12-O-Tetradecanoylphorbol-13-acetate-induced Apoptosis in LNCaP Cells Is Mediated through Ceramide Synthase

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ABSTRACT

Protein kinase C (PKC) activation is often antiapoptotic, although in a few cell types PKC initiates apoptosis by an unknown mechanism. Recent investigations showed that activation of PKCα by 12-O-tetradecanoylphorbol-13-acetate (TPA) induces apoptosis in LNCaP prostate cancer cells. The present studies examine the mechanism of this effect and show that TPA-induced apoptosis in LNCaP cells was mediated through ceramide synthase activity. Ceramide synthase activity was increased by 70%, and ceramide synthase inhibitors blocked TPA-induced apoptosis.

INTRODUCTION

Protein kinase C (PKC) family of serine/threonine kinases is involved in signal transduction of a myriad of biological effects. Responses demonstrated to occur secondary to phorbol ester-mediated PKC activation include cellular transformation, proliferation, differentiation, and resistance to apoptosis. Phorbol ester treatment protects cells from undergoing apoptosis caused by TNF-α, chemotherapy, growth factor withdrawal, and irradiation, whereas inactivation of PKC with specific inhibitors can induce cell death directly. Alternatively, phorbol esters have been shown to induce apoptosis in a few cell types via PKC activation.

One cell line in which phorbol ester activates PKC and signals apoptosis is the human prostate cancer cell LNCaP. Studies indicate that the phorbol ester, TPA, causes selective membrane translocation of PKC (15). Exposure of LNCaP cells to analogues of the lipid second messenger ceramide also induces apoptosis (16, 15). Because exposure of LNCaP cells to analogues of the lipid second messenger ceramide also induces apoptosis, it was hypothesized that ceramide might mediate apoptosis in response to TPA.

Ceramide has emerged as a signal for induction of apoptosis by a variety of stresses in numerous cellular types. In most instances, cytokines, hormones, and environmental stresses induce rapid catabolism of membrane-bound sphingomyelin to ceramide by the action of neutral or acidic sphingomyelinases.

MATERIALS AND METHODS

Materials

Fatty acid-free BSA, palmitoyl-CoA, β-benzamidine (Hoechst 33258), ceramide Type III, FB1, DMSO, OPA, and sphinganine were purchased from Sigma Chemical Co. D-erythro-C24-sphingosine was from Matreya, Inc. TPA was purchased from LC Laboratories. C2-ceramide, C2-dihydroceramide, and Escherichia coli diacylglycerol kinase were from Biomol. Cardiolipin was purchased from Avanti Polar Lipids. [1-14C]Palmitoyl-CoA (57.1 mCi/mmol) and [γ-32P]ATP (3000 Ci/mmol) were obtained from DuPont (NE). Octyl-β-D-glucopyranoside was from Calbiochem. C2-ceramide was stored at −20°C and dissolved in DMSO (final concentration, 0.4%) just prior to each experiment. FB1 was diluted in sterile saline (0.9%) and stored at 4°C until use. TPA was dissolved in DMSO, aliquoted, and stored at −20°C until use.

Cell Culture

LNCaP cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. For apoptosis and lipid studies, cells were plated at a density of 1.5–2.5 x 10^5 cells/ml onto either 6- or 12-well plates and treated 36 h later. Cells (20 x 10^6 point) were homogenized in lysis buffer [5 mM NaCl, 4 mM Tris-HCl (pH 7.4), 2 mM EDTA, 5 mM EGTA, 1 mM MgCl2, 2 mM DTT, 10 μg/mL each leupeptin, aprotinin, and soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride] at 4°C, and cytoplasm (supernatant) and membrane (pellet) fractions were separated by centrifugation at 100,000 x g for 1 h.

PKC Studies

Cells (20 x 10^6 point) were homogenized in lysis buffer [5 mM NaCl, 4 mM Tris-HCl (pH 7.4), 2 mM EDTA, 5 mM EGTA, 1 mM MgCl2, 2 mM DTT, 10 μg/mL each leupeptin, aprotinin, and soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride] at 4°C, and cytoplasm (supernatant) and membrane (pellet) fractions were separated by centrifugation at 100,000 x g for 1 h.
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1 h. The membrane was resuspended in lysis buffer containing 1% Triton X-100, incubated on ice with frequent mixing for 30 min, and then centrifuged for 30 min at 100,000 × g to remove insoluble material. Cytoplasmic and membrane-bound PKCs were partially purified by passage through a DEAE-cellulose column, and PKC activity was measured using the BIOTRAK Protein Kinase C Enzyme Assay System according to the manufacturer's instructions (Amersham International, Buckinghamshire, United Kingdom).

**Lipid Studies**

**DAG Kinase Assay.** Following incubation with TPA, FB, or both, LNCaP cells, which are typically loosely adherent, were detached by gentle pipetting. The cells were pelleted by centrifugation (500 × g for 5 min), washed with ice-cold PBS, and extracted with 2 ml of chloroform:methanol:1 n HCl (100:100:1 v/v/v), 540 µl of buffered saline solution (135 mM NaCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, and 10 mM HEPES, pH 7.2), and 60 µl of 100 mM EDTA. Lipids in the organic phase were extracted from lipid extracts using a CA18 column (60 A, 0.5 µm, 2 × 20 mm; Waters), and subjected to mild alkaline hydrolysis (0.1 n methanolic KOH for 1 h at 37°C) to remove glycerophospholipids. Samples were re-extracted, and the organic phase was dried under N2. Ceramide was quantified by the DAG kinase assay as described (23).

**HPLC Assay.** Cell ceramide content was also determined by a non-enzymatic method, which is a modification of a technique used for amino acid analyses (23). Lipids were extracted as described for the DAG kinase assay above. Ceramide in the organic phase was measured after deacylation to sphingoid base and derivitization with OPA as described by Merrill et al. (23). Briefly, aliquots of the organic phase were mixed with d-erythro-C14-sphingosine, which was used as an internal standard. The organic phase was dried under N2, resuspended in 100 µl methanol, and mixed with 100 µl of OPA reagent, which was prepared fresh daily by mixing 99 ml of 3% (w/v) boric acid in water (pH adjusted to 10.5 with KOH) and 1 ml of ethanol containing 50 µg of OPA and 50 µg of 2-mercaptoethanol. After incubation for 5 min at 22°C, 500 µl of methanol:5 mM potassium phosphate (pH 7.0; 90:10, v/v) were added, and the samples were clarified by brief centrifugation. Aliquots (20 µl) were quantified by reverse-phase HPLC using a Nova Pak C18 column (60 Å, 4 µm, 3.9 × 150 mm; Waters). Fluorescent lipids were eluted isocratically with methanol:5 mM potassium phosphate, pH 7.0 (90:10), at a flow rate of 0.6 ml/min, detected by spectrofluorometer (excitation wavelength, 340 nm; emission wavelength, 455 nm), and corrected for recovery using the d-erythro-C14-sphingosine internal standard. Ceramide levels were determined by comparison with a concomitantly run standard curve of known amounts of ceramide (Sigma type III). The retention time for C14-sphingosine and D-erythro-C14-sphingosine were 4 and 10 min, respectively.

**Direct Comparison of Ceramide Levels by the DAG Kinase and HPLC Assays.** A recent study has questioned the validity of the DAG kinase assay for measuring ceramide levels (25). To address this issue, we compared the quantity of ceramide measured by the DAG kinase assay with the HPLC method using lipid extracts from LNCaP cells treated with TPA (10 ng/ml) or diluent for various times between 0 and 12 h. Identically treated samples were then assayed for ceramide levels using either the DAG kinase or HPLC methods. The ceramide levels determined by these methods correlated closely, as determined by linear regression analysis (r = 0.943). Using the t test for correlation, this value was found to be highly significant (P < 0.005), indicating that both methods register the same answer.

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**Fig. 1.** Ceramide determinations by DAG kinase and HPLC correlate linearly. Lipids were extracted from 0.5 × 10⁶ cells treated with TPA (10 ng/ml) or diluent for various times between 0 and 12 h. Identically treated samples were then assayed for ceramide levels using either the DAG kinase or HPLC methods. The ceramide levels determined by these methods correlated closely, as determined by linear regression analysis (r = 0.943). Using the t test for correlation, this value was found to be highly significant (P < 0.005), indicating that both methods register the same answer.

**Statistical Analysis**

Statistical analysis was performed by Student's t test and f test for correlation coefficient. Linear regression analysis was by the method of least squares.

**RESULTS**

TPA Activates PKCα and Induces Apoptosis in LNCaP. Initial studies compared the effect of TPA on PKC activation and apoptosis. These experiments extended the investigations of Powell et al. (15), which showed, by Western analysis, that TPA induced selective and prolonged translocation of PKCα from cytoplasm to membrane. Similarly, we demonstrated translocation of greater than 90% of PKC activity from cytoplasm to membrane by 15 min of TPA stimulation (data not shown). This resulted in a 6–7-fold increase in membrane-bound activity. Increased activity in the membrane persisted for up to 24 h; the ED₅₀ for this event was 3–4 ng/ml TPA (data not shown).

We next examined the apoptotic response of LNCaP to TPA treatment. For these studies, cells were treated with 1–100 ng/ml TPA for the indicated times, followed by bis-benzimide staining to assess apoptosis. Apoptosis was evident after 12 h of stimulation with TPA.
ceramide generation at 6 h was similar to that for TPA-induced apoptosis. Three to 10 ng/ml produced maximal elevation in ceramide; an ED_{50} for this event was \(~1~ng/ml\) (Fig. 2d). These studies indicate that ceramide generation occurs early after TPA treatment and precedes the onset of apoptosis by many hours.

**Mechanism of Ceramide Generation in LNCaP Cells.** Stimulated elevation in cellular ceramide levels has been shown to occur by hydrolysis of sphingomyelin to ceramide via the action of neutral or acidic sphingomyelinases or by activation of the synthetic enzyme, ceramide synthase (18). Initial studies examined the effect of a maximal dose of TPA for induction of apoptosis on neutral and acidic sphingomyelinase activities. LNCaP cells display detectable levels of neutral and acidic sphingomyelinase, with maximal velocities of 100 pmol/mg protein/min and 1200 pmol/mg protein/min, respectively. At no time between 1 min and 24 h was either sphingomyelinase activity elevated (n = 5). We therefore investigated whether the TPA effect represented de novo synthesis of ceramide via activation of ceramide synthase. Cells were treated with TPA for 45 min, and microsomal membranes were prepared. The kinetics of ceramide synthase activity with increasing sphinganine concentrations are depicted in Fig. 3a. Eadie-Hofstee transformation of these data revealed that LNCaP cells manifest a basal level of ceramide synthase activity with a maximal velocity of 100 pmol/min/mg protein and a K_{m} of 1.4 \muM. A similar maximal velocity was obtained using palmitoyl-CoA as substrate (data not shown); the K_{m} for palmitoyl-CoA was 1.0 \muM. A 60% increase in the V_{max} with no change in K_{m} occurred in response to 100 ng/ml TPA (Fig. 3b). Similar results were obtained at 16 h of TPA treatment and with a dose of 10 ng/ml TPA (data not shown). A TPA

and was progressive for 48 h (Fig. 2a). TPA-induced apoptosis was concentration dependent (Fig. 2b), with 3–10 ng/ml TPA eliciting maximal cell death; the ED_{50} for this event was \(~2~ng/ml\) TPA (Fig. 2b). Thus, the ED_{50} for apoptosis correlated closely with the ED_{50} for translocation of PKCz activity to membrane upon TPA treatment. Removal of TPA after 20 min did not affect the level of apoptosis at 24 h (data not shown). Morphological changes of apoptosis were accompanied by DNA ladder formation (data not shown). The delay in induction of LNCaP apoptosis after TPA suggests that an intermediary event might be involved.

**TPA Induces Ceramide Elevation in LNCaP Cells.** To determine whether ceramide elevation occurred prior to TPA-induced apoptosis, we measured ceramide levels after treatment with this agent. As shown in Fig. 2c, exposure of LNCaP cells to TPA (10 ng/ml) resulted in a 40% increase in ceramide levels within 3 h. A smaller increase in cellular ceramide levels was routinely detected by 1 h of TPA stimulation (data not shown). Ceramide generation was linear for 12 h, at which point the level of ceramide had increased 100% over baseline. Increased ceramide levels were sustained for at least 24 h (data not shown). The dose-dependence of TPA-induced

Fig. 2. Effect of TPA on ceramide levels and apoptosis in LNCaP cells. A, apoptosis time course. Intact LNCaP cells were exposed to 10 ng/ml TPA or diluent DMSO (final concentration, 0.4%). Quantification of morphological changes of nuclear apoptosis was performed by staining the cells with the DNA-specific fluorochrome Hoechst-33258. Segmentation of the nucleus into three or more chromatid fragments was considered apoptotic. Each value represents the mean of 32 determinations and was progressive for 48 h (data not shown). B, apoptosis dose response. LNCaP cells were treated as in A for 24 h with 1–10 ng/ml TPA. Cells were scored for apoptosis as in A. Each value represents the mean \pm range of duplicate determinations from three independent experiments; bars, SE. A minimum of 400 cells was scored for the incidence of apoptosis in each experiment. C, time course of ceramide generation. LNCaP cells were exposed to 100 ng/ml TPA for 6 h, and ceramide was quantified as in C. Each value represents the mean of 32 determinations and was progressive for 48 h (data not shown). D, dose dependence of ceramide generation. LNCaP cells were exposed to 1–10 ng/ml TPA for 6 h, and ceramide was quantified as in C. Each value represents the mean of 32 determinations and was progressive for 48 h (data not shown). A TPA dose of 10 ng/ml TPA (Fig. 3b). Similar results were obtained at 16 h of TPA treatment and with a dose of 10 ng/ml TPA (data not shown). A TPA...
The present studies define a mechanism by which TPA signals apoptosis in LNCaP prostate cancer cells. TPA induced ceramide elevation within 1 h of treatment, and the level of cellular ceramide increased linearly for 12 h. Thus, ceramide elevation preceded apoptosis, which was detected at 12 h. There was a close correlation between the dose dependence of PKCa activation, ceramide elevation, and apoptosis, all of which manifested ED50s between 1 and 4 ng of TPA/ml. The enzymatic mechanism of TPA-induced ceramide elevation was determined and involved an increase in the activity of ceramide synthase, the regulatory enzyme for ceramide synthesis. TPA signaled ceramide synthase activation within 45 min, and the dose dependence for this event correlated closely to ceramide generation. Ceramide synthase appeared obligatory for induction of apoptosis by TPA because FB1 blocked TPA-induced ceramide generation and cell death, and ceramide analogues bypassed the blockade and restored the apoptotic response. These studies comprise the first evidence for activation of ceramide synthase via a signal transduction pathway.

Most often, phorbol esters have been shown to promote an antiapoptotic state (27), perhaps via activation of the mitogen-activated protein kinase cascade (28). However, in a few instances, in addition to LNCaP, phorbol esters have been shown to be proapoptotic. In this regard, EBV-infected Burkitt Lymphoma cells display substantial apoptosis in response to phorbol esters, whereas uninfected Burkitt cells are resistant (29). Furthermore, immature thymocytes appear to be susceptible to phorbol ester-induced apoptosis (13). Perhaps most relevant to the present studies are the findings of de Vente et al. (14). These investigators overexpressed PKCa in MCF-7 breast cancer cells and compared the effects of TPA on the parental and modified lines. The MCF-7 parental line was growth arrested in response to treatment, whereas the MCF-7-PKCa line exhibited a cytotoxic response. These studies indicate that the status of PKCa may regulate the type of response elicited by phorbol ester treatment.

Our studies on the susceptibility of LNCaP cells to TPA-induced...
apoptosis are consistent with and extend the findings of other laboratories (11, 12, 15). Powell et al. (15) demonstrated that TPA induced selective and prolonged translocation of PKCα to the membrane prior to apoptosis. Zhao et al. (30) showed that the action of TPA involved Rb dephosphorylation and G₀-G₁ arrest, events also reported to occur in response to ceramide treatment in other cell types (31, 32). Whether G₀-G₁ arrest, like apoptosis, is signaled through ceramide in LNCaP cells requires further investigation.

The present study expands the potential role of ceramide synthase activation as a mechanism for generation of ceramide during induction of apoptosis. In previous studies from our laboratory, evidence that daunorubicin-induced apoptosis, presumably secondary to DNA damage, was mediated by ceramide synthase activation. Witty et al. (20) corroborated this finding, showing that daunorubicin-induced, but not UV-induced, apoptosis was abolished by FB₁ in hen granulosa cells. Recently, Suzuki et al. (21) showed that FB₁ could inhibit apoptosis secondary to treatment of murine fibroblasts with the chemotherapeutic agent CPT-11 and molecularly ordered ceramide generation upstream of caspases. Whether ceramide generated via the synthase interacts with the same targets as ceramide generated through sphingomyelinases is presently uncertain. However, the recognition that ceramide synthase is preferentially concentrated in mitochondria and that ceramide analogues induce generation of reactive oxygen species in isolated mitochondria by inhibition of complex III and permeability transition strongly suggest a role for ceramide synthase as a regulator of apoptosis at the mitochondrial level (33–35).

Prostate cancer is the most common cancer in men in the United States and accounts for 42,000 deaths annually (36). Although surgery and radiation are effective for treatment of localized disease, there is presently no effective chemotherapeutic regimen for metastatic prostate cancer. The present study constitutes the first evidence of a signaling pathway for the death response in prostate cancer. Further understanding of pathways involved in the death of prostate cancer cells might allow for the development of new systemic therapies for this disease. Whether ceramide is more generally involved in the death response of prostate cells to other stresses, including androgen-withdrawal and ionizing radiation, is presently unknown.

REFERENCES

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