Protein Kinase C Inhibition Induces Apoptosis and Ceramide Production through Activation of a Neutral Sphingomyelinase

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Abstract

We report that WEHI-231 undergo apoptosis following exposure to the protein kinase C inhibitors chelerythrine chloride and calphostin C. Following the addition of chelerythrine or calphostin C to WEHI-231 cells, ceramide production increased over baseline levels with a concurrent decrease in sphingomyelin. More detailed examinations determined that the ceramide accumulation resulted from activation of neutral, but not acidic, sphingomyelinase. These results suggest an antagonistic relationship between protein kinase C activity and ceramide in the signaling events preceding apoptosis.

Introduction

Recent investigations indicate that signaling events following cellular exposure to tumor necrosis factor α, Fas ligand, IgM cross-linking, irradiation, and other DNA-damaging agents may trigger apoptosis via the hydrolysis of membrane sphingomyelin generating ceramide (1–3). Activation of PKC by phorbol esters or growth factors opposes ceramide-induced apoptosis, and indirect evidence suggests that PKC activation may limit ceramide production (4–6). One potential action of ceramide and its metabolite, sphingosine, is to prevent activation of specific PKC isoforms (7–10). Taken together, these studies suggest that PKC activation may oppose the actions of ceramide production in the apoptotic pathway.

To understand the potential interactions between PKC activity, ceramide production, and apoptosis, we used two inhibitors of PKC, chelerythrine chloride and calphostin C. Chelerythrine chloride and calphostin C compete for the conserved catalytic sites and regulatory domains of PKC, respectively, and are potent and specific inhibitors of the PKC isoforms (α, β, δ, and ε) found in WEHI-231 cells (7, 11–14). We report herein that PKC inhibition with chelerythrine or calphostin C induces apoptosis. Furthermore, treatment of cells with chelerythrine or calphostin C enhances ceramide production following the activation of a neutral sphingomyelinase, suggesting that PKC and the lipid second messenger ceramide may play opposing roles in determining survival following diverse cellular signals.

Materials and Methods

Drugs, Reagents, and Cell Culture. Chelerythrine [IC50 (PKC) = 0.66 µM; IC50 (PKA) = 170 µM] and calphostin C [IC50 (PKC) = 0.51 nM; IC50 (PKA) = 50 µM] show high specificity for the PKC family of enzymes (Sigma Chemical Co., St. Louis, MO). These inhibitors are at least 30-fold more specific at blocking PKC activity as opposed to the relatively nonselective yet widely used agents staurosporine and 1-(5-isouquinolinesulfonyl)-2-methylpiperazine. They do not appear to inhibit other kinases, such as the PKA, at concentrations used for our experiments (11, 13). 7-Amino-actinomycin D, ATP, and propidium iodide were purchased from Sigma Chemical Corp. 3H-palmitic acid (60 Ci/mmol), [3H]sphingomyelin (60 Ci/mmol), and [γ-32P]ATP were purchased from DuPont NEN. All solvents were high-performance liquid chromatography grade. WEHI-231 JM cells were grown at a density of 3–6 X 106 cells/ml in RPMI 1640 culture medium, 1% penicillin-streptomycin, 10% heat-inactivated fetal calf serum (Life Technologies, Inc., Grand Island, NY), and 5 X 10−5 M β-mercaptoethanol (Sigma) in 5% CO2.

DAPI Staining for Nuclear Visualization. WEHI-231 cells (5 X 106) were resuspended in approximately 100 µl of DAPI, Sigma D9542, 1 µg/ml in PBT (PBS + 1% Triton X-100). The cells were viewed by fluorescence microscopy using an Olympus BX-40 microscope with a 100 Watt Mercury lamp, a X40 fluorite objective, N.A. 0.75 Leco #1-UB527, and a UV filter cube (ex 330–385 nm, em 420 nm, wide band pass; Leco #U-M536).

DNA Laddering Assay. The assay was identical to that reported previously (15). Briefly, following appropriate treatment, 24-well plates, cells were collected and lysed in 0.5 ml lysis buffer (0.6% SDS + 10 mM EDTA, pH 7.0). NaCl was added to 1M, mixed by inversion, left for 12 h in 4°C, and spun at 14,000 X g for 30 min. Samples were precipitated in chloroform (1:1) and ethanol, etherophoresed in a 3% agarose gel, and visualized with ethidium bromide.

Ceramide and DAG Quantification by DAG Kinase Reaction. Quantitation of ceramide by DAG kinase was similar to that described by Dressler and Kolesnick (16). After irradiation, lipids were extracted and resuspended by bath sonication in 10 µl of 7.5% n-octyl-β-d-glucopyranoside, 5 mM EDTA, and 1 mM DETAPAC. Seventy µl of a reaction mix were added to give a final concentration of 0.05 M imidazole/HC1 (pH 6.6), 0.05 M NaCl, 12.5 mM MgCl2, 1 mM EGTA, and DAG kinase at a concentration of 0.7 unit/ml. The reaction was started by the addition of 10 µl of [γ-32P]ATP (1.0 µCi/tube) in 5 mM ATP, incubated at room temperature for 30 min, and stopped by the extraction of lipids with 450 µl of CHCl3:CH2OH (1:2) and 20 µl of 1% HClO4. The monophase was mixed, and after 10 min, 150 µl CHCl3 and 150 µl of 1% HClO4 were added; then the tubes were vortexed and centrifuged at 5000 X g for 5 min. The lower organic phase was washed twice with 1% HClO4 and then dried in a Speed Vac apparatus. The phosphorylation products, ceramide-1-phosphate and phosphatidic acid (from DAG), were resolved on a 10-cm X 10-cm L-HK TLC plate (Whatman). Data was calculated as pmol of ceramide/106 cells by scintillation counting.

Labeling, Extraction, and Analysis of [3H]Palmitate-labeled Lipids. Cells were prelabeled with [3H]palmitate (10 µCi/ml) for 24 h to isotopic equilibrium in 15 ml glass tubes to minimize changes in cellular ceramide and sphingomyelin due to handling and changing of media (17). Following treatment of cells with chelerythrine, total lipids were extracted via a modified Folch method. Briefly, 1 ml of MeOH:2 N HCl (100:6, v/v) was used to resuspend the cell pellet. Two ml of chloroform and 0.6 ml of water were added to each tube, vortexed three times, and centrifuged for 20 min at 1000 X g, producing a bilayer. An equal amount of the organic phase was taken from each tube, dried, and reconstituted with 30 µl of chloroform: methanol (95:5). A 10-µl aliquot was streaked on a 10 cm X 10-cm L-HK TLC plate (Whatman), previously run in a wash solvent of CHCl3:CH2OH:H2O (60:40:10, v/v). Sphingolipids were resolved in CHCl3:MeOH:CH3COOH:H2O (32:5:12.5:4:2.25), iodine stained, sprayed with En3Hance

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3 The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; DAPI, 4',6-iamidino-2-phenylindole; PKA, cyclic-AMP dependent protein kinase; ROS, reactive oxygen species.
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(DuPont), and autoradiographed. Autoradiographs were quantitated using an Epson 1200c 30-bit scanner and NIH Image software (public domain program developed at the NIH and available from the Internet via FTP from zippy.n-imh.nih.gov). Both counting and density analysis provided similar results.

Assay for Neutral and Acidic Sphingomyelinase Activity. The mixed micellar sphingomyelinase assay using \(^{14}C\)-labeled sphingomyelin was performed as described by Wiegmann et al. (18) with minor modifications. Exponentially growing cells (2 \(\times\) 10\(^7\)) were treated as indicated, collected, and lysed in 200 \(\mu\)l of either neutral buffer (20 mM HEPES (pH 7.4), 10 mM MgCl\(_2\), 2 mM EDTA, 5 mM DTT, 100 mM Na\(_2\)VO\(_4\), 100 mM NaMO\(_4\), 10 mM \(\beta\)-glycerolphosphate, 750 \(\mu\)M ATP, 1 mM phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and 2% Triton X-100) or acid buffer (2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, leupeptin, and pepstatin). After incubation for 5 min at 4°C, cells were homogenized by sonication, and cell debris was removed by low-speed centrifugation at 800 \(\times\) g. Protein (50 \(\mu\)g) were incubated for 2 h at 37°C in a buffer final volume, 50 ml) containing 20 mM HEPES, 1 mM MgCl\(_2\), and 0.9 \(\mu\)l [\(N\)-methyl-\(^{14}\)C]sphingomyelin. The reaction was linear at this protein concentration. Phosphorylcholine was then extracted with 800 ml of chloroform:methanol (2:1, v/v), and 250 ml H\(_2\)O radioactive phosphorylcholine was measured by scintillation counting and expressed as a percentage of control.

Results and Discussion
Chelerythrine and Calphostin C Induce Apoptosis. Twenty-four h following the addition of chelerythrine (10 \(\mu\)M) or calphostin C (250 nM) to WEHI-231 growing in complete media, propidium iodide fluorescence-activated cell sorting analysis demonstrated greater than 90% cell death. Gel electrophoresis of low molecular weight DNA and DAPI fluorescent staining revealed DNA laddering (Fig. 1A) and nuclear condensation (Fig. 1B). Taken together, these results established that WEHI-231 undergo apoptosis following treatment with inhibitors of PKC.

Chelerythrine and calphostin C were found to act in synergy with exogenous ceramide analogues to induce apoptosis (7). This finding is consistent with previous reports demonstrating that phorbol esters limit ceramide toxicity in WEHI-231 and other cell lines (5, 19, 20). Since activation of PKC may limit ceramide production (4–6), we investigated whether decreased PKC activity leads to its accumulation.

Ceramide Production following Protein Kinase C Inhibition Is Due to the Activation of a Neutral Sphingomyelinase. To test whether PKC inhibition alters ceramide production, we used the DAG kinase assay to quantify intracellular ceramide following chelerythrine and calphostin C treatment (16, 21). Fig. 2, A and B, depicts the time course of ceramide production following the addition of 10 \(\mu\)M chelerythrine to exponentially growing cell cultures. Approximately 30 min following the addition of chelerythrine, ceramide generation increased 100% over baseline and returned to a near pretreatment level within hours. Thus, PKC inhibition induces ceramide accumulation within the first hour following treatment with the inhibitor.

To investigate whether the increased ceramide occurs through the hydrolysis of its respective precursors or from new lipid synthesis, we examined the sphingomyelin levels in WEHI-231 following treatment with chelerythrine. Cells (4 \(\times\) 10\(^5\)) were labeled with [\(\beta\)]H]palmitate (10 \(\mu\)Ci/ml) for 24 h, and total lipids were extracted and separated using TLC as detailed previously (1). Fig. 2A demonstrates that sphingomyelin mass decreased concurrently with the increase in ceramide, reaching its lowest levels 60 min (55% of control) following treatment with the inhibitor. As shown in Fig. 2C, calphostin C had similar effects on ceramide production and sphingomyelin levels. Thus, inhibition of PKC causes not only increased intracellular ceramide but induced a concurrent decrease in sphingomyelin. These experiments suggested that ceramide is produced through the hydrolysis of its precursor by a sphingomyelinase.

To confirm that ceramide accumulation was due to increased sphingomyelinase activity, we used a mixed micellar assay to quantify in vitro enzyme activity. Fig. 2D illustrates that neutral, but not acidic, sphingomyelinase activity increases to 235% (±38 SD) of control within 30 min of exposure to chelerythrine. Calphostin C, like chel-
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A 250
200
150
100
50
0
Time (minutes)

% change in sphingolipid mass

Ceramide

Fig. 2. Inhibition of PKC causes an increase in ceramide production through the activation of a neutral sphingomyelinase and decreases in sphingomyelin levels. A, ceramide production was quantitated using the DAG kinase assay for WEHI-231 JM cells, and total lipid extraction was used to assay sphingomyelin mass. Following the addition of 10 μM chelerythrine, cells were lysed at the appropriate time points as described above. Each data point represents the average percentage change in ceramide (C-1-P) or sphingomyelin (SM) generation from baseline derived from at least eight independent experiments for all time points, with the exception of those marked with * (n = 4); bars, SD. No change in viability or cell size was detectable at the 2-h time point as assayed by propidium iodide exclusion and FACS. B, autoradiograph from the DAG kinase assay demonstrating ceramide production (CER) following the addition of water (–) or 10 μM of chelerythrine (CH) to JM cells. Lanes depict triplicate determinants from a single representative experiment. C, calphostin C induces ceramide production and decreased sphingomyelin levels. Ceramide production and sphingomyelin mass were quantitated identical to that for chelerythrine. Cells were lysed 2 h following the addition of 300 nM calphostin C. The average percentage change in ceramide and sphingomyelin generation from baseline is derived from at least three independent experiments; bars, SD. D, graph of neutral and acidic sphingomyelinase activity normalized to a percentage of control. Cells (2 × 10⁶) were treated with chelerythrine (10 μM) for 30 min or light-activated calphostin C (300 nM) prior to being assayed for neutral or acidic sphingomyelinase activity. Data from four individual experiments, with duplicate determinants for chelerythrine and 3 experiments for calphostin C, are shown; bars, SD.

eythrine, induced a 209% (±9 SD) increase in neutral but not acidic sphingomyelinase 2 h following treatment, suggesting that a majority of the ceramide accumulation results from the hydrolysis of sphingomyelin following inhibition of PKC. Inactivated chelerythrine or calphostin C had no effect on cell viability or sphingomyelinase activation. Furthermore, these inhibitors tested, in the absence of cellular preparations, did not induce hydrolysis of labeled sphingomyelin, demonstrating that increased cellular sphingomyelinase activity is responsible for sphingomyelin hydrolysis.

The mechanisms by which ceramide may mediate cell death is yet unknown. Previous studies have demonstrated multiple signaling targets for ceramide, including a serine/threonine kinase (CAPK) and phosphatase (CAPP), PKC isoforms (22), p54 (23), and phospholipase D (24, 25). Since changes in oxidative status induce a wide range of cellular responses, including growth and apoptosis in WEHI-231 and other lymphocytes, we tested whether inhibition of PKC and a resulting accumulation of ceramide would affect the level of reactive oxygen species within the cell using the dye dichlorodihydrofluorescein and FACS analysis. As shown in Fig. 3A, surface IgM cross-linking induces a characteristic activation of PKC and generation of ROS consistent with data reported previously (26). The addition of 30 μM exogenous ceramide (Fig. 3B) or 10 μM chelerythrine (Fig. 3C) for 15 min reduced the ROS in the cells, suggesting that the accumulation of ceramide acts as a reducing agent. Although nontoxic oxidation may promote cell growth, we speculate that intracellular reduction may lead to the activation of cysteine proteases, which are proposed mediators of apoptosis. This hypothesis is currently under investigation by our laboratory.

Our results demonstrate that inhibition of PKC activity may induce apoptosis in WEHI-231 cells, in part, through activation of a neutral sphingomyelinase and subsequent accumulation of ceramide. We have previously established that PKC activation by phorbol esters limits ceramide generation and protects cells from apoptosis induced by sphingomyelin hydrolysis (5, 27). These results indicate that PKC activity and ceramide signaling play opposing roles in determining the fate of a cell. The production of ceramide and activation of its apoptotic signaling cascade may represent potentially novel therapeutic targets for enhancing apoptosis in tumor cells.
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References


Fig. 3. Exogenous ceramide and PKC inhibition induce oxidative changes in WEHI-231 cells. WEHI-231 cells (1 × 10⁶) were treated with anti-IgM antibodies for 2 h (A), 30 µM exogenous ceramide for 15 min (B), or 10 µM chelerythrine for 15 min (C). The ROS in the cells was then measured using dichlorodihydrofluorescein and quantified using fluorescence-activated cell sorting analysis. The histogram analysis of fluorescence is plotted as LOG function. The gray area represents the control cells, while the overlaid line plot demonstrates the treated cells.
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