Activation of JNK and p38 but not ERK MAP Kinases in Human Skin Cells by 5-Aminolevulinate-Photodynamic Therapy

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ABSTRACT

5-Aminolevulinate (ALA) photodynamic therapy (PDT) is being used clinically for the treatment of skin cancers. ALA is applied as a precursor of porphyrins serving as endogenous photosensitizers. Irradiation of HaCaT cells preincubated with 1 mm ALA for 24 h with red light of 570-750 nm at a dose of 4.5 J/cm² leads to a 6-fold elevation of cellular c-Jun N-terminal kinase activity; phosphorylation of p38 mitogen-activated protein kinase (MAPK) is enhanced to a similar extent. In contrast, neither activation nor increased phosphorylation of the extracellular stimulus-regulated kinase MAPKs is detected. p38 is also phosphorylated by ALA-PDT in the human melanoma cell lines Bro and SkMel-23, applying doses that lead to 80-95% cell death after 24 h. Hence, the effects of ALA-PDT on MAPKs are similar to stresses like UV irradiation or exposure to hydrogen peroxide with respect to activation of JNK and p38 MAPKs. They are different, however, in that extracellular stimulus-regulated kinase activity is not raised by ALA-PDT.

Of the 830 pmol porphyrins/mg protein that were present at 24 h in HaCaT cells, 99 pmol/mg were intracellular. When extracellular porphyrins had been removed by washing, p38 responses were retained. Thus, intracellular porphyrins synthesized from ALA are sufficient to elicit activation of p38 on photosensitization.

INTRODUCTION

Photosensitization on irradiation of porphyrin-enriched tissue leads to the formation of reactive species and to cell death. This effect is successfully exploited clinically in PDT. The efficiency of PDT is highly dependent on the cellular localization of the respective photosensitizer. In addition, the accumulation of the sensitizer in target tissue relative to nontargeted tissue should be high to avoid unwanted tissue destruction.

PDT based on the application of the porphyrin precursor ALA before irradiation with red light fulfills these requirements. The metabolism of ALA has been studied in several cell lines. It circumvents the ALA synthase reaction, the rate-limiting step in mammalian metabolism of ALA has been studied in several cell lines. It circumvents the ALA synthase reaction, the rate-limiting step in mammalian metabolism of ALA. However, ALA can reach extracellular levels, and extracellular porphyrins may contribute to the observed effects. Photosensitization on irradiation of porphyrin-enriched tissue leads to the formation of reactive species and to cell death. This effect is successfully exploited clinically in PDT. The efficiency of PDT is highly dependent on the cellular localization of the respective photosensitizer. In addition, the accumulation of the sensitizer in target tissue relative to nontargeted tissue should be high to avoid unwanted tissue destruction.

Cell Culture. HaCaT keratinocytes and Bro and SkMel-23 melanoma cells were a kind gift from Dr. B. Farthmann (Dermatological Clinic, Benjamin Franklin University, Berlin, Germany). Cells were kept at 37°C in a humidified atmosphere of 5% CO₂ in DMEM culture medium (Life Technologies, Inc.) supplemented with 1% (w/v) l-glutamine, 10% (v/v) FCS, penicillin (400 µg/ml), and streptomycin (50 µg/ml).

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Western Blotting. After irradiation, cells grown on 30-mm dishes were incubated at 37°C for 30 min. With 0.9% NaCl, and lysed by scraping in 2 X SDS-PAGE lysis buffer (125 mm Tris, 4% (w/v) SDS, 20% (v/v) glycerol, 100 mm DTT, 0.2% (w/v) bromophenol blue, pH 6.8). The lysates were heated at 95°C for 5 min and used for SDS-PAGE or frozen until use. Samples (8-16 µl) were subjected to gel electrophoresis on 12% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes (ECL nitrocellulose; Amersham). Immunodetection of phosphorylated ERK1/2 and p38 with a-phospho-MAPK and a-phospho-p38 antibodies, respectively; an a-p38 antibody served as a gel loading and protein control. Incubation with an

Porphyrin Assay. Porphyrins were determined according to Piomelli et al. (15) with minor modifications. Directly after treatment, cells were homogenized and extracted in 1 N perchloric acid/methanol (1:1, v/v). After a 10-min centrifugation, porphyrins were measured in the supernatants by fluorescence (excitation, 405 nm, emission, 598 nm) using uroporphyrin as a calibration standard. The amount of protein in the pellets was determined according to Lowry et al. (16).

MATERIALS AND METHODS

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α-rabbit secondary antibody conjugated to horseradish peroxidase was followed by chemiluminescence detection. All antibodies were from New England Biolabs (Schwalbach, Germany). Densitometric analysis used Scan Pack software from Biometra (Göttingen, Germany).

**JNK and ERK activity.** JNK activity was determined as described previously (see Ref. 13). Briefly, HaCaT cells were grown to near confluency in 60-mm dishes and treated as desired. After irradiation, the cells were incubated at 37°C for 30 min, washed with 0.9% NaCl of room temperature and lysed with 300 μl of ice-cold RIPA buffer [50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1% (w/v) NP40, 0.5% (w/v) sodium desoxycholate, 0.1% (w/v) SDS, 0.2 mM Na,VO₄, 0.8 mM PMSF, 1 μg/ml aprotinin, 2 μg/ml leupeptin]. The lysed cells were scraped off and centrifuged at 4°C. Supernatants were normalized for protein content (DC-Protein Assay: Bio-Rad), and JNKs were immunoprecipitated from cell lysates (100 μg of protein in a total volume of 100 μl of RIPA lysis buffer) using 5 μl of a rabbit antiserum (diluted 1:10 in distilled water), which was a generous gift from Dr. Peter E. Shaw (Department of Biochemistry, University of Nottingham, Nottingham, United Kingdom).

Immune complexes were collected with Protein A-Sepharose, and washed with both ice-cold RIPA buffer and kinase buffer (10 mM Tris/Cl pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM DTT). The kinase assay was performed as described previously (see Ref. 13), and the phosphorylated substrate GST-cJun (1–79; Alexis, Grünberg, Germany) was analyzed by electrophoresis on a 12% SDS-polyacrylamide gel, identified by autoradiography and quantified by phosphoimaging with a Fuji-BAS-1500 reader (raytest Isotopenmessgeräte, Straubenhardt, Germany).

ERK activity was determined analogously, but using an anti-ERK 1/2 antibody (Upstate Biotechnology, Inc., Lake Placid, NY) as the precipitating agent (1 μg/sample) and myelin basic protein (Sigma Chemical Co., Deisenhofen, Germany) as the substrate (1 mg ml⁻¹/assay) for immunoprecipitated ERK.

**RESULTS**

**Porphyrin Synthesis in HaCaT Cells after Incubation with ALA and Cell Viability after Irradiation.** In ALA-PDT, irradiation of the skin with red light is performed within a few hours after exposure to ALA because an optimum ratio of the porphyrin level accumulated in cancerous tissue to the level in healthy tissue is reached. In the present cell culture study, using only one type of cell, irradiation was performed at 24 h, when the maximum concentration of endogenous porphyrins was detected.

HaCaT cells incubated with 1 mM ALA for 24 h produced a total (cells + incubation medium) of 830 ± 83 (n = 5) pmol fluorescent porphyrin/mg cellular protein. The cells had an average content of 99 ± 30 (n = 6) pmol fluorescent porphyrin/mg protein. The porphyrins undergo photobleaching; after irradiation with 4.5 J/cm² of red light only 49 ± 12 (n = 4) pmol/mg protein in the cellular fractions were measured (Table 1).

Cells were irradiated at 4.5 J/cm² and 7.5 J/cm², the former resulting in a viability of 65 ± 4% (n = 4), the latter leaving only 12% of the cells viable directly after irradiation (Table 1).

**Effect of Irradiation on MAPKs in HaCaT cells.** Endogenous JNK activity was substantially increased on irradiation (4.5 J/cm²) of HaCaT cells preincubated with ALA, whereas control cells showed no increase in JNK activity. Phosphoimaging analysis revealed an average induction of about 6-fold. In parallel, the phosphorylation of p38 is enhanced about 5-fold at 7.5 J/cm² and at 4.5 J/cm², with higher variability in the latter case (4.6 ± 3.4-fold induction; n = 4). However, no activation or increased phosphorylation of the ERK-MAPKs was observed (Fig. 1; Table 1).

**Activation of p38-MAPK in Melanoma Cells.** Bro and SkMel-23 melanoma cell lines preincubated with ALA for 24 h were irradiated with red light of doses resulting in viabilities comparable with those found in HaCaT cells irradiated with 7.5 J/cm² after preincubation with ALA; twenty-four hours after irradiation, cells had viabilities of 6% (Bro; 42 J/cm²) to 23% (SkMel-23; 24 J/cm²). Incubation with 1 mM ALA for 24 h resulted in a total (cells + incubation medium) of 174 ± 18 (Bro; n = 3) and 662 ± 90 (SkMel-23; n = 3) pmol fluorescent porphyrin/mg cellular protein. The cells had an average content of 26 ± 6 (Bro; n = 3) and 127 ± 5 (SkMel-23; n = 3) pmol fluorescent porphyrin/mg protein, respectively. Irradiation of Bro and SkMel-23 cells with the above-mentioned doses leads to a pronounced activation of p38-MAPK as can be seen from its dual phosphorylation status 30 min after irradiation (Fig 24).

**Intracellular Porphyrins Are Sufficient for p38 Activation.** As can be seen from the porphyrin data above (total versus cellular porphyrins generated), there is an appreciable amount of porphyrins released into the culture medium. Only 12% and 15% of the total porphyrins determined in HaCaT and Bro cells, respectively, is intracellular. However, washing HaCaT (Fig. 2) and Bro cells (not shown) before irradiation did not diminish the activation of p38 by ALA-PDT effects relative to unwashed controls, pointing to intracellular porphyrins as the photosensitizers responsible for the effects observed. Additionally, transferring culture medium from ALA-treated HaCaT cells to untreated control cells and irradiation of the latter yielded no p38 activation (data not shown), thus supporting the above conclusion.

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**Table 1** Induction of MAPK activity, porphyrin content, and viability of HaCaT cells treated with ALA, light, or ALA plus red light

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ALA</th>
<th>Light</th>
<th>ALA + light</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNK Activity</td>
<td>1</td>
<td>1.1 ± 0.3</td>
<td>1 ± 0.3</td>
<td>5.6 ± 1.6</td>
</tr>
<tr>
<td>ERK Activity</td>
<td>1</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>p38 Phosphorylation</td>
<td>1</td>
<td>0.9 ± 0.1</td>
<td>1.2 ± 0.4</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>Cellular porphyrins</td>
<td>3.8 ± 2</td>
<td>99 ± 30</td>
<td>32 ± 1</td>
<td>49 ± 12</td>
</tr>
<tr>
<td>(pmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viability (%)</td>
<td>100</td>
<td>91 ± 2</td>
<td>81 ± 5</td>
<td>65 ± 4</td>
</tr>
</tbody>
</table>

* Irradiations with 4.5 J/cm².
DISCUSSION

The data presented here show that irradiation with red light of human keratinocytes (HaCaT) preincubated with ALA to generate endogenous porphyrins (HaCaT) preincubated with ALA to generate endogenous porphyrins leads to activation of JNK and p38, but not ERK-MAPKs. p38 is also activated in the human melanoma cell lines Bro and SkMel-23. This is in line with experiments showing that "O2, which is generally considered as a key agent in PDT (17), is capable of activating JNKs in human skin fibroblasts but seems to leave ERKs unactivated (13).

The above pattern of MAPK activation on application of a (oxidative) stressful stimulus to cells has, as yet, only been found in murine keratinocytes treated with benzoporphyrin derivative and irradiated subsequently (14). Many stressful stimuli are capable of activating JNK-MAPKs; however, different from the MAPK activation pattern induced by ALA-PDT presented here, stimuli such as hydrogen peroxide or UV C irradiation can activate ERK-MAPKs (18, 19); on the other hand, unspecific oxidative stress is also capable of down-regulating instead of enhancing p38 activity (20).

Regarding signaling events known to be induced on PDT, irradiation of mouse lymphoma cells in the presence of a phthalocyanine sensitizer increases overall tyrosine phosphorylation, one phosphorylated protein being identified as HS1, a substrate of src-like protein tyrosine kinases (21). Furthermore, enhanced transcription of the proto-oncogenes c-fos and c-jun and an increased AP-1 binding affinity were observed in HeLa cells treated with photofrin and red light (22, 23). Both induction of c-fos/c-jun and of AP-1-like activity are mediated by the activation of MAPKs; specifically, p38 seems to play a prominent role in transcriptional activation of c-jun. JNKs, in turn, phosphorylate and thereby activate c-Jun and ATF2 (24–26). An increased cellular JNK/p38 to ERK activity ratio has been connected with an enhanced tendency of undergoing apoptosis (27), which has, in fact, been shown to occur in response to both ALA-PDT (28) and treatment with "O2 (11, 29) in various cell culture systems.

The observed kinase activation pattern is regarded as a stress response. Cells not killed directly by ALA-PDT will turn to apoptosis. The synthesis of the inflammatory cytokines, tumor necrosis factor (TNF)α, IL-1, and IL-6 are induced by PDT (22, 23), pointing to the induction of inflammatory and immunological responses. IL-1 and IL-6, in turn, are known to induce the expression of metalloproteinases degrading the extracellular matrix, such as interstitial collagenase (MMP-1; Ref. 30). Degradation of extracellular matrix by MMP-1 may serve to facilitate the removal of cells killed on PDT or serve to increase the accessibility of tumor cells not killed to inflammatory agents or phagocytes (31). Both IL-1 and IL-6 and MMP-1 are also induced on treatment of cells with singlet "O2 (8, 10).

Cancer cells are more susceptible to ALA-PDT than nontransformed cells as they synthesize and accumulate photosensitizing porphyrins more effectively, mostly protoporphyrin IX (2, 3). This has been successfully exploited clinically in skin tumor therapy (1, 32).
EFFECT OF ALA-PDT ON MAPKs


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