The Protein Kinase C Activators Phorbol Esters and Phosphatidylserine Inhibit Neutral Sphingomyelinase Activation, Ceramide Generation, and Apoptosis Triggered by Daunorubicin

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ABSTRACT

To address the role of protein kinase C (PKC) in the regulation of ceramide production, we evaluated the impact of the PKC activators 12-O-tetradecanoylphorbol-13-acetate and phosphatidylserine on the apoptotic signaling pathway triggered by the chemotherapeutic drug daunorubicin. Treatment of U937 and HL-60 cells with 0.5–1 μM daunorubicin induced a greater than 30% activation of neutral sphingomyelinase activity within 4–10 min with concomitant sphingomyelin hydrolysis and ceramide generation. Activation of PKC by 12-O-tetradecanoylphorbol-13-acetate and phosphatidylserine inhibited daunorubicin-induced neutral sphingomyelinase activation, sphingomyelin hydrolysis, ceramide generation, and apoptosis. The apoptotic response could be restored by the addition of 25 μM cell-permeant C6-ceramide. In conclusion, PKC emerges as a potentially critical negative regulator of the anthracycline-activated sphingomyelin-ceramide apoptotic pathway.

INTRODUCTION

Anthracyclines are one of the most active antitumor compounds used in clinical oncology, especially in the treatment of acute leukemias. Despite intense efforts, their mechanism of action is still not fully understood. Indeed, it has been documented that anthracyclines induce membrane alterations through lipid peroxidation; generate radical oxygen species due to electron transfer from the semiquinone ring; disturb mitochondrial homeostasis; intercalate into nuclear DNA; and interact with both topoisomerase I and II, leading to covalent binding of these enzymes to DNA and subsequent DNA breaks (for a review, see Ref. 1). More recently, it has been shown that anthracyclines induce apoptosis in some, but not all, cellular models (2, 3). Present knowledge does not allow us to determine whether apoptosis simply reflects membrane and/or DNA lesions or represents an independent cytotoxic mechanism triggered by a specific signaling pathway (for a review, see Ref. 4).

In a recent report, we demonstrated that daunorubicin activates the sphingomyelin-ceramide cycle. Indeed, daunorubicin stimulated neutral sphingomyelinase activity responsible for sphingomyelin hydrolysis and subsequent ceramide generation in U937 and HL-60 human leukemia cells (5). The fact that cell-permeant ceramides, as well as natural ceramide (generated by exposure of the cells to bacterial sphingomyelinase), induce apoptosis in these cells suggests strongly that ceramide was the mediator of daunorubicin-induced apoptosis. Such an apoptotic signaling pathway has also been described in vincristine, ionizing radiation, anti-Fas, and tumor necrosis factor α-induced apoptosis (for a review, see Ref. 6).

The sphingomyelin-ceramide pathway appears to be efficiently regulated downstream of ceramide generation. For example, we reported recently that Bel-2 overexpression blocked both daunorubicin and cell-permeant ceramide-induced apoptosis of leukemic cells but did not affect daunorubicin-induced ceramide generation (7). Previous studies, Jarvis et al. (8) showed that not only PKC³ activators including phorbol esters (TPA) and diacylglycerol inhibited the ability of cell-permeant ceramides to induce apoptosis but also that PKC inhibitors enhanced ceramide-induced apoptosis (9). From these studies, it has been suggested that PKC is a key negative regulator of ceramide-induced apoptosis.

Little is known about the role of PKC in the regulation of ceramide production. It has been reported recently that PKC inhibitors triggered neutral sphingomyelinase, suggesting that PKC may also play an important role by regulating basal sphingomyelinase activity (10). These findings are consistent with those reported by Haimovitz-Friedmann et al. (11), who observed that TPA inhibited ceramide generation and apoptosis in irradiated bovine endothelial cells.

In this study, we present evidence that daunorubicin-triggered sphingomyelinase activity, ceramide generation, and apoptosis in myeloid leukemia cells can be inhibited by the PKC activators TPA and phosphatidylserine.

MATERIALS AND METHODS

Drugs and Reagents. Daunorubicin was obtained from the National Cancer Institute Drug Repository. TPA and bovine brain phosphatidylserine were from Sigma Chemical Co. (Paris, France). All drugs were diluted in ethanol (final dilution, <0.01%).

Cell Culture. The human monocytic leukemia cell lines U937 and HL-60, purchased from the American Type Culture Collection (Rockville, MD), were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Eurobio, Les Ulis, France). Cell stocks were screened routinely for Mycoplasma (Stratagene Mycoplasma PCR kit; Stratagene, La Jolla, CA).

Cytochemical Staining. Changes in cellular nuclear chromatin were evaluated by fluorescence microscopy with DAPI staining (12).

DNA Analyses. DNA was resolved on a 1.8% agarose gel and visualized with ethidium bromide as described previously (13). Quantitative DNA fragmentation was determined by the spectrofluorometric DAPI procedure as described previously (14, 15).

Metabolic Cell Labeling and Sphingolipid Quantitation. Sphingomyelin quantitation was performed by labeling cells to isotopic equilibrium with 0.4 μCi/ml of [methyl-³H]choline (specific activity 81.0 Ci/mmol, DuPont-NEN, Les Ulis, France) for 48 h in complete medium as described previously (5, 16). Cells were then washed and resuspended in serum-free medium for kinetic experiments. Aliquots were taken for protein determination (17).

⁴ These abbreviations are: PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; DAPI, 4′,6′-diamidino-2-phenylindole.

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Total cellular ceramide quantitation was performed by labeling cells to isotopic equilibrium with 1 μCi/ml of [9,10-3H]palmitic acid (53.0 Ci/mmol, Amersham, Les Ulis, France) for 48 h in complete medium as described previously (5). Cells were then washed and resuspended in serum-free medium for kinetic experiments. Lipids were extracted and resolved by TLC (20), and ceramide was scraped and quantitated by liquid scintillation spectrometry. Statistical analyses were performed by Student’s t test.

Neutral and Acid Sphingomyelinase Assay. Sphingomyelinase activities were determined as described previously (21) using [choline-methyl-14C]sphingomyelin (54.5 mCi/mmol; DuPont-NEN; 120,000 dpm/assay) as substrate (5).

PKC Assay. Total PKC activity was determined by measuring the incorporation of 32P into myelin basic protein (22). Briefly, 5 × 10⁶ cells were lysed in 20 mM Tris-HCl (pH 7.4), 60 mM glycerophosphoric acid, 10 mM EGTA, 20 mM MgCl₂, 0.1 mM sodium fluoride, 2 mM DTT, 1 mM sodium orthovanadate, 20 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After 15 min, the assay was initiated by the addition of 0.1 mM ATP, 2.5 mg/ml myelin basic protein, and [γ-32P]ATP (3000 Ci/mmol). After 30 min at 30°C, the reaction was stopped by the addition of 10% trichloroacetic acid and spotted on phosphocellulose discs. Following extensive washes with 10% trichloroacetic acid, water, and finally ethanol, the incorporation of 32P into myelin basic protein was measured by scintillation counting.

RESULTS AND DISCUSSION

To ascertain the potential effects of PKC activators on daunorubicin-induced neutral sphingomyelinase stimulation, U937 cells were preincubated with 100 nM TPA for 3 h or 50 μg/ml phosphatidylserine for 30 min, after which daunorubicin was added. These were considered as optimal conditions for PKC activation of U937 cells in complete media (Fig. 1, A and B), which remained above 200% for at least 8 h (data not shown). At several time points, cells were washed and both neutral and acid sphingomyelinase activity was measured. As shown in Fig. 2A, treatment of U937 cells with 1.0 μM daunorubicin led to a >30% increase in neutral sphingomyelinase activity, which peaked at 5 min. However, pretreatment of cells with TPA or phosphatidylserine completely inhibited neutral sphingomyelinase stimulation but had no effect on basal levels (approximately 43 pmol/h/mg of protein). Acid sphingomyelinase activity levels were unchanged in the presence of daunorubicin, and PKC activators had no effect on this activity (data not shown). Similar results were obtained in HL-60 cells (data not shown).

To determine whether the inhibition of daunorubicin-induced sphingomyelinase stimulation may influence sphingomyelin hydrolysis and ceramide generation, we measured both sphingomyelin and ceramide content in U937 cells pretreated with TPA and phosphatidylserine, compared to untreated U937 cells. As shown in Fig. 2B, 1.0 μM daunorubicin triggered a sphingomyelin hydrolysis cycle within 4–10 min. Total percentage of sphingomyelin hydrolysis in U937 cells averaged 25%. As expected, in daunorubicin-treated cells, a significant burst in intracellular ceramide generation (about 35%) was observed concurrently with sphingomyelin hydrolysis at approximately 10 min (Fig. 2C). Similar results were observed when intracellular ceramide was quantitated by the diacylglycerol kinase method (data not shown). However, pretreatment with TPA or phosphatidylserine completely abolished both sphingomyelin hydrolysis and ceramide generation (Fig. 2, B and C).

To evaluate the effects of the inhibition of ceramide generation on daunorubicin-induced apoptosis, U937 cells, pretreated with TPA or phosphatidylserine and incubated further for 1 h with daunorubicin, were washed and resuspended in drug-free medium. At various time points, cells were analyzed for apoptosis. Daunorubicin treatment led to cell shrinkage and chromatin condensation in more than 90% of cells by 6 h, as observed by fluorescence microscopy (DAPI staining; Fig. 3). Quantitative analysis revealed that daunorubicin induced about 55% of DNA fragmentation (Table 1). Both TPA and phosphatidylserine significantly inhibited (>95%) the morphological features of daunorubicin-triggered apoptosis and completely blocked both DNA laddering (Fig. 4) and fragmentation (Table 1). Interestingly, the inhibition by TPA and phosphatidylserine of daunorubicin-triggered apoptosis could be relieved partially by treating cells with 25 μM of cell-permeant C6-ceramide (Table 1). Although the effect of the C6-ceramide was not as potent as that of daunorubicin (17% compared to 55%, respectively, in controls) when used in complete media, these observations nevertheless confirmed that cell permeant-ceramides can mediate an apoptotic effect. Higher C6-ceramide concentrations (50–100 μM) led to necrotic features (data not shown). Similar results were obtained in HL-60 cells (data not shown). To further demonstrate the apparent link between PKC activation and inhibition of daunorubicin-induced apoptosis, dose-effect studies were performed. As shown in Fig. 5, significant effects on both ceramide generation and DNA fragmentation were observed at concentrations
that stimulated PKC activity by at least 150%. Moreover, complete inhibition was seen with 250% PKC activation. These results further showed a close dose-effect correlation of TPA and phosphatidylserine on PKC activation and inhibition of daunorubicin-triggered ceramide generation and DNA fragmentation. Finally, neither TPA nor phosphatidylserine presented any significant effect on daunorubicin uptake or retention (data not shown).

In this study, we showed that PKC activators TPA and phosphatidylserine inhibited daunorubicin-induced neutral sphingomyelinase...
stimulation, sphingomyelin hydrolysis, ceramide generation, and apoptosis. Only the classic (α, β, and γ) and novel (δ, ε, θ, and μ) PKC isoforms present a phorbol ester-binding site, whereas all of the PKCs have a NH2-terminal regulatory domain containing a phosphatidylserine-binding site (for a review see Ref. 23). Therefore, our study presents evidence that daunorubicin-induced neutral sphingomyelinase stimulation can be manipulated negatively by one or several classic and/or novel PKC isoforms but likely not by atypical (ζ, Α, and ω) isoforms activatable by lipid cofactors, such as phosphatidylinositol-3 phosphate and ceramide itself (24–27).

The mechanism by which PKC regulates sphingomyelinase activity remains to be elucidated. The fact that PKC activation had no influence on basal sphingomyelin activity suggests that PKC regulates signaling events triggered by daunorubicin upstream of sphingomyelinase stimulation. Recently, it has been suggested that proteolytic events precede daunorubicin-induced sphingomyelinase stimulation and ceramide generation (28, 29). Therefore, it can be speculated that sphingomyelinase activation is controlled by both phosphorylation and proteolytic processes; e.g., it could be possible that sphingomyelinase is recruited to the plasma membrane after phosphorylation and proteolytic processing or degradation of an inhibitory protein (30). Alternatively, it could be possible that phosphorylation is needed to activate a critical protease responsible for the cleavage of a proform into an active form of the sphingomyelinase.

Regardless of the mechanism by which PKC regulates sphingomyelinase stimulation, our findings may have important implications in anthracycline pharmacology. Indeed, a number of intrinsic and environmental factors strongly influence PKC activity and, therefore, may contribute to resistance to these drugs. For example, cytokines and growth factors, such as tumor necrosis factor α, interleukin 3, or granulocyte-macrophage colony-stimulating factor, induce the diacylglycerol formation through hydrolysis of phosphatidylcholine (31–34). Furthermore, oncogenes, such as c-Abl variants, may interact directly with phospholipase C and contribute to accelerated diacylglycerol turnover (35, 36). Because diacylglycerol activates PKC, it is conceivable that paracrine or autocrine production of cytokines, as well as abnormal oncogene expressions that may lead to constitutive phospholipase C activation, may limit sphingomyelinase stimulation, ceramide generation, and apoptosis in anthracycline-treated cells. One could speculate that such a mechanism could account for the lack of apoptotic response to daunorubicin observed in certain leukemia cells (2).

Finally, PKC emerges as a critical negative regulator of anthracycline-activated sphingomyelin-ceramide apoptotic pathway. PKC may potentially act through different mechanisms: inhibition of the apoptotic effect of ceramide (37); stimulation of sphingosine kinase activity (38), resulting in accumulation of sphingosine-1-phosphate, a potent apoptosis inhibitor (39); inhibition of ceramide production (as in this study); and perhaps other mechanisms. These findings may explain the results of previous studies that showed a correlation between PKC activity and clinical resistance to anthracyclines (1), as well as the induction of resistance to doxorubicin by phorbol esters (40).

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Fig. 5. Dose effect of TPA and phosphatidylserine on daunorubicin-triggered ceramide generation and DNA fragmentation. U937 cells were treated with different concentrations of TPA for 3 h (Δ) or phosphatidylserine for 30 min (Δ), followed by a 60-min incubation with or without 1.0 μM daunorubicin. A, ceramide levels were estimated in U937 cells pretreated with [9,10-3H]palmitic acid for 48 h. B, quantitative DNA fragmentation was determined by the spectrophotofluorometric DAPI procedure as described in “Materials and Methods.” Results represent peak ceramide generation observed at 4–10 min. (B), quantitative DNA fragmentation was determined by the spectrophotofluorometric DAPI procedure as described in “Materials and Methods.” Results are means of triplicate determinations (bars, SE).


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