Advances in Brief

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). PKC activation 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 PKC inhibitors 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 (a, ß, S, and e) found in WEHI-231 cells (7, 11–14). We report that PKC inhibition with chelerythrine or calphostin C enhances ceramide production following the addition of chelerythrine or calphostin C to WEHI-231 cells. We report that PKC inhibition with chelerythrine or calphostin C enhances ceramide production following the addition of chelerythrine or calphostin C to WEHI-231 cells.

Materials and Methods

Drugs, Reagents, and Cell Culture. Chelerythrine [IC50 (PKC) = 0.66 μM; IC50 (PKA) = 170 μM] and calphostin C [IC50 (PKC) = 0.50 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 nonslective yet widely used agents staurosporine and I-(5-isoquinolinesulfonyl)-2-methylpiperazone. 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 × 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 × 10⁻⁵ M β-mercaptoethanol (Sigma) in 5% CO₂.

DNA Staining for Nuclear Visualization. The assay was identical to that described previously (15). Briefly, following appropriate treatment in 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 × g for 30 min. Samples were precipitated in chloroform (1:1) and ethanol, electrophoresed 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 20 μl of 7.5% n-octyl-β-D-glucopyranoside, 5 mM sodium lauryl sulfate, and 1 mM DETAPAC. Seventy μl of a reaction mix were added to give a final concentration of 0.05 mM imidazole/HC1 (pH 6.6), 0.05 mM 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:CH₃OH (1:2) and 20 μl of 1% HClO₄. The monophosphate was mixed, and after 10 min, 150 μl CH₃Cl and 150 μl of 1% HClO₄ were added; then the tubes were vortexed and centrifuged at 5000 × 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 × 10-cm LH-P TLC plate (Whatman). Data was calculated as pmoles of ceramide/10⁶ cells by scintillation counting.

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2. 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.
PROTEIN KINASE C INHIBITION, CERAMIDE, AND APOPTOSIS

(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 3H-labeled sphingomyelin was performed as described by Wiegmann et al. (18) with minor modifications. Exponentially growing cells (2 x 10^7) were treated as indicated, collected, and lysed in 200 µl of either neutral buffer (20 mM HEPES (pH 7.4), 10 mM MgCl₂, 2 mM EDTA, 5 mM DTT, 100 mM Na₃VO₄, 100 mM NaMoO₄, 10 mM β-glycerophosphate, 750 µM 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 x g. Protein (50 µg) were incubated for 2 h at 37°C in a buffer final volume, 50 ml) containing 20 mM HEPES, 1 mM MgCl₂, and 0.9 µl [N-methyl-3H]sphingomyelin). The reaction was linear at this protein concentration. Phosphorylcholine was then extracted with 800 µl of chloroform:methanol (2:1, v/v), and 250 ml H₂O 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 µM) 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 µM 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 x 10⁶) were labeled with [3H]palmitate (10 µCi/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. 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 quantitate 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-

Fig. 1. Chelerythrine and calphostin C cause apoptosis. A. analysis of low molecular weight DNA by agarose electrophoresis (3%) from 5 X 10⁵ cells exposed to chelerythrine or calphostin C. Lanes 2–5, control with 0.2% DMSO (–), 10 µM chelerythrine (CH), and 250 nM calphostin C (CA), respectively. A molecular weight standard of 123 bp was run in Lanes 1 and 6 (BP). B. DAPI fluorescent staining of DNA revealing 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.
PROTEIN KINASE C INHIBITION, CERAMIDE, AND APOPTOSIS

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 three independent experiments; bars, SD. D, graph of neutral and acidic sphingomyelinase activity normalized to a percentage of control. Cells (2 x 10⁷) 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.

Chelerythrine, induced a 209% (±9 SD) 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.
Fig. 3. Exogenous ceramide and PKC inhibition induce oxidative changes in WEHI-231 cells. WEHI-231 cells (1 x 10^6) 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 quantitated using fluorescence-active cell sorting analysis. The histogram analysis of fluorescence is plotted as LOG function. The gray area represents the control cells, while the overlaid line demonstrates the treated cells.
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