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Activation of protein kinase C is required for protection of cells against apoptosis induced by singlet oxygen

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Abstract We evaluated the role of protein kinase C (PKC) in the regulation of apoptosis triggered by singlet oxygen. Activation of PKC by short-term 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) treatment inhibited apoptosis, whereas inhibition of PKC with several inhibitors potentiated this process. The antiapoptotic effect of TPA was accompanied by phosphorylation of extracelluar signal-regulated kinase 1/2 (ERK1/2). Pretreatment of cells with MEK inhibitor, PD98059, inhibited TPA-induced phosphorylation of ERK1/2 and the cytoprotective ability of TPA. These results suggest that activation of PKC in HL-60 cells confers protection against apoptosis induced by singlet oxygen and that ERK1/2 mediates antiapoptotic signaling of PKC.

Key words: Protein kinase C; Apoptosis; Singlet oxygen; Extracellular signal-regulated kinase; Rose bengal; HL-60 cell

1. Introduction

Apoptosis is believed to play an important role in destruction of malignant cells treated with photodynamic therapy (PDT) [1]. The mechanism of this therapy involves the formation of reactive oxygen species (ROS) following the uptake of a photosensitive dye and subsequent activation of the dye with visible light. The types of ROS generated in this process depend on the photosensitizing drug, its intracellular location and the light wavelength. We have previously shown that excitation of rose bengal (RB), a photosensitizer localizing in the plasma membrane, with UVA irradiation produced radicals and singlet oxygen, whereas with visible light irradiation it produced only singlet oxygen [2]. Further studies indicated that both species may be associated with induction of apoptosis [3].

The signaling mechanisms that mediate apoptosis in response to PDT are being studied and are still unclear. A early observation indicated the PDT-induced apoptosis did not involve new protein or RNA synthesis in L5178 mouse lymphoma cells [4], whereas an increase in production of ceramide was associated with DNA fragmentation [5]. Recently, Luo et al. [6] reported that PDT-induced apoptosis could be attenuated by inhibition of serine/threonine phosphatase 1 and 2A and potentiated by a serine/threonine kinase inhibitor, suggesting that serine/threonine dephosphorylation is required for the initiation of apoptosis. In other words, activation of one or more serine/threonine kinases could protect cells against apoptosis. Although several serine/threonine kinases are linked to antiapoptotic signaling [7–9], little is known about the serine/threonine kinases involved in regulation of singlet oxygen-induced apoptosis.

Protein kinase C (PKC) is a well known serine/threonine kinase, consisting of three groups, cPKC (α, β, β2 and γ), nPKC (δ, ε, η and θ) and aPKC (ζ and ι/λ), and is involved in many cellular functions, such as proliferation and differentiation. Recently, a number of studies have shown that PKC also participates in the regulation of apoptosis induced by many stimuli, such as antitumor drugs, tumor necrosis factor α (TNFα) and ionizing irradiation [10–12]. However, the regulatory effect of PKC on apoptosis is still controversial; PKC activation and PKC inhibition can, depending upon the cell types and inducers used, either prevent or induce apoptosis [13]. In HL-60 cells, downregulation of PKC potentiates ara-C-induced apoptosis and activation of PKC by exposure to PMA (phorbol-12-myristate 13-acetate) opposes apoptosis induced by topoisomerase I and II inhibitors [14,15]. In contrast to these findings, a stimulatory role of PKC is suggested by the reported ability of PMA to enhance nitric oxide-induced fragmentation of genomic DNA [16].

In this study, we have investigated the potential role of PKC in regulation of apoptosis induced by singlet oxygen and found that short-term treatment of cells with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), a PKC activator, protects cells against apoptosis, whereas pharmacological inhibition of PKC with various inhibitors potentiates this process, suggesting that PKC functions as a negative regulator in singlet oxygen-induced apoptosis. Further studies demonstrated that ERK1/2 mediates the antiapoptotic signaling of PKC.

2. Materials and methods

2.1. Chemicals

TPA, H7, staurosporine and diamidino phenyl indole (DAPI) were purchased from Sigma, GF109203X was from Calbiochem-Novabiochem, PD98059 was from Alexis and rose bengal was from Aldrich. Rabbit polyclonal ERK (ERK1-CT) antibody was purchased from Upstate Biotechnology and phospho-specific p44/42 MAP kinase antibody was from New England Biolabs. Rabbit monoclonal Bcl-2 antibody was obtained from Santa Cruz Biotechnology.

2.2. Cell culture and treatment

Early passage human promyelocytic leukemia HL-60 cells, obtained from the ATCC, were grown in suspension in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) in the absence of antibiotics. Cells were passaged twice a week and used between passages 20 and 40. For cell photosensitization, cell suspensions in RPMI 1640 without phenol red and FBS were incubated with 3 μM RB for 15 min at room temperature and then exposed to visible light (514 nm) provided by a continuous wave argon laser (Innova 100, Coher-

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Abbreviations: PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MEK, ERK kinase; JNK, c-jun NH2-terminal kinase; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; DMEM, Dulbecco's modified Eagle's medium; RB, rose bengal

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ent). The irradiance at the level of cells was 30 mW/cm^2. After irradiation, cells were transferred to RPMI 1640 medium with 20% FBS and incubated at 2×10^5 cells/ml for the indicated times before harvesting. When required, concentrated TPA (100 μg/ml) was added to the medium to give a final concentration of 100 ng/ml.

2.3. Morphology observations

Cell morphology was evaluated by fluorescence microscopy following DAPI staining. Apoptotic cells were identified by features characteristic of apoptosis (e.g. nuclear fragmentation and condensation, formation of membrane blebs and apoptotic bodies). Necrotic cells were determined by staining with 0.4% trypan blue. Five hundred cells were counted for each sample and the numbers of apoptotic or necrotic cells were expressed as the percentage of the total cell population.

2.4. Analysis of DNA fragmentation

Cells (2×10^6/ml) were pelleted by centrifugation at $200\times g$ for 5 min. Cell pellets were resuspended in lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 1% SDS) and incubated in proteinase K (10 μg/ml) at 56°C overnight. The lysates were then incubated with ribonuclease A (10 μg/ml) for 2 h at 37°C. DNA preparations were precipitated with 2-propanol and subsequently washed with 75% ethanol. After air-drying, the DNA pellets were resuspended in 30 μl of TE buffer, separated by horizontal electrophoresis on 1.5% agarose gels, stained with 0.5 μg/ml ethidium bromide, and visualized under ultraviolet light.

2.5. Western blot analysis

Cells were centrifuged for 5 min at $200\times g$, washed once with phosphate-buffered saline without calcium chloride and magnesium chloride and then suspended in the lysis buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 1 mg/ml bromophenol blue, and 0.5% 2-mercaptoethanol). Equal amounts of total cellular protein lysates were separated on 10% polyacrylamide gels. For detection of the phosphorylated form of Bcl-2, 12% polyacrylamide gels were used and electrophoretically transferred to nitrocellulose membranes. After treatment of the membrane with 5% skim milk at 4°C overnight, the membranes were incubated sequentially with the indicated primary antibodies followed by an appropriate horseradish peroxidase-conjugated secondary antibody. Bound antibodies were visualized with chemiluminescence detection on autoradiographic film.

3. Results

We have investigated apoptosis initiated by singlet oxygen in HL-60 cells. Singlet oxygen was generated by RB photosensitization. As shown in Fig. 1, exposure of cells to visible light (514 nm) at a dose of 300 mJ/cm^2 in the presence of 3 μM RB induced typical morphological features of apoptosis as characterized by fragmented nuclei and the formation of apoptotic bodies following 2.5 h incubation. In contrast, neither visible light nor RB alone increased the number of apoptotic cells compared to control samples (data not shown). Consistent with these results, agarose gel electrophoresis revealed that photosensitization with RB caused DNA cleavage with the characteristic pattern of internucleosomal fragmentation produced by apoptosis whereas DNA from cells irradiated by visible light in the absence of RB or exposure of cells to RB without irradiation was unfragmented (data not shown). These results suggest that generation of singlet oxygen by photosensitization can induce apoptosis in HL-60 cells.

Analysis of the time course showed that DNA fragmentation was barely visible at 1 h after photosensitization and increased progressively with incubation time (Fig. 2A); laddering was most apparent at 4 h and decreased with further incubation. Staining of photosensitized cells with trypan blue, indicative of necrosis, increased with incubation time (1–5 h) (Fig. 2B). DNA fragmentation induced by singlet oxygen also

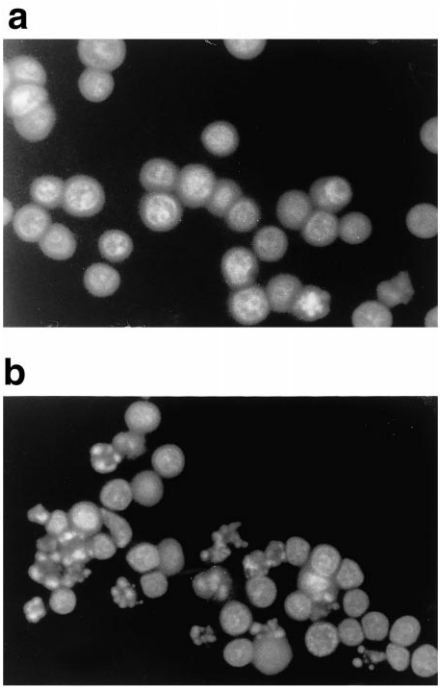

Fig. 1. Singlet oxygen generated by photosensitization with rose bengal and 514 nm light induces apoptosis in HL-60 cells. HL-60 cells (2×10^6/ml) were treated with vehicle or RB (3 μM) for 15 min and then either left untreated or irradiated with 514 light at a dose of 300 mJ/cm^2. After incubation in the complete medium for 2.5 h, cells were harvested and stained with DAPI. a: Control; b: photosensitization with RB plus light (PS). Results are representative of three independent experiments.

increased with light dose, with a maximum effect produced by 300–420 mJ/cm^2. In this experiment, photosensitization with RB was followed by 2.5 h incubation; DNA ladders became apparent at 180 mJ/cm^2 (Fig. 2C). Doses of light over 540 mJ/cm^2 inhibited formation of DNA laddering although trypan blue staining of cells increased in a dose-dependent fashion (Fig. 2D). These results indicate that lower doses of light or shorter incubation time following photosensitization produce apoptosis, but higher light doses or longer incubation time are associated with necrosis. For our studies of the regulatory mechanism of singlet oxygen-induced apoptosis, 300 mJ/cm^2 of 514 light and 2.5 h of incubation were used for the following experiments.

It has been reported that the alteration of PKC activity is associated with modulation of apoptosis induced by a variety of stimuli [13,17]. We were interested in determining whether PKC is involved in the regulation of apoptosis induced by singlet oxygen. To investigate the effect of PKC on singlet oxygen-induced apoptosis, we first examined the effect of TPA, a potent PKC activator. It is well known that TPA can activate c and nPKC after short-term treatment [18]. As shown in Fig. 3, incubation of cells with TPA dramatically inhibited the expression of apoptotic morphology in response to visible light plus RB. In contrast to the effect of TPA, 4-α-TPA, an analogue of TPA without the ability to activate PKC, had no effect. These results suggest that the activation of PKC protects cells against apoptosis induced by singlet oxygen.

The role of PKC in singlet oxygen-induced apoptosis was further evaluated by using three PKC inhibitors. H7 and

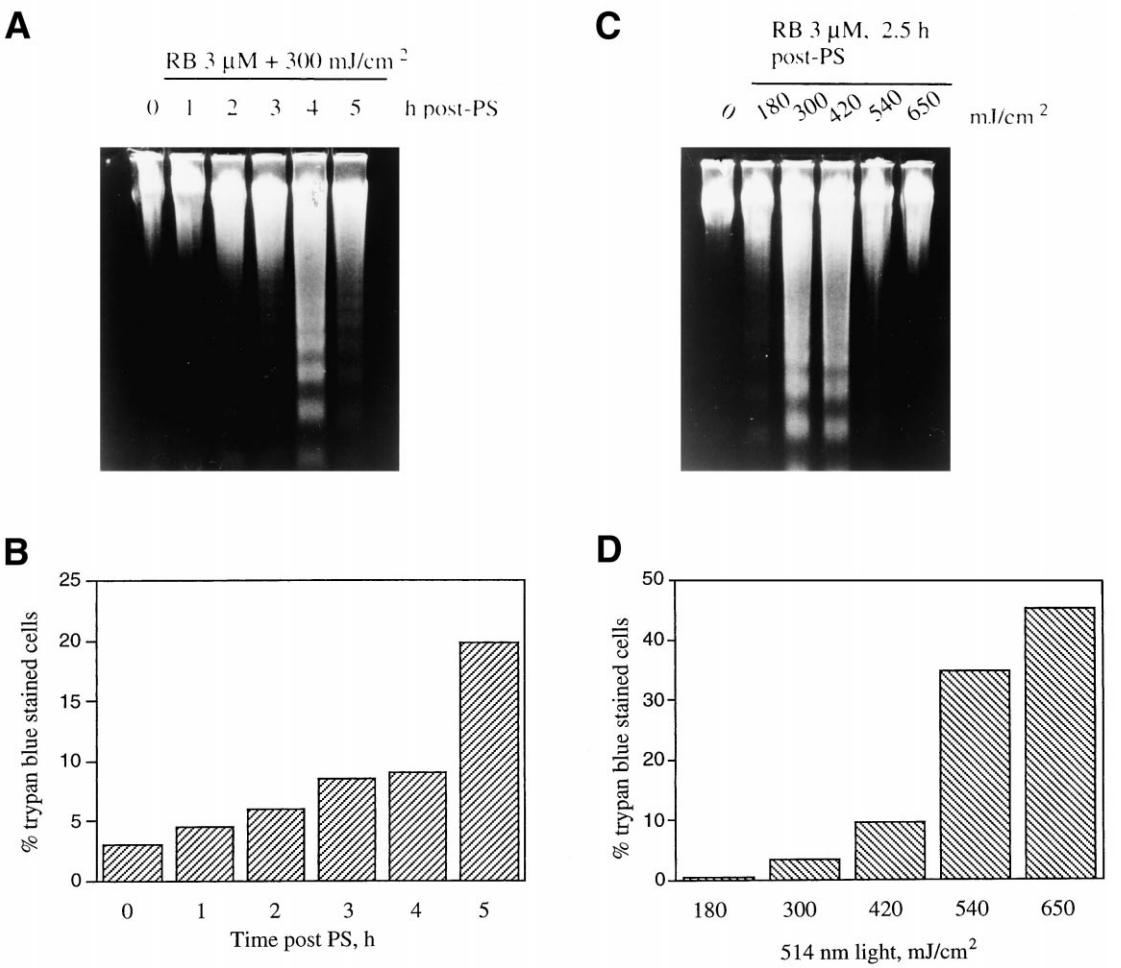

Fig. 2. Time course for singlet oxygen-induced cell death as estimated by DNA fragmentation and trypan blue staining. Cells (2×10^6/ml) were treated with RB (3 μM) for 15 min and irradiated with 300 mJ/cm^2 of 514 nm light. After incubation for the indicated times, cells were harvested for DNA analysis on a 1.5% agarose gel (A) or stained with 0.4% trypan blue and then counted (B). Cells (2×10^6) were treated with RB as above and then irradiated with a variety of doses of 514 nm light as indicated. After incubation for 2.5 h, cells were harvested for DNA fragmentation analysis (C) or stained with trypan blue and then counted (D). Results are representative of three independent experiments.

staurosporine are non-specific PKC inhibitors, which have been shown to be able to induce apoptosis in HL-60 cells [19]. In contrast, GF109203X is a highly selective PKC inhibitor [20]. To investigate the effect of these PKC inhibitors on singlet oxygen-induced apoptosis, cells were pretreated with each agent for 1 h, and then exposed to light plus RB. As shown in Fig. 4, all the PKC inhibitors potentiated the apoptotic response to singlet oxygen, with the greatest effect in staurosporine-treated cells. Incubation of cells with each inhibitor alone for 2.5 h only had a minor effect. Thus, these data, together with the effect of short-term TPA treatment, suggest that PKC mediates antiapoptotic signaling in singlet oxygen-induced apoptosis.

Since the activation of ERK has been reported to play an antiapoptotic role in several systems [21–23], we asked whether the antiapoptotic action conferred by TPA was through the activation of ERK pathways. To test this possibility we investigated the effect of a highly specific MEK inhibitor, PD98059, on TPA-mediated protection in singlet oxygen-induced apoptosis. As shown in Fig. 5A, incubation of cells in RPMI 1640 medium containing 20% FBS caused a baseline ERK1/2 phosphorylation (lane 1 and 5); photosensi-

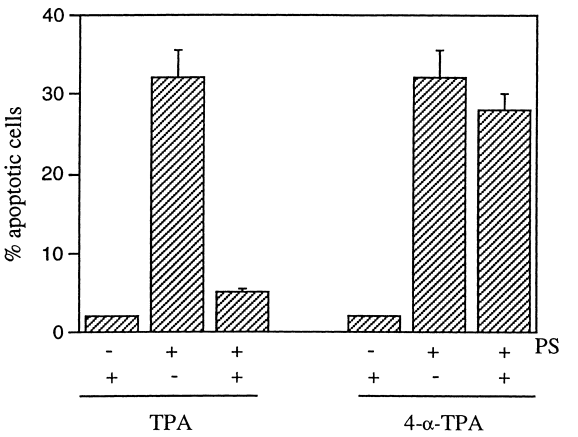

Fig. 3. Activation of PKC by TPA protects cells from singlet oxygen-induced apoptosis. After photosensitization with 3 μM RB plus 300 mJ/cm^2 514 nm light, cells were incubated in complete medium containing TPA (100 ng/ml) or 4-α-TPA (100 ng/ml) for 2.5 h and then harvested. Apoptotic cells were assessed as described in Section 2. Values are means ± S.D. of the data from three independent experiments. PS indicates photosensitization with RB plus 514 nm light.

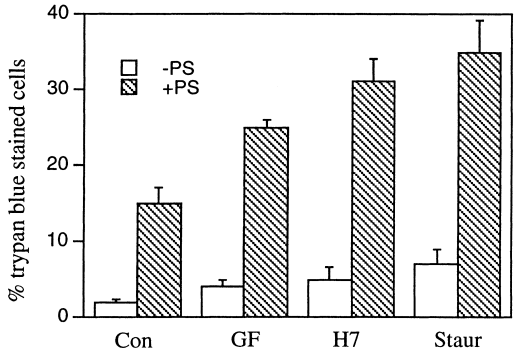

Fig. 4. PKC inhibitors enhance singlet oxygen-induced apoptosis. Cells were treated with vehicle (Con), 5 μM GF109203X (GF), 10 μM H7 or 100 nM staurosporine (Staur) for 1 h followed by photosensitization (PS) with 3 μM RB plus 300 mJ/cm^2 light. After incubation for 2.5 h in the presence of the agents, cells were harvested and stained with DAPI. Apoptotic cells were scored as described in Section 2. Values are means ± S.D. of the data from three independent experiments.

tization with RB had no further effect (lane 2). However, pretreatment of cells with TPA induced a phosphorylation of ERK1/2 above baseline (lane 3). This phosphorylation could be abolished by MEK inhibitor, PD98059 (lanes 4 and 7).

Fig. 5B shows that treatment of cells with PD98059 also abolished the antiapoptotic ability of TPA. Incubation of cells with 100 ng/ml of TPA led to inhibition of singlet oxygen-induced apoptosis by 52%. In the presence of PD98059, TPA-treated cells resumed almost their full ability to undergo apoptosis in response to singlet oxygen. PD98059 itself slightly enhanced apoptosis. These results indicate that activation of ERK1/2 is required for mediating the antiapoptotic effect of PKC.

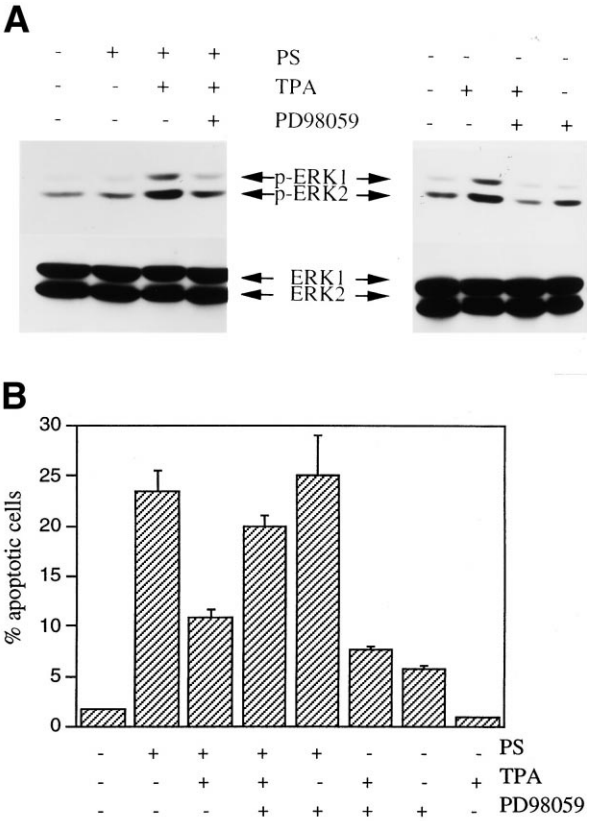

Fig. 5. PD98059 inhibits TPA-mediated ERK1/2 phosphorylation and abrogates the antiapoptotic effect of TPA. Cells were treated with vehicle or 20 μM PD98059 for 30 min followed by photosensitization (PS) with 3 μM RB plus 300 mJ/cm^2 light. After incubation for 2.5 h in the presence or absence of 100 ng/ml TPA, cell lysates were subjected to SDS-PAGE analysis and blotted with antibodies to phospho-ERK1/2 or ERK1/2 (representative of three experiments) (A) or apoptotic cells were evaluated as described in Section 2 after staining with DAPI. Values are means ± S.D. of the data from three independent experiments (B).

4. Discussion

The induction of apoptosis by PDT depends on the formation of ROS, including singlet oxygen and radicals. We have previously shown that singlet oxygen generated in plasma membrane is more effective than radicals produced from RB in the same site for initiation of apoptosis in HL-60 cells [3]. In this study, we have shown that activation of PKC by TPA suppresses singlet oxygen-induced apoptosis and that the cytoprotective ability of TPA is associated with activation of ERK1/2.

Although several reports have demonstrated that activation of PKC protects cells against apoptosis [10–12], the mechanisms for this antiapoptotic activity are not well defined. It has been demonstrated that expression of Bcl-2, an antiapoptotic protein, is increased following short-term phorbol ester treatment in the immature Ramos B-cell line, and up-regulation of expression of Bcl-2 increases cell resistance to apoptosis [24,25]. In HL-60 cells, induction of apoptosis by ara-C has been shown to be associated with down-regulation of Bcl-2 [26]. However, apoptosis induced by photosensitization is a rapid process, which does not require new protein synthesis [4]. Consistent with this finding, the Bcl-2 level was unchanged throughout the 5 h time course examined following photosensitization (data not shown). In addition, incubation with TPA also did not alter Bcl-2 expression in photosensitized cells. These results indicate that suppression of apoptosis by TPA was not through regulating expression of Bcl-2. Recently, May et al. have reported that PKC activation results in the rapid phosphorylation of Bcl-2, which is required for its antiapoptotic function in murine myeloid cell line, FDC-P1/ER and NFS/N1.H7 [27]. However, Bcl-2 phosphorylation was not seen following TPA treatment in HL-60 cells (data not shown), suggesting that the antiapoptotic effect exerted by PKC activation does not involve Bcl-2 phosphorylation in this cell line.

In addition to Bcl-2, PKC also interacts with other signaling molecules which can mediate cell survival. This is illustrated by observations such as those of Kyriakis et al. [28] where PKC activation results in the rapid phosphorylation and activation of c-raf kinase, which in turn sequentially activates MEK and ERK kinases. In addition, reports from several laboratories show that ERK mediates an antiapoptotic signal in various stimuli-induced apoptosis [21–23]. In this study, we have demonstrated that activation of PKC by TPA induced ERK1/2 phosphorylation and subsequently inhibited apoptosis, and that these effects were abolished by blockade of ERK1/2 activation (Fig. 5), suggesting that ERK is necessary for mediating the antiapoptotic effect of PKC in HL-60 cells.

How ERK activation inhibits apoptosis remains to be determined. Xie et al. [23] have reported that ERK and JNK/p38 kinases play opposing effects on apoptosis induced by NGF withdrawal in rat pheochromocytoma PC-12 cell, activation of ERK and suppression of JNK/SAPK promoting cell survival, while activation of JNK and p38 and inhibition of ERK inducing apoptosis. This model was further extended by Spiegel et al. in HL-60 cells indicating that a balance between levels of sphingolipid metabolites, sphingosine-1-phosphate (SPP) and ceramide, and their effects on the ERK and JNK pathways may be critical in determining whether a cell survival or undergoes apoptosis [29]. It has been reported that photosensitization of cells stimulated an increase in synthesis of ceramide and activated JNK/p38 [5,30], while activation of PKC by TPA could inhibit neutral sphingomyelinase activation and ceramide production and stimulate sphingosine kinase activity and increased SPP [29,31]. Thus, it is reasonable to speculate that the TPA-mediated antiapoptotic effect might be mediated by an increase of SPP and subsequent activation of ERK and inhibition of the stress proteins, JNK or p38.

Agarwal et al. [32] have reported that apoptosis in mouse lymphoma cells photosensitized with aluminum phthalocyanine was associated with phospholipase activation, and the inhibitors for phospholipase C (PLC) could block the formation of fragmentation of nuclear DNA, implying that activation of PLC is associated with initiation of apoptosis. Since activated PLC hydrolyzes the lipids, resulting in the production of DAG, a direct endogenous activator of PKC, it seems that in this system PKC might function as a potential downstream target that links intracellular lipid signal to induction of apoptosis by PDT. If this is case, it would be contrary to our results. A possible explanation for this difference is that the activation of PLC by photosensitization may mainly signal to other downstream molecules which trigger apoptosis. It has been reported that PLC is not only associated with activation of PKC, but also with production of ceramide, a mediator for apoptosis induced by various stimuli [33]. In addition, It is also possible that production of different types of ROS by photosensitization of different photosensitizers, may trigger distinct signaling pathways responsible for apoptosis.

In summary, the findings of this study indicate that activation of PKC is able to confer resistance to apoptosis induced by singlet oxygen generated in the plasma membrane and that ERK is required for transducing the antiapoptotic signaling. How activation of ERK affects antiapoptotic signaling needs further investigation.

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