Published in final edited form as:
J Cell Biochem. 2010 November 1; 111(4): . doi:10.1002/jcb.22786.

Protein Kinase Cδ and Caspase-3 Modulate TRAIL-Induced Apoptosis in Breast Tumor Cells

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Abstract

This report describes that protein kinase C delta (PKCδ) overexpression prevents TRAIL-induced apoptosis in breast tumor cells; however, the regulatory mechanism(s) involved in this phenomenon is (are) incompletely understood. In this study, we have shown that TRAIL-induced apoptosis was significantly inhibited in PKCδ overexpressing MCF-7 (MCF7/PKCδ) cells. Our data reveal that PKCδ inhibits caspase-8 activation, a first step in TRAIL-induced apoptosis, thus preventing TRAIL-induced apoptosis. Inhibition of PKCδ using rottlerin or PKCδ siRNA reverses the inhibitory effect of PKCδ on caspase-8 activation leading to TRAIL-induced apoptosis. To determine if caspase-3-induced PKCδ cleavage reverses its inhibition on caspase-8, we developed stable cell lines that either expresses wild-type PKCδ (MCF-7/cas-3/PKCδ) or caspase-3 cleavage-resistant PKCδ mutant (MCF-7/cas-3/PKCδ mut) utilizing MCF-7 cells expressing caspase-3. Cells that overexpress caspase-3 cleavage-resistant PKCδ mutant (MCF-7/cas-3/PKCδmut) significantly inhibited TRAIL-induced apoptosis when compared to wild-type PKCδ (MCF-7/cas-3/PKCδ) expressing cells. In MCF-7/cas-3/PKCδmut cells, TRAIL-induced caspase-8 activation was blocked leading to inhibition of apoptosis when compared to wild-type PKCδ (MCF-7/cas-3/PKCδ) expressing cells. Together, these results strongly suggest that overexpression of PKCδ inhibits caspase-8 activation leading to inhibition of TRAIL-induced apoptosis and its inhibition by rottlerin, siRNA, or cleavage by caspase-3 sensitizes cells to TRAIL-induced apoptosis. Clinically, PKCδ overexpressing tumors can be treated with a combination of PKCδ inhibitor(s) and TRAIL as a new treatment strategy.

Keywords

PKC-δ; CASPASE-3; APOPTOSIS; TRAIL; BREAST CANCER

The protein kinase C (PKC) family of serine/threonine kinases regulates many cellular functions including proliferation and survival [Newton, 1995; Mackay and Twelves, 2007]. PKCδ is activated by numerous apoptotic stimuli and is emerging as an important modulator of apoptosis in human cancers [Brodie and Blumberg, 2003; Jackson and Foster, 2004]. Most studies reported a pro-apoptotic function for PKCδ in response to various stimuli, such as H_2O_2 [Kaul et al., 2005], tumor necrosis factor-α [Emoto et al., 1995], etoposide [Blass et al., 2002], cisplatin [Basu et al., 2001], and UV radiation [Chen et al., 1999]. On the other hand, studies by Humphries et al. [2006] demonstrated that apoptosis was suppressed in irradiated salivary glands in PKCδ-deficient mice, suggesting that PKCδ plays a major role

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in the regulation of apoptosis both in vitro and in vivo. Different apoptotic stimuli have been shown to induce caspase-dependent cleavage of PKCδ, resulting in the generation of a constitutively active catalytic fragment [Ghayur et al., 1996; D'Costa and Denning, 2005]. The cleavage of PKCδ has been implicated in pro-apoptotic function [Ghayur et al., 1996; DeVries et al., 2002; D'Costa and Denning, 2005; DeVries-Seimon et al., 2007] while PKCδ has been shown to negatively regulate apoptosis. We have shown that PKCδ overexpression contributes to antiestrogen resistance in mammary tumor cells [Nabha et al., 2005], in non-small cell lung cancer cells [Clark et al., 2003] it promotes survival and chemotherapeutic drug resistance. The mechanisms by which PKCδ regulates pro- and anti-apoptosis remain unclear.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a novel member of TNF family. Ashkenazi and Dixit [1998] demonstrated that TRAIL ligand has selective toxicity on cancer cells, but not normal cells suggesting the potential as a therapeutic agent.

The binding of TRAIL to DR5 and DR4 receptors leads to the formation of a death-inducing signaling complex (DISC) and this rapidly association with the adaptor protein Fas-associated death domain (FADD). This then conjugates with the initiator caspase-8, mediating apoptosis either by direct activation of downstream effectors caspase-3, −6, −7 cascade or by the cleavage of apoptotic molecules, such as Bid and Bcl-xl leading to activation of the caspase-9 complex [Herr and Debatin, 2001]. The caspases function to cleave cellular proteins critical for life, and the cleavage of caspase substrates sets the stage for the morphological and biochemical changes that are hallmarks of apoptosis [Earnshaw et al., 1999].

In this study, we report the role of PKCδ in TRAIL-induced apoptosis using MCF-7 breast cells and analyzed the molecular mechanism(s) that regulates pro- and anti-apoptotic function in breast cells. Our data clearly demonstrate that overexpression PKCδ in MCF-7 cells (MCF-7/PKCδ cells) increases the expression of death receptors DR4 and DR5 in comparison to vector transfected MCF-7 cells but does not increase TRAIL-induced apoptosis. We show that expression of caspase-3 cleavage-resistant PKCδ (MCF-7/cas-3/PKCδmut) protects the MCF-7 cells from TRAIL-induced apoptosis by inhibiting caspase-8 activation in response to TRAIL treatment leading to inhibition of both death receptors (extrinsic pathway) and mitochondria (intrinsic pathway). However, overexpression of wild-type PKCδ (MCF-7/cas-3/PKCδ) leads to cleavage and activation of PKCδ (enhances apoptosis). This cleavage of PKCδ by caspase-3 reverses the PKCδ-induced casapase-8 inhibition and significantly enhances TRAIL-induced apoptosis. Additionally, TRAIL-induced apoptosis can be reversed by blocking caspase-3 suggesting that caspase-3 induced PKCδ cleavage could play a major role in regulating TRAIL-induced apoptosis.

MATERIALS AND METHODS

CELL LINES AND REAGENTS

The human breast carcinoma cell lines MCF-7 and T47D were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FCS, 10 μg/ml insulin (Sigma), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco). Caspase-3 stably transfected MCF-7 (MCF-7/Cas-3) cells were generously provided by Dr. Xiaohe Yang (University of Oklahoma Health Sciences Center, OK, USA). The plasmids of full-length PKCδ (PKCδ), caspase-3 cleavage-resistant PKCδ D327A mut (PKCδmut), and control vector were generously provided by Dr. C. Brodie (Henry Ford Hospital, MI, USA). DR4/TRAILR1 monoclonal antibody and DR5/TRAILR2 polyclonal antibody was purchased from Imgenex (San Diego, CA). Monoclonal antibodies of PKCδ, PARP, Bid, caspase-8 were purchased from BD Biosciences (San

Diego, CA). Akt, phospho-Akt, Caspase-3, and caspase-9 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) rabbit polyclonal was obtained from Trevigen (Gaithersburg, MD). Rottlerin was purchased from Calbiochem (San Diego, CA), TRAIL and caspase-3 inhibitor was purchased from R&D Systems (Minneapolis, MN). Cell Death Detection ELISA^{PLUS} was purchased from Roche (Indianapolis, IN). Reagents for protein concentration analysis and protein gel electrophoresis were obtained from Bio-Rad (Hercules, CA). Mammalian PKCδ siRNA expression plasmid (pKD-PKCδ-v2) and random siRNA (pKD-NegCon-v1) were purchased from Upstate (Temecula, CA). Tunicamycin and all other chemicals, unless otherwise specified, were obtained from Sigma in the highest suitable purities.

ISOLATION OF STABLE TRANSFECTANTS

MCF-7 or MCF-7/cas-3 cells were transfected with PKCδ, PKCδmut, or control vector using Lipofectamine™ 2000 Reagent (Invitrogen). Stable transfectants were selected in the presence of 500 μg/ml Geneticin (G418; Gibco) after 2–3 weeks. Individual antibiotic-resistant colonies were isolated and screened for the expression of the corresponding protein by Western blot analysis using anti-PKCδ antibody (BD Biosciences). The pools made by mix six positive clones were named MCF-7/Vector, MCF-7/PKCδ, MCF-7/Cas-3/Vector, MCF-7/Cas-3/PKCδ, and MCF-7/Cas-3/PKCδmut cell lines were developed and used for experiments. All cell lines were routinely tested for mycoplasma contamination and found to be negative.

IMMUNOHISTOCHEMICAL ANALYSIS (IHC)

The specimens used for this study were collected under an IRB approved protocol (HIC#113003MP4E) such that the specimens could not be linked to the human subject and were considered exempt. Immunohistochemistry (IHC) was performed on tumor tissue sections placed on glass slides using the standard laboratory protocols as previously described [Visscher et al., 1997]. Briefly, after deparaffinizing and hydrating with phosphate-buffered saline (PBS) buffer (pH 7.4), tissue sections were pretreated with hydrogen peroxide (3%) for 10 min to remove endogenous peroxidase, followed by antigen retrieval via steam bath for 20 min in EDTA. A primary antibody was applied, followed by washing and incubation with the biotinylated secondary antibody for 30 min at room temperature. Detection was performed with diaminobenzidine (DAB) and counterstained with Mayer hematoxylin followed by dehydration and mounting. A score of zero indicates no staining relative to background, 1+= weak staining, 2+= moderate staining, and 3+= strong staining. For comparison of staining among tissues, the results were quantified by calculation of a complete H-score which considers both staining intensity and the percentage of cells stained at a specific range of intensities. A complete H-score was calculated by summing the product of the percentage cells stained (0–100%) and staining intensity (0–3) according to Kerfoot et al. [2004]. Statistical analysis of the complete H-scores obtained for the normal tissue and re-occurring breast tumors was carried out by using the two-tailed Student's *t*-test with unpaired data of equal variance.

WESTERN IMMUNOBLOT ANALYSIS

MCF-7 and T47D cells were grown in DMEM with 5% or 10% fetal bovine serum, to near confluence in the presence or absence of various treatments. Cells were lysed and Western blotting was performed as described previously [Nabha et al., 2005] using a standard protocol. In brief, cell extracts were obtained by lysing the cells in RIPA buffer (20 mM HEPES, 100 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% deoxycholate, 1 mM Na_3VO_4, 1 mM EGTA, 50 mM NaF, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl

fluoride, and 1× protease inhibitor mixture). Samples containing 100 μg of total protein were resolved on 10% or 15% SDS–polyacrylamide gels and proteins were electrophoretically transferred onto a PVDF membrane. Membranes were probed with antibodies as indicated, followed by HRP-conjugated mouse or rabbit secondary antibodies (Amersham). Anti-G3PDH was used for loading controls.

RNA INTERFERENCE ASSAY

Cells were plated in six-well tissue culture plates, at a density of 3×10^5/well, in DMEM containing 5% FBS. After 24 h, cells were transfected with 4 μg pKD-PKCδ plasmid or pKD-NegCon-v1 (non-specific siRNA with scrambled sequence) plasmid using Lipofectamine transfection reagent according to the manufacturer's instructions. After 72 h of transfection, cells were treated with or without TRAIL for an additional 4 h at 37°C. Cells were harvested and extracts prepared for Western blot analysis.

APOPTOSIS ASSAY

Apoptosis was assessed using the Cell Death Detection ELISA^{PLUS} kit (Roche) according to the manufacturer's instructions. This kit quantitatively detected cytosolic histone-associated DNA fragments. In brief, 5×10^5 cells were grown in six-well tissue culture plates for 24 h at 37°C in CO_2 incubator. After 24 h, cells were pretreated with either rottlerin (3 μM) or caspase-3 inhibitor (10 μm) for 1 h and then TRAIL (10 ng/ml) was added for 4 h. Cells were harvested and apoptosis measured according to the manufacturer's instructions. Apoptosis was quantified by ELISA and normalized to values measured in untreated cells. Data are mean ± SE of triplicate determinations.

RESULTS

PKCδ IS OVEREXPRESSED IN HUMAN BREAST TUMORS

We examined PKCδ expression in re-occurring human and compared with normal adjacent breast tissue using IHC. Interpretation of slides was performed by microscopic examination by a pathologist. Staining patterns of tumor at various stages of tumor development are shown in Figure 1. Results indicate a strong level of PKCδ expression in ductal carcinoma in situ (DCIS) and a moderate level of expression in invasive breast tissue when compared to surrounding normal breast tissue. The staining intensity of each specimen was judged relative to the intensity of a control slide containing an adjacent normal section. The staining of the section labeled with the negative reagent control was considered background. The summary of PKCδ staining intensity in re-occurring human breast cancers and adjoining normal breast tissue is given in Table I. The data suggest that re-occurring human breast tumors over-express PKCδ compared to surrounding normal breast tissue.

PKCδ OVEREXPRESSION PROTECTS CELLS FROM TRAIL-INDUCED APOPTOSIS

To determine the mechanism by which PKCδ regulates both pro- and anti-apoptotic process in breast tumor cells, we stably transfected PKCδ cDNA into MCF-7 cells. MCF-7 cells normally express low levels of PKCδ (Supplementary Fig. 1). We have previously demonstrated that PKCδ overexpression does not alter other PKC isoforms in MCF-7 cells [Nabha et al., 2005]. Overexpression of PKCδ in MCF-7 (MCF-7/PKCδ) cells was associated with increased expression of death receptors DR4 and DR5 when compared to vector transfected (MCF-7/vector) control cells (Fig. 2A). In addition, we utilized an endoplasmic reticulum stress inducer tunicamycin (10 μg/ml) to upregulate the expression of death receptors. Increased DR4 and DR5 receptors do not increase TRAIL-induced apoptosis in PKCδ overexpressing MCF-7/PKCδ cells (Fig. 2B), suggesting the possibility that PKCδ overexpression protects MCF-7 cells from TRAIL-induced apoptosis. To confirm

that PKCδ overexpression protects TRAIL-induced apoptosis in MCF-7 cells, we inhibited PKCδ with rottlerin (3 μM), a pharmacologic inhibitor of PKCδ. Our data show that rottlerin significantly inhibits PKCδ levels and TRAIL had minimal effect on its expression in MCF-7/PKCδ cells (Fig. 2A). The combination of rottlerin and TRAIL significantly reduced PKCδ levels and enhanced apoptosis as shown in Figure 2A,B. Currently, the use of rottlerin as a PKCδ inhibitor remains controversial. To further confirm the role of PKCδ in TRAIL-induced apoptosis, we knocked down PKCδ using PKCδ siRNA in MCF-7/PKCδ cells. Inhibition of PKCδ by siRNA in the presence of TRAIL significantly reduced phosphorylated Akt (p-Akt), while increasing both Bid and PARP cleavage when compared to random siRNA-treated cells (Fig. 3A) and increased apoptotic activity. These results suggest that PKCδ overexpression protects MCF-7 cells from TRAIL-induced apoptosis and this inhibition enhances apoptosis (Fig. 3B).

ROLE OF CASPASE-3 IN PKCδ-INDUCED PRO- AND ANTI-APOPTOSIS

Caspase-3 is a member of the cysteine protease family, which plays a crucial role in the apoptotic pathway by cleaving a variety of key cellular proteins including PKCδ. Previous studies suggest that pro-apoptotic function of PKCδ is typically associated with its cleavage by caspase-3 in the hinge region, resulting in a phospholipid-independent activation of the enzyme [Emoto et al., 1995; Ghayur et al., 1996; Janicke et al., 1998; Okhrimenko et al., 2005]. Devarajan et al. [2002] showed that 75% of human breast tumors lack caspase-3 transcripts, but other caspases, such as caspase-8 and −9 were normal. MCF-7 cells were ideally suited for these proposed studies because they lack caspase-3 expression as a result of a functional deletion mutation in the casp-3 gene, while expression of other caspases-8 and −9 remains normal. In order to determine if restoration of caspase-3 enhances TRAIL-induced apoptosis in breast tumors, we developed stably transfected MCF-7 cell lines that express both PKCδ and caspase-3 (MCF-7/cas-3/PKCδ), and evaluated the role of caspase-3 in PKCδ and TRAIL-induced apoptosis. The introduction of caspase-3 gene in PKCδ over-expressing cells (MCF-7/cas-3/PKCδ) significantly enhanced the cleavage of the PKCδ, caspase-3, and PARP cleavage in the presence of TRAIL when compared to untreated cells (Fig. 4A). Similar results were observed in PKCδ/T47D cells (Supplementary Fig. 2). Inhibition of caspase-3 activity by its inhibitor Z-DEVD-fmk reversed PKCδ and PARP cleavage and blocked TRAIL-induced apoptosis in MCF-7/cas-3/PKCδ cells (Fig. 4B). These data suggest that caspase-3-induced cleavage of PKCδ may play a significant role in enhancing TRAIL-induced apoptosis in breast tumor cells.

PKCδ REGULATES TRAIL-INDUCED PRO- AND ANTI-APOPTOSIS

Studies were undertaken to determine the mechanism(s) by which PKCδ overexpression regulates TRAIL-induced pro- and anti-apoptosis. For this stable MCF-7 cell lines that express wild-type PKCδ (MCF-7/cas-3/PKCδ cells) or caspase-3 cleavage-resistant PKCδmut (PKCδD327A) (MCF-7/cas-3/PKCδmut cells) expressing cell were developed utilizing MCF-7 cells expressing caspase-3. Cells that overexpress caspase-3 cleavage-resistant PKCδ mutant (MCF-7/cas-3/PKCδmut) significantly inhibited TRAIL-induced apoptosis when compared to wild-type PKCδ (MCF-7/cas-3/PKCδ) expressing cells. Western blot analysis shows that in MCF-7/cas-3/PKCδmut cells, TRAIL-induced caspase-8 activation was blocked leading to inhibition of downstream signaling molecules such as caspase-3, −9, Bid, and PARP cleavage (Fig. 5A) and apoptosis (Fig. 5B) compared to wild-type PKCδ (MCF-7/cas-3/PKCδ) expressing cells. In addition, our data also show that MCF-7/cas-3/PKCδmut cells have significantly reduced intracellular FADD levels when compared to wild-type PKCδ expressing cells (Fig. 5B), suggesting a role for PKCδ upstream of caspase-8 processing and activation. These data suggest that overexpression of PKCδ protects these cells from TRAIL-induced apoptosis by inhibiting caspase-8 activation

and its cleavage by caspase-3 reverses this inhibition and enhances TRAIL-induced apoptosis.

DISCUSSION

In this study, we explored the signaling pathway(s) by which PKCδ regulates TRAIL-induced apoptosis in human breast cancer cells. Despite the potential of TRAIL as a useful tool in anti-cancer therapy, very little remains known about the mechanisms regulating the sensitivity to TRAIL-induced apoptosis in PKCδ overexpressing breast tumor cells. It has been suggested that the decoy receptors TRAIL-R3 and -R4 may regulate the sensitivity of cells to TRAIL [Sheridan et al., 1997; Pan et al., 1997a]. However, analyses of TRAIL receptor expression in a number of human tumor cell lines have indicated no correlation between TRAIL sensitivity and decoy receptor TRAIL-R3 and -R4 mRNA expression [Griffith and Lynch, 1998]. There is a possibility that resistance to TRAIL-induced apoptosis may be mediated by intracellular pro- and anti-apoptotic molecules such as PKCδ, Akt, and caspases that can block or activate apoptotic signaling. In this study, we demonstrated that over-expression of PKCδ in MCF-7 (MCF-7/PKCδ) protects these cells from TRAIL-induced apoptosis. Inhibition of PKCδ by rottlerin or PKCδ siRNA sensitized the cells to TRAIL-induced apoptosis suggesting PKCδ overexpression protects the MCF-7 cells from TRAIL-induced apoptosis. Some of the mechanisms by which PKCδ inhibition increases TRAIL-induced DNA damage and apoptosis in MCF-7 cells which lack caspase-3 expression as a result of mutation in caspase-3 gene [Janicke et al., 1998] may be associated with: (i) although classic apoptotic models are involved in the activation of caspase-3, evidence indicates that apoptosis can proceed independently of caspase-3 through cathepsin B, etc. [Nylandsted et al., 2000; Foghsgaard et al., 2001; Lin et al., 2006] and (ii) PKCδ was also shown to induce ornithine decarboxylase in response to oxidation damage induced by hydrogen peroxide. The impairment of an ornithine decarboxylase response to DNA damage can explain the persistent DNA fragmentation observed in cells treated with PKCδ antisense oligonucleotides [Otieno and Kensler, 2000; McCracken et al., 2003]. In addition, there are data to suggest that rottlerin suppresses the NF-κB and enhances the caspase-processing leading to apoptosis [Zhang et al., 2005]. Rottlerin was also shown to affect the mitochondrial function independent of PKCδ, thereby sensitizing the cells to TRAIL-induced apoptosis [Tillman et al., 2003]. The mechanisms that are involved in the protective effect of PKCδ in human breast tumors remain unclear. Other studies have shown that caspase-3-dependent cleavage of PKCδ leads to the generation of a constitutively active catalytic fragment that is associated with PKCδ apoptotic function [Ghayur et al., 1996; Basu et al., 2001; Blass et al., 2002]. In an attempt to determine the role of caspase-3 cleavage on PKCδ and TRAIL-induced apoptosis, we expressed wild-type or caspase-3 cleavage-resistant PKCδmut in caspase-3 expressing MCF-7 cells. Our data clearly show that overexpression of caspase-3 cleavage-resistant PKCδ mutant (MCF-7/cas-3/PKCδmut) prevents caspase-8 activation, a first step in TRAIL-induced apoptosis, thereby blocking TRAIL-induced apoptosis. In contrast, overexpression of wild-type PKCδ (MCF-7/cas-3/PKCδ) leads to caspase-8 activation and PKCδ, Bid, caspase-3, and −9, and PARP cleavage resulting in a significant increase in apoptosis (Fig. 5). The above data suggest that PKCδ overexpression protects the cells from TRAIL-induced apoptosis and its inhibition by rottlerin, siRNA, or cleavage by caspase-3 reverses PKCδ-induced caspase-8 inhibition which then leads to enhanced TRAIL-induced apoptosis.

These data show that caspase-3 mRNA expression levels in breast tumors were at least 10–50 times lower than those in normal breast tissues [Devarajan et al., 2002] representing a clinically significant observation which has the potential to prevent TRAIL-induced apoptosis in PKCδ overexpressing breast tumors. Table I shows that overexpression of PKCδ was statistically significant in re-occurring breast tumors when compared to the

adjacent normal breast tissue. Previously, we showed that tamoxifen-resistant breast tumor cells overexpress PKCδ [Nabha et al., 2005], suggesting that PKCδ plays a major role in a subset of breast tumors and these tumors do not respond to TRAIL and other drug treatments.

The involvement of the death adaptor protein FADD and apoptosis-initiating caspase-8 in death signaling by DR4 and DR5 are controversial. Some studies suggest that dominant-negative FADD inhibited TRAIL-induced apoptosis [Chaudhary et al., 1997; Schneider et al., 1997; Sprick et al., 2000]. Our studies also show significant reduction of FADD levels in MCF-7/cas-3/PKCδmut cells in the presence of TRAIL. In other studies, overexpression of dominant-negative FADD did not prevent TRAIL-induced apoptosis [MacFarlane et al., 1997; Pan et al., 1997a,b]. Co-immunoprecipitation of overexpressed cytoplasmic domains of DR4 and DR5 with FADD support a role for FADD in TRAIL-induced apoptosis [Chaudhary et al., 1997; Schneider et al., 1997]. Murine embryonic fibroblasts (MEF) from FADD-deficient mice underwent apoptosis upon expression of human DR4 [Yeh et al., 1998]. Thus, the role of FADD in TRAIL-induced apoptosis in PKCδ overexpressing cells needs further studies.

The ability of cells to evade apoptosis is one of the essential hallmarks of cancer cells. Thus, loss or reduction of caspase-3 and/or other caspases may serve as an important step in the survival of tumor cells. For example, complete inactivation of caspase-8 gene in neuroblastomas was reported and these cells were resistant to doxorubicin- and death receptor-induced apoptosis [Teitz et al., 2000]. In addition, there are data to suggest that caspase-3 mRNA were undetectable in breast and cervical cancers and substantially decreased in ovarian cancer [Devarajan et al., 2002]. Similarly, 80% of prostate tumors were shown to have lost caspase-1 protein expression and reduced levels of caspase-3 expression [Winter et al., 2001]. Taken together these results suggest that a lack of caspases can attenuate TRAIL-induced apoptosis and this may represent an important mechanism of cell survival.

In conclusion, our results demonstrate that PKCδ overexpression protects breast tumor cells by inhibiting caspase-8 activation, a first step in TRAIL-induced apoptosis, thus preventing TRAIL-induced apoptosis. Our experimental evidence suggests that PKCδ over-expression protects the cells from TRAIL-induced apoptosis and its inhibition by rottlerin, siRNA, or its cleavage by caspase-3 reverses PKCδ-induced inhibition of caspase-8 activation leading to enhanced TRAIL-induced apoptosis. Clinically, PKCδ overexpressing tumors can be treated with a combination of PKCδ inhibitor(s) and TRAIL as a potential cancer treatment strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant sponsor: National Institute of Health; Grant number: RO1 CA 118089.

Abbreviations used

TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
DR4	death receptor 4
DR5	death receptor 5

PKC-δ	protein kinase C delta
PARP	poly(ADP-ribose) polymerase
FADD	Fas-associated protein with death domain
Cas-3	caspase-3

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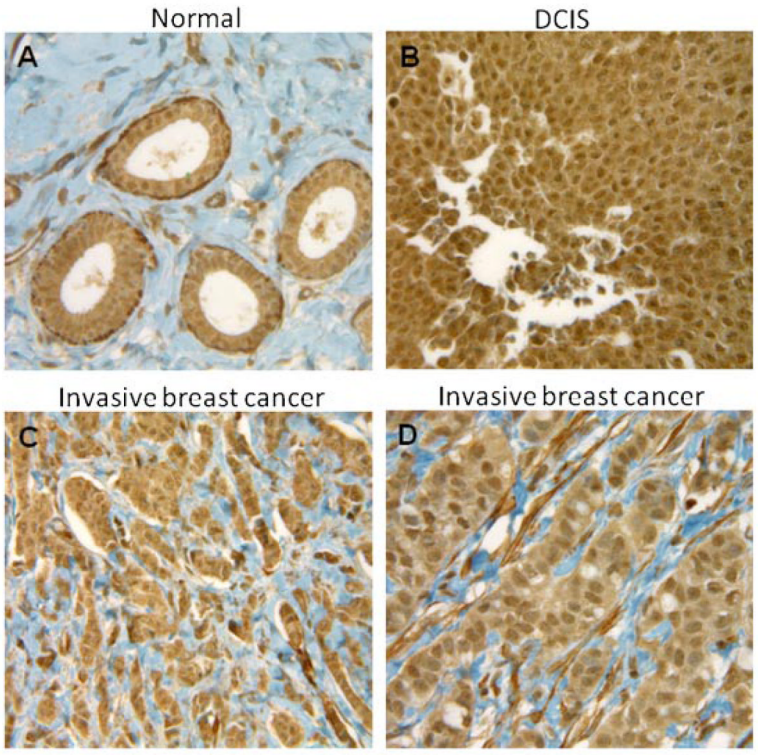

Fig. 1.

PKCδ immunohistochemical staining in breast tissue specimens (40×). A: Normal adjacent breast epithelia shows mild, (B) DCIS breast cancer shows strong staining, and (C,D) invasive breast cancer showed moderate staining.

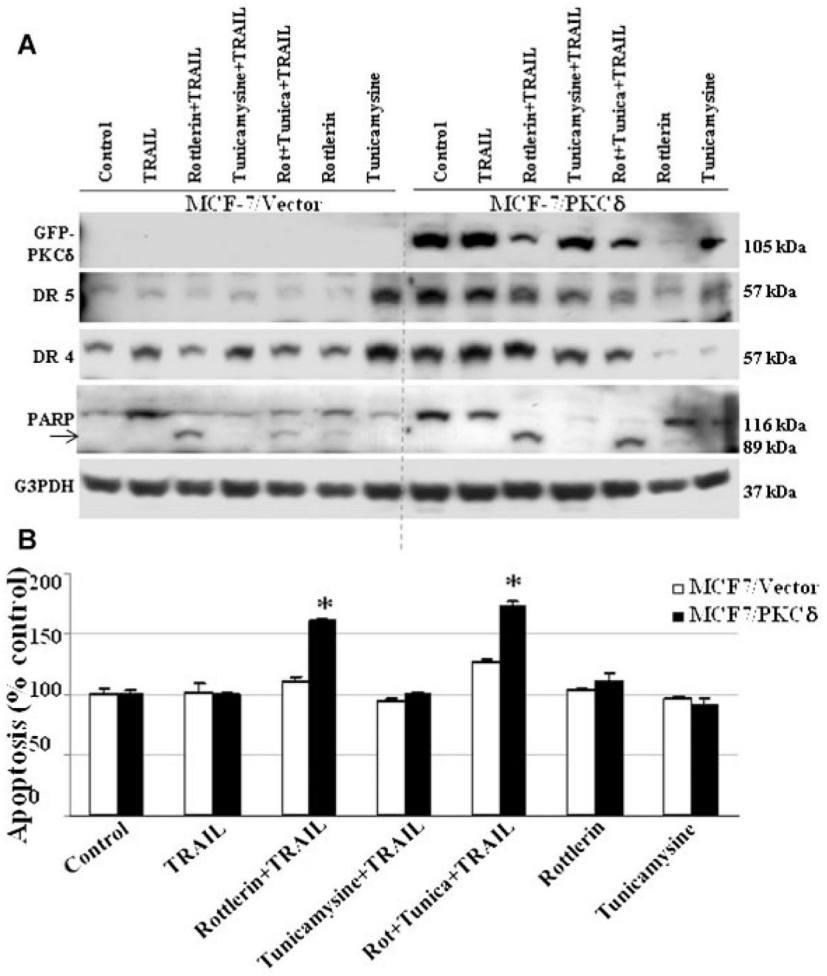

Fig. 2.

A: PKCδ overexpression protects the MCF-7 cells from TRAIL-induced apoptosis. MCF-7 cells overexpressing PKCδ (MCF-7/PKCδ) or vector alone (MCF-7/vector) were treated with TRAIL (10 ng/ml), rottlerin (3 μM), tunicamycin (10 μg/ml) alone, or in combination with TRAIL for 4 h. After 4 h PKCδ, DR4, DR5, and PARP cleavage were evaluated using specific antibodies by Western blots. MCF-7/PKCδ cells showed enhanced DR4 and DR5 expression in comparison to vector-transfected control cells. However, addition of TRAIL was unable to induce a significant increase in PARP cleavage in comparison to controls. Combination of TRAIL and rottlerin (PKCδ inhibitor) significantly enhanced PARP cleavage. Equivalent protein loading was assessed by stripping the membrane and re-probing with G3PDH antibody. Arrow indicates cleaved fragment. B: PKCδ inhibition enhances TRAIL-induced apoptosis in MCF-7 cells. Inhibition of PKCδ by rottlerin (3 μM) enhances TRAIL-induced apoptosis in MCF-7 cells. Apoptosis was quantified by ELISA and normalized to values measured in untreated cells. MCF-7/PKCδ cell showed a significant increase in TRAIL-induced apoptosis in the presence of rottlerin in comparison to untreated cells ($^{(}P < 0.001$). Data are mean +SE of triplicate determinations.

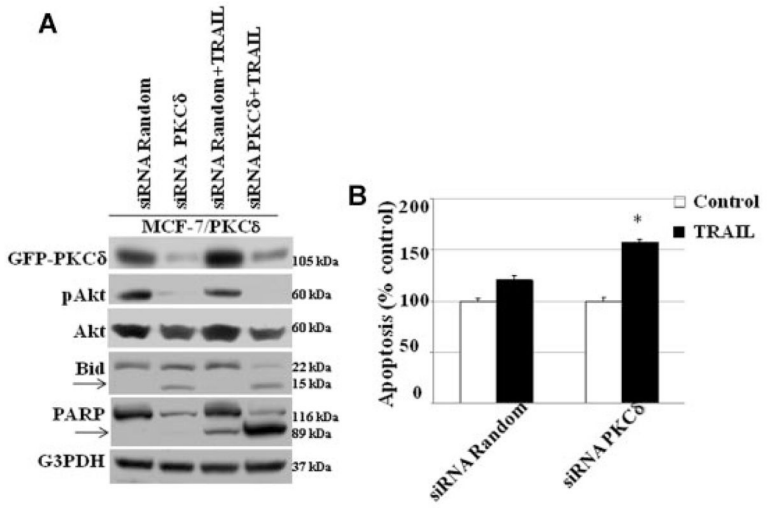

Fig. 3.

PKCδ siRNA enhances TRAIL-induced apoptosis: MCF-7/PKCδ cells were transfected with PKCδ siRNA or random siRNA alone for 72 h and then cells were treated with TRAIL for 4 h. A: Western blot shows PKCδ siRNA significantly reduces PKCδ, p-Akt levels, and enhances Bid cleavage, and PARP cleavage. Equivalent protein loading was assessed by stripping the membrane and re-probing with G3PDH antibody. Arrow indicates cleaved fragment. B: PKCδ inhibition by siRNA enhances TRAIL-induced apoptosis compared to random transfected cells in the presence of TRAIL.

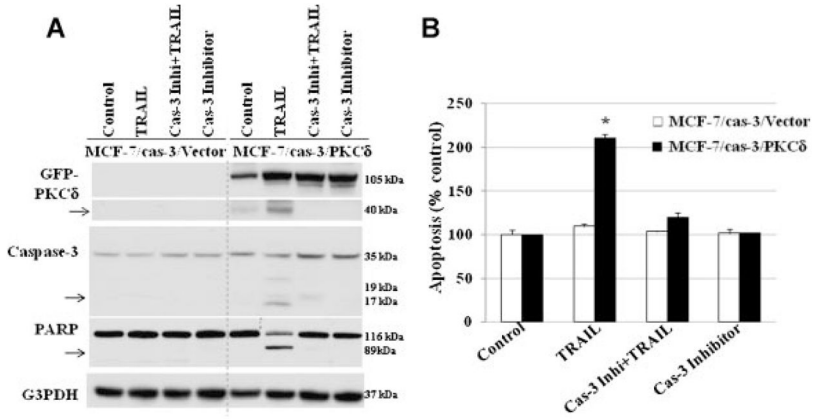

Fig. 4.

Caspase-3 is essential for TRAIL-induced apoptosis. A: Effect of TRAIL on MCF-7/Cas-3/PKCδ expressing and control cells. Addition of TRAIL enhances PKCδ cleavage (activated), caspase-3 cleavage (activated), and PARP cleavage compared to untreated cells. Inhibition of caspase-3 by Z-DEVD-fmk reverses TRAIL-induced biochemical changes and apoptosis (A,B). Equivalent protein loading was assessed by stripping the membrane and re-probing with G3PDH antibody. Arrow indicates cleaved fragment.

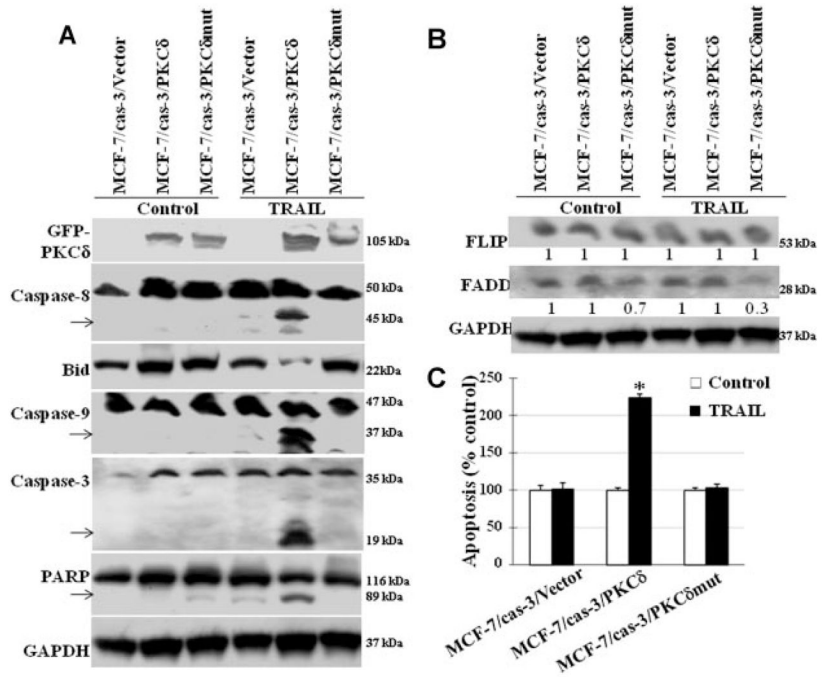

Fig. 5.

Caspase-3-resistant PKCδ reverses TRAIL-induced apoptosis. We used three MCF-7 cell lines: (i) caspase-3 expressing MCF-7 cells (MCF-7/cas-3/vector), (ii) caspase-3 and PKCδ expressing MCF-7 cells (MCF-7/cas-3/PKCδ), and (iii) caspase-3 and PKCδ mutant that prevents caspase-3-induced PKCδ cleavage (MCF-7/cas-3/PKCδmut) in the presence or absence of TRAIL. In MCF-7/cas-3/PKCδ cells TRAIL significantly induces Bid cleavage, caspase-8, caspase-3, caspase-9 activation and PARP cleavage in comparison to control cells. However, MCF-7/cas-3/PKCδmut cells blocked TRAIL-induced pro-apoptotic biochemical changes. B: MCF-7/cas-3/PKCδmut cells show a significant reduction in FADD in the presence of TRAIL in comparison to untreated cells. C: TRAIL-induced apoptosis is blocked in MCF-7/cas-3/PKCδmut cells. MCF-7/cas-3/PKCδ cell showed a significant increase in TRAIL-induced apoptosis compared to untreated cells ($^{(}P < 0.001$). However, MCF-7/cas-3/PKCδmut cells blocked TRAIL-induced apoptosis suggesting PKCδ cleavage plays a major role in TRAIL-induced apoptosis. Bars: mean +SE of triplicate determinations.

TABLE I

Summary of PKCδ Immunohistochemical Staining Intensity in Normal and Cancer Breast Tissue

		Staining intensity				
Breast tumors	**n**	**1+**	**2+**	**3+**	**Mean complete H-score**	
Normal tissue	6	3	3	0	95	P <0.01*
Breast tumors (re-occurring)	7	0	6	1	161.4	