunoprecipitated by incubation of 500 µg of Pam212 cell lysate in a microcentrifuge tube. To this was added an equal volume of 6% NETF (100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 50 mM NaF, and 6% Nonidet P-40) and then 20 µl of protein A-Sepharose (1:1 slurry) for 30 min to pre-clear. The GST-cJun (1–169) protein was denatured by incubating the gel in buffer (Light) and then in buffer B (25 mM Hepes, pH 7.4, 10 mM MgCl₂, 100 mM sodium vanadate, and 5 mM β-mercaptoethanol) at room temperature for 2 h. The protein was renatured in buffer A containing 0.04% Tween 20 and 2 µl dithiothreitol at 4 °C for 2 × 16 h and then in buffer B (25 mM Hepes, pH 7.4, 10 mM MgCl₂, 100 mM sodium vanadate, and 5 mM β-mercaptoethanol) at room temperature for 30 min. The kinase assay was performed by shaking the gel in 25 ml of buffer B containing 50 µM ATP and 10 µCi/ml of [γ-32P]ATP at room temperature for 1 h. The reaction was terminated by adding 25 µl of 5% trichloroacetic acid and 10 mM sodium pyrophosphate. The gel was then subjected to extensive washing, followed by drying and autoradiography.

RESULTS

PDT Induces Tyrosine Phosphorylation of a 40-kDa Protein—To investigate the signal transduction pathways involved in the PDT-induced cellular responses, we analyzed, by Western blotting, the tyrosine phosphorylation of cellular proteins in whole-cell extracts prepared from PDT-treated Pam212 cultures. Since UV irradiation induces tyrosine phosphorylation of a number of cellular proteins (3, 14, 15), cell cultures that had been UV irradiated were included as positive controls. Cells exposed to light only, or receiving BPD without light, were used as negative controls. In UV- and PDT-treated samples, there was an induction of tyrosine phosphorylation of a protein at 40 kDa (Fig. 1A). This inductive effect was not observed in samples treated with light or BPD only. Since the MAPK family members undergo tyrosine phosphorylation and are in this molecular weight range, we performed a Western blot using antibodies against SAPK/JNK, p38 HOG1, ERK1, and ERK2. We found that the p38 HOG1 protein showed a similar migration pattern to the tyrosine-phosphorylated protein following UV and PDT treatment (Fig. 1B). This indicated that members of the MAPK family might be involved in PDT-induced early signaling events.

PDT Causes a Dose- and Time-dependent Induction of GST-CJun (1–169) and hsp27 Phosphotransferase Activity—Phosphotransferase activity toward MBP, GST-cJun (1–169), and heat shock protein 27 (hsp27) were examined in PDT-treated Pam212 cells (Fig. 2). MBP is an efficient substrate for ERK1 and ERK2, whereas GST-cJun (1–169) includes the activating phosphorylation sites Ser-63 and Ser-73, which have been phosphorylated by ERK1 and ERK2 (29, 30).

In-gel Kinase Assay—Approximately 100 µg of Pam212 lysate proteins were resolved on 11% SDS-PAGE in the presence of 40 µg/ml GST-cJun (1–169). After electrophoresis, the gel was washed twice for 30 min in 100 ml of buffer A (50 mM Hepes, pH 7.4, and 5 mM β-mercaptoethanol) containing 20% propanol. This was followed by incubation in 250 ml of buffer A containing 2 µl dithiothreitol for 1 h at room temperature. The protein was denatured by incubating the gel in buffer A containing 6 M guanidine-HCl for 2 h. The protein was renatured in buffer A containing 0.04% Tween 20 and 2 µl dithiothreitol at 4 °C for 2 × 16 h and then in buffer B (25 mM Hepes, pH 7.4, 10 mM MgCl₂, 100 mM sodium vanadate, and 5 mM β-mercaptoethanol) at room temperature for 30 min. The kinase assay was performed by shaking the gel in 25 ml of buffer B containing 50 µM ATP and 10 µCi/ml of [γ-32P]ATP at room temperature for 1 h. The reaction was terminated by adding 25 µl of 15% SDS-PAGE sample buffer for further analysis by SDS-PAGE.

In-gel Kinase Activity—Using MBP as a substrate, extracts from PDT-treated keratinocytes did not show any significant increase in phosphotransferase activity compared to controls. However, there was
a significant increase in MBP phosphotransferase activity in PMA-treated cells. UV irradiation induced only a modest increase in MBP phosphotransferase activity. When the GST-cJun (1–169) was used as a substrate, there was a strong induction of phosphotransferase activity in both UV- and PDT-treated samples. The same inductive effect was not observed in extracts derived from PMA-treated cells and control cells. Using hsp27 as a substrate, an increase in hsp27 phosphotransferase activity was observed with extracts derived from UV- and PDT-treated but not light- or BPD-only treated cells. PMA treatment caused a weak induction of hsp27 phosphotransferase activity. These results indicate that, like UV irradiation, PDT may activate SAPK and MAPKAPK2 in murine keratinocytes. Since PDT does not cause significant phosphorylation of MBP, which may be an indication of ERK activation, the activation of MAPKAPK2 by PDT is likely via p38 HOG1.

We next investigated the relationship between the dose of PDT and the induction of GST-cJun (1–169) and hsp27 phosphotransferase activity. The light dose was fixed at 2 J/cm², whereas the BPD dose was titrated from 10 ng to 250 ng/ml. Twenty-five min after PDT treatment, cells were lysed, and the crude cell extract was subjected to the GST-cJun (1–169) and hsp27 phosphotransferase assay. The results suggested a correlation between the extent of induction of both GST-cJun (1–169) and hsp27 phosphotransferase activity and the amount of BPD used for the PDT treatment. The maximum inductive effect was observed when 200 ng/ml BPD was used, and the minimum effective dose was between 25 and 50 ng/ml (Fig. 3, A–D).

The time course of PDT-induced activation of GST-cJun (1–169) and hsp27 phosphotransferase was established by an in vitro kinase assay using cell extracts prepared at different times following PDT treatment at a dose of 150 ng/ml BPD and 2 J/cm² of light. With GST-cJun (1–169) as a substrate, the earliest response was observed between 5 and 10 min following PDT. The maximum activation of phosphotransferase activity was observed at 30 min following PDT. The duration of activation persisted up to 2 h but was significantly reduced after 90 min (Fig. 3, E and F). Using hsp27 as a substrate, the activation of phosphotransferase activity was observed within 2.5 min after PDT treatment. The activity peaked at 20 min following PDT treatment. Thereafter, there was a gradual decrease in hsp27 phosphotransferase activity, and the activity returned to the basal level after 2 h (Fig. 3, G and H).

PDT Stimulates Stress-activated Protein Kinase—We investigated the involvement of SAPK/JNK in PDT-induced signaling events. SAPK was immunoprecipitated from crude extracts using an anti-JNK1 antibody, and the immune complex was assayed for GST-cJun (1–169) phosphotransferase activity. Incubation of the anti-JNK1 immune complex with the GST-cJun (1–169) and [γ-32P]ATP resulted in a significant phosphorylation of GST-cJun (1–169) in the samples derived from PDT- and UV-treated cells (Fig. 4A). The GST-cJun (1–169) phosphotransferase activity was also increased, although to a much less extent, in the sample derived from PMA-treated cells. Negligible activity was seen in the samples prepared from cells treated with light or BPD. A Western blot of the immunoprecipitated SAPK/JNK1 revealed that the amount of SAPK/JNK1 from untreated control. The data is representative of two separate experiments. For the kinetics study, Pam212 cells were treated with 200 ng/ml BPD plus 2 J/cm² of light. After PDT, cultures were incubated at 37 °C for an additional period of time ranging from 2.5 min to 2 h. The phosphotransferase activity toward GST-cJun (1–169) (E and F) and hsp27 (G and H) by the whole-cell extract prepared at different times after PDT was determined.
the variously treated samples was approximately the same (Fig. 4B).

The activation of SAPK by PDT treatment was also confirmed by a solid-phase kinase assay. This assay was based on the fact that SAPK binds to a small 30-amino acid segment called the δ subdomain that is amino-terminal to the Ser-63 and Ser-73 phosphorylation site on c-Jun. The GST-cJun (1–169) bead complexes were washed and then incubated with [γ-32P]ATP. After 30 min, the reaction was terminated, and the phosphorylation of GST-cJun (1–169) was analyzed by SDS-PAGE (C). The in-gel kinase assay was performed by resolving 100 μg of cell extracts prepared from PMA-, UV-, light-, and PDT-treated Pam212 cells on 11% SDS-PAGE in the presence of GST-cJun. The proteins were denatured, renatured, washed and exposed to film as described under “Experimental Procedures” (D).

GST-cJun (1–169). After electrophoresis, the proteins were reoxidized in the gel and incubated in a kinase buffer containing [γ-32P]ATP. In the samples from PDT- and UV-treated cells, there was a significant increase in phosphorylation of proteins at 54 to 55, and 46 kDa, which may represent the SAPK α, β, and γ isoforms, respectively (Fig. 4D). These results indicate that PDT, like UV irradiation, may activate all three SAPK isoforms.

PDT Stimulates p38 HOG1—To investigate the involvement of p38 HOG1 in PDT-induced signaling events, we performed immunoprecipitation of p38 HOG1 using HOG1-NT antibody. The immune complex was analyzed by Western blot using the 4G10 antiphosphotyrosine monoclonal antibody and anti-HOG1-NT (Fig. 5A). Anti-HOG1-NT immunoprecipitated equivalent amounts of p38 HOG1 after various treatments (Fig. 5B). However, there was a significant increase in tyrosine phosphorylation of p38 HOG1 in PDT- and UV-treated cells. Aliquots of immunoprecipitates were assayed for ATF2 phosphotransferase activity. ATF2 has been shown to be phosphorylated by activated p38 HOG1 (31). In our system, we detected a significant increase in ATF2 phosphotransferase activity in the immune complexes derived from UV- or PDT-treated samples compared with controls (Fig. 5, C and D). This stimulation of p38 HOG1 by Photodynamic Therapy 27111
experiment confirmed that PDT treatment can lead to the activation of p38 HOG1.

**PDT Did Not Cause Significant Activation of ERK1 and ERK2**—To investigate the effect of PDT on ERK1 and ERK2, we immunoprecipitated ERK1 and ERK2 using anti-ERK1 and ERK2 antibodies, respectively. The immune complexes were further analyzed for tyrosine phosphorylation by Western blot using 4G10 antiphosphotyrosine monoclonal antibody (Fig. 6A). There was a significant increase in tyrosine phosphorylation of both ERK1 and ERK2 in immune complexes derived from the cells treated with PMA, EGF, or H2O2. However, there was no significant increase in tyrosine phosphorylation of either ERK1 or ERK2 in untreated control or PDT-treated cells, although the amount of ERK1 and ERK2 was similar. In the cells receiving EGF plus PDT treatment, we observed a similar increase in ERK1 and ERK2 tyrosine phosphorylation relative to the cells treated with EGF only. This indicated that PDT treatment did not interfere with the normal response of Pam212 cells to EGF stimulation with respect to ERK1 and ERK2 activation. These results were further confirmed by immune complex kinase assays in which PMA, EGF, or PDT plus EGF caused significant increases in MBP phosphotransferase activity, whereas PDT treatment alone had no significant stimulatory effect.

The effect of PDT or UV irradiation on ERK1 and ERK2 activity was monitored at various times following the treatment (Fig. 6, B and C). At the dose which strongly activated both SAPK and p38 HOG1, PDT treatment did not cause significant increases in tyrosine phosphorylation and MBP phosphotransferase activities of ERK1 or ERK2 up to 2 h after PDT. The representative data from the immune complex kinase assay showed that at 0, 5, 15, and 30 min after PDT, the kinase activity of ERK1 and ERK2 was no more than 1.3-fold increased relative to untreated control. Statistical analysis of the data derived from three independent experiments showed no statistically significant increase in ERK1 or ERK2 activity at these time points except for a 1.3-fold increase in ERK1 activity immediately after PDT (p < 0.01). In the cells treated with UV irradiation at the dose that strongly activated SAPK and p38 HOG1, we observed a transient increase in tyrosine phosphorylation of ERK2 at 15 min after treatment. This increase in tyrosine phosphorylation coincided with a 1.8-fold increase in ERK2 activity in the representative experiment. The data derived from three independent experiments showed an average of 1.6-fold increase in ERK2 activity at 15 min after UV treatment, and it is significantly different from untreated control (p < 0.05). This evidence indicated that PDT treatment did not cause significant activation of ERK1 and ERK2, whereas UV irradiation induced a transient but minimal activation of ERK2.

**Reactive Oxygen Intermediates Played a Significant Role in PDT-induced SAPK and p38 HOG1 Activation**—Thus far, we have demonstrated that, like UV irradiation, PDT caused a dose- and time-dependent induction of the SAPK and p38 HOG1 pathways. However, under the same experimental conditions, appreciable activation of ERK1 and ERK2 by PDT was not observed. The mechanism for the UV-induced activation of SAPK and p38 HOG1 was not clear. However, reactive oxygen intermediates generated during UV irradiation may play a role in the UV-induced signaling events. This is supported by the observation that N-acetyl-cysteine (NAC), an antioxidant, par-
Stimulation of SAPK and p38 HOG1 by Photodynamic Therapy

DISCUSSION

We have shown for the first time that PDT specifically induces a transient and dose-dependent activation of SAPK and p38 HOG1. However, under the same experimental conditions, PDT treatment did not cause significant activation of ERK1 or ERK2. The activation of different MAP kinases was established by: 1) demonstrating elevated phosphotransferase activity toward specific substrates; and 2) increased tyrosine phosphorylation in the immune complexes derived from immunoprecipitation using antibodies against specific MAPK. These approaches were based on the fact that the members of the MAPK family are activated by dual phosphorylation at specific serine and threonine and tyrosine residues, and activated MAPKs exerted their phosphotransferase activity toward specific substrates.

We have shown that PDT induced a strong activation of SAPK/JNK. The results from different approaches, including anti-JNK1 immunoprecipitation and the solid-phase kinase assay, showed that UV and PDT treatment activated SAPK. The GST-cJun (1–169) fusion protein contains Ser-63 and Ser-73, which are the phosphorylation sites specific for the SAPK. Therefore, the in vitro phosphotransferase assay using GST-cJun (1–169) as substrate was used to measure SAPK activity. GST-cJun (1–169) also binds SAPK specifically through its δ subdomain. This specific interaction provides the basis for the solid-phase kinase assay in which Sepharose bead-conjugated antigen was used to capture SAPK in the cell extract.

The GST-cJun (1–169) phosphotransferase activity was measured in proteins at molecular masses similar to the SAPK isomers in an in-gel phosphotransferase assay following PDT. However, demonstration of direct evidence to prove the involvement of specific isomers in PDT-induced SAPK activation. However, more convincing evidence for the involvement of specific isomers in PDT-induced SAPK activation. However, demonstration of GST-cJun (1–169) phosphotransferase activity in proteins at molecular masses similar to the SAPK isomers in an in-gel kinase assay support the possibility of the involvement of all three isomers.

We obtained the evidence for the activation of p38 HOG1 following PDT. We have shown an increase in hsp27 phosphotransferase activity in the crude cell extract derived from the cells treated with PDT. The in vitro kinase assay using hsp27 as a substrate was used to measure MAPKAPK2 activity. Since MAPKAPK2 lies downstream of p38 HOG1 (29), an increase in hsp27 phosphotransferase activity can be used as indirect evidence for p38 HOG1 activation. However, other upstream kinases present in crude cell extracts may also activate MAPKAPK2. ERK1 and ERK2 have been shown to phosphorylate MAPKAPK2 in vitro (30), although a recent report indicated that phosphorylation of MAPKAPK2 at Thr-25 by ERK1 and ERK2 did not result in activation of MAPKAPK2 (32). In our system, PDT did not cause appreciable activation of ERK1 and ERK2. Therefore, we argued that an increase in hsp27 phosphotransferase activity following PDT may indicate the activation of p38 HOG1. However, more convincing evidence for the PDT induction of p38 HOG1 was derived from the anti-HOG1 immunoprecipitation experiment. We observed a significant inhibition of UV-induced c-Jun transcription and c-Src activation (14). We speculated that since the generation of singlet oxygen and other reactive oxygen intermediates seems to be the main mode of action of PDT, it was possible that PDT-induced SAPK and p38 HOG1 activation might be partly mediated by ROI. To test this hypothesis, we investigated the effect of antioxidants on PDT-induced activation of GST-cJun (1–169) phosphotransferase and hsp27 phosphotransferase.

Pam212 cells were pretreated with antioxidants L-His or NAC at the indicated concentration 1.5 h before PDT. Different doses of PDT were tested by titrating the BPD concentration. After PDT, cell lysates were prepared and assayed for GST-cJun (1–169) and hsp27 phosphotransferase activity as described previously. Data from three independent experiments were combined and processed for statistical analysis. The results were summarized in Fig. 7. As described previously, there was a dose-dependent induction of GST-cJun (1–169) and hsp27 phosphotransferase activity in the cells treated with PDT doses of 50, 75, and 150 ng/ml BPD plus 2 J/cm² of red light. With the presence of L-His or NAC at the concentration of 25 or 50 mM, we observed an almost complete inhibition of both GST-cJun (1–169) and hsp27 phosphotransferase activity in the cells treated with PDT at the dose of 75 ng/ml BPD. However, in the cells treated with PDT at 150 ng/ml BPD, pretreatment with 25 or 50 mM NAC had no inhibitory effect on both GST-cJun (1–169) and hsp27 phosphotransferase activity, whereas pretreatment with 25 or 50 mM L-His led to 50 and 80% inhibition, respectively. We also investigated the possibility that there might be a direct inhibition of enzymatic reactions by the antioxidant used. Different antioxidants were added to the in vitro GST-cJun (1–169) and hsp27 phosphotransferase reaction mixture. The results showed that the addition of antioxidants at the concentration effective in inhibiting PDT-induced c-Jun and hsp27 phosphotransferase activity in vitro had no direct inhibitory effect in an in vitro assay (data not shown). Therefore, the inhibitory effect of the antioxidants on PDT-induced GST-cJun (1–169) and hsp27 phosphotransferase activity was not likely to be the result of direct inhibition of enzyme activity by antioxidants but rather the result of their anti-ROI effect. These results indicated that ROI played a significant role in PDT-induced SAPK and p38 HOG1 activation.

![Fig. 7. ROI played a significant role in PDT-induced GST-cJun (1–169) and hsp27 phosphotransferase activity.](http://www.jbc.org/)
increase in tyrosine phosphorylation of p38 HOG1 in PDT- and UV-treated cells but not in light-only and BPD-only treated cells. We also demonstrated a significant increase in p38 HOG1 activity by measuring ATF2 phosphotransferase activity in the anti-p38 HOG1 immune complex. Based on the fact that activated p38 HOG1 phosphorylates ATF2 and MAPKAPK2 and phosphorylation of MAPKAPK2 by p38 HOG1 leads to the induction of hsp27 phosphotransferase activity of MAPKAPK2, we conclude that PDT, like UV irradiation, activates the p38 HOG1 pathway.

The kinetic study for SAPK and p38 HOG1 activation was performed by measuring GST-c-Jun (1–169) phosphotransferase activity and indirectly via the assay of hsp27 phosphotransferase activity in crude cell extracts. PDT-induced SAPK and p38 HOG1 activation followed different time courses. In both cases, induction was within minutes after PDT, and the response lasted two or more hours. Compared with the kinetics of GST-c-Jun (1–169) phosphotransferase activation, the induction of hsp27 phosphotransferase activity was more short lived and had an earlier onset. This may reflect differences in the activation and negative-feedback pathways that regulate SAPK and p38 HOG1 in response to PDT. SAPK is believed to be activated through dual phosphorylation by SAPK/ERK kinase, which may in turn be activated by a MAP kinase kinase kinase, MKKK1 (33, 34). p38 HOG1 is dual phosphorylated and activated by MKK3 (MAP kinase kinase-3) (31). The signal pathways further upstream of MEKK1 and MKK3 are not clear at this time, although several recent reports indicate involvement of members of the p21-activated kinases that may in turn be activated by Rac and Cdc42, which are members of the Rho family of small guanosine 5-triphosphate (GTP)-binding proteins (35–37). PDT-induced SAPK and p38 HOG1 activation described in this report provides a useful system to study upstream signaling events for both pathways.

It is interesting that both SAPK and p38 HOG1 seem to be responsive to similar stimuli including inflammatory cytokines, UV irradiation, PDT, and other environmental stresses such as heat, change in the osmolarity, and increase in intracellular ROIs. The common signaling components that link all these diverse stimuli to the SAPK and p38 HOG1 pathways are unknown. In the case of inflammatory cytokines, TNF-α and interleukin-1, the induction of sphingomyelinase, generation of ceramide, and activation of a ceramide-activated protein kinase have been documented (38, 39). Treating cells with TNF-α or interleukin 1 causes activation of both SAPK and p38 HOG1. There is some evidence indicating that ceramide is involved in the regulation of SAPK (40). In addition, TNF-α treatment increases production of ROIs in mitochondria (9). Treating cells with PMA, EGF, or H2O2 resulted in significant activation of both ERK1 and ERK2 at the level similar to that following EGF treatment alone. These results indicated that the action of PDT is specific toward the stress-regulated signaling pathways. This phenomenon of differential activation of certain members of the MAPK family has been reported previously. The depletion of nerve growth factor led to down-regulation of the ERK pathway and up-regulation of SAPK and p38 HOG1 pathways in PC-12 cells (42). Cross-linking surface IgM on B cells resulted in an activation of the ERK pathway, whereas cross-linking CD40 caused the activation of both SAPK and p38 HOG1 (43). It is likely that cells respond to diverse stimuli by activating different MAPK signaling pathways. In the case of stress stimuli, activation of certain MAPK pathways may be required for the protective stress response or for the initiation of apoptosis.

The possible role of ROI in PDT-induced SAPK and p38 HOG1 activation was tested by using the antioxidants 1-His and NAC. 1-His is an efficient scavenger for singlet oxygen. NAC is the precursor for glutathione, which participates in the enzymatic detoxification of H2O2 as well as lipid peroxide. The results showed that both 1-His and NAC almost completely inhibited the activation of SAPK and p38 HOG1 induced by PDT at the dose of 75 ng/ml PDT. These results indicated that ROIs played a significant role in mediating these PDT-induced signaling events. However, our results also showed that a higher concentration of 1-His is required for the inhibitory effect in the cells given a higher dose of PDT (150 ng/ml BPD), whereas NAC failed to inhibit SAPK and p38 HOG1 activation induced by the same dose of PDT. We have no experimental evidence that may explain the apparent difference in the inhibitory effect of 1-His and NAC at high dose PDT treatment. We speculate that this might be attributed to differential uptake or subcellular localization of the two scavengers. Since the two scavengers, which are different in their mode of action, were able to exhibit similar inhibitory effects at a lower dose of PDT (75 ng/ml BPD), we were not able to attribute the different effects between the two scavengers to the role of particular ROI species in PDT-induced SAPK and p38 HOG1 activation. Further work is required to clarify these issues.

In our study, cells briefly exposed to UV light were used as the positive control. UV irradiation has been shown to cause strong activation of both SAPK and p38 HOG1 and is also known to activate Src, Ras, Raf, ERK1, and ERK2. The activation of the Ras/Raf/MEK/ERK pathway was proposed due to UV-induced growth factor receptor phosphorylation and activation (15). Under the conditions described, we observed a similar activation of SAPK and p38 HOG1 by UV and PDT. However, in our system, PDT did not cause significant activation of ERK1 and ERK2, whereas the UV effect on ERK pathway was minimal. These similarities between UV and PDT in their effects on the MAPK pathways led us to believe that PDT and UV can be used as useful experimental tools to investigate the effect of oxidative stress on the signaling components upstream of MAPK in Pam212 cells. By this kind of parallel study, we may be able to gain further insight into the upstream events leading to the activation of both SAPK and p38 HOG1. In particular, it may allow us to identify a common mediator for both pathways and to define where both pathways diverge.

The role of SAPK and p38 HOG1 in apoptosis has been implicated recently. Xia et al. (42) reported a protective effect of ERK and a promoting effect of SAPK and p38 HOG1 on apoptosis in growth factor-deprived PC-12 pheochromocytoma cells. PDT has been reported to induce apoptosis in some cell lines (28). We also observed that the dose-dependent induction of SAPK and p38 HOG1 correlated with PDT cytotoxicity. The
Stimulation of SAPK and p38 HOG1 by Photodynamic Therapy

scavenger, L-histidine, which blocked induction of SAPK and p38 HOG1, protected the cells from PDT-induced death (data not shown). Future work will address the possible induction of apoptosis in Pam212 cells by PDT and the relationship between the activation of SAPK and p38 HOG1 and the induction of apoptosis. This may be a useful system to study signaling events leading to programmed cell death.

In conclusion, our results have uncovered a new form of stimulus for the activation of the SAPK and p38 HOG1 pathways. This system can be used as an effective tool to study the upstream signaling events leading to the activation SAPK and p38 HOG1, activation in some of the PDT-induced cellular responses in-... 

Acknowledgments—We thank Dr. Stephen Ulrich for providing the Pam212 cell line, Dr. Peter Young for providing CSBP2 antibody, and Dr. Michael Gold for his advice and scientific support during the course of this work. We also thank Dr. Bill Salt for help in the performance of some of the experiments. Dr. Philippe Margaron and Mark Curry for advice and stimulating discussion, and Fred Wong and Monika Aluwa-lia for valuable assistance.

REFERENCES

1. Davis, R. J. (1994) Trends Biochem. Sci. 19, 470–473
2. Pelech, S. L., Siao, Y. L., and Koide, H. B. (1995) Protein Kinase Circuitry in Neuronal Cell Signalling, in press, CRC Press, Boca Raton, FL
3. Derijard, B., Hibi, M., Wu, I-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
4. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Ruble, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) Nature (Lond.) 369, 156–160
5. Slus, H. K., Barrett, T., Derijard, B., and Davis, R. J. (1994) Mol. Cell. Biol. 14, 8376–8384
6. Han, J., Lee, J.-D., Bihb, L., and Ulevitch, R. J. (1994) Science 265, 808–811
7. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) J. Biol. Chem. 270, 7420–7426
8. Hallwell, B., and Gutteridge, J. M. C. (1989) Protection against Oxidants in Biological Systems: the Superoxide Theory of Oxygen Toxicity in Free Radicals in Biology and Medicine, p. 86, Clarendon Press, Oxford
9. Schulze-Osthoff, K., Beyaert, R., Vandevoorde, Y., Haegeman, G., and Fiers, W. (1993) EMBO J. 12, 3095–3104
10. Mai, S., Stein, B., Van den Berg, S., Kaina, B., Lucke-Huhle, C., and Ponta, H. (1989) Mechanisms of the ultraviolet light response in mammalian cells. J. Cell Sci. 94, 605–615
11. Weichselbaum, R. R., Hallahan, D., Fuks, Z., and Kufe, D. (1994) Int. J. Radiat. Oncol. Biol. Phys. 30, 229–234
12. Giorgetti, A. (1990) in Photodynamic Therapy of Neoplastic Disease (Kessel, D., ed), Vol. 1, p. 229, CRC Press, Boca Raton, FL
13. Karin, M. (1991) Curr. Opin. Cell. Biol. 3, 467–473
14. Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. (1992) The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell 71, 1081–1091
15. Sachsenmaier, C., Radler-Pohl, A., Zinck, R., Nordheim, A., Herrlich, P., and Rahmstorf, H. J. (1994) Cell 76, 963–972
16. Agarwal, M. L., Clay, M. E., Harvey, E. J., Autuone, A. R., and Oleinick, N. L. (1991) Cancer Res. 51, 5983–5996
17. Henderson, B. W., and Donovan, J. M. (1989) Cancer Res. 49, 6896–6900
18. Evans, S., Matthews, W., Perry, B., Fraker, D., Norton, J., and Pass, H. I. (1990) Effect of photodynamic therapy on tumor necrosis factor production by murine macrophages. J. Natl. Cancer Inst. 82, 34–39
19. Kruftmann, J., Atheta, M., Mendel, D. B., Khan, I. U., Gure, P. M., Mukhtar, H., and Elmets, C. A. (1989) J. Biol. Chem. 264, 11407–11413
20. Gomer, C. J., Ferrario, A., Rucker, N., Wong, S., and Lee, A. S. (1991) Cancer Res. 51, 6574–6579
21. Gomer, C. J., Luna, M., Ferrario, A., and Rucker, N. (1991) Photobiol. Photobiol. 53, 275–279
22. Gomer, C. J., Ferrario, A., Hayachi, N., Rucker, N., Szirth, B. C., and Murphy, A. L. (1988) Lasers Surg. Med. 8, 450–463
23. Curry, P. M., and Levy, J. G. (1993) Photochem. Photobiol. 58, 374–380
24. Nickeloff, B., and Turk, J. A. (1989) Am. J. Pathol. 135, 325–331
25. Lanza, M. C., Wong, S., and Gomer, C. J. (1994) Cancer Res. 54, 1374–1380
26. Legrand-Pouls, G., Bouv, R., Piret, B., Pih, M., Epe, B., Rentier, B., and Pette, J. (1993) J. Biol. Chem. 270, 6935–6939
27. Rye, P. W., and Gomer, C. J. (1995) Photochem. Photobiol. 58, 753–756
28. Agarwal, M. L., Larkin, H. E., Zaidi, S. A., Mukhtar, H., and Oleinick, N. (1993) Cancer Res. 53, 5879–5902
29. Freshney, N. N., Rawlinson, L., Guesdon, J. J., Cowley, S., Hsuan, J., and Sakkatvala, J. (1994) Cell 78, 1093–1049
30. Stokoe, D., Campbell, D. G., Nienkamp, S., Ishida, K., Leevers, S. J., Marshall, C., and Cohen, P. (1992) EMBO J. 11, 3985–3994
31. Derijard, B., Raingeaud, J., Barrett, T., Wu, I., Han, J., Ulevitch, R. J., and Davis, R. J. (1994) Science 267, 682–684
32. Ben-Levy, R., Leighton, I. A., Doza, Y. N., Attwood, P., Horrice, N., Marshall C. J., and Cohen, P. (1995) EMBO J. 14, 5920–5930
33. Sanchez, I., Hughes, R. T., Mayer, R. J., Ye, R. E., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) Nature 372, 794–798
34. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) Nature 372, 798–800
35. Cose, C. A., Chiariello, M., Xu, Q., and Holbrook, N. J. (1996) Curr. Opin. Cell. Biol. 8, 1137–1146
36. Minden, A., Lin, A., Claret, F., Abe, A., and Karin, M. (1995) Cell 81, 1147–1157
37. Zhang, S., Han, J., Selle, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J., and Bokoch, G. M. (1995) J. Biol. Chem. 270, 23934–23936
38. Schutze, S., Potashoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Krones, M. (1992) Cell 71, 765–776
39. Kolesnic, R., and Golde, D. W. (1994) Cell 77, 325–328
40. Verheij, M., Bose, R., Lin, X. H., Yao, B., Jaxiv, W. D., Grand, S., Birrer, M. J., Stabs, E., Zon, L. I., and Kaneski, J. S., and Kolesnic, R. N. (1996) Nature 380, 75–79
41. Guyton, R. J., Liu, Y., Gores, M., Xu, Q., and Holbrook, N. J. (1995) J. Biol. Chem. 270, 4138–4142
42. Xia, Z., Dickens, M., Rainaung, J., Davis, R. J., Greenburg, M. E. (1995) Science 270, 1326–1331
43. Sutherland, C. L., Heath, A. W., Pelech, S. L., Young, P. R., and Gold, M. R. (1996) J. Immunol. in press

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
Stimulation of Stress-activated Protein Kinase and p38 HOG1 Kinase in Murine Keratinocytes following Photodynamic Therapy with Benzoporphyrin Derivative
Jing-song Tao, Jasbinder S. Sanghera, Steven L. Pelech, Geraldine Wong and Julia G. Levy

J. Biol. Chem. 1996, 271:27107-27115.
doi: 10.1074/jbc.271.43.27107

Access the most updated version of this article at http://www.jbc.org/content/271/43/27107

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 14 of which can be accessed free at http://www.jbc.org/content/271/43/27107.full.html#ref-list-1