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Singlet Oxygen-induced Attenuation of Growth Factor Signaling: Possible Role of Ceramides

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Singlet oxygen, an electronically excited form of molecular oxygen, is a primary mediator of the activation of stress-activated protein kinases elicited by ultraviolet A (UVA; 320–400 nm). Here, the effects of singlet oxygen ($^1O_2$) on the extracellular signal-regulated kinase (ERK) 1/2 and Akt/protein kinase B pathways were analyzed in human dermal fibroblasts. While basal ERK 1/2 phosphorylation was lowered in cells exposed to either $^1O_2$, UVA or photodynamic treatment, Akt was moderately activated by photochemically generated $^1O_2$ in a phosphoinositide 3-kinase (PI3K)-dependent fashion, resulting in the phosphorylation of glycogen synthase kinase-3 (GSK3). The activation of ERK 1/2 and Akt as induced by stimulation with epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) was inhibited by $^1O_2$ generated intracellularly upon photoexcitation of rose Bengal (RB). Photodynamic therapy (PDT)-induced apoptosis is known to be associated with increased formation of ceramides. Likewise, both $^1O_2$ and UVA induced ceramide generation in human skin fibroblasts. The attenuation of EGF- and PDGF-induced activation of ERK 1/2 and Akt by $^1O_2$ was mimicked by stimulation of fibroblasts with the cell-permeable C_2-ceramide. Interestingly, EGF-induced tyrosine phosphorylation of the EGF receptor was strongly attenuated by $^1O_2$ but unimpaired by C_2-ceramide, implying that, although ceramide formation may mediate the above attenuation of ERK and Akt phosphorylation induced by $^1O_2$, mechanisms beyond ceramide formation exist that mediate impairment of growth factor signaling by singlet oxygen. In summary, these data point to a novel mechanism of $^1O_2$ toxicity: the known $^1O_2$-induced activation of proapoptotic kinases such as JNK and p38 is paralleled by the prevention of activation of growth factor receptor-dependent signaling and of anti-apoptotic kinases, thus shifting the balance towards apoptosis.

Keywords: Singlet oxygen; Oxidative stress; Ceramides; Growth factors; MAPK; ERK

Abbreviations: ALA, δ-aminolevulinic acid; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; HPTLC, high performance thin layer chromatography; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PDT, photodynamic therapy/treatment; PI3K, phosphoinositide 3-kinase; RB, rose Bengal; UVA, ultraviolet A (320–400 nm)

INTRODUCTION

Reactive oxygen species (ROS) have been shown to be involved in a variety of cellular signaling processes both under physiological conditions and in stressed cells.[1–4] The non-radical ROS, singlet oxygen ($^1O_2$), an electronically excited form of molecular oxygen, may be generated metabolically, e.g. as part of the immune response in inflamed tissues, or photochemically: $^1O_2$ has been shown to be a primary mediator of biological effects of ultraviolet A (UVA, 320–400 nm) radiation and of photodynamic therapy (PDT), including their cytotoxicity[5–7] and several

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cellular signaling processes (see Ref. [8] for review), such as the activation of p38 MAPK and JNK signaling cascades,[9–11] the activation of transcription factor AP-1[12] and the expression of various genes (see Ref. [13] for a comprehensive list).

Recently, ceramides were characterized as mediators of UVA/singlet oxygen-induced signaling, mediating the activation of transcription factor AP-2 and AP-2-dependent upregulation of intercellular adhesion molecule-1 expression in response to UVA/$^1O_2$ in normal human keratinocytes.[14,15] Ceramides were found to be generated non-enzymatically upon exposure of cells to singlet oxygen.[15] In line with the emerging concept of ceramides as second messengers in the cellular stress response towards photochemically generated oxidative stress, generation of ceramides in response to UVA or PDT was demonstrated to be associated with the activation of stress kinases and with apoptosis in various mammalian cell types.[16,17–19]

If $^1O_2$ is capable of activating proapoptotic signaling, i.e. the generation of ceramide and the activation of stress kinases associated with apoptosis, then what is the influence of $^1O_2$ on signaling cascades that are also activated by various stressful stimuli but that are regarded as largely anti-apoptotic and promoting cellular survival? Two such cascades that are activated upon stimulation of growth factor receptors result in the activation of extracellular signal regulated kinase (ERK) 1 and ERK2 and in the activation of the serine/threonine kinase Akt (protein kinase B). Both pathways are known to be activated by ROS, such as H_2O_2[20,21] or peroxynitrite.[22,23] Activation of ERK 1/2 is regarded as promoting cellular proliferation and growth, which is due to substrates of ERK 1/2 that include transcription factors and proteins regulating translation as well as the biosynthesis of nucleotide precursors.[24] The ERK pathway is known to be activated upon stimulation of receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor or the platelet-derived growth factor (PDGF) receptor, and activation is via the small G-protein Ras, the Ser/Thr kinase Raf and the dual-specificity kinases MEK 1 and MEK 2. The activation of Akt is similarly caused by stimulation of growth factor receptors, the tyrosine phosphorylation of which triggers the binding of phosphoinositide-3-kinase (PI3K) and the stimulated production of 3′-phosphorylated phosphoinositides that ultimately entail the phosphorylation and activation of Akt. Substrates of Akt include proapoptotic proteins that are inactivated upon phosphorylation, such as Bad or forkhead trancription factors,[25] and Raf, which was described as being negatively regulated by Akt,[26] thus establishing another link between the ERK and Akt pathways.

We demonstrate here that ceramide generation and growth factor signaling are linked in the cellular response to $^1O_2$. Exposure of human skin fibroblasts leads to the generation of ceramides and results in an attenuation of the activation of the ERK1/2 and Akt pathways elicited by growth factors such as EGF and PDGF. This attenuation is mimicked by exposure to C_2-ceramide, pointing to ceramides as potential mediators of the singlet oxygen-induced down-regulation of growth factor signaling.

MATERIALS AND METHODS

Reagents

C_2-ceramide (*N*-acetylsphingosine) was purchased from Calbiochem (La Jolla, CA, USA). Human recombinant PDGF-AB and EGF were purchased from R&D Systems (Minneapolis, MN, USA), rose Bengal (RB) was from Sigma-Aldrich (St Louis, MO, USA) and LY294002 (LY) and wortmannin were from Alexis (San Diego, CA, USA).

Cell Culture

Human dermal fibroblasts from fetal foreskin ("HFFF2") and "1306" human dermal fibroblasts were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK; No. 86031405 and No. 90011887, respectively), neonatal human primary skin fibroblasts were obtained from Clonetics (Walkersville, MD, USA) and BioWhittaker Europe (Taufkirchen, Germany). Further, skin fibroblasts isolated from human foreskin biopsies derived from circumcision surgeries were used. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin/streptomycin, and were grown on plastic petri dishes in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

For exposure to singlet oxygen, confluent cells were washed twice and covered with PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) or Hanks' balanced salt solution (HBSS) containing rose Bengal (RB), followed by irradiation for 10 min with a commercially available 500 W halogen lamp from a fixed distance. Approximately 130 μM (cumulative concentration) of singlet oxygen were generated during 10 min of irradiation of 0.3 μM RB under similar conditions.[10] Incubation of cells with RB without irradiation and irradiation in the absence of RB served as controls. Irradiation with UVA was with a UVA700 (Waldmann, Villingen, Germany), exposure to UVA1 (340–400 nm) with a Sellamed 24000 A (Dr Sellmeier, Sellas GmbH, Gevelsberg, Germany) irradiation device. UVA-delta-aminolevulinic acid (ALA)-PDT was performed as described.[7]

In experiments with inhibitor treatments, the cells were preincubated for 30–75 min with either DMSO (control) or the respective inhibitor, which were

present also during treatment with singlet oxygen and during postincubation. Hydrogen peroxide was either added as a bolus of up to 1 mM H_2O_2 in HBSS and the cells incubated for 30 min or it was generated enzymatically *in situ* using glucose oxidase (GOx; Sigma), which was added to HBSS (containing 5.55 mM glucose) for 30 min.

Western Blotting

Western blotting was performed as described previously.[10,27] Phosphorylated ERK 1/2 and p38 MAPK were detected with polyclonal anti-active-MAP kinase antibody from Promega (Mannheim, Germany) and polyclonal anti-phospho-p38 MAPK (Cell Signaling Technology, Frankfurt am Main, Germany) antibodies, respectively. For detection of phosphorylated (Ser^{473} or Thr^{308}) and total Akt as well as phosphorylated glycogen synthase kinase-3 (GSK3)-α/β, rabbit polyclonal antibodies from Cell Signaling Technology (Beverly, MA, USA) were used at the dilutions recommended by the supplier. Detection of EGFR and phosphorylated EGFR (Tyr 1068) was with antibodies from Upstate Biotechnology (Lake Placid, NY, USA) and BioSource International (Camarillo, CA, USA), respectively. Incubation with an anti-rabbit secondary antibody conjugated to horseradish peroxidase was followed by chemiluminescence detection (ECL, Amersham, Braunschweig, Germany). After stripping, the membrane was reprobed with polyclonal anti-MAPK (Promega) and polyclonal anti-Akt (Cell Signaling Technology) antibodies, which served as gel loading and protein controls.

Determination of Ceramide by HPTLC

For determination of ceramide formation, the cell pellet was suspended in water and an amount corresponding to 500 μg of protein (Bradford) was used for Folch extraction and mild alkaline hydrolysis. Thereafter, lipid extracts were separated as described previously:[15] Samples and standards were applied to high performance thin layer chromatography (HPTLC) plates using a CAMAG Linomat IV (CAMAG, Berlin, Germany) in bands of 0.8 cm with a spray-on technique (10 μl per sample) using nitrogen carrier gas. Visualization was done with an acidified manganese chloride solution [60 ml $MnCl_2$ (33 mM in H_2O) + 60 ml methanol + 4 ml concentrated H_2SO_4] in an automated dipping device. After heating the plate for 20 min at 120°C in a temperature-controlled oven the plate was dried and scanned using a *CAMAG* TLC Scanner lll (CAMAG) and CATS software (CAMAG). Quantitation was done measuring absorption at 550 nm with a plot of peak area versus weight spotted for a series of standards using a second-order polynomial calibration. The employed method does not discern between C2, C6, C12 and C16 and higher ceramides which all migrate with similar R_f values.

RESULTS

Singlet Oxygen Interferes with Growth Factor Signaling

Singlet oxygen activates JNK and p38 MAPK in human skin fibroblasts, but not ERK 1/2.[10] In fact, exposure of human skin fibroblasts to $^1O_2$ generated photochemically by irradiation of solutions of RB with white light even resulted in a loss of basal phosphorylation of ERK 1/2 (Fig. 1, top row). The same effect was seen in cells prestimulated with growth factors, PDGF, EGF, or with fetal calf serum (Fig. 1, top row): the strong activation of ERK 1/2 by any of the growth factors was prevented by prior exposure of the cells to $^1O_2$. This was not due to loss of cellular protein or cell death, as concluded from the gel loading control (total Akt, 3rd row in Fig. 1) and from p38 activation (4th row) by $^1O_2$,[10] which was unimpaired.

Different from ERK 1/2, phosphorylation of Akt was induced by the exposure to RB plus light, as

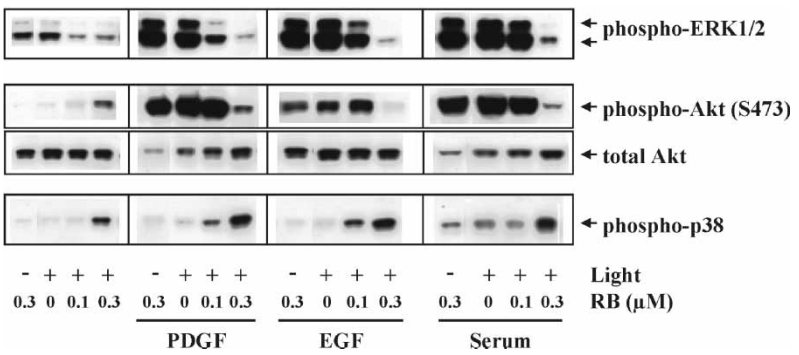

FIGURE 1 Singlet oxygen interferes with growth factor signaling. Human skin fibroblasts were exposed to singlet oxygen [generated by irradiation of rose Bengal (RB) solutions in HBSS for 10 min with white light] and posttreated for 30 min with buffer (HBSS) or growth factors: PDGF, platelet-derived growth factor AB (100 ng/ml); EGF, epidermal growth factor (100 ng/ml); fetal calf serum. Samples were analyzed by Western blotting with antibodies against phosphorylated ERK 1/2, phosphorylated Akt (Ser473), total Akt or phosphorylated p38.

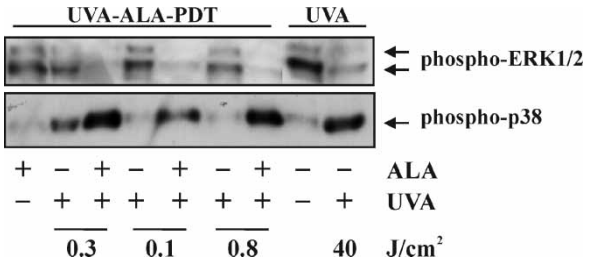

FIGURE 2 ERK phosphorylation is attenuated in cells undergoing UVA irradiation or UVA-ALA-PDT. Western analysis for ERK and p38 phosphorylation in skin fibroblasts undergoing UVA-ALA-PDT (i.e. irradiation of cells exposed to 1 mM ALA for 24 h, followed by irradiation with UVA at the given doses) as well as skin fibroblasts irradiated with UVA only.

demonstrated in row 2 (Fig. 1). Like ERK 1/2 phosphorylation, however, the strong phosphorylation of Akt induced by growth factors was prevented by prior exposure to $^1O_2$, which, as above, was not due to cytotoxic effects.

The observed decrease in basal ERK 1/2 phosphorylation upon exposure to $^1O_2$ was also observed in cells undergoing UVA-ALA-PDT, or in cells exposed to UVA (Fig. 2). In UVA-ALA-PDT, cells are exposed to ALA to support cellular synthesis of photosensitizing protoporphyrin IX, followed by exposure to UVA at low doses.[7] This is in line with the fact that cytotoxicity and signaling effects of both UVA-ALA-PDT and UVA are mediated by $^1O_2$.[7,8,13]

For unknown reasons, however, no activation of Akt was found after UVA-ALA-PDT or in cells exposed to UVA (not shown). This may be due to the fact that Akt is only moderately activated upon exposure to $^1O_2$ (Fig. 3A), as can be seen from comparison of Akt phosphorylation after exposure to RB/light with the extent of activation elicited upon treatment with growth factors (Fig. 1, 2nd row) as well as with the strong activation elicited by exposure to hydrogen peroxide both applied as a bolus (Fig. 3D) or generated *in situ* by action of glucose oxidase (Fig. 3E). Akt activation by $^1O_2$ was mediated by PI3K, as it was completely prevented in the presence of two structurally unrelated inhibitors of PI3K, LY294002 or wortmannin (Fig. 3B). Activation of p38 was unimpaired in the presence of the inhibitors, excluding an interaction between the inhibitors and $^1O_2$ (Fig. 3B). Though moderate, the activation of Akt by $^1O_2$ resulted in phosphorylation of endogenous substrates of Akt, such as GSK3 (Fig. 3C).

Ceramide Generation and Interference with Growth Factor Signaling

Exposure of human keratinocytes to $^1O_2$ or UVA is known to result in the generation of ceramides.[15] Similarly, enhanced ceramide levels were observed in human skin fibroblasts exposed to RB/light (Fig. 4A) or UVA1 (340–400 nm; Fig. 4B). Ceramide formation may account for the above-mentioned (Fig. 1) prevention of growth factor-induced activation of ERK 1/2 and Akt: pretreatment of human

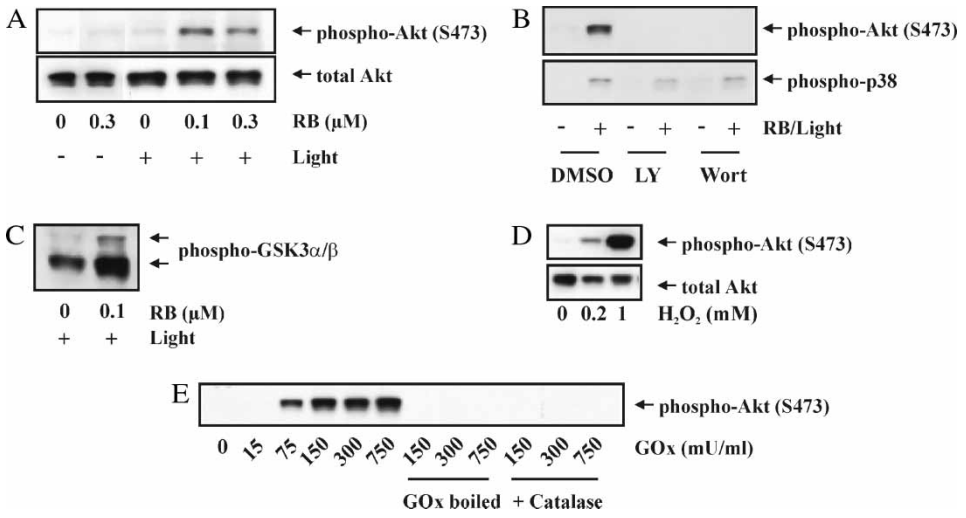

FIGURE 3 Singlet oxygen activates the PI3K/Akt pathway. (A) Human skin fibroblasts were irradiated in the presence or absence of the photosensitizer rose Bengal (RB) for 10 min, incubated in serum-free medium for 30 min and lysed and processed for Western analysis. The figure shows phosphorylated and total Akt as detected by Western blotting analysis (see "Materials and methods", section). (B) Singlet oxygen-induced phosphorylation of Akt is blocked in the presence of the PI3K inhibitors LY294002 (LY) and wortmannin (wort); DMSO, vehicle control. Activation of p38 served as control for interaction of the inhibitors with singlet oxygen and was not influenced. (C) Singlet oxygen-induced phosphorylation of GSK-3 α (51 kDa) and β (47 kDa) as detected by Western blot analysis. (D) Exposure of human skin fibroblasts to hydrogen peroxide (in HBSS for 30 min) results in a strong activation of Akt. (E) Hydrogen peroxide was generated *in situ* with glucose (from HBSS: 5.55 mM) and glucose oxidase (GOx). Fibroblasts were incubated with varying amounts of GOx for 30 min. To control for other reaction products or for a simple protein effect, boiled GOx was added in control treatments. Also, catalase (100 U/ml) was added together with GOx to control for hydrogen peroxide formation.

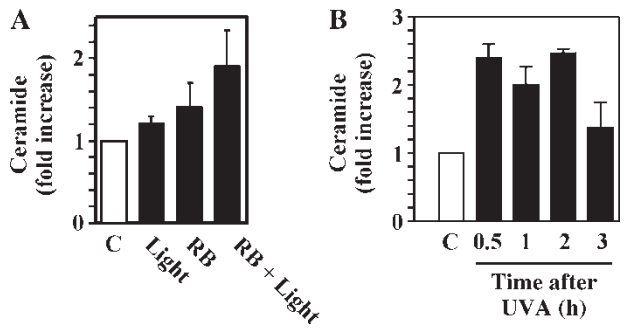

FIGURE 4 Ceramide generation in human skin fibroblasts after exposure to photochemically generated singlet oxygen (rose Bengal, RB, plus light) and UVA1. (A) Human skin fibroblasts were exposed to RB plus light for 10 min, followed by posttreatment in HBSS for 15 min and lysis for ceramide analysis as described in "Materials and methods", section. Data are means ± SEM ($n = 8$). (B) Human skin fibroblasts were covered with PBS and exposed to UVA1 (30 J/cm^2), followed by postincubation in medium for the given times and lysis for ceramide analysis. Data are means ± SD, $n = 3$.

skin fibroblasts with cell permeable C_2-ceramide rendered cells refractory to growth factor-induced ERK and Akt activation (Fig. 5). Treatment with EGF and PDGF resulted in a strong dual phosphorylation of ERK (Fig. 5A) and phosphorylation of Akt at Ser473 and Thr308 (Fig. 5B), respectively. ERK and Akt activation were prevented if exposure to growth factors was preceded by treatment with C_2-ceramide for 30 min.

Ceramide alone neither activated ERK (not shown) nor Akt (Fig. 5B), implying that Akt *activation* by $^1O_2$ is independent from ceramide generation. On the contrary: it appears that dephosphorylation of both ERK 1/2 and Akt was promoted by preincubation with C_2-ceramide.

Singlet Oxygen and Ceramide Differ in their Affecting the EGF Receptor

A possible explanation for the attenuation of growth factor-induced phosphorylation of ERK and Akt by exposure to $^1O_2$ is that $^1O_2$ might block the activation of the respective growth factor receptor. In order to test this hypothesis, phosphorylation of the EGF receptor at tyrosine-1068 by EGF was tested for after exposure to singlet oxygen (Fig. 6A) or C_2-ceramide (Fig. 6B). Indeed, EGF-induced EGFR tyrosine phosphorylation was diminished in fibroblasts exposed to RB/light (Fig. 6A). This effect was accompanied by a second phenomenon, i.e. the apparent loss of the EGFR itself (Fig. 6A, bottom row). Different from these singlet oxygen effects, however, C_2-ceramide neither impaired EGF-induced EGFR phosphorylation (Fig. 6B, top row) nor did it induce a loss in EGFR (bottom row). Thus, in addition to Akt activation, the attenuation of EGFR phosphorylation as well as the loss of EGFR are singlet oxygen-effects that do not rely on the intermediate formation of ceramide.

DISCUSSION

Singlet oxygen is known to be a proapoptotic stimulus: the activation of p38 in human promyelocytic leukemia cells exposed to RB/light may trigger cleavage of bid, leading to cytochrome c release from mitochondria, which, in turn, results in activation of caspase 3.[28] JNK, which are also known to be activated upon treatment of cells with $^1O_2$,[9] were shown to be required for $^1O_2$-induced activation of

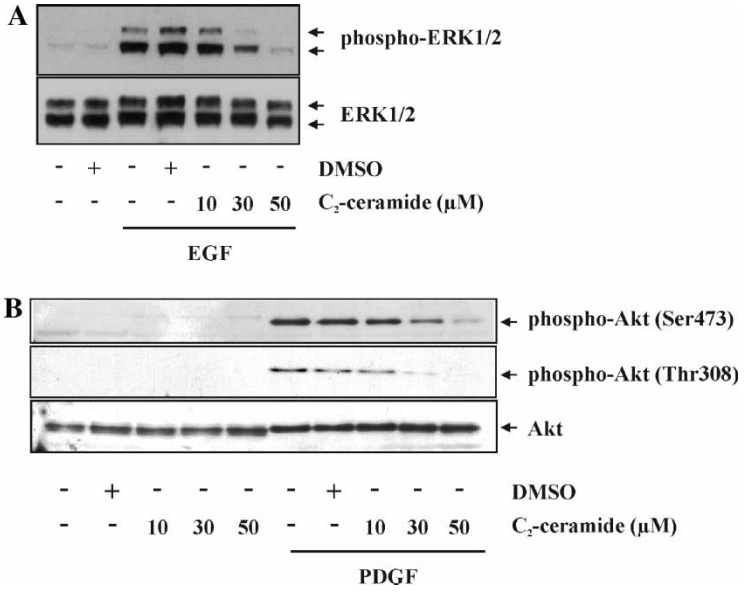

FIGURE 5 C_2-ceramide interferes with growth factor signaling. Human skin fibroblasts were exposed to C_2-ceramide at the given concentrations or DMSO (vehicle) for 30 min in serum-free medium, followed by addition of EGF (A, 100 ng/ml) or PDGF-AB (B; 50 ng/ml) for another 30 min. Cells were then lysed and analyzed by Western blotting.

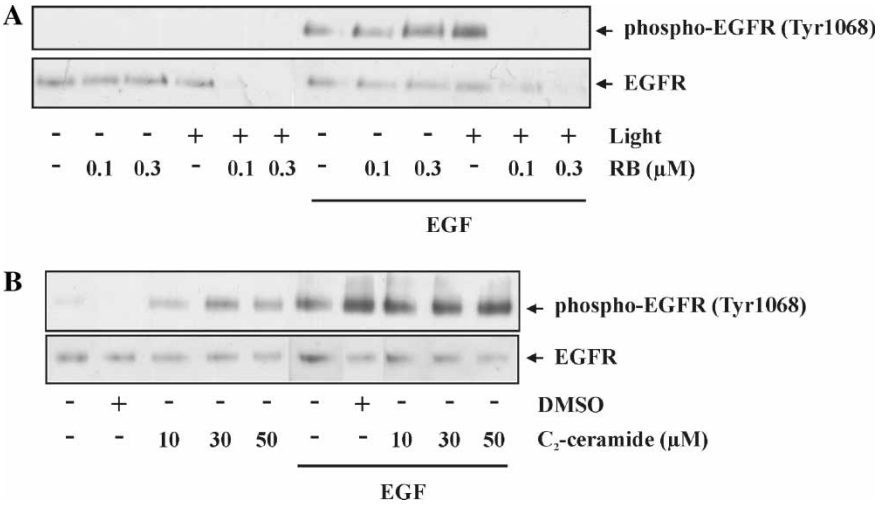

FIGURE 6 Singlet oxygen, but not C_2-ceramide, affects EGF-induced phosphorylation of the EGF-receptor. 1306 human skin fibroblasts were grown to confluency, serum-starved overnight and exposed to singlet oxygen [generated by irradiation of rose Bengal (RB) solutions for 10 min with white light] (A) or to C_2-ceramide or DMSO (vehicle control) for 30 min (B) prior to incubation with EGF (100 ng/ml) for 30 min, followed by lysis and Western analysis of EGF receptor (EGFR) phosphorylation.

caspase 3 in human epidermoid carcinoma cells.[11] Different from p38 and JNK, the activation of ERK 1/2 is usually regarded as promoting proliferation and anti-apoptotic (see "Introduction", section), although there are exceptions, such as *cis*-platinum-induced apoptosis, where ERK 1/2 appear to promote rather than prevent cell death.[29] Hence, to lower basal activity and to prevent the activation of ERK 1/2 (Figs. 1 and 2) with their anti-apoptotic action would be in line with the recognized proapoptotic properties of $^1O_2$. Similarly, Akt is an anti-apoptotic protein (see "Introduction", section) the activation of which by growth factors is prevented by prior exposure to $^1O_2$ (Fig. 1). Interestingly, $^1O_2$ per se moderately activates Akt (Fig. 3). Yet this activation, although leading to phosphorylation (and thus the inhibition) of GSK3 (Fig. 3C), does not significantly affect cellular survival after exposure to $^1O_2$, which was unchanged in the presence of inhibitors of PI3K (not shown). At present, it is not known what mediates the activation of the PI3K/Akt pathway by $^1O_2$ (Fig. 3). Growth factor receptors such as the EGFR or the PDGFR mediate the activation of the pathway by hydrogen peroxide[21] and peroxynitrite,[23], respectively. Neither ceramide production nor the inactivation of a regulating tyrosine phosphatase are responsible for PI3K/Akt activation. Ceramides can be excluded as activators because the addition of C_2-ceramide did not induce any phosphorylation of Akt (Fig. 5B). The inactivation of (a) tyrosine phosphatase(s) negatively regulating growth factor receptor tyrosine kinases was made responsible for UV- and hydrogen peroxide-induced net activation of receptor tyrosine kinases,[30,31] but can be excluded here because the inactivation of such a phosphatase by $^1O_2$ would render the system more susceptible to the action of growth factors and would result in an enhanced rather than attenuated phosphorylation of Akt by $^1O_2$ (Fig. 1). A role of growth factor receptors in Akt activation by singlet oxygen in murine fibroblasts was excluded by Zhuang and Kochevar.[32]. In this respect, exposure of cells to singlet oxygen resembles treatment with Cu^{2+} which similarly activates Akt independent of growth factor receptors.[33]

Apoptosis caused by UVA[34] and by PDT (for review, see Ref. [35]) is, at least in part, mediated by the photochemical generation of $^1O_2$. Both irradiation with UVA[15,18,19] and PDT[16,17,36] as well as chemically generated $^1O_2$[15] result in the generation of ceramide, which is associated with apoptotic death of the targeted cells.

The sphingomyelin-ceramide pathway is involved in the regulation of a variety of cellular functions such as growth, differentiation and cell death, depending on stimulus and cell type. A number of signaling molecules were identified as targets of ceramides, including kinases such as PKCzeta[37,38] and others (for review, see Ref. [39]) or phosphatases: the Ser/Thr protein phosphatases PP1 and PP2A were described as ceramide-activated phosphatases.[40–43]

Interestingly, the exposure of cells to water-soluble ceramides (C_2- or C_6-ceramide) results in a MAPK activation pattern resembling that of human skin fibroblasts exposed to $^1O_2$: JNKs are activated,[38,44] but the dephosphorylation of ERK 1/2 is promoted,[44,45] similar to the results presented in Figs. 1, 2 and 5.

In addition to the activation of Ser/Thr phosphatases by ceramides, the activation of tyrosine phosphatases[46] as well as the impairment of the interaction of PKCepsilon with ERKs and Raf-1,[47] a kinase upstream of ERK 1/2, were discussed as

possible mechanisms for attenuated activation of ERKs in the presence of ceramides.

Similarly, phosphorylation of Akt is attenuated by ceramides: ceramide-promoted Akt dephosphorylation was made responsible for loss in Akt activity, although the inhibition of PI3K by ceramides in rat fibroblasts, as well as the activation of PKCzeta by ceramides and the interaction of this kinase with Akt was also shown to occur and hypothesized to cause loss in Akt activity and phosphorylation after exposure to ceramides.[48–50]

Interestingly, and in line with our hypothesis that Ser/Thr phosphatases might be involved in the ceramide-mediated regulation of ERK and Akt activation by singlet oxygen (Fig. 7), okadaic acid, an inhibitor of PP1 and PP2a, was demonstrated to prevent a singlet oxygen-induced decrease in ERK and Akt phosphorylation in immortalized human keratinocytes.[51] These authors also described the caspase-3-dependent degradation of the EGFR as well as the dephosphorylation of the EGFR in cells exposed to singlet oxygen, which is in line with the disappearance of the EGFR and phospho-EGFR signals in Fig. 6A. This latter effect, however, is not seen with C_2-ceramide (Fig. 6B), which renders it likely that control of growth factor signaling by singlet oxygen is at two levels, (i) at the level of the growth factor receptor, which is independent of ceramide formation, and (ii) at the level of ERK/Akt dephosphorylation, which is mimicked by C_2-ceramide. At this point, a contribution of EGFR dephosphorylation to singlet oxygen-induced loss of EGF activation of ERK and Akt cannot be excluded.

In analogy to the generation of ceramides in keratinocytes and sphingomyelin-containing liposomes exposed to $^1O_2$,[15] we hypothesize that ceramides are generated non-enzymatically in fibroblasts exposed to photochemically generated $^1O_2$. Thus, inhibitors of sphingomyelinases or ceramide synthase were not employed to block the demonstrated $^1O_2$-effects.

In summary, we suggest that ceramide generation upon exposure of human skin fibroblasts to $^1O_2$ may be partly responsible for the loss in responsiveness of ERK 1/2 and Akt to stimulation by growth factors. This points to a role of ceramides as mediators of the $^1O_2$-induced inhibition of growth factor-induced activation of cellular survival pathways and to a novel mechanism of $^1O_2$ toxicity: the known $^1O_2$-induced activation of proapoptotic kinases such as JNK and p38 is paralleled by the prevention of activation of anti-apoptotic kinases (ERK, Akt), thus shifting the balance toward apoptosis.

$^1O_2$-induced activation of the PI3K/Akt pathway is in parallel to, and independent from, the generation of ceramides, but the latter may control the extent of Akt phosphorylation (Fig. 7).

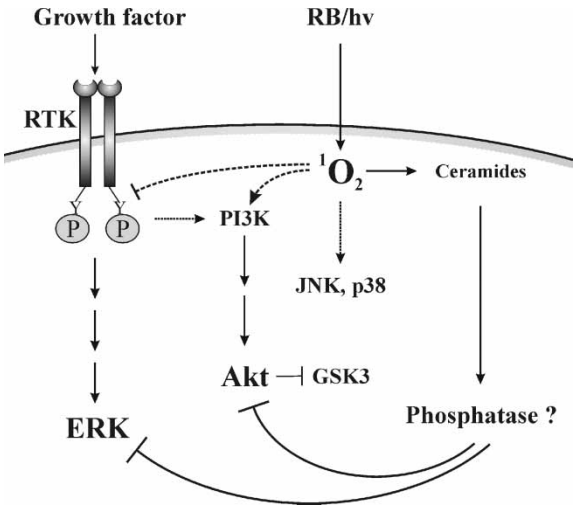

FIGURE 7 Hypothetical interaction between growth factor and ceramide signaling pathways in the cellular response to photochemically generated singlet oxygen. Exposure of human skin fibroblasts to rose Bengal (RB) plus light results in a moderate activation of Akt via phosphoinositide 3-kinase (PI3K). Akt, upon activation, phosphorylates and thereby inactivates glycogen synthase kinase-3 (GSK3). Ceramides are generated upon exposure to singlet oxygen and negatively control Akt and ERK phosphorylation, that was induced by growth factor activation of receptor tyrosine kinases (RTK). Negative control by singlet oxygen is at two levels, (i) the inhibition of RTK tyrosine phosphorylation by singlet oxygen and (ii) via phosphatase activation by ceramides.

Acknowledgements

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