Endometrial cancer cell survival and apoptosis is regulated by protein kinase C $\alpha$ and $\delta$

James M Haughian, Twila A Jackson, David M Koterwas and Andrew P Bradford

Division of Basic Reproductive Sciences, Department of Obstetrics and Gynecology, University of Colorado Health Sciences Center at Fitzsimons, Mail Stop 8309, PO Box 6511 12800 E. 19th Avenue, Aurora, Colorado 80045, USA

Requests for offprints should be addressed to A P Bradford; Email: andy.bradford@uchsc.edu

Abstract

Endometrial cancer is the most common invasive gynecologic malignancy but the molecular mechanisms underlying its onset and progression are poorly understood. Paradoxically, endometrial tumors exhibit increased apoptosis, correlating with disease progression and poor patient prognosis. Endometrial tumors also show altered activity and expression of protein kinase C (PKC) isoforms, implicated in the regulation of programmed cell death; however, PKC modulation of apoptosis in endometrial cancer cells has not been investigated. We detected nine out of ten PKC isoforms in Ishikawa endometrial cancer cell lines, and demonstrated expression of both PKC$\alpha$ and $\delta$ in human endometrial tumors. To determine the functional roles of PKC$\alpha$ and $\delta$ in apoptosis in endometrial cancer, Ishikawa cells were treated with selective PKC inhibitors or adenoviral constructs encoding wild-type or isoform-specific, dominant-negative mutants. Apoptosis was assessed by DNA fragmentation and caspase-mediated poly-(ADP-ribose)-polymerase cleavage. The inhibition of PKC$\delta$ suppressed etoposide-induced apoptosis, while overexpression of PKC$\delta$ enhanced it. In contrast, inhibition of PKC$\alpha$ elevated basal levels of apoptosis and potentiated etoposide-induced cell death. Etoposide treatment also selectively activated PKC$\alpha$, but resulted in both cytosolic translocation and decreased activity of PKC$\alpha$. A fraction of PKC$\delta$ also underwent caspase-dependent cleavage, in response to etoposide. Our results suggest that changes in apoptosis and PKC expression in endometrial cancer are mechanistically linked, such that PKC$\delta$ is required for DNA damage-induced apoptosis, while PKC$\alpha$ mediates a survival response. Thus, PKC$\alpha$ and $\delta$ expression and signaling may be important in endometrial tumorigenesis and could serve as potential prognostic indicators and/or novel targets for therapeutic intervention.

Endocrine-Related Cancer (2006) 13 1251–1267

Introduction

Endometrial cancer is the most common invasive gynecologic malignancy and the fourth most common cancer in women in the United States. In 2005, it was estimated that there would be over 40 000 new cases and more than 7300 deaths (Jemal et al. 2005), making endometrial cancer an important health issue for women. Despite the large number of individuals affected by this disease, the molecular mechanisms involved in the pathophysiology of this gynecologic cancer remain largely unknown and understudied.

The protein kinase C (PKC) family of serine/threonine kinases has been shown to regulate a broad range of key cellular pathways including growth, survival, differentiation, and apoptosis (Fishman et al. 1998, Morse-Gaudio et al. 1998, Zeidman et al. 1999, Cross et al. 2000, Musashi et al. 2000, Ventura & Maioli 2001, Brodie & Blumberg 2003, Gutcher et al. 2003, Hofmann 2004). To date, at least ten PKC isoforms have been identified, which differ in their expression patterns, substrate specificity, and response to extracellular stimuli (Clemens et al. 1992, Lucas & Sanchez-Margalet 1995, Jaken 1996, Nishikawa et al. 1997, Newton 2001). PKCs have been implicated in neoplastic transformation, the growth and metastasis of tumors, and response to therapy in a variety of tissues including breast, prostate, liver, colon, skin, stomach, and respiratory tract (Goodnight et al. 1994, Kiley...
et al. 1996, Cornford et al. 1999, Gomez et al. 1999, Spitaler et al. 1999, Watters & Parsons 1999, Musashi et al. 2000, Koivunen et al. 2006). In endometrial cancers, the total PKC activity was significantly higher when compared with normal endometrial tissue (Fujimoto et al. 1995) and differential overexpression of PKC isoforms has been linked to the proliferative potential of endometrial cancer cell lines and tumor pathogenesis (Gretz et al. 1994, Bamberger et al. 1996, 1997, 1998, Fujimoto et al. 1996, Connor et al. 1997). In an analysis of endometrial tumors, PKCα was more highly expressed in higher-grade endometrial tumors exhibiting lower levels of estrogen receptor (ERα) and poorer prognosis (Fournier et al. 2001). However, studies of PKC in endometrial tumors are limited, largely correlative, and the functional role of specific PKC isoforms in the pathophysiology of endometrial cancer has not been determined.

Specific PKC isoforms have also been shown to play a critical role in the regulation of apoptosis and may be either pro- or anti-apoptotic, dependent on cell type and stimulus (Leszczynski 1995, Lucas & Sanchez-Margalef 1995, Whelan & Parker 1998, Musashi et al. 2000, Brodie & Blumberg 2003). Paradoxically, studies of endometrial tumors report an increase in apoptotic index during the progression from endometrial hyperplasia, through atypical endometrial hyperplasia, to endometrial adenocarcinoma (Ioffe et al. 1995, Whelan & Parker 1998, Musashi et al. 2001). Moreover, undifferentiated and poorly differentiated endometrial carcinomas exhibited higher apoptotic indices when compared with well-differentiated tumors, and higher apoptotic indices correlated inversely with prognosis (Heatley 1997, Kokawa et al. 2001a). Thus, increased rates of apoptosis have been proposed to be a morphological indicator of potentially malignant endometrial tumors (Arends 1999, Stewart et al. 1999, Kokawa et al. 2001a).

Given the above evidence of aberrant apoptosis and changes in PKC expression in endometrial cancers and that PKCs modulate apoptosis in other tumors of epithelial origin, we investigated the potential role of PKC in the regulation of apoptosis in endometrial cancer cells. Herein, we provide evidence that PKCα and δ differentially regulate apoptosis and survival in Ishikawa endometrial cancer cells such that PKCα is a critical mediator of apoptosis, while PKCδ is important in cell survival. Our results demonstrate distinct functional roles for PKCα and δ in endometrial cancer cells and provide, for the first time, a potential mechanistic link between the reported changes in apoptotic index and PKC expression and/or activity, concomitant with progression from hyperplasia to malignancy in the endometrium.

Materials and methods

Cell culture

Ishikawa endometrial adenocarcinoma cells were a generous gift from Dr K K Leslie (University of New Mexico, Albuquerque, NM, USA). Cells were grown in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 12.5% horse serum, 2.5% fetal calf serum, 10 units/ml penicillin, 10 μg/ml streptomycin, and 200 μM l-glutamine and maintained at 37 °C in 5% CO2 in air. Before treatment, the cells were serum-deprived overnight by culturing in DMEM without added serum. Drugs used in experiments were solubilized in dimethyl sulphoxide (DMSO). Etoposide, rottlerin, G6976, phorbol ester (TPA), and benzylxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD-FMK) were purchased from Calbiochem (San Diego, CA, USA).

Western blot analysis

The cells were harvested by washing twice in ice-cold PBS and lysing them in 150 μl buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Triton X-100) supplemented with protease inhibitor cocktail (Roche Diagnostics). Lysate protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories) and an equal volume of Laemmli sample buffer (20% glycerol, 2% (w/v) SDS, 5% β-mercaptoethanol, 62.5 mM Tris (pH 6.8), 1% (w/v) bromophenol blue dye) was added. Between 20 and 75 μg total cellular protein was resolved by electrophoresis on 10% polyacrylamide-SDS gels in electrophoresis buffer (25 mM Tris (pH 8.3), 192 mM glycine, 0.1% (w/v) SDS), transferred to polyvinylidene difluoride (PVDF) membrane in transfer buffer (25 mM Tris (pH 8.3), 192 mM glycine, and probed using specific antibodies. The PKC antibodies used for immunoblotting include PKCα (sc-208), PKCβ (sc-209), PKCβII (sc-210), PKCδ (sc-937), PKCε (sc-214), PKCγ (sc-211), PKCη (sc-215), PKCλ (sc-1091), PKC0 (sc-212), PKCζ (sc-216), and secondary anti-rabbit (sc-2004) or anti-mouse (sc-2005) IgG-HRP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The poly(ADP-Ribose)-polymerase (PARP) cleavage fragment-specific antibody was purchased from BD Biosciences Pharmingen (San Jose, CA, USA). The actin loading controls were assessed using a monoclonal anti-β-actin (A5316) antibody (Sigma). The primary antibody binding was visualized using species-specific secondary antibody conjugated to HRP, chemiluminescent substrate, and exposure to autoradiographic film. The molecular weight of proteins was estimated by
comparison with the Full Range Rainbow recombinant protein molecular weight marker from Amersham Biosciences. Quantitation of band intensity was performed by densitometry using Quantity One software (v. 4.5.1) and a GelDoc imaging system (Bio-Rad).

Immunohistochemistry

Endometrial tumor tissue that had been formalin-fixed and paraffin-embedded was obtained from the Department of Pathology at the University of Colorado Health Science Center. The tissue was sectioned into 5 μm thick slices and blocked for endogenous peroxidase activity using 3% hydrogen peroxide. Antigen retrieval was performed in citrate buffer (20 mM, pH 6.0) for 10 min at 60 °C. The sections were incubated with antibodies specific for PKCδ (C-20, 0.17 μg/ml) and PKCζ (H-7, 1.275 μg/ml), and stained using an indirect avidin biotin immunoperoxidase method on a DAKO Autostainer (DakoCytomation, Carpinteria, CA, USA) as described (Tringler et al. 2006). The specificity of staining was verified using subclass-matched IgG1 (BD PharMingen) in place of PKC antibodies and through blocking studies with peptide antigen (Santa Cruz Biotechnology).

Chromatin condensation and DNA fragmentation assay

Nuclear DNA in healthy and apoptotic cells was stained using Hoechst 33342 dye as described (Mpoke & Wolfe 1997). Ishikawa cells were plated on glass cover slips in serum-containing media and allowed to adhere before treatments. Following treatment of cells, the cover slips were washed twice in PBS so that only the adherent cells remained. After the PBS washes, the cells were covered for 5 min with a solution containing 5 μg/ml Hoechst 33342 dye from Sigma. Following staining, the cells were washed a final time in PBS and the stained nuclei were examined at 10× magnification under u.v. light. The proportion of apoptotic cells was determined by counting cells in four random fields per condition in three separate experiments. Quantitation of extranuclear fragmented DNA was performed using the Cell Death Detection ELISA kit as per the manufacturer’s recommended protocol (Roche).

Adenoviral vectors

PKC adenoviral constructs were a generous gift of Drs Lee Carpenter and Trevor Biden (Garvan Institute of Medical Research, St Vincent’s Hospital, Sydney, Australia) and Dr Mary Reyland (University of Colorado Health Sciences Center, Denver, CO, USA). Information pertaining to the creation and use of these constructs in other cell types has been published previously (Reyland et al. 1999, Carpenter et al. 2002). Dominant-negative PKC constructs were created by introducing a single amino acid mutation in the ATP-binding site of the catalytic domain, specifically a (K376R) mutation in PKCδ from rat and (K368R) mutation in PKCζ from mouse. For adenovirus infections, 3.0×10⁶ cells were plated on 60 mm dishes and allowed to adhere for 18 h or more. After removing the serum-containing media, adenovirus was added to the adherent cells in 1 ml serum-free media at a predetermined multiplicity of infection (MOI) based on viral titer. The plates were gently agitated every 10 min for 1 h, then serum free media was added to a final volume of 3 ml. The infected cells were then incubated for 18–24 h in the serum-free media to enable adequate protein expression before drug treatments.

Immunoprecipitations and PKC kinase assays

Immunoprecipitations were performed using protein A/G PLUS-agarose beads from Santa Cruz. PKC kinase assays included PKC lipid activator from Upstate (Lake Placid, NY, USA), histone (Sigma), and [γ-32P] ATP (Amer sham). The cells were harvested by washing once in ice-cold PBS and lysing cells in lysis buffer (25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA (pH 8.0), 0.5 mM dithiothreitol, 0.1% Triton X-100) amended at each use with phosphatase and protease inhibitors (20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride, and protease inhibitor cocktail). Cell lysates were vortexed and frozen before clearing insoluble cell debris by centrifugation at 14 000 g for 10 min at 4 °C. The total cellular protein was quantitated and 1000 μg (PKCζ) or 500 μg (PKCδ) total cellular protein was combined with 5 μg PKCζ (sc-208) or PKCδ (sc-937) primary antibody in a total volume of 300 μl. After agitating overnight at 4°C, protein A/G agarose beads were added as recommended and agitated with the samples for 2 h. The immune complexes were pelleted and washed four times in lysis buffer. After the final lysis buffer wash, the pellet was washed twice in kinase assay buffer (40 mM Tris (pH 7.4), 20 mM MgCl₂, 20 μM ATP, 2.5 mM CaCl₂). The final pellet was resuspended in 22 μl kinase assay buffer and a 2 μl aliquot of the suspension was acquired to be used as a loading control by western blotting. The final reaction
was performed in a 40 μl volume containing the resuspended immunoprecipitates, 5 μl PKC lipid activator, 20 μg histone, and 5 μCi \([\gamma^32P]ATP\). Before addition to the reaction, the PKC lipid activator containing 0.05 mg/ml phosphatidylserine and 0.05 mg/ml diacylglycerol was sonicated on ice for 30 s. All reaction components were combined on ice and then moved to a 30 °C water bath. After 20 min, the reactions were immediately placed on ice and Laemmli sample buffer was added before heating the samples for 5 min at 95 °C. Phosphorylated histone protein was visualized by electrophoresis on a 12.5% polyacrylamide gel followed by drying and exposing the gel to film. Quantitation of radioactivity in each band was determined using a Storm 860 Phosphor-Imager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

**Cell fractionation**

Soluble and particulate cell fractions were isolated as described (Jackson et al. 2001), briefly, after treatment adherent cells were washed twice in ice-cold PBS and harvested in lysis buffer A (20 mM Tris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 0.1% β-mercaptoethanol, and protease inhibitor cocktail) using a cell scraper. The cells were then sonicated for 10 s (output 4, constant duty cycle) using a Branson Sonicator (Branson Ultrasonics Corp., Danburg, CT, USA) and pelleted at 70 000 × g for 1.5 h. The supernatant (soluble fraction) was removed to a new tube and the remaining pellet was resuspended in lysis buffer B (lysis buffer A + 1% Triton X-100) by sonicating for 10 s. Following sonicating, the sample was pelleted again by centrifugation at 13 000 × g for 15 min at 4 °C and the supernatant harvested as the Triton-soluble, particulate fraction.

**Statistical analysis**

Values shown in figures are given as the mean ± S.D. or S.E.M. The data were analyzed using a paired Student’s t-test. P values < 0.05 were considered significant.

**Results**

Ishikawa cells are derived from a well-differentiated endometrial tumor (Nishida et al. 1996, Leslie et al. 1997) and retain expression of both estrogen and progesterone receptors (Dai et al. 2002). They represent the most widespread and well-characterized human cell-based model for endometrial cancer and have been used extensively to elucidate molecular mechanisms of hormone action, signal transduction pathways, and tumorigenesis (Vollmer 2003). Expression of PKC isoforms was determined by western blot analysis using isoform-specific antibodies (Santa Cruz Biotechnology). As shown in Fig. 1A, Ishikawa cells express the conventional isoforms PKCα, βII, and γ; novel PKCs δ, ε, η, and θ; and the atypical PKCζ. The conventional PKCβI was present at low levels, detectable after prolonged exposure, and the atypical PKC isoform τ/ω was not detected. β-Actin was used a loading control.

PKCα is the predominant isoform associated with cell survival or suppression of apoptosis (Whelan & Parker 1998, Li et al. 1999, Deng et al. 2001). Its expression has previously been shown in partially purified extracts of endometrial tumors (Tonetti et al. 2000) and linked to estrogen-dependent proliferation (Tonetti et al. 1998, Wu et al. 2005). PKCδ, the predominant pro-apoptotic isoform in other epithelial cell types (Basu 2003, Brodie & Blumberg 2003, Gutcher et al. 2003), was expressed in EnCa101 endometrial cancer cells (Tonetti et al. 1998); however, its expression had not been shown in endometrial tumors. Using immunohistochemistry, we observed the expression of both PKCα and δ in paraffin sections of human endometrial tumors and normal tissue. PKCα and δ were detected in each of the 24 samples of endometrial tumors and in each of the eight sections derived from proliferative, secretory, and atrophic normal endometrium (Fig. 1B and C; data not shown). Given their functional role in modulating apoptosis in numerous epithelial cell types (Brodie & Blumberg 2003, Gutcher et al. 2003, Hofmann 2004) and expression in human endometrial tumors, we hypothesized that PKCα and δ are important regulators of apoptosis in endometrial cancer.

To address the functional roles of PKCα and δ in regulating apoptosis in endometrial cancer cells, we utilized selective PKC inhibitors and determined their effects on etoposide-induced apoptosis, assessed by changes in cell morphology, DNA fragmentation, and caspase activation. The topoisomerase II inhibitor etoposide has been used as a chemotherapeutic agent in endometrial cancer (Poplin et al. 1999), and is a potent inducer of DNA damage and apoptosis (Ruvolo et al. 1998). First, Ishikawa cells were stained with Hoechst fluorescent dye 33342, which is indicative of membrane disruption, organelle acidification, and chromatin condensation characteristic of the initial stages of apoptosis (Mpoke & Wolfe 1997, Chen et al. 2005, Wada et al. 2005). Cells were visualized under u.v. and visible light and results quantitated by counting multiple fields. Under basal conditions, approximately 24% of Ishikawa cells stained positive
Figure 1 PKC isoform expression profile in Ishikawa endometrial cancer cell line. (A) Cell extracts were probed with the indicated isoform-specific PKC antibodies. Uniform protein loading was confirmed by blotting for β-actin. (B) and (C) Immunohistochemical staining of (B) PKCα and (C) PKCδ in normal secretory and proliferative endometrium and a pathological grade II, endometrial tumor. Representative images were acquired from the same location within a single tumor. Larger fields are magnified 16×, inset 25×. IgG is included as a control for non-specific immunostaining.
(Fig. 2A). Treatment with 50 µM etoposide increased the fraction of Hoechst 33342 staining cells to over 43%. To determine the roles of PKCα and δ, we utilized the PKC inhibitors Gö6976 and rottlerin, which are selective for the conventional PKCs (α, βI, βII, and γ) and PKCδ respectively (Gschwendt et al. 1994, Keenan et al. 1997). As indicated in the representative fields (Fig. 2A) and associated quantitation (Fig. 2B), pretreatment with rottlerin had no effect on basal apoptosis in Ishikawa cells but significantly reduced the proportion of Hoechst 33342-positive cells in response to etoposide from 43 to 32%. In contrast, treatment with Gö6976 alone increased the basal apoptotic index from 23.9 to 29.1% and potentiated the effect of a lower submaximal dose (10 µM) of etoposide (Fig. 2C), significantly increasing the fraction of apoptotic cells from 31 to 47%. These results suggest that the inhibition of PKCδ attenuated etoposide-induced apoptosis in endometrial cancer cells, whereas inhibition of conventional PKCs

**Figure 2** PKC inhibitors regulate etoposide-induced apoptosis. Ishikawa cells were pretreated for 30 min with pharmacological inhibitors rottlerin (PKCδ-selective inhibitor) and Gö6976 (conventional PKC inhibitor) to block kinase activity during etoposide treatment. Cellular apoptosis was assessed 24 h later by Hoechst 33342 staining of condensed nuclear chromatin and representative fields of select treatments are shown in A. Bar graphs depict the proportion of apoptotic cells following treatments with rottlerin and 50 µM etoposide (B), or Gö6976 and a lower (10 µM) dose of etoposide (C). The proportion of apoptotic cells was determined by dividing the number of positive cells by the total number of cells in a field. Results shown are mean ± S.E.M. derived from counting four fields per treatment, in three separate experiments.
increased basal levels of apoptosis and enhanced the response to etoposide.

To corroborate our results obtained by Hoechst staining, we examined the effects of PKC inhibitors on etoposide-induced DNA fragmentation using a Cell Death Detection ELISA kit (Roche) that quantifies the amount of low molecular weight, histone-bound DNA in the cytoplasm, characteristic of cells undergoing apoptosis (Reyland et al. 1999). As shown in Fig. 3, treatment of Ishikawa cells with etoposide resulted in an increase in DNA fragmentation. Again, consistent with PKCδ being pro-apoptotic, pretreatment with rottlerin markedly reduced DNA fragmentation in a dose-dependent manner (Fig. 3A). Conversely, pretreatment of cells with Gö6976 enhanced the amount of DNA fragmentation detectable after etoposide treatment (Fig. 3B). Neither inhibitor alone significantly affected DNA fragmentation in this assay. Thus, Ishikawa cells treated with rottlerin, a PKCδ-selective inhibitor, showed a reduced apoptotic response to etoposide, suggesting that PKCδ is pro-apoptotic. In contrast, Gö6976 enhanced etoposide-induced apoptosis, suggesting a pro-survival role for conventional PKC isoforms.

Gö6976 does not distinguish between the conventional PKC isoforms (α, β, and γ), and, despite its widespread use, concern has been expressed regarding the specificity of rottlerin in vitro (Davies et al. 2000). Thus, in order to more specifically manipulate cellular activities of PKCα and δ, we used adenoviral expression constructs expressing wild-type (WT) or dominant-negative (DN), kinase dead forms of these enzymes that have been shown to increase or suppress endogenous isoform-specific PKC activity respectively (Reyland et al. 1999, 2000). Cleavage of PARP, an essential DNA repair enzyme, is another hallmark indicator of cells undergoing apoptosis. PARP becomes inactivated when the apoptotic effector caspase-3 and caspase-7 cleave the full-length PARP protein (116 kDa) releasing an 89 kDa fragment (Soldani & Scovassi 2002). Western blotting to detect the appearance of the 89 kDa PARP cleavage fragment has thus been used as an indirect assay of intracellular caspase-3 and caspase-7 activity (Yu et al. 2001).

As shown in Fig. 4A, 48 h following transduction with adenovirus at the specified MOI of 50, both wild-type and dominant-negative PKC were overexpressed relative to the level of endogenous PKCδ evident in cells infected with GFP adenovirus as a control (Fig. 4A). Etoposide treatment did not significantly affect expression of endogenous or adenoviral PKCδ expression. Figure 4B shows Ishikawa cell extracts, treated with or without etoposide and probed for the PARP cleavage fragment. In cells transduced with WT PKCδ adenovirus, a marked increase in basal PARP cleavage, relative to green fluorescent protein (GFP) control is observed (Fig. 4B and C) reaching levels comparable with that observed in GFP cells treated with 50 µM etoposide. Furthermore, overexpression of PKCδ significantly enhanced PARP cleavage induced by etoposide. Conversely, expression of dominant-negative PKC alone suppressed any detectable basal PARP cleavage when compared with GFP controls and significantly attenuated the effects of etoposide (Fig. 4B and C). Of note, the PKCδ WT and DN constructs differ in only one amino acid and were expressed at similar levels. Thus, it is unlikely that the observed effects are a consequence of non-specific viral toxicity.

To confirm the apparent synergistic pro-apoptotic effect of PKCδ overexpression in combination with etoposide, Ishikawa cells infected at a constant MOI were challenged with increasing doses of etoposide (Fig. 4D). Consistent with our initial experiment (Fig. 4B and C), overexpression of PKCδ alone induced PARP cleavage and this effect was increased by etoposide in a dose-dependent manner. PARP cleavage in GFP adenovirus transduced control cells was not detectable until treatment with 25 µM etoposide, while cells overexpressing PKCδ exhibited increased apoptotic PARP cleavage in the presence of only 2 µM etoposide. As a control, expression of dominant-negative PKCδ considerably suppressed PARP cleavage at both 25 and 50 µM doses of the etoposide (Fig. 4D). These results are completely consistent with observations using PKC inhibitors.

**Figure 3** PKC inhibitors modulate DNA fragmentation. Ishikawa cells were pretreated for 30 min with the PKCδ inhibitor rottlerin (A) or PKCα inhibitor Gö6976 (B) before treatment with etoposide (50 µM). After 24 h, internucleosomal DNA fragmentation, a hallmark of apoptosis, was assayed and quantified using the Cell Death Detection kit (Roche) as per the manufacturer’s protocol. The results are expressed as fold change in absorbance relative to etoposide-treated cells; mean ± s.e.m. of three experiments.

Endocrine-Related Cancer (2006) 13 1251–1267
Figure 4 Overexpression of wild-type PKCδ induces apoptosis, whereas dominant-negative PKCδ is protective. Ishikawa cells were infected at an MOI of 50 with adenoviral constructs encoding wild-type PKCδ (δWT), dominant-negative PKCδ (δDN), or green fluorescent protein (GFP) as control. One day following infection, these cells were challenged with 50 µM etoposide and harvested 24 h later. The western blot in (A) depicts adenovirally mediated overexpression of PKCδ protein relative to the level of endogenous expression seen in GFP-infected controls. In (B), representative western blot using an antibody that specifically recognizes the 89 kDa cleavage fragment of PARP protein produced by activated caspases. (C) Western blots of cleaved PARP and actin from four separate experiments were quantitated by densitometry. Data presented are the ratio of intensity between cleaved PARP and β-actin respectively, from the same western blot. Values are mean ± S.E.M. (D) Ishikawa cells were infected as described and challenged with increasing concentrations of etoposide as indicated. Equal amounts of protein (50 µg) were loaded and cellular apoptosis was again determined by blotting specifically for the PARP cleavage fragment.
Figure 5 Overexpression of dominant-negative PKCα enhances etoposide-induced apoptosis. Ishikawa cells were infected at an MOI of 50 with adenoviral constructs encoding wild-type PKCα (αWT), dominant-negative PKCα (αDN), or green fluorescent protein (GFP) as control. One day following infection, the cells were challenged with 50 µM etoposide and harvested for western blot 24 h later. The representative western blots in (A) depict the level of cellular apoptosis as determined by Western blotting with an antibody that specifically recognizes the 89 kDa cleavage fragment of PARP protein produced by activated caspases. Also shown in (A) is the level of adenovirally mediated overexpression of PKCα protein relative to the level of endogenous expression seen in GFP-infected controls. β-Actin serves as a control for equal protein loading. (B) Western blots from four separate experiments were quantitated by densitometry. Data presented are the ratio of intensity between cleaved PARP and β-actin respectively, from the same western blot. Values are mean ± S.E.M. (C) PARP cleavage in Ishikawa cells infected with PKCα adenoviral constructs at an increasing MOI in combination with a low dose (5 µM) of etoposide.
and suggest that PKCδ is a critical component of the pro-apoptotic pathway in endometrial cancer cells.

To determine the role of PKCα in etoposide-mediated apoptosis, Ishikawa cells were infected with adenoviral constructs encoding wild-type or dominant-negative kinase (PKCαDN). GFP adenovirus was again used as a control. Increased PARP cleavage was apparent in untreated cells overexpressing dominant-negative PKCα relative to the level of basal apoptosis seen in control Ishikawa cells expressing GFP (Fig. 5A). Following etoposide treatment, PARP cleavage was increased to a greater degree in cells overexpressing dominant-negative PKCα, suggesting that inhibition of PKCα sensitized the cells to etoposide-induced apoptosis. Overexpression of wild-type PKCα, however, elicited no change in PARP cleavage relative to GFP control cells treated with etoposide. Thus, while endogenous PKCα activity is clearly an important mediator of cell survival, increased PKCα expression was not sufficient to confer resistance to etoposide.

It is evident that 50 μM etoposide treatment apparently increased the level adenoviral PKCα expression (Fig. 5A, panel 2), possibly due to increased viral promoter activity or protein stabilization. No significant change in endogenous levels of PKCα, in response to etoposide, was observed. However, since both wild-type and dominant-negative constructs are affected, the observed effects on apoptosis cannot be attributed to non-specific effects of viral protein overexpression. Nevertheless, to further confirm the role of PKCα, we conducted a second experiment where the Ishikawa cells were challenged with a tenfold lower dose of etoposide (5 μM) and infected with adenovirus at an increasing MOI (10, 25, 50). As seen in Fig. 5C, panels 2 and 4, infecting cells with wild-type or dominant-negative PKCα adenovirus at increasing MOI resulted in the expected dose-dependent increase in the level of PKCα protein, but the lower dose of etoposide did not influence virally mediated PKCα expression. In vehicle-treated cells expressing dominant-negative PKCα, PARP cleavage increased in a MOI-dependent fashion (panel 1; lanes 4–6), whereas no increase in PARP cleavage was apparent in cells overexpressing wild-type PKCα (panel 1; lanes 1–3). Treatment of cells overexpressing PKCα, with 5 μM etoposide, induced minimal PARP cleavage, which was not affected by increased expression of PKCα (panel 4; lanes 1–3). In contrast, etoposide resulted in a robust, MOI-dependent, increase in PARP cleavage in cells expressing dominant-negative PKCα, over and above levels induced by expression of dominant-negative PKCα.

Figure 6 In vitro kinase assay of PKCδ and α activities following etoposide treatment. Ishikawa cells treated with 50 μM etoposide or 100 nM TPA were harvested at the times indicated. (A), top panel is an autoradiograph of histone protein phosphorylated by PKCδ immunoprecipitated from 500 μg total cellular protein. The kinase activity in the immunoprecipitate was determined by co-incubation at 30 °C for 20 min with radiolabeled ATP, lipid co-activators (phosphatidyl serine, diacylglycerol), and histone as substrate. Uniform immunoprecipitation of PKCδ and α at each time point was verified by western blotting. (B) Equivalent conditions to (A) except that PKCα protein was immunoprecipitated from 1000 μg of total cellular protein. Quantitation of radioactivity in each experiment was determined using a phosphorimager. Values are normalized as a proportion of activity at 0 min and are presented as mean ± S.E.M. of three separate experiments *P<0.05.
alone (compare panels 1 and 4; lanes 3–6), indicating that inhibition of PKCα dramatically sensitized the cells to this DNA damaging agent.

In summary, apoptotic PARP cleavage in untreated and etoposide-treated Ishikawa cells was consistently increased by expression of dominant-negative PKCδ, while overexpression of the wild-type kinase had no apparent effects. Considering our results from both the pharmacological inhibitors and the adenoviral-mediated overexpression, we conclude that PKCδ is mediating a pro-survival signal in Ishikawa endometrial cancer cells, such that inhibition of PKCδ induces apoptosis and sensitizes cells to etoposide treatment.

We next determined changes in PKCδ and α activities in response to an apoptotic stimulus. Immunocomplex kinase assays were used to examine the effect of etoposide on PKCδ and α activities in Ishikawa cells. As shown in Fig. 6A, in response to etoposide treatment, PKCδ kinase activity is rapidly increased within 15 min, reaching a maximum level at 30 min, and remains moderately elevated at 2 h. Conversely, PKCα activity apparently decreases over this same period of time, reaching a nadir at 30 min and remaining below basal levels at the 2 h time point (Fig. 6B). Western blot analysis confirmed uniform PKCδ and δ loadings in the kinase assay. No histone kinase activity was detected in control immunoglobulin immunoprecipitates.

The activation of PKCs is frequently associated with the movement from cytosol to membrane fractions and translocation is used as an indirect assessment of activity (Mackay & Mochly-Rosen 2001). To correlate the apparent etoposide-induced changes in PKC kinase activity with translocation, lysates of Ishikawa cells were fractionated by differential centrifugation as described previously (Jackson et al. 2001), and the soluble and the particulate fractions probed for PKCα and δ. Under basal conditions, the majority of PKCδ protein (∼75%) was localized to the particulate fraction in untreated cells (Fig. 7A). While TPA treatment resulted in translocation from soluble to membrane fractions, somewhat surprisingly, etoposide induced a small but significant transient increase in PKCδ in cytosolic fractions (Fig. 7A). This rapid cytosolic translocation of PKCδ in response to etoposide occurred over a similar time course to that of the elevated kinase activity (Fig. 6A). Such stimulus-specific translocation of PKCδ to the cytoplasm has been reported previously and may reflect the formation of activated lipid-independent kinase activity (Rybin et al. 2004, Yang et al. 2006).

In contrast, PKCα was predominantly (∼85%) localized in the soluble fraction (Fig. 7B). Consistent with the reduction in kinase activity (Fig. 6B), etoposide treatment resulted in a complete loss of PKCα from the particulate fraction and a corresponding increase in soluble enzyme. Treatment with the phorbol ester TPA, a known activator of PKCα (Ohno et al. 1991), resulted in a rapid and robust translocation to the particulate fraction (Fig. 7B). Thus, taken together, these results are consistent with PKCδ

Figure 7 Translocation of PKCδ and α following etoposide treatment. Lysates from Ishikawa cells treated with 50 μM etoposide, or 100 nM TPA (positive control) were harvested in detergent-free buffer, sonicated, and centrifuged at 70 000 g for 1.5 h. The soluble fraction (supernatant) was collected and the remaining pellet was resuspended and sonicated in buffer containing 1.0% Triton X-100 to create the Triton-soluble, particulate fraction. Western blotting of particulate and soluble fractions indicates the relative amount of PKCδ (A) or PKCα (B) protein in each fraction following treatment. The band intensities were determined by densitometry and proportions shown in bar graphs represent the band intensity from one fraction divided by the sum total band intensity of the two fractions. Values are mean ± S.E.M. from three separate experiments *P<0.05.
PKCs modulate apoptosis in endometrial cancer

Recent evidence from other cell types suggests that PKCδ is not only a mediator, but also a target of the apoptotic pathway. In cells undergoing apoptosis, PKCδ can undergo caspase-dependent proteolytic cleavage, between the regulatory and the catalytic domains, releasing a catalytically active fragment (Emoto et al. 1995, Mizuno et al. 1997, Denning et al. 1998, Reyland et al. 1999). To determine if PKCδ is a target of caspases in endometrial cancer cells, lysates from etoposide-treated Ishikawa cells were harvested over a 24-h time course and probed for PKCδ protein using an antibody that recognizes an epitope in the C-terminal catalytic domain. As shown in Fig. 8, a 40 kDa band (indicated by the arrow), consistent with the previously reported size of the PKCδ catalytic fragment, increases in abundance over time in response to etoposide. It is important to note that this band is only detectable after a prolonged exposure of the Western blot, and therefore seems to represent a relatively small fraction of the total PKCδ protein in the cell. Treatment of Ishikawa cells with the caspase inhibitor, Z-VAD-FMK, blocked the formation of 40 kDa catalytic fragment indicating that cleavage of PKCδ is caspase-dependent (Fig. 8). A second minor band migrating at higher molecular weight was observed with varying intensity in some samples and was not suppressed by inhibition of caspases. Treatment with Z-VAD-FMK alone had no effect on full-length or cleaved PKCδ levels (data not shown).

In summary, we have demonstrated that PKCα and δ can differentially regulate endometrial cancer cell survival and apoptosis. PKCδ is a critical component of the apoptotic pathway in Ishikawa cells, whereas PKCα activity is required for cell survival. Accordingly, etoposide treatment and induction of apoptosis resulted in activation and caspase-dependent cleavage of PKCδ accompanied by a reduction in PKCα kinase activity. Thus, the balance of PKCα and δ expression and/or activities may be important in the survival of endometrial cancer cells and modulate response to chemotherapeutic agents.

**Figure 8** Full-length PKCδ protein is cleaved by active caspases. Time course of Ishikawa cells treated with 50 μM etoposide in combination with 10 μM of the general caspase inhibitor, Z-VAD-FMK. A western blot of 20 μg of total cellular protein using a PKCδ-specific antibody is shown at an exposure time of 15 s (top) or the same blot exposed for 1 h (1 h; bottom). Both full-length PKCδ protein (PKCδ FL, 75 kDa) and a PKCδ catalytic fragment (PKCδ Cat, 40 kDa) sensitive to Z-VAD-FMK (denoted by the arrow) were apparent. For each western blot, a standard marker was used to approximate molecular weight (denoted by the arrow) were apparent. For each western blot, a standard marker was used to approximate molecular weight and β-actin was used to confirm uniform protein loading.

**Discussion**

In the endometrium, apoptosis plays a critical role in normal menstrual cycle physiology and is regulated in response to estrogen and progesterone (Arends 1999). Aberrant apoptosis was present in dysfunctional uterine bleeding (Stewart et al. 1999) and increasing cellular apoptosis was observed upon progression from endometrial hyperplasia through atypia to adenocarcinoma (Ioffe et al. 1998). Moreover, undifferentiated and poorly differentiated endometrial carcinomas paradoxically exhibited higher apoptotic indices when compared with well-differentiated tumors; these high indices correlated inversely with prognosis (Heatley 1995, Kokawa et al. 2001b). The highest rates of apoptosis were observed in the rare but aggressive papillary serous and clear cell endometrial carcinomas, characterized by late-stage presentation, extra-uterine invasion, and poor prognosis (Kokawa et al. 2001a). Thus, increased rates of apoptosis have been proposed to be an early morphological indicator of progressively abnormal endometrial growth (Arends 1999, Stewart et al. 1999, Kokawa et al. 2001a).

Differential overexpression of PKC isoforms has also been implicated in endometrial tumor pathogenesis and patient prognosis (Bamberger et al. 1996, 1997, 1998, Tonetti et al. 1998, 2000, Wu et al. 2005), and members of the PKC family have been shown to regulate apoptosis in a variety of other epithelial cell types and tumors (Musashi et al. 2000, Mandil et al. 2001, Brodie & Blumberg 2003). In general, PKCα appears to suppress or protect against apoptosis (Whelan & Parker 1998, Li et al. 1999, Deng et al. 2001), while the majority of studies show PKCδ involvement in the induction of programmed cell death (Basu 2003, Brodie & Blumberg 2003, Gutcher et al. 2003). However, despite evidence that aberrant apoptosis and PKC isoform expression are phenotypic markers of carcinogenesis and tumor progression in the endometrium, the role of PKC in the regulation of apoptosis in endometrial tumors has not been elucidated.

In this study, we demonstrate, for the first time, a potential mechanistic link between the reported altered PKC expression/activity and aberrant apoptosis.
characteristic of endometrial tumors and suggest a functional role for specific PKC isoforms in the pathogenesis of endometrial cancer. We show that PKCζ and δ differentially modulate apoptosis in endometrial cancer cells, such that PKCδ is required for etoposide-induced cell death, while PKCζ is a critical component of a cell survival pathway. Thus, inhibition of PKCδ confers resistance to the chemotherapeutic drug etoposide (Figs 3 and 4), whereas inhibition of PKCζ is sufficient to induce apoptosis and sensitize cells to a genotoxic insult (Figs 3 and 5). Conversely, overexpression of PKCδ increased basal levels of apoptosis and enhanced the effect of etoposide (Fig. 4). Consistent with these observations, treatment with etoposide and induction of apoptosis resulted in the activation of PKCδ and the concomitant inhibition of PKCζ (Fig. 6). While inhibition of PKCζ or expression of a dominant-negative construct induced apoptosis and potentiated the response to etoposide, overexpression of wild-type PKCζ did not confer resistance to etoposide-induced cell death (Fig. 5). These data suggest that, while PKCζ is a critical component of a cell survival pathway in Ishikawa cells, it may not act directly to antagonize apoptotic signals. Our results indicate that relative expressions and/or activities of PKCζ and δ can modulate apoptosis and survival in endometrial cancer cells and that isoform-specific PKC signaling pathways may be important in endometrial tumorigenesis. These studies focus on PKCζ and δ, the principal isoforms implicated in the regulation of apoptosis. However, Ishikawa cells express additional PKCs (Fig. 1), which have also been shown to modulate cell survival and apoptosis (Musashi et al. 2000, Gutcher et al. 2003). Whilst only PKCζ expression has been shown to correlate with patient prognosis and response to hormonal therapy (Tonetti et al. 1998, 2000), the functional role of these additional PKC isoforms remains to be established.

The mechanism by which PKCs modulate apoptosis in the endometrium remains to be established. However, aberrant expression of Bcl-2 proteins in endometrial cancers is associated with increased malignancy and poor prognosis (Loffe et al. 1998, Ouyang et al. 1998, Sakuragi et al. 2002). Decreased levels of the anti-apoptotic Bcl-2 and upregulation of its pro-apoptotic partner Bax, correlate with increased apoptosis and progression from hyperplasia to malignancy in the endometrium (Chiang et al. 1996, Henderson et al. 1996, Kuwashima et al. 1996, Saegusa et al. 1996, Mozzetti et al. 2000, Kokawa et al. 2001a, Peiro et al. 2001, Sakuragi et al. 2002). PKCζ has been shown to phosphorylate Bcl-2, which may be required for optimal anti-apoptotic function (Ruvolo et al. 1998, Deng et al. 2001). Similarly, the tumor suppressor PTEN is a negative regulator of PI3-kinase/Akt prosurvival signal transduction pathway (Kennedy et al. 1997, Vazquez & Sellers 2000) that is frequently mutated or downregulated in endometrial tumors (Maxwell et al. 1998, Ali 2000, Mutter et al. 2000). Ishikawa cells do not express functional PTEN protein (Wan et al. 2002), resulting in constitutive phosphorylation and activation of Akt (Lilja et al. 2001). The present evidence indicates that PKCζ and δ can be both regulators and targets of the PI3K/Akt signaling pathway regulating cell survival (Mandil et al. 2001, Brodie & Blumberg 2003, Lu et al. 2004, Greco et al. 2006). PI3K-dependent activation of PKCδ is an important mediator of invasion of mammary epithelial cells (Woods Ignatoski et al. 2003) and Akt modulates antiapoptotic PKCζ signaling in breast cancer cells. Conversely, PKCs have been shown to modulate PI3k-dependent signal transduction (Wen et al. 2003). PKCζ typically stimulates Akt phosphorylation and activity, while PKCδ has been shown to induce dephosphorylation and inactivation of Akt in a variety of cell types (Mao et al. 2000, Li et al. 2004, 2006, Zhu et al. 2004). In LNCap prostate cancer cells, which, like endometrial cancers, lack functional PTEN, both PKCζ and δ activation results in dephosphorylation of Akt and induction of apoptosis (Tanaka et al. 2003). We did not detect changes in the levels or phosphorylation states of Akt in response to overexpression or inhibition of PKCδ in Ishikawa cells (data not shown). However, potential interactions between the PTEN/Akt and PKC-dependent signaling pathways in endometrial cancer cells remain to be investigated.

The characterization of the functional roles of specific PKC isoforms in endometrial cancer cells may provide new diagnostic or prognostic markers to identify aggressive, malignant tumors and provide a rational basis for novel therapeutic strategies based upon PKC modulating drugs presently under development (Carter 2000, Goekjian & Jirousek 2001, Hofmann 2004). Given the differential effects of PKCζ and δ in endometrial cancer cells reported herein, such approaches require the development of isoform-specific PKC activators or inhibitors.

Acknowledgements

We thank Drs Mary Reyland, Arthur Gutierrez-Hartmann, William Wood, and John Tentler for critical reading of this manuscript and Dr Ken Shroyer (Department of Pathology, UCHSC) for assistance with immunohistochemistry staining.
Funding

This work was supported by NCI-CA10487 and the University of Colorado Department of Obstetrics & Gynecology. The authors have no conflicts of interest.

References


Arends M 1999 Apoptosis in the endometrium.

Histopathology 35 174–178.


Brodie C & Blumberg PM 2003 Regulation of cell apoptosis by protein kinase c delta. Apoptosis 8 19–27.


Greco S, Storelli C & Marsigliante S 2006 Protein kinase C (PKC)-delta/epsilon mediate the PKC/Akt-dependent phosphorylation of extracellular signal-regulated kinases 1 and 2 in MCF-7 cells stimulated by bradykinin. *Journal of Endocrinology* **188** 79–89.


Hoffmann J 2004 Protein kinase C isozymes as potential targets for anticancer therapy. *Current Cancer Drug Targets* **4** 125–146.


Reyland ME, Barzen KA, Anderson SM, Quissell DO & Matassa AA 2000 Activation of PKC is sufficient to induce an apoptotic program in salivary gland acinar cells. *Cell Death and Differentiation* **7** 1200–1209.


