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

Mark Garzotto, Margaret White-Jones, Youwei Jiang, Adriana Haimovitz-Friedman, Zvi Fuks, and Richard Kolesnick


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) induced apoptosis in LNCaP prostate cancer cells. The present studies examine the mechanism of this effect and show that de novo ceramide generation through the enzyme ceramide synthase is required. TPA induced rapid ceramide generation, which was detectable by 1 h and increased linearly for 12 h. TPA-induced apoptosis was measurable by 12 h and was progressive for 48 h. Investigations into the mechanism of TPA-induced ceramide generation revealed that acid and neutral sphingomyelinase activities were not enhanced. However, TPA induced an increase in ceramide synthase activity that persisted for at least 16 h. Treatment with fumonisin B1, a specific natural inhibitor of ceramide synthase, abrogated both ceramide production and TPA-induced apoptosis. Ceramide analogues bypassed fumonisin B1 inhibition to initiate apoptosis directly. Thus, ceramide appears to be a necessary signal for TPA-induced apoptosis in LNCaP cells. This represents the first description of a pathway by which PKC may signal apoptosis.

INTRODUCTION

The 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 (1, 2). Phorbol ester treatment protects cells from undergoing apoptosis caused by TNF-α (3, 4), chemotherapy (5), growth factor withdrawal (6), and irradiation (7), whereas inactivation of PKC with specific inhibitors can induce cell death directly (8–10). Alternatively, phorbol esters have been shown to induce apoptosis in a few cell types via PKC activation (11–14).

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 PKCα, and pretreatment with PKC inhibitors blocks apoptosis (11, 15). Because exposure of LNCaP cells to analogues of the lipid second messenger ceramide also induced apoptosis (16), we 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 (17, 18). Stimulated elevation in cellular ceramide has been shown to occur by two distinct pathways. 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 (17, 18). Alternatively, ceramide may be synthesized de novo via the condensation of the sphingoid base sphinganine and fatty acyl-CoA catalyzed by the enzyme ceramide synthase (also termed sphingosine N-acyltransferase; EC2.3.1.24) to form dihydroceramide, which is subsequently oxidized to ceramide.

Boe et al. (19) reported that prolonged activation of ceramide synthase can be induced by daunorubicin treatment of P388 and U937 cells (19). The V₅₀ of ceramide synthase was increased by 70%, resulting in sustained elevations of ceramide (and sphingomyelin) levels for at least 8–12 h. Furthermore, FB₈, a fungal toxin that specifically inhibits ceramide synthase, blocked daunorubicin-induced ceramide generation and apoptosis. Because FB₈ did not block TPA-induced ceramide generation that occurred by sphingomyelinase activation, FB₈-pretreated apoptotic death was concluded that the mechanism of cell death involving ceramide synthase activation was specific for daunorubicin. Recently, FB₈ has been shown to abrogate daunorubicin-induced apoptosis in hen granulosa cells (20) and CPT-11-induced apoptosis in LNCaP cells (21).

In the present studies, we explored the mechanism by which phorbol ester signals the apoptotic response in LNCaP cells. We have defined an obligatory role for ceramide generation via activation of ceramide synthase. These studies represent the first description of a mechanism by which activation of the PKC pathway can signal apoptosis.

MATERIALS AND METHODS

Materials

Fatty acid-free BSA, palmitoyl-CoA, bis-benzimide (Hoechst 33258), ceramide Type III, FB₈, DMSO, OPA, and sphingamine were purchased from Sigma Chemical Co. d-erythro-C₃₅H₃₆P 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. [1-³²P]ATP (3000 Ci/mmol) and [γ-³²P]ATP (3000 Ci/mmol) were obtained from DuPont (NEN). Octyl-β-D-glucopyranoside was from Calbiochem. C₂-ceramide was stored at −20°C and dissolved in DMSO (final concentration, 0.4%) prior to each experiment. FB₈ 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 × 10⁵ cells/ml onto either 6- or 12-well plates and treated 36 h later. For ceramide synthase assays, cells were plated at a similar density onto 100-mm dishes.

PKC Studies

Cells (20 × 10⁶/pool) 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 phenylmethysulfonyl fluoride) at 4°C, and cytoplasm (supernatant) and membrane (pellet) fractions were separated by centrifugation at 100,000 × g for 2h.

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2 These authors made significant and independent contributions.
3 To whom requests for reprints should be addressed, at Laboratory of Signal Transduction, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: (212) 639-8573; Fax: (212) 639-2767.
4 The abbreviations used are: PKC, protein kinase C; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; FB₈, fumonisin B₁; OPA, o-phthalaldehyde; DAG, diacylglycerol; HPLC, high-performance liquid chromatography.

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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 X 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 dried and subjected to mild alkaline hydrolysis (0.1 n methanolic KOH for 1 h at 37°C) to remove glycosylphosphatidylinositols. Samples were re-extracted, and the organic phase was dried under N2. Ceramide was quantified by the DAG kinase assay as described (22).

**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, dissolved 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 ethanolic containing 50 μg of OPA and 50 μl 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 X 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 spectrophorometer (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 times for C14-sphingosine and D-erythro-C14,3,-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 r value was found to be highly significant (P < 0.005), indicating that both methods register the same answer.

**Ceramide Synthase Assay.** Microsomal membranes were prepared as described previously (19). Briefly, cells were washed with ice-cold PBS, scraped off the plate, and resuspended in 300 μl homogenization buffer (25 mM HEPES (pH 7.4), 5 mM EGTA, 50 mM NaF, and 10 μg/ml each of leupeptin and soybean trypsin inhibitor). Cells were disrupted by sonication on ice, and lysates were pelleted at 800 X g for 5 min. The postnuclear supernatant was centrifuged at 250,000 X g for 35 min. The microsomal membrane pellet was resuspended in 0.5 ml of homogenization buffer. Assays of ceramide synthase activity were performed as described previously (19). Briefly, microsomal membrane protein (75 μg) was incubated with 2 mM MgCl2, 20 μM fatty acid-free BSA, indicated concentrations of sphinganine, 70 μM unlabeled palmitoyl-CoA, and 0.2 μCi of [1-14C]palmitoyl-CoA at 37°C for 1 h. Following extraction of the lipids with 2 ml of chloroform:methanol (1:2), radiolabeled dihydrosphingosine was resolved by TLC using a solvent system of chloroform:methanol:3.5 n ammonium hydroxide (85:15:1, v/v/v), detected by iodine vapor staining based on comigration with commercially available ceramide (Sigma type III), and quantified by liquid scintillation counting. Under the conditions used, the substrate was not rate limiting, and the reaction was linear for time and enzyme concentration.

**Quantification of Apoptotic Cells.** Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bis-benzimide trihydrochloride (Hoechst-33258), as described previously (26). Briefly, LNCaP monolayers were detached with 0.25% trypsin and 0.02% EDTA in HBSS and combined with the floating population. The cell pellet was then washed in PBS, resuspended in 50 μl of 3% paraformaldehyde, and incubated for 10 min at 22°C. The fixative was removed, and the cells were resuspended in 20 μl of PBS containing 8 μg/ml RNAse A and 3% paraformaldehyde. and incubated for 10 min at 22°C. The fixative was removed, and the cells were resuspended in 20 μl of PBS containing 8 μg/ml RNAse A and 3% paraformaldehyde. After a 15-min incubation at 22°C, an 8-nl aliquot was placed on a glass slide, and 400 cells were scored for the incidence of apoptotic chromatin changes under an Olympus BH2 fluorescence microscope using a BH2-DMU2UV Dich Mirror Cube filter. Cells displaying at least three apoptotic bodies were scored as apoptotic.

**Statistical Analysis**

Statistical analysis was performed by Student's t test and t 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.

![Fig. 1. Ceramide determinations by DAG kinase and HPLC correlate linearly. Lipids were extracted from 0.5 X 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 r value was found to be highly significant (P < 0.005), indicating that both methods register the same answer.](image-url)
CERAMIDE SYNTHASE SIGNALS LNCaP APOPTOSIS

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 chromatins fragments was considered apoptotic. Each value represents the mean ± 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. 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 ± range of duplicate determinations from two independent experiments; bars, SE. C, time course of ceramide generation. LNCaP cells were treated as in A for the indicated times. Lipids were extracted, and ceramide levels were quantified by the DAG kinase reaction as described in “Materials and Methods.” Each value represents the mean ± range of duplicate determinations from three experiments; bars, SE. 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 triplicate determinations from one of three similar experiments; bars, SE.

Fig. 3. Measurement of ceramide synthase activity in response to TPA. A, ceramide synthase activity. TPA (100 ng/ml) was added to LNCaP cells for 45 min, and microsomal membranes were prepared as in “Materials and Methods.” The ceramide synthase reaction mixture containing microsomal membrane protein (75 μg), 2 mm MgCl2, 20 μM fatty acid-free BSA, 0.5–10 μM sphinganine, 70 μM unlabeled palmitoyl-CoA, and 0.2 μCi of [1-14C]palmitoyl-CoA was incubated at 37°C for 1 h. Lipids were extracted, and radio-labeled dihydroceramide was resolved by TLC using a solvent system of chloroform:methanol:3.5 n ammonium hydroxide (85:15:1), detected by iodine vapor staining based on comigration with commercially available ceramide (Sigma type III), and quantified by liquid scintillation counting. Each value represents the mean of four separate experiments; bars, SE. B, determination of Km and Vmax from A using Eadie-Hofstee analysis. C, dose response. LNCaP cells were exposed to 1–100 ng/ml TPA and assayed for ceramide synthase activity as described in A using 5 μM sphinganine. Each value represents the mean of triplicate determinations from one of three separate experiments; bars, SE.

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 ED50 for this event was ~2 ng of TPA/ml (Fig. 2b). Thus, the ED50 for apoptosis correlated closely with the ED50 for translocation of PKCa 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 ceramide generation at 6 h was similar to that for TPA-induced apoptosis. Three to 10 ng/ml produced maximal elevation in ceramide; an ED50 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 Km of 1.4 μM. A similar maximal velocity was obtained using palmitoyl-CoA as substrate (data not shown); the Km for palmitoyl-CoA was 1.0 μM. A 60% increase in the Vmax with no change in Km 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
cells were treated with exogenous C2-dihydroceramide, a ceramide analogue that lacks the trans C4–5 double bond on the sphingoid backbone. Exogenous dihydroceramide is poorly converted to ceramide in mammalian cells (26), and at equimolar concentrations (up to 50 μM), C2-dihydroceramide failed to induce apoptosis (data not shown).

**DISCUSSION**

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 synthesis appeared obligatory for induction of apoptosis by TPA because FB, 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 anti-apoptotic 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...
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 was presented 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 FB in hen granulosa cells. Recently, Suzuki et al. (21) showed that FB 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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