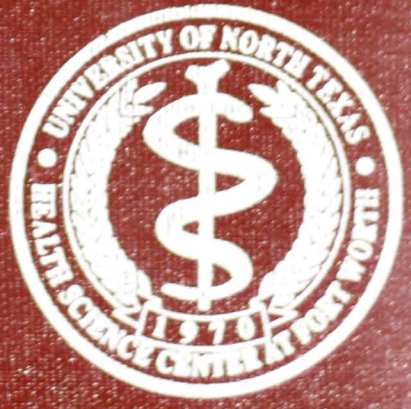
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Protein kinase C-ε (PKCε), a novel PKC, has been shown to attenuate tumor necrosis factor-α (TNF)-induced apoptosis in breast cancer cells. The purpose of this dissertation is to delineate the mechanism(s) by which PKCε exerts its antiapoptotic effect.

Comparison of PKCε level in several breast cancer cells revealed that PKCε level alone could not explain sensitivity of breast cancer cells to TNF. Protein kinase B/Akt (Akt) was constitutively active in breast cancer cells resistant to TNF. Inhibition of phosphatidyl-inositol 3-kinase (PI3-K) by Ly294002 increased TNF-mediated apoptosis in MCF-7 cells that overexpress Akt and sensitized BT-20 and SKBR-3 cells that express constitutively-active (CA) Akt to TNF. PKC inhibitor bisindolylmaleimide (BIM) also sensitized BT-20 and MCF-7 cells to TNF. Overexpression of CA-Akt in MCF-7 cells attenuated TNF-induced apoptosis. Therefore, both PKCε and Akt are important for deciding TNF sensitivity.

The cross-talk between PKCε and Akt was examined in MCF-7 cells. PKCε overexpression increased basal Akt phosphorylation and enhanced TNF-induced Akt activation. Knockdown of PKCε by siRNA decreased TNF-induced Akt activation. Depletion of Akt abolished the antiapoptotic effect of PKCε. Akt was constitutively associated with PKCε and DNA-dependent protein kinase (DNA-PK), and this association was increased by TNF. Knockdown of DNA-PK diminished the effect of

PKCε on Akt phosphorylation and increased TNF-mediated apoptosis. These results suggest that PKCε activates Akt via DNA-PK to mediate its antiapoptotic function.

We also investigated whether PKCε regulates mitochondrial cell death pathway by inhibiting the proapoptotic function of Bcl-2 family member Bax. Overexpression of wild-type but not dominant-negative PKCε inhibited TNF-mediated mitochondrial depolarization. Depletion of Bax inhibited TNF-induced apoptosis. PKCε overexpression abolished Bax dimerization and translocation to mitochondria, while PKCε depletion had the opposite effect. Bax was associated with PKCε in PKCε-overexpressing cells. These results indicate that PKCε attenuates mitochondrial cell death pathway by inhibiting Bax translocation.

These findings demonstrate that PKCε activates Akt via DNA-PK and inhibits proapoptotic Bax to mediate its antiapoptotic effect in breast cancer cells. An understanding of the mechanism(s) by which PKCε inhibits apoptosis in breast cancer cells is important for developing more effective cancer therapies.

REGULATION OF TNF-MEDIATED CELL DEATH

IN BREAST CANCER CELLS

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LIST OF ABBREVIATIONS

2D Gel	2-dimensional gel electrophoresis
ANOVA	Analysis Of Variance Between Groups
Apo2L/TRAIL	TNF-related apoptosis-inducing-ligand
ASK-1	Apoptosis signal-regulating kinase-1
ATM	Ataxia telangiectasia mutated gene
ATR	ATM-related
Bcl-2	B-cell lymphoma protein 2
BH domain	Bcl-2 homology domain
BIM	Bisindolymaleimide
CA	constitutively-active
CDK	Cyclin-Dependent Kinase
Cyt *c*	Cytochrome *c*
DAG	Diacylglycerol
DISC	death inducing signaling complex
DN	dominant-negative
DNA-PK	DNA dependent protein kinase
DNA-PKcs	DNA dependent protein kinase catalytic subunit
EGF	Epidermal growth factor
ERK	Extracellular signal-related kinase

FADD	Fas-associated death-domain-containing protein
FASL	Fas ligand
FRET	Florescence resonance energy transfer
GFP	Green florescence protein
GSK3α/β	glycogen synthase kinase-3α/β
HEK	human embryonic kidney
HSP	heat shock protein
IKK	NF-κB kinase
ILK	integrin-linked kinase
IP6	Inositol hexaphosphate
JNK	c-Jun N-terminal kinase
JNKK	JNK kinase
MAPK	mitogen-activated protein kinases
MAPKAP kinase-2	mitogen-activated protein kinase-activated protein kinase 2
MEKK1	MAPK kinase kinase-1
NF-κB	nuclear factor kappa-B
NIK	NF-κB -inducing kinase
PDGF	platelet-derived growth factor
PDK1	Phosphoinositide-dependent protein kinase 1
PDK2	Phosphoinositide-dependent protein kinase 2
PH domain	pleckstrin homology domain
PI	propidium iodide

PI3-K	phosphatidyl-inositol 3-kinase
PIKK	PI3-kinase-related kinase
PIP2	phosphotidylinositol (4,5) diphosphate
PIP3	phosphotidylinositol (3,4,5) trisphosphate
PKA	cyclic-AMP dependent protein kinase
PKB/Akt	protein kinase B/Akt
PKC	protein kinase C
PTEN	phosphatase and tensin homolog
RIP	receptor-interacting protein
siRNA	small interfering RNA
TIM	TRAF-interacting motifs
TNF	tumor necrosis factor-α
TRADD	TNF-R associated DD
TRAF	TNF-R associated factor
WT	wild-type

CHAPTER I

INTRODUCTION

Breast cancer

Breast cancer is the second leading cause of cancer related deaths in women of the Western world. Nearly 211,240 women in the United States were reported to have invasive breast cancer and about 40,410 women died from the disease in 2005, and over 180,000 new cases are diagnosed annually. Currently it is estimated that there are over 2 million women living in the US who have been treated for breast cancer. Despite significant improvements in cancer diagnosis and treatment about a quarter of breast cancer patients are expected to die from the disease. Therefore, the development and application of new, molecular based therapies are of utmost importance. The key to the development of such rational therapeutic approaches lies in the identification of genes and biochemical pathways involved in breast tumorogenesis and drug resistance. (www.cancer.org)

Apoptosis

Apoptosis, or programmed cell death is a cell-suicide mechanism that has essential role in controlling cell number in many developmental and physiological settings (1). Apoptosis eliminates individual cells when they are no longer needed or have become seriously damaged. Failure to undergo apoptosis is associated with cancer, suggesting

that disruption of apoptotic function contributes to the transformation of a normal cell into a tumor cell. Apoptosis is also an important phenomenon in chemotherapy-induced tumor-cell killing (2). However, the deregulation in apoptosis results in treatment failure or resistance (3). The switching on/off of apoptosis is determined by the balance between proapoptotic and antiapoptotic signals.

Apoptotic cells are characterized by cell shrinkage, plasma membrane blebbing, nuclear condensation, chromatin aggregation, and endonucleocytic degradation of DNA into nucleosomal fragmentations (4). The key to this ordered destruction is the activation of a class of proteases, known as caspases (5).

Caspases, a family of cysteinyl aspartate specific proteases, are activated as a cascade. All caspases exist as inactive zymogens (procaspases) sharing a common domain structure consisting of a prodomain, a large (p20) subunit and a small (p10) subunit (5). Based on the phylogentic analysis of the caspase domain, caspases segregate into two major subfamilies, the apoptotic caspases (caspase-8, -10, -9, -3, -6, and -7) and the inflammatory caspases (caspase-1, -4, -5, -11, and -12) (6). Two distinct groups are divided according to the structure of the prodomain and the function: initiator and executioner caspases (5). Initiator caspases are characterized by a long prodomain which allows for the recruitment of the procaspase into an activating protein complex. The long prodomain containing caspases are caspases-1, -2, -4, -5, -9, -11 and -12 with an N-terminal caspase recruitment domain (CARD), and caspase-8 and -10 with an N-terminal death effector domain (DED) (7). In contrast to the initiator caspases, the executioner caspase-3, -6 and -7 lack the large N-terminal non-enzymatic domain, and they are

responsible for the cleavage of critical cellular proteins during apoptosis (8, 9). The executioner caspases share their short prodomain with caspase-14, which is involved in keratinocyte maturation (10). Caspase-8, -9 and –10 participate in the initiation of apoptosis whereas caspase-3, -6 and –7 are involved in the execution of apoptosis. Caspase-2 can function as both initiator and executioner caspase (5). Two principal pathways for apoptosis have been identified: the death receptor-mediated extrinsic pathway and the mitochondria-apoptosome-mediated intrinsic pathway. Both of these pathways lead to caspase activation and cleavage of critical cellular substrates to cause cell death (Figure 1).

In the extrinsic pathway, cell death is mediated by death receptors, a subgroup of the tumor necrosis factor (TNF) receptor superfamily (11). The TNF superfamily includes TNF, Fas ligand (FasL) and TNF-related apoptosis-inducing-ligand (Apo2L/TRAIL). Binding of the death receptors by their ligands leads to the formation of death-inducing signaling complex (DISC) (12). DISC consists of adaptor proteins such as Fas-associated death-domain-containing protein (FADD) and initiator caspases, pro-caspase-8 or –10. Close proximity of these procaspases in the DISC leads to activation of their catalytic activity, presumably by an allosteric mechanism, involving dimerization between two caspase-8 or -10 molecules (13, 14). Activated initiator caspases start the caspase cascade that leads to apoptosis.

Activation of intrinsic apoptotic pathway starts from mitochondria. Central to this intrinsic pathway is the formation of an intracellular caspase-9-activating complex, the apoptosome (15). DNA damage-inducing chemotherapeutic drugs and other stress

signals engage the mitochondria to release apoptotic factors, such as Cytochrome *c* (*Cyt c*), Smac/DIABLO, HtrA2/Omi, into cytosol (16). *Cyt c* binds to monomeric apoptotic protease-activating factor 1 (Apaf-1) promoting its oligomerization to form apoptosome. The apoptosome binds to proform of caspase-9, leading to its oligomerization to form active caspase-9, which in turns causes cleavage of caspase-3 or –7 and other downstream caspases (17).

The extrinsic and intrinsic cell death pathways are linked by the cleavage of B-cell lymphoma protein 2 (Bcl-2) family member Bid by caspase-8. Bcl-2 family of proteins have a central role in controlling the integrity of the mitochondrial membrane (18). It consists of antiapoptotic members like Bcl-2, Bcl-X_L, Bcl-w, Bcl-G, Mcl-1, Bfl-1/A1 and proapoptotic members like Bax, Bak, Bok (Mtd), Bad, Bid, Bik and Bim1 (19). Several models suggest that Bcl-2 family members form channels that facilitate protein transport and interact with other mitochondrial proteins such as voltage-dependent anion channel (VDAC), and also induce the rupture of the outer mitochondrial membrane (20-22).

TNF signaling

TNF, a multifunctional cytokine, is originally characterized by its anti-tumor activity (23, 24) and it is the first cytokine to be employed for cancer biotherapy. Cloning of TNF gene in 1984 led to an era of clinical trails (25). TNF has two distinct receptors, TNFR1 (CD120a; p55/60) and TNFR2 (CD120b; p75/80) (26-28). TNFR1 is expressed on all cell types while TNFR2 is only expressed on immune and endothelial cells (29). Neither receptor exhibits enzymatic activity.

TNF family receptors can be classified into three major groups. TNFR1 belongs to the first group that contains a death domain (DD) in the cytoplasmic tail. Activation of these DD-containing receptors by their corresponding ligands leads to the recruitment of intracellular DD-containing adaptors such as FADD and TNFR-associated DD (TRADD), which together form the DISC. These molecules not only cause activation of the caspase cascade by activating caspase-8 or caspase-10 and induction of apoptosis, but can also recruit TNFR-associated factor (TRAF) family members. The second group of receptors contains one or more TRAF-interacting motifs (TIM) in the cytoplasmic tail. Activation of TIM-containing TNFR leads to the direct recruitment of TRAF family members, which ultimately activate multiple signal transduction mediators, such as mitogen-activated protein kinases (MAPK) (*e.g.* c-Jun N-terminal kinase (JNK), p38 MAPK, extracellular signal-related kinase (ERK)), inhibitor of nuclear factor kappa-B (NF-κB) kinase (IKK) and phosphatidyl-inositol 3-kinase/protein kinase B/Akt (PI3-K/Akt). The third group of TNF receptor family members does not contain functional intracellular signaling domains or motifs. Although these "decoy" receptors cannot provide intracellular signaling, they can effectively compete with the other two receptor groups for their corresponding ligands.

TNF mediates cell death signal mainly through TNFR1. Binding of TNF with TNFR1 induces association of the receptor with TRADD via DD (Figure 2). TRADD functions as a platform adapter that recruits several signaling molecules to the activated receptor: FADD, TRAFs, and receptor-interacting protein (RIP). FADD recruits caspase-8 and initiates a caspase cascade. There are two types of cells-Type I and Type II. In Type I

cells, activation of caspase-8 is sufficient to trigger cell death via the extrinsic pathway (30). In Type II cells, the extent of caspase-8 activation is low and cleavage of Bid by caspase-8 triggers mitochondria-mediated intrinsic pathway to amplify the death signals. Signals through other adaptor proteins, such as TRAFs and RIP, lead to activation of NF-κB and JNK/AP-1. TRAFs and RIP activate the NF-κB-inducing kinase (NIK), which in turn activates the inhibitor of IκB kinase complex, IKK. IKK phosphorylates IκB leading to degradation of IκB and allowing NF-κB to translocate to the nucleus to activate transcription (31). The pathway from TRAF-2 and RIP to JNK involves a cascade that includes the MAP kinases MEKK1 (MAP3K MEK kinase 1), JNKK (JNK kinase) and JNK (32), as well as AP-1. TNF also binds to TNFR2, a member of the non-death domain-containing subgroup of the TNF receptor family. TNFR2 itself does not induce apoptosis, but may play an important role in the regulation of apoptosis through TNFR1. Several investigators have reported that TNFR2 potentiates TNFR1-mediated apoptosis although the mechanism is not clear (33, 34). The apoptotic TNFR cross talk is based on TNFR2-mediated abrogation of antiapoptotic TRAF2-dependent signaling pathways initiated by TNFR1, but not Apo1/Fas or the apoptotic TNF-related apoptosis-inducing ligand receptors (33, 34). Therefore, TNF not only triggers cell death through caspase cascade (26-28), but also induces activation of NF-κB to trigger antiapoptotic signals.

Although TNF mediates apoptosis in cancer cells, including breast cancer, some cancer cells are resistant to TNF. Protein kinases play critical roles in the regulation of apoptosis. This study will delineate the signaling pathways by which breast cancer cells

acquire resistance to TNF. We will specifically focus on the role of protein kinase B (PKB/Akt) and protein kinase C (PKC) on TNF-induced cell death.

PKB/Akt (Akt)

Akt, also known as PKB, the cellular homologue of oncogene v-Akt, is a family of the serine/threonine kinases (35). Akt belongs to a subfamily of protein kinases termed AGC protein kinases, which include cyclic-AMP-dependent protein kinase (PKA), cyclic GMP-dependent kinase (PKG) and protein kinase C (PKC) (36). Akt family consists of three members, PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (37). All three members contain an N-terminal phosphotidylinositol (3,4,5) trisphosphate (PtdIns(3,4,5)P3)- and PtdIns(3,4)P2-binding pleckstrin homology (PH) domain and a C-terminal kinase catalytic domain (Figure 3). All three isoforms are activated by growth factors such as platelet-derived growth factor (PDGF) and insulin in a PI3-K-dependent manner, and are inhibited by phosphatase and tensin homologue tumor suppressor PTEN. PI3-K generates PtdIns(3,4,5)P3, a lipid second messenger essential for the translocation of Akt to the plasma membrane where it is phosphorylated and activated. Following activation of PI3-K, Akt isoforms are recruited from the cytosol to the plasma membrane through association with PtdIns(3,4,5)P3 and/or PtdIns(3,4)P2 (Figure 4). One phosphorylation site of Akt is Thr308 in the T-loop, also known as the activation loop in the kinase domain. The other phosphorylation site Ser473 is located in a non-catalytic region termed hydrophobic motif. Mutagenesis studies showed that phosphorylation of both sites is required for Akt activity. Phosphoinositide-dependent protein kinase 1 (PDK1)

phosphorylates Akt at Thr308 (38-40). Although a kinase that phosphorylates Akt at Ser473 has been tentatively designated PDK2, its nature remains unclear. It has been reported that phosphorylation of Akt at Ser473 may be mediated by PDK1, autophosphorylation, integrin-linked kinase (ILK) or mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP kinase-2) (35, 41, 42). Recent evidence suggests that phosphorylation of Akt at Ser473 may be mediated by rictor-mTOR complex or DNA-dependent protein kinase (DNA-PK) (43, 44).

The identification of Akt as a key regulator of cellular survival implicates the role of Akt in oncogenesis. A number of oncogenes and tumor suppression genes upstream of Akt have been found to influence cancer progression by regulating Akt activity. PI3-K has been identified as the enzymatic component of oncogene vP3K. Cells transformed by vP3K have constitutive Akt activity, while transfection with dominant-negative Akt can revert oncogenic transformation by vP3K (45). Overexpression of constitutively-active forms of PI3-K catalytic subunit activates Akt in both mammalian and insect cells (45). A recent study showed that concomitant activation of PI3-K/Akt and Ras/ERK signaling pathway was essential for transformation by v-SEA oncogene (46).

Overexpression of wild type Akt also causes oncogenesis, as it has been found that Akt2 is amplified with high frequency in ovarian, breast and pancreatic and lung cancers. Membrane-targeted Akt2 and Akt3 could induce oncogenic transformation (47). Inactivating mutant of PTEN, the important human tumor suppressor, has been found to be associated in numerous malignancies, including breast cancer, glioblastoma and germ cell tumors (48). The finding that PTEN level is inversely correlated with

phosphorylated Akt confirms a role for Akt in cancer progression (49). Akt is constitutively active in many types of human cancers, including breast cancer (50). All three isoforms of Akt have been associated with breast cancer. PKBα/Akt1 is overexpressed in breast cancer MCF-7 cells (51). PKBβ/Akt2 gene is amplified in 3% of breast cancers (52) and is frequently activated in primary human breast carcinoma (53). Upregulation of PKBγ/Akt3 may contribute to the more aggressive clinical phenotype of the estrogen receptor-negative breast cancers and androgen-insensitive prostate carcinomas (54). Clinical studies show that breast cancer patients associated with high levels of Akt have poor prognosis for endocrine treatment (55). Although the mechanisms have not yet been fully characterized, constitutively active Akt is believed to promote proliferation and increase cell survival and thereby contributing to cancer progression.

Akt promotes cancer progression by two cooperative mechanisms: one is to promote proliferation under the conditions in which cells should normally be in growth arrest, and the other is to inhibit apoptosis by cell death inducing agents. Akt exerts antiapoptotic effects in a variety of ways. One mechanism by which Akt functions to inhibit apoptosis is through phosphorylation and inactivation of proapoptotic proteins such as Bad, caspase-9, and possibly Apaf-1 (56-59). Another mechanism by which Akt mediates cell survival is through regulation of transcription factors such as Forkhead transcription factor-FKHRL1 and NF-κB (60-62).

Phosphorylation of Bad at Ser136 by Akt enables it to interact with 14-3-3 proteins, which sequestrate Bad into cytoplasm and prevent the binding of Bad to Bcl-X_L to

perform its proapoptotic function (57). Akt also phosphorylates procaspase-9 at Ser196, thereby diminishing caspase-9 activation and formation of the apoptosome with Apaf-1. Akt has also been shown to inhibit *Cyt c* release from mitochondria independent of phosphorylation of Bad and procaspase-9. There may be some mitochondrial targets of Akt involved in this process. Recently, Bax, another proapoptotic member of Bcl-2 family has been reported to be a substrate of Akt. The phosphorylation at Ser184 site inhibits Bax dimerization and translocation from cytosol to outer mitochondrial membrane to perform its proapoptotic function (63). Mitochondrial Raf-1 may be one of the candidates, as activation of Raf-1 has been shown to be involved in antiapoptotic effects of Akt in haematopoietic cells (64).

Akt not only attenuates the mitochondria-mediated intrinsic apoptosis pathway, but also inhibits the extrinsic apoptosis pathway. Akt has been shown to induce transcription of FLIP (FLICE-inhibitory protein), which is an inhibitor of caspase-8 to block apoptosis (65). Akt also regulates *de novo* gene expression in apoptotic pathway. Forkhead family members (FKHR, FKHRL1/Af6q21 and AFX) are all targets of Akt. Phosphorylation of FKHRL1 at Thr32 and Ser253 by Akt promotes its association with 14-3-3 and sequestration in the cytoplasm, thereby inhibiting the transcription of cell death genes, such as Fas Ligand (66). Akt can also activate NF-κB through direct phosphorylation of IKK at Thr23, which phosphorylates IκB and induces its degradation. Activated NF-κB regulates the transcription of variety of survival factors, including pro-inflammatory cytokines (67). A recently identified substrate of Akt is the apoptosis signal-regulating kinase-1 (ASK-1), which stimulates the MAPKK that activates the JNK and p38 MAP

kinase (68). Akt phosphorylates ASK-1 at Ser83 and inhibits ASK-1 activity. Therefore, Akt plays an important role in cancer progression and is a potential target for anticancer therapy.

DNA-PK

DNA-dependent protein kinase (DNA-PK) is a potential PDK2 that phosphorylates Akt at Ser473 site. DNA-PK is a nuclear serine/threonine protein kinase that is activated upon DNA damage generated by ionizing radiation or UV irradiation. It is a three-protein complex consisting of a 470-kDa catalytic subunit (DNA-PKcs) and the regulatory DNA binding subunits, Ku heterodimer (Ku70 and Ku80) (69). Binding of Ku heterodimer to double-strand DNA breaks results in recruitment of its catalytic subunit and additional factors necessary for DNA repair. DNA-PK plays important role in DNA repair as well as protection against apoptosis (70). DNA-PKcs is a very abundant nuclear protein in human cells and a very large polypeptide of 4127 amino (71). The C-terminus of DNA-PKcs is similar to PI3-K family members, including ataxia telangiectasia mutated gene (ATM), ATM-related (ATR), and p110 PI3-K (69). However, DNA-PKcs acts as protein kinase, but not a lipid kinase (69).

While the activation of DNA-PK requires DNA ends, its activity can be regulated by several mechanisms including phosphorylation. Autophosphorylation of DNA-PKcs has been shown to inhibit the DNA-PK activity (72, 73). PKCδ and c-Abl have been reported to interact with DNA-PK. Phosphorylation of DNA-PK by these kinases results

in inhibition of the ability of DNA-PK to form a complex with DNA (74). PP2-like protein phosphatases dephophorylate DNA-PK (72).

DNA-PK phosphorylates many protein substrates on Ser/Thr residues which are followed by glutamine, the S/TQ motif. It can also phosphorylate proteins at non-S/TQ sites with a preference for Ser/Thr followed by a hydrophobic amino acid (75). DNA-PK has been shown to colocalize with Akt on the cell membrane and phosphorylate Akt at Ser473 in a PI3-K-dependent manner (43). The mechanisms by which DNA-PK localizes to the membrane is not clear, but it may involve interaction with epidermal growth factor (EGF) receptor (76). Inositol phosphate, especially inositol hexaphosphate (IP6) has been reported to serve as a potent co-factor for DNA-PK activation and it may play a significant role in modulating the localization of DNA-PK (77, 78).

DNA-PK is believed to be an attractive molecular target for cancer therapy because it plays a vital role in radiation-induced DNA damage (79, 80). Also DNA-PK complexes are upregulated in cancer cells that are resistant to radiation (81-83). In addition, the finding that DNA-PK activates Akt in the plasma membrane indicates that DNA-PK may also be involved in the antiapoptotic signaling of Akt.

Protein kinase C (PKC)

PKC is a family of phospholipid-dependent serine/threonine kinases that consist of at least 10 isozymes that have distinct and in some cases opposing roles in cell survival and apoptosis (23, 84). The PKCs are divided into three groups based on the structural and activation characteristics: conventional (or classical) PKCs (cPKCs: α, βI, βII and γ),

novel PKCs (nPKCs: δ, ε, η and θ) and atypical PKCs (aPKCs: ζ and λ/ι) (85-87). While activation of cPKCs requires Ca^{2+} and diacylglycerol (DAG)/phorbol esters, nPKCs are Ca^{2+}-independent, but need DAG/phorbol esters for their activation; aPKCs are insensitive to both Ca^{2+} and DAG/phorbol esters.

Members of the PKC family are single polypeptides, comprised of an N-terminal regulatory region and a C-terminal catalytic region joined by a hinge region. There are four conserved domains in PKCs: C1, C2, C3 and C4 (88). The C1 domain contains a Cys-rich motif, duplicated in most isozymes, which forms the DAG/phorbol ester binding site (89); this domain is immediately followed by an auto-inhibitory pseudosubstrate sequence (90). C2 domain contains the recognition site for acidic lipids and, in some isozymes, the Ca^{2+}-binding site (91). The C3 and C4 domains form the ATP- and substrate-binding pockets of the kinase core (92). Binding of the pseudosubstrate sequence to the substrate-binding site keeps PKC in an inactive conformation. DAG/phobol esters serve as hydrophobic anchors to recruit PKC to the membrane. Cofactor binding (e.g. phosphotidylserine) induces conformational change and releases the pseudosubstrate binding to facilitate PKC activation. Proteolytic separation of the regulatory and catalytic domains is another mechanism to activate PKC (93, 94). Phosphorylation of PKC at Ser and/or Thr in activation loop, hydrophobic site and autophosphorylation site at C-terminal is required for its full activation.

The downstream events following PKC activation are little known. The main pathway, which is activated by PKC, is the MEK-ERK pathway. It has been proposed that PKCα, δ and ε activate the MEK-ERK pathway via Raf1 (95, 96). Activated Raf1

phosphorylates MEK1 and MEK2, which activate the mitogen-activated protein kinase cascade, ultimately resulting in transcription of genes involved in cell proliferation (97). PKCα, βI and γ specifically inactivate GSK-3β by phosphorylation, leading to derepression of the c-Jun transcription factor (98). PKCθ has been reported to synergize with the Ca^{2+}-dependent phosphatase calcineurin to stimulate JNK1 via Rac1 (99).

PKC plays an important role in processes relevant to neoplastic transformation, carcinogenesis and tumor cell invasion. Therefore, PKC is a potential target for anticancer therapy. Early studies suggested a role for PKC isozymes in tumor promotion (100). Increased levels of PKC have been associated with malignant transformation in a number of cell lines including breast (101), lung (102) and gastric carcinomas (103). The role of PKCs in tumorigenesis is tissue and cell specific. PKCα has no effect on skin tumor promotion. PKCδ reduces papilloma development (104). PKCβ has a partial oncogenic effect in fibroblasts (105). PKCε seems to be involved in tumor development and tumor cell invasion and metastasis in several tissues. This study will focus on the role of PKCε in the regulation of TNF-mediated cell death.

PKCε

PKC-epsilon (PKCε), a novel PKC, has an actin-binding motif (AA 223-228) located between the first and second Cys-rich regions of the C1 domain (Figure 5). The association of PKCε with actin filaments in response to extracellular stimuli is independent of phosphatidylserine (106, 107). Binding to actin can maintain the catalytically active conformation of PKCε (106). Phosphorylation at conserved sites

(Thr566 in the activation loop, Ser729 in the C-terminal hydrophobic domain and Thr710, an autophosphorylation site in the C-terminal domain) is required for PKCε activation by second messengers (108).

The essential role of PKCε has been established in many signaling systems including proliferation, differentiation, gene expression, muscle contraction and metabolism (109-113). PKCε also plays an important role in apoptosis and tumorigenesis. PKCε shows oncogenic effects when overexpressed in fibroblast, colonic and prostatic epithelial cells (114-116). Its oncogenic activity seems to be exerted through the ras-signaling cascade at the level of Raf-1 activation (96, 117). Inhibition of PKCε may suppress tumor promotion (118). PKCε can also contribute to tumor metastasis (104). PKCε is required for the cell spreading mediated by integrin β1 through interaction with RACK1. The association with F-actin via the actin binding motif of PKCε mediates increased adhesion and mobility of cancer cells (119). PKCε also activates the Raf-1/MEK/ERK cascade, and plays a role in malignant progression mediated by chronic hypoxia (120). PKCε has also been implicated in ultraviolet-induced apoptosis and tumor promotion (121, 122). Overexpression of the catalytic fragment of PKCε via *de-novo* synthesis has been reported in lung carcinoma cells (123). PKCε has been reported to be cleaved by caspase-3/-7 or calpain during apoptosis (124, 125).

Our lab showed that overexpression of PKCε attenuated TNF-induced apoptosis in breast cancer MCF-7 cells (124). In addition, breast cancer cells containing high level of PKCε were sensitized to TNF by PKC inhibitor BIM (bisindolylmaleimide) (126).

However, the level of PKCε was not sufficient to explain breast cancer cell sensitivity to TNF (126) .

Both Akt and PKC belong to AGC kinase family and they exhibit significant similarities in their structure, function and regulation (37). Both PKC and Akt function downstream of PI3-K and are involved in TNF signaling (127). TNF can cause proteolytic activation of antiapoptotic proteins PKCε and PKCζ (128-131). TNF can also activate Akt by increasing its phosphorylation at Ser473 (132). There is evidence that PKC could regulate Akt activity. It has been reported that PKCζ negatively regulates growth factor-mediated Akt phosphorylation and activation, although both PKCζ and Akt are prosurvival proteins (133). PKCβ but not other PKCs has been reported to negatively regulate Akt (134). Inhibition of PKCε by dominant-negative PKCε (DN-PKCε) is associated with inhibition of Akt phosphorylation by insulin, demonstrating that PKCε activity is required for Akt phosphorylation (135). Akt has been reported to be a downstream effector of PKCε for ethanol-induced cardioprotection (136). The association between PKCε and Akt has been reported in proteomics study in cardiomyocytes (137). It is not clear if PKC and Akt interact with each other and if there is cross-talk between these two pathways during TNF-mediated apoptosis.

Bcl-2 family

Bcl-2 family proteins are the key regulators of mitochondria-mediated intrinsic cell death pathway (18). They consist of about 20 homologues, that can either be pro- or antiapoptotic members. The established mode of function of the individual members is to

either preserve or disupt mitochondrial integrity, thereby inducing or preventing release of apoptogenic factors like *Cyt c* from the mitochondria. The Bcl-2 homologues fall into three classes, all of which share at least one conserved Bcl-2 homology (BH) domain (Figure 6).

The first group, the Bcl-2-like antiapoptotic proteins are described as "membrane-bound scavengers of proapoptotic proteins" (18). They contain three to four BH domains that are required for their antiapoptotic function, and this group includes Bcl-2, Bcl-X_L, Bcl-w, A1/Bfl-1, Boo/Diva/Bcl-B and Mcl-1. The BH1–BH4 domains mediate interactions with other proteins and the molecules localized to the cytoplasmic side of intracellular membranes, such as the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope. The BH1–BH3 domains form a hydrophobic groove, and the N-terminal BH4 domain stabilizes this structure (138). The hydrophobic groove can bind the BH3α-helix of an interacting (proapoptotic) Bcl-2 family member (20). As a response to certain apoptotic stimuli, Bcl-2-like survival factors are converted into proapoptotic proteins after proteolytic removal of the N-terminal BH4 domain (139).

The second group, Bax-like death factors contain BH1–BH3 domains and they play proapoptotic role via conformational change and pore formation. This subfamily of Bcl-2 homologues consists of the mammalian family members Bax, Bak and Bok/Mtd. While Bax and Bak are widely expressed (140, 141), Bok expression appears to be restricted to reproductive tissues (142). Bak and Bok, are exclusively membrane-bound in healthy cells and do not require translocation in apoptotic cells (143, 144). Unlike other channel-forming proapoptotic Bcl-2 proteins including Bax and Bak, Bok preferentially dimerizes

with antiapoptotic proteins Mcl-1 and Bfl-1 but does not interact with Bcl-2, Bcl-w, and Bcl-X_L (143). Mouse genetic data suggest that the presence of either Bax or Bak is essential for apoptosis in many cell types (145, 146). In Bax, the BH3 helix is less packed to the hydrophobic core than in Bcl-X_L, and this flexibility of the BH3 domain allows it to bind to the hydrophobic grooves of Bcl-2-like survival factors (18, 147). The C-terminal tail of Bax is folded back into its hydrophobic pocket mimicking the binding of proapoptotic BH3 peptides to the hydrophobic pocket of antiapoptotic Bcl-2 family members (18, 147). This folding prevents Bax from binding to membranes, and unleashing the C-terminus results in mitochondrial translocation upon apoptotic stimulation (148). Once Bax is attached loosely to the mitochondrial membrane, Bax-like death factors undergo an activating conformational change which leads to their stable insertion into the membrane (18). These molecules then either form channels (149) or interact with channel-forming proteins to increase the permeability of the outer mitochondrial membrane (150, 151).

The third group, the BH3-only death factors share only the short BH3 domain with the other Bcl-2 family subclasses. The 10 or more so far identified BH3-only proteins (Bim, Bmf, Hrk, Bik, Bid, Bad, Noxa and Puma) in mammals represent homologues of the proapoptotic *C. elegans* protein EGL1. With the exception of Bid and possibly Bim, they are thought to act by binding to and neutralizing the Bcl-2-like survival factors (152). Bid might also inactivate antiapoptotic Bcl-2 family members but, in addition, it seems to transduce cell death signals by activating Bax and Bak (153, 154). The results for Bim are contradictory. The intact Bim protein binds and antagonizes Bcl-2 and Bcl-

X_L function and seems unable to interact with Bax or Bak. However, the isolated Bim BH3 domain, when removed from the context of the entire protein, is capable of inducing oligomerization of Bak and Bax to release *Cyt c*, like the Bid BH3 domain (155). Bim has also been suggested to directly interact with and activate the voltage-dependent anion channel VDAC (156) and/or to activate Bax by damaging the mitochondrial membrane directly (157). BH3-only proteins act upstream of Bax-like death factors because they are not able to induce cell death in the absence of Bax and Bak (145). They are described as the "sensors and mediators of apoptosis" and individual BH3-only proteins might transduce different and specific death signals to the multidomain Bcl-2 family members (158). In healthy mammalian cells, BH3-only proteins are kept inactive (152). In response to proapoptotic signals, they become transcriptionally upregulated and/or posttranslationally modified (or relocalized) to gain their full proapoptotic potential. Bad and Bik are two BH3-only proteins which are regulated by phosphorylation. In cells which are protected against apoptosis by cytokines, Bad is phosphorylated at several serine residues (Ser 112, Ser 135 and Ser 155 by different kinases such as Akt and Raf) (18); Phosphorylation of Bad leads to its association with 14-3-3 scaffold proteins and sequestration in the cytoplasm (159). Upon cytokine or extracellular matrix withdrawal, Bad is dephosphorylated and released from 14-3-3 (160). The molecule is now able to interact with Bcl-2-like survival proteins such as Bcl-X_L and to neutralize their antiapoptotic interaction with Bax-like death factors.

Bid is cleaved by caspase-8 in response to death receptor activation (or by Granzyme B) (161, 162). Proteolysis results in exposure of the Bid BH3 domain which is normally

hidden in the full-length protein (163, 164). The cleaved (p7/p15) complex is myristoylated on p15 and translocated to mitochondria (165). Bid seems to form homotrimers in the mitochondrial membrane which then may induce mitochondrial Bax and/or Bak to oligomerize (154). Thus, Bid provides a link between the receptor-mediated pathway and the mitochondrial pathway (161, 162).

In all cases, the net balance between the pro-survival (Bcl-2-like) and the proapoptotic BH3-only proteins seems to determine the cell's fate and the decision whether to live or to die.

Bax

In healthy primary cells, Bax is a monomeric cytosolic protein which, upon apoptotic stimulus, changes its conformation and translocates to the mitochondria (18). Intriguingly, in most cultured cells a significant proportion of Bax is already located in mitochondria in the absence of apoptotic stimuli (166). In the cytoplasm, the hydrophobic carboxy-terminal Bax helix occludes its hydrophobic groove formed by the BH1, -2 and -3 domains (147). Stress signals then provoke the C terminus to flip out and mediate Bax integration into the mitochondria and its subsequent oligomerization. Whether such mitochondrial Bax oligomers form Bax pores or influence existing mitochondrial channels remains an open issue but, in any case, release of apoptogenic factors like *Cyt c* from the mitochondria activates caspase-9 and downstream caspases (152).

Recent evidence implicates Bax in cancer cell response to death receptor-mediated apoptosis. Bax-deficient human colon carcinoma HCT116 cells are resistant to TRAIL-, FasL- and TNF-induced cell death, which is in stark contrast to their Bax-expressing wild-type counterparts (167). Moreover, mutational inactivation of the proapoptotic Bcl-2 homolog Bax induces tumor-cell resistance to death receptor-mediated apoptosis (168). Another set of experiments suggested that Bax and Bak differentially regulate the release of Cyt *c* and Smac/Diablo from mitochondria (169). The important role of Bax in tumor cell resistance to apoptosis is supported in a further study wherein Bax-deficient HCT116 cells overexpressing proapoptotic Bik and Bid were refractory to various apoptotic stimuli, UV, staurosporine, and thapsigargin (170).

According to the results from cell culture and animal models, down-regulation or inactivation of Bax is observed in several human cancers. The decreased Bax levels in tumors are not surprising given the fact that Bax is a transcriptional target of the tumor suppressor p53 which is mutated in the majority of human cancers (171). Impaired Bax expression has been reported in breast cancer (172), hepatocellular carcinomas (173), chemoresistant B-CLL (174) and in a number of other tumor types. Somatic frameshift mutations in the *bax* gene have been described in colon cancers (175) and certain hematopoietic malignancies have been shown to possess loss-of-function mutations of *bax* (176). For several different tumors, low Bax expression level was demonstrated as a negative prognostic factor for patients' survival (177-179).

Bax conformational change and translocation are recently found to be regulated by protein kinases. Akt has been shown to suppress Bax translocation to mitochondria

(180). Furthermore, Akt phosphorylates Bax at Ser184 and regulates Bax activation and apoptosis in neurophils (63). This is a novel mechanism by which the PI3-K/Akt pathway may promote cell survival. Bax has been shown to be associated with PKCε and the interaction between these two molecules promotes cell survival in recurrent prostate cancer cells (181). Thus, Bax might be a common target for Akt and PKCε to regulate TNF-mediated cell death in breast cancer cells.

Project hypothesis and Specific Aims

Both PKCε and Akt have oncogenic potential and function as antiapoptotic proteins. PKCε overexpression protects breast cancer cells from TNF-mediated cell death. However, the mechanism by which PKCε transduces its antiapoptotic signal in breast cancer cells is not clear. The goal of this dissertation is to understand the molecular pathway(s) by which PKCε mediates the antiapoptotic signals in breast cancer cells. It is hypothesized that PKCε plays an antiapoptotic role in breast cancer cells through activating Akt and inhibiting the proapoptotic Bcl-2 family member, Bax. The hypothetical model is illustrated in Figure 7. The significance of this study lies in understanding the signal transduction pathways that mediate resistance to TNF cytotoxicity. Ultimately, this study will provide important information valuable for cancer therapy.

To test the proposed hypothesis, the following specific aims were addressed:

Specific Aim 1 To examine whether activation of Akt leads to breast cancer cell resistance to TNF.

The protein level and phosphorylation status of Akt in various breast cancer cell lines was determined by Western blot analysis. The effect of pharmacological inhibitor of PI3-K/Akt (Ly294002) on Akt phosphorylation was examined by Western blot, and the cell death mediated by TNF was detected by flow cytometric analysis. To increase or decrease Akt activity, constitutively-active Akt (CA-Akt) or dominant-negative Akt (DN-Akt) was transfected into breast cancer cells. TNF-induced cell death was analyzed using flow cytometric analysis after the transfection.

Specific Aim 2 To determine whether PKCε acts upstream of Akt to regulate TNF-mediated cell death in breast cancer cells.

The effect of TNF on Akt phosphorylation/activity was examined by Western blot analysis. PKCε level/activity was altered by overexpression of wild-type or dominant-negative constructs of PKCε or knockdown of PKCε by small interfering RNA (siRNA) into breast cancer MCF-7 cells and and the impact on Akt phosphorylation/activity was determined by Western blot as well as by *in vitro* kinase assay. The effect of CA-Akt and Akt depletion by siRNA on cell death was monitored by flow cytometric analysis. The physical association of Akt and PKCε and/or DNA-PK, a potential PDK2, was detected by co-immunoprecipitation.

Specific Aim 3 To detect whether Bcl-2 family member Bax is a downstream target of PKCε.

First, the effect of PKCε on TNF-mediated mitochondrial depolarization was determined using mitochondrial potential sensor-JC-1 staining. The effect of Bax depletion by siRNA on TNF-mediated cell death was determined using a flow cytometric analysis. Effect of PKCε overexpression or depletion on Bax dimerization and translocation was examined by Western blot analysis and immunocytochemistry. Co-immunoprecipitation was performed to detect the association between PKCε and Bax.

Figure 1. Extrinsic and intrinsic Cell death pathways

The extrinsic pathway is activated *in vivo* by death ligands that engage death receptors, resulting in adaptor proteins recruitment and activation of initiator caspases-8 or -10 that start the caspase cascade and cell death. Caspase-8 also cleaves Bid, which induces translocation of truncated Bid (tBid) to mitochondria and initiate the cell-intrinsic pathway. The intrinsic pathway is activated by diverse stress signals which cause release of cytochrome c (*Cyt c*). *Cyt C* together with the adaptor protein Apaf-1 participate in apoptosome formation and activation of caspase-9 which in turn activate effector caspases and cell death. In addition, stimulated mitochondria can release other apoptotic factors such as Smac/DIABLO and $HtrA_2$/Omi that bind inhibitors of apoptosis (IAPs). Bcl-2 can prevent release of mitochondrial apoptogenic factors.

Figure 1.

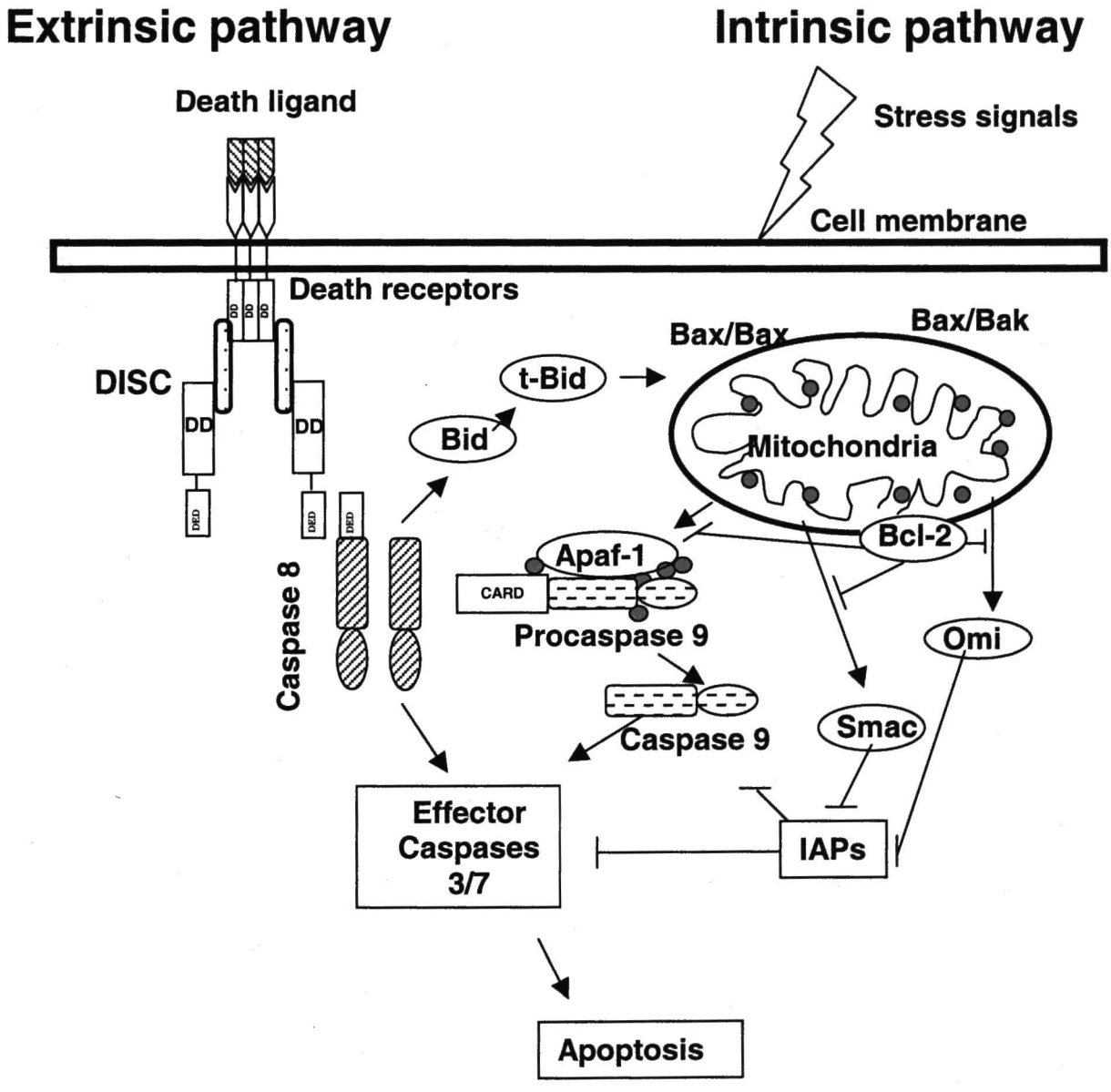

Extrinsic pathway
Intrinsic pathway
Death ligand
Stress signals
Cell membrane
Death receptors
DISC
DD
DD
DD
DED
DED
DED
Caspase 8
Bid
t-Bid
Bax/Bax
Bax/Bak
Mitochondria
Bcl-2
Apaf-1
CARD
Procaspase 9
Caspase 9
Omi
Smac
Effector
Caspases
3/7
IAPs
Apoptosis

Figure 2. TNF signaling pathways

TNF binds TNFR1 inducing association of receptor DDs with TRADD. TRADD functions as a platform adapter that recruits several signaling molecules to the activated receptor: FADD, TRAFs, and receptor-interacting protein (RIP). FADD recruits caspase-8 and initiates an apoptosis caspase cascade. Signals through other adaptor proteins, such as TRAFs and RIP, lead to activation of NF-κB and JNK/AP-1. TRAFs and RIP activate the NF-κB-inducing kinase (NIK), which in turn activates the inhibitor of IκB kinase complex, IKK. IKK phosphorylates IκB leading to degradation of IκB and allowing NF-κB to translocate to the nucleus to activate transcription. The pathway from TRAF-2 and RIP to JNK involves a cascade that includes the mitogen-activated protein (MAP) kinases MEKK1 (MAP/ERK kinase kinase-1, JNKK (JNK kinase), c-Jun N-terminal kinase (JNK), as well as AP-1 activation. TNF-also binds TNFR2, and TNFR2 binds TRAF-2 and signals activation of NF-κB and AP-1. Modified from Guseva NV, Taghiyev AF, Rokhlin OW, and Cohen MB. Death receptor-induced cell death in prostate cancer. Journal of Cellular Biochemistry, 2004; 91:70–99.

Figure 2.

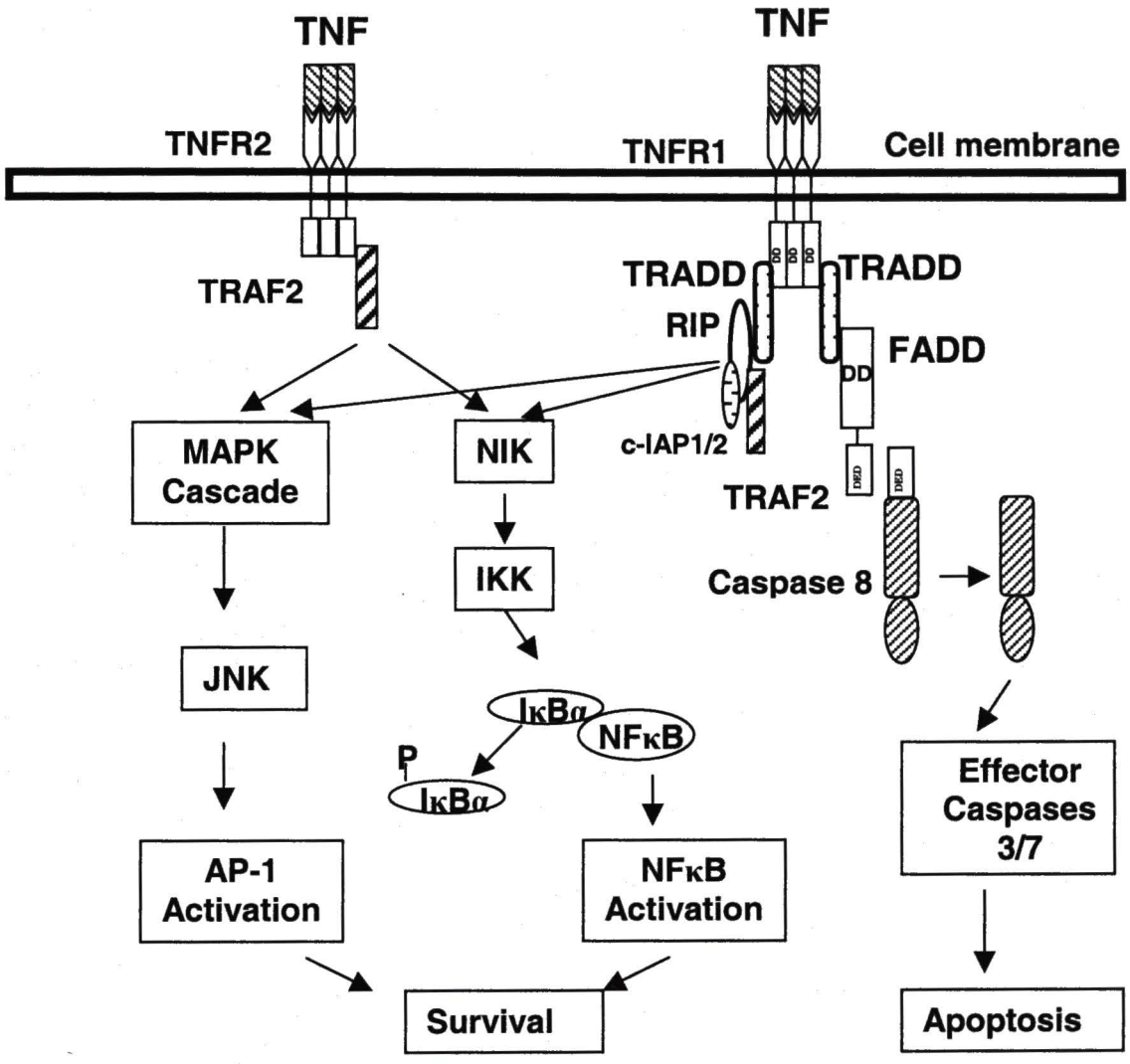

TNF
TNF
TNFR2
TNFR1
Cell membrane
TRAF2
TRADD
TRADD
RIP
FADD
DD
c-IAP1/2
TRAF2
DED
DED
MAPK Cascade
NIK
IKK
Caspase 8
JNK
IκBα
NFκB
P
IκBα
Effector Caspases 3/7
AP-1 Activation
NFκB Activation
Survival
Apoptosis

Figure 3. Structure of the three major Akt/PKB isoforms

All Akt/PKB variants contain a plecsktrin homology domain (PH), a catalytic domain, and a putative regulatory fragment at the C-terminus. Phosphorylation at both threonine and serine sites is required to induce Akt activation.

Figure 3.

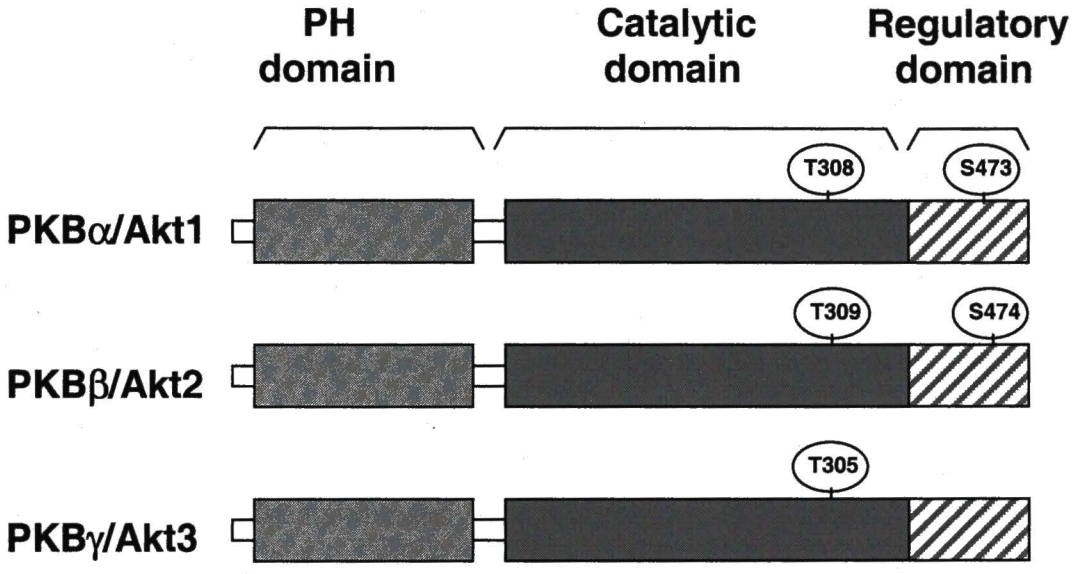

PH domain
Catalytic domain
Regulatory domain
T308
S473
PKBα/Akt1
T309
S474
PKBβ/Akt2
T305
PKBγ/Akt3

Figure 4. Current model of Akt activation by growth factors

Growth factor(s) binding to tyrosine kinase receptor (RTK) elicits recruitment and activation of PI3-kinase (PI3-K). Upon activation, PI3-K catalyzes production of phosphoinositides phosphorylated at position 3. PI(3,4,5)P3 then binds with high affinity to the PH domain of PDK1 and Akt. The action of PI3-K is counteracted by the lipid phosphatases such as PTEN that reduce the intracellular level of PI(3,4,5)P3. Akt is anchored to the plasma membrane. This is accompanied by a conformational change that relieves the inhibitory function of the PH domain and exposes Akt to subsequent activation by other kinases. PI(3,4,5)P3 increases PDK1 and PDK2, which phosphorylate Akt on threonine 308 and serine 473, respectively. The identity of PDK2 is still questionable. Once activated, Akt translocates to the cytoplasm and the nucleus to function as an antiapoptotic protein.

Figure 4.

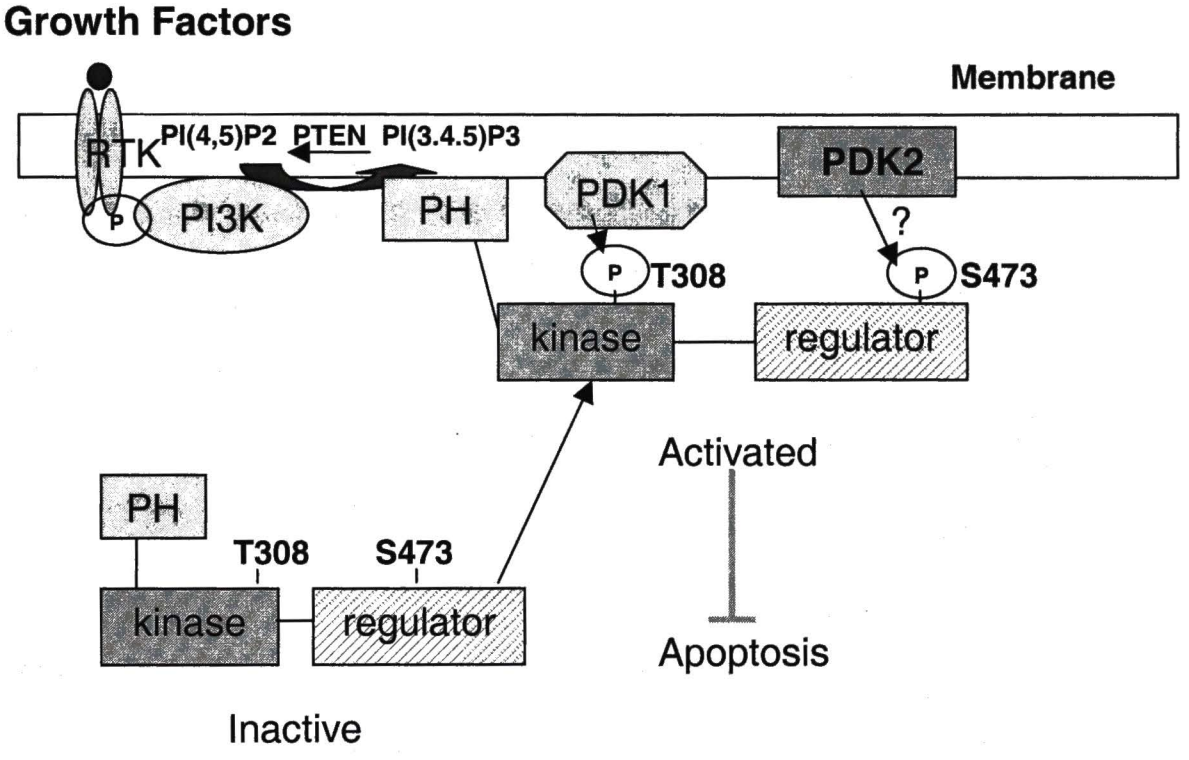

Growth Factors
Membrane
RTK
PI(4,5)P2
PTEN
PI(3.4.5)P3
PI3K
PH
PDK1
PDK2
?
P
T308
P
S473
kinase
regulator
Activated
PH
T308
S473
kinase
regulator
Apoptosis
Inactive

Figure 5. Structure of PKCε

The full-length PKCε is 84-kDa and contains a regulatory domain at the NH_2-terminus and a catalytic domain at the COOH-terminus separated by the hinge region. PKCε contains C3 and C4 regions at the catalytic domain and a C1 region at the regulatory domain. The C1 region contains two cysteine-rich zinc finger structures and is responsible for binding DAG/phorbol esters. Uniquely, PKCε has an actin-binding motif (AA 223-228) located between the two cysteine-rich regions. PKCε lacks the classical C2 region that binds Ca^{2+} but contains a C2-like region at the N-terminal end. A pseudosubstrate sequence between C1 and C2-like domain maintains it in an inactive conformation. PKCε contains three phosphorylation sites: Thr566 at the activation loop, Ser729 in C-terminal hydrophobic site and Thr710 at an autophosphorylation site. Modified from Akita Y. Protein Kinase C-epsilon (PKC-ε): Its unique structure and function. J Biochem 2002; 132: 847-52.

Figure 5.

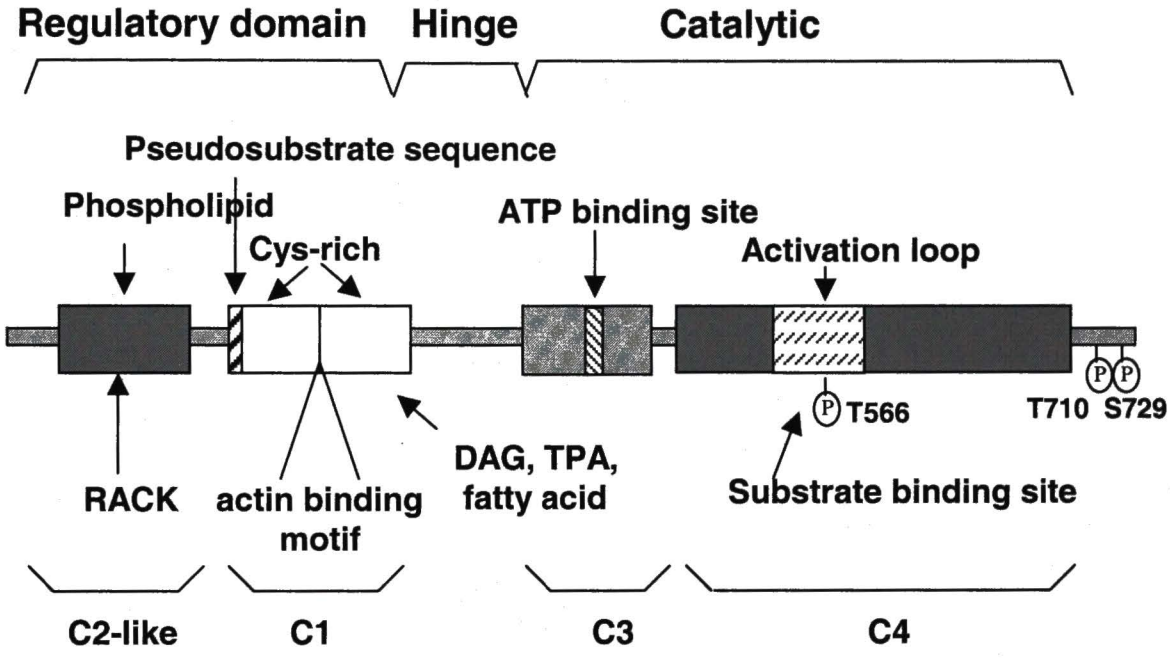

Regulatory domain
Hinge
Catalytic
Pseudosubstrate sequence
Phospholipid
Cys-rich
ATP binding site
Activation loop
P
T566
T710
P
P
S729
RACK
actin binding motif
DAG, TPA, fatty acid
Substrate binding site
C2-like
C1
C3
C4

Figure 6. Bcl-2 family members

Three groups of Bcl-2 family members have been identified. Group 1 is an antiapoptotic group of proteins, such as Bcl-2 and Bcl-X_L. BH1 to BH4 are conserved sequence motifs. Group 2 proteins are proapoptotic and lack a functional BH4 domain. This group includes Bax and Bak. Group 3 is a large group of proapoptotic molecules such as Bid and Bad that contains only the BH3 domain. BH1, BH2, and BH3 domains are responsible for homo- or hetero-dimerization. The TM domain is important for localization to membrane structure. Modified from Chan S-L and Yu VC. Proteins Of The Bcl-2 Family In Apoptosis Signalling: From Mechanistic Insights To Therapeutic Opportunities. Clinical and Experimental Pharmacology and Physiology 2004; 31:119-28.

Figure 6.

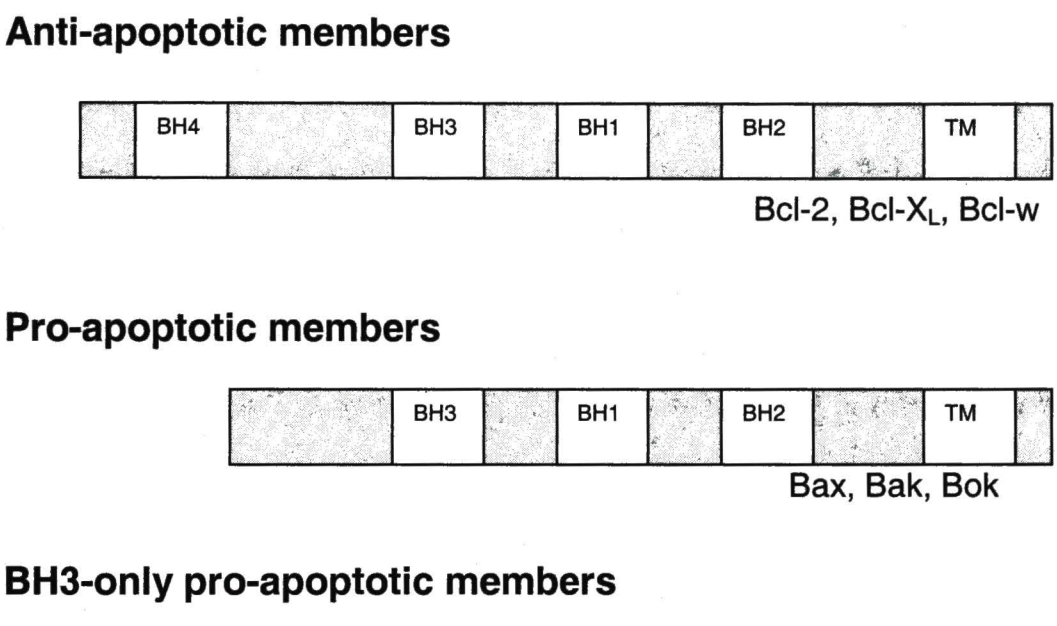
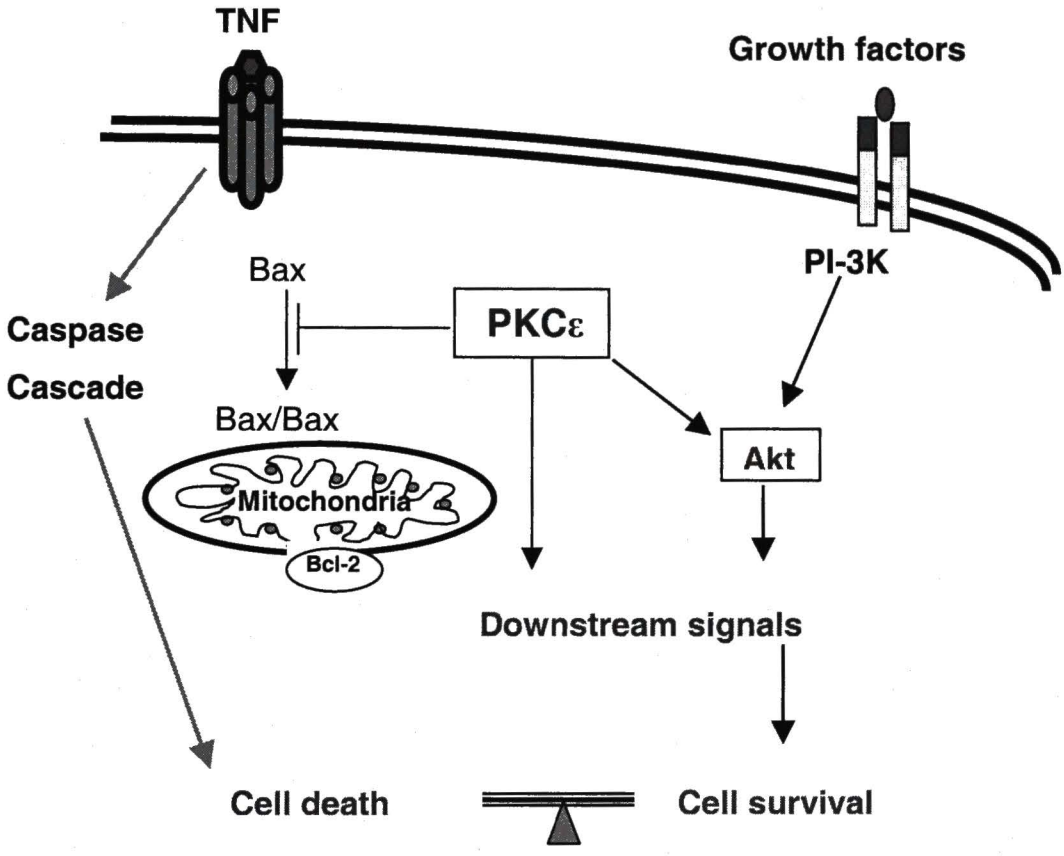

Anti-apoptotic members
BH4
BH3
BH1
BH2
TM
Bcl-2, Bcl-X_L, Bcl-w
Pro-apoptotic members
BH3
BH1
BH2
TM
Bax, Bak, Bok
BH3-only pro-apoptotic members
BH3
TM
Bim, Bmf, Hrk, Bik
BH3
Bid, Bad, Noxa, Puma

Figure 7. Hypothetical model of the project

TNF triggers apoptosis mainly through a caspase cascade and amplifies the cell death signal through mitochondrial pathway which is regulated by Bcl-2 family members. Both PKCε and Akt are antiapoptotic molecules. They may inhibit TNF-mediated cell death through linear pathway or parallel pathway, or they may have common downstream targets to mediate cell survival signals. The hypothesis tested in this dissertation is that PKCε acts upstream of Akt and inhibits proapoptotic function of Bcl-2 family member Bax, to play its antiapoptotic role in breast cancer cells. The outcome of the balance between cell death and survival signals is hypothesized to represent the primary determinant of cell fate.

Figure 7.


TNF
Growth factors
PI-3K
Bax
PKCε
Caspase
Cascade
Bax/Bax
Akt
Mitochondria
Bcl-2
Downstream signals
Cell death
Cell survival

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CHAPTER II

DEREGULATION OF PKB INFLUENCES ANTIAPOPTOTIC SIGNALING BY PKC IN BREAST CANCER CELLS

PREFACE

Breast cancer cells have different sensitivity to TNF, and PKC has been shown to regulate TNF-mediated cell death. The PKC inhibitor bisindolylmaleimide (BIM) potentiated TNF-induced apoptosis in MCF-7, BT-20 and MDA-MB-231 breast cancer cells. The effect of BIM was most pronounced in MDA-MB-231 cells which has high level of PKCε. Overexpression of PKCε protected MCF-7 cells from TNF-mediated cell death, while introduction of DN-PKCε induced cell death even without TNF. This suggests that PKCε activation is required for its antiapoptotic function. However, the breast cancer cells with low level of PKCε such as SKBR-3 cells were not sensitive to TNF as we predicted. Thus, PKCε level alone is not sufficient to explain TNF sensitivity. Akt has been shown to be overexpressed or activated in breast cancer. Thus, the objective of the work in the following section was to determine the level and the activity status of Akt in different breast cancer cells and to examine if Akt activation causes TNF resistance.

DEREGULATION OF PKB INFLUENCES ANTIAPOPTOTIC SIGNALING BY PKC IN BREAST CANCER CELLS

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Key words: Akt/PKB, PKC, TNF, apoptosis, breast cancer

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ABSTRACT

We have previously shown that novel protein kinase C (nPKC) isozymes, such as nPKCε, negatively regulate TNF-induced apoptosis in breast cancer cells although the level on nPKCs did not correlate with cellular sensitivity to TNF. In the present study, we examined if the level/activation status of Akt/PKB influences antiapoptotic signaling by nPKCε. While MCF-7 cells overexpressed PKB, BT-20 and SKBR-3 cells expressed constitutively phosphorylated PKB, and MDA-MB-231 cells expressed unphosphorylated PKB. Ly294002, an inhibitor of PI-3 kinase, induced cell death in SKBR-3 cells, which contained little nPKCs. Although Ly294002 by itself had only a modest effect on cell death in BT-20 and MCF-7 cells, it potentiated sensitivity of these cells to TNF. In contrast, Ly294002 either alone or in combination with TNF had little effect on cell death in MDA-MB-231 cells. These results suggest that the status of PKB in breast cancer cells influences antiapoptotic signaling by PKC.

INTRODUCTION

Tumor necrosis factor-α (TNF) was originally identified as a cytokine that induces necrosis in tumors and regression of cancer in animals (1). TNF exerts its effects by binding to its cell surface receptors, TNFR1 or TNFR2 (2-4). TNFR1 is believed to be responsible for the transduction of death signal (2-4). There is, however, no correlation between receptor number, affinity and TNF-mediated cellular responses (5, 6). Cell death by TNF involves activation of caspases, a family of cysteine proteases that cleave proteins after aspartate residues (7). Caspase-8, -9 and -10 participate in the initiation phase of apoptosis whereas caspase-3, -6 and -7 are involved in the execution phase of apoptosis (8). A failure to undergo apoptosis can lead to cancer.

TNF has been shown to cause activation of Akt/PKB, which is the cellular homologof the oncogene product v-Akt (9). It acts downstream of PI3-kinase and mediates the survival effects of different growth factors, cytokines and oncogenes (10). PKB is deregulated in many human cancers, including breast cancer (11-14). Three isoforms of PKB have been identified-Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ (22). All three PKB isoforms have been associated with breast cancer. Akt1 is overexpressed in breast cancer, including MCF-7 breast cancer (13). It has been reported that AKT2 is amplified in 3% of breast cancer (11) and is frequently activated in primary human breast carcinoma (14). Upregulation of AKT3 may contribute to the more aggressive clinical phenotype of the estrogen receptor-negative breast cancers (15). It has been proposed

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that the kinase activity of Akt plays an important role in the development and progression of human cancers (13).

Phosphorylation appears to be critical for its kinase activity since dephosphorylation of Akt causes its inactivation (16, 17). The tumor suppressor protein PTEN that dephosphorylates the 3 position of phosphoinositides and thereby inactivates PKB is also deregulated in 10% of breast carcinoma (18). Activation of PKB by growth factors is associated with its phosphorylation at Thr308 in the activation loop and Ser473 in the C-terminal domain. Phosphorylation at both sites is necessary for its full activation. Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates PKB at Thr308 whereas little is known about the phosphorylation of PKB at Ser 473 (16, 17, 19). A putitive PDK2 is believed to phosphorylate PKB at Ser473 (17). It has also been reported that phosphorylation of PKB at Ser473 may be mediated by PDK1, autophosphorylation, ILK or MAPKAP-2 (9, 19, 20). TNF has been shown to induce phosphorylation of PKB at Ser473 but not at Thr308 (21).

PKB exhibits significant similarities with PKC in their structure, function and regulation (22). The PKC signal transduction pathway has also been implicated in breast cancer pathogenesis (23-27). We and others have shown that PKC negatively regulates cell death by TNF (28-32). PKC represents a family of 11 isozymes that have been categorized into three groups: conventional (α, βI, βII and γ), novel (δ, ε, η, θ and μ) and atypical (ζ and ι/λ) (33). These isozymes differ in structure function and biochemical properties. nPKCε is the only PKC isozyme that has been reported to have oncogenic potential (34, 35). Several members of the PKC family have been shown to regulate cell

death by apoptosis. TNF can cause proteolytic activation of nPKCε and aPKCζ, which function as anti-apoptotic proteins (29, 36-38).

Both PKC and PKB function downstream of PI3-kinase (17). It has been reported that aPKCζ regulates growth factor-mediated Akt phosphorylation and activation (39). Although both aPKCζ and PKB have been recognized as prosurvival proteins, PKCζ negatively regulates Akt activity. Wen et al. implicated PKCβ but not -δ or -ε in the negative regulation of PKB (40). On the other hand, Matsumoto *et al.* (41) reported that inhibition of PKCε by DN-PKCε was associated with inhibition of PKB phosphorylation by insulin, heat-shock and hydrogen peroxide whereas other PKC isozymes had no effect, demonstrating that PKCε activity was required for PKB phosphorylation. Recently, it has been reported that PKB may be a downstream effector of PKCε for EtOH-induced cardioprotection (42). Thus, how PKC and PKB interact with each other may depend on the cellular context.

We have shown that overexpression of novel PKCε attenuated TNF-induced apoptosis in breast cancer MCF-7 cells (43). In addition, breast cancer cells containing high level of PKCε were sensitized to TNF by PKC inhibitor (32). However, some breast cancer cells that expressed low level of PKCε were resistant to TNF-induced apoptosis, suggesting that the level of nPKCε was not sufficient to explain breast cancer cell sensitivity to TNF (32). Since both PKC and PKB can function as anti-apoptotic proteins and they can interact with each other, we investigated if the status of PKB influences antiapoptotic signaling by PKC. Our results show that cells that contain constitutively active Akt are resistant to TNF-induced apoptosis regardless of the status of novel PKCs.

However, the ability of PKB inhibitor to induce cell death depends not only on the status of PKB but also on PKC. Thus, PKC could collaborate with PKB to regulate cell survival and cell death.

MATERIALS AND METHODS

Materials

Ly294002 was obtained from Alexis Biochemicals (San Diego, CA). BIM was purchased from CalBiochem (San Diego, CA) and TNF from R&D Systems (Minneapolis, MN). Polyclonal antibody phospho-PKB (Ser473) and monoclonal antibody to PARP were obtained from PharMingen (San Diego, CA). Polyclonal antibody to Akt/PKB and phospho-PKB (Ser 473) were obtained from Cell Signaling (Beverly, MA). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmuno Research Lab. Inc. (West Grove, PA). Poly (vinylidene difluoride) membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

Cell Culture

Breast cancer cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, and kept in a humidified incubator at 370C with 95% air and 5% CO2.

Immunoblot Analysis

Equivalent amounts of total cellular extracts were electrophoresed by SDS-PAGE and transferred electrophoretically to poly(vinylidene difluoride) membrane. Immunoblot analyses were performed as described before (44).

Assessment of Apoptosis by Flow Cytometric Analysis

Cells were treated with or without PKB and PKC inhibitor, and then treated with TNF as indicated in the text. At the end of the incubation, cells were harvested and washed with phosphate-buffered saline. Nuclei were isolated, stained with propidium iodide and DNA content was analyzed by a flow cytometer (Coulter Epics) (45).

Comparison of the Level and Phopshorylation Status of Akt/PKB in Breast Cancer Cells.

The ability of cancer cells to escape TNF-induced apoptosis can lead to malignant transformation. Several members of the PKC family have been shown to function as antiapoptotic proteins. We have previously shown that novel PKCs rather than conventional or atypical PKCs are important in regulating cell death in breast cancer cells (46). For example, the PKC-specific inhibitor bisindolylmaleimide (BIM) at concentrations that inhibit cPKCs, as well as Gö6976, a specific inhibitor of cPKCα and –βI, had little effect on TNF-induced cell death, suggesting that cPKCs may not be important in regulating cell death by TNF. PKC activator TPA or PDBu that do not bind to aPKCs protected cells against TNF cytotoxicity, suggesting that inhibition of apoptosis by PKC activators may not be mediated by aPKCζ. Breast cancer cells containing high level of PKCε were sensitized to TNF by PKC inhibitor (32). In addition, overexpression of nPKCε and -η attenuated TNF-induced apoptosis in MCF-7 cells (31,43). Furthermore, TNF caused proteolytic activation of PKCε and activation of PKCε was associated with its anti-apoptotic function (43). While these results underscore the importance of novel PKC, especially PKCε as an anti-apoptotic protein, the level of novel PKCs was not sufficient to explain cellular sensitivity/resistance to TNF (32). The PKC isozyme profile of MCF-7 and BT-20 cells was similar; they both expressed conventional PKCα, novel PKCδ, -ε and -η, and atypical PKCζ and -ι but BT-20 cells were more

resistant to TNF compared to MCF-7 cells. Furthermore, breast cancer cells, such as SKBR-3, CAMA-1 and Hs578t cells that contained little novel PKCε and -η were resistant to TNF.

The oncogene product Akt/PKB is well recognized for its pro-survival/antiapoptotic function and is often deregulated in breast cancer. Therefore, we examined if the status of PKB influences the anti-apoptotic function of PKC. Since the state of phosphorylation of PKB is an indication of its activation, we monitored PKB phosphorylation. Thr308 is phosphorylated by PDK1, which is often constitutively active (19, 41). It has been reported that TNF influences Ser473 phosphorylation (21). Therefore, we have used an antibody that specifically recognizes Ser473. Figure 1 shows that while MCF-7 cells overexpressed PKB, BT-20, CAMA-1, SKBR-3 and Hs578t cells contained phosphorylated PKB. These results may explain why SKBR-3, CAMA-1 and Hs578t cells, which contained little nPKCs compared to MCF-7 cells were resistant to TNF. The abundance of PKB in BT-20, SKBR-3 and MDA-MB-231 cells was equivalent but MDA-MB-231 cells did not contain any phospho-PKB. Since growth factors present in the fetal bovine serum (FBS) may induce PKB phosphorylation, we compared phospho-PKB status in cells cultured in serum-free versus normal serum (10% FBS)-containing media. PKB phosphorylation was not dependent on the presence of serum in the media, suggesting that PKB was constitutively phosphorylated in SKBR-3 and BT-20 cells (data not shown).

Effect of Ly294002 on PKB inhibition and TNF-Induced Cell Death

To determine if the status of PKB influences cellular sensitivity to TNF, we examined the effect of Ly294002, a pharmacological inhibitor of PI3-kinase which is widely used to inhibit PKB activity, on TNF-induced cell death. Treatment of breast cancer cells with Ly294002 resulted in a concentration-dependent inhibition of PKB phosphorylation, such that 25 μM Ly294002 abolished PKB phosphorylation in MCF-7, SKBR-3 and BT-20 cells (Fig. 2). Therefore, we have mostly used 25 μM Ly294002 to inhibit PKB activity in subsequent studies.

We then compared the effect of Ly294002 on TNF-induced cell death in breast cancer cells that differ in the level/activation status of PKB. MCF-7 cells lack functional caspase-3, which is required for DNA fragmentation and morphological changes associated with apoptosis (47). Therefore, we have used cleavage of PARP, a substrate for executioner caspase-3 and -7, as an indicator of apoptosis. When cells undergo apoptosis, 116-kDa intact PARP is cleaved to an 85-kDa form. Ly294002 alone induced cleavage of PARP in SKBR-3 cells, which contained high level of constitutively active PKB, suggesting that PI-3 kinase/Akt pathway plays an important role in the survival of these breast cancer cells. The inhibition of PKB phosphorylation by Ly294002 paralleled cleavage of PARP (Fig. 3). Since the levels of nPKCs were very low in SKBR-3 cells, inhibition of PKB may be sufficient to induce cell death in these cells. In contrast, Ly294002 alone had little effect on PARP cleavage in MCF-7 and BT-20 cells but it potentiated TNF-induced PARP cleavage in both cell types.

Since it is difficult to quantify cell death by PARP cleavage, we also monitored apoptosis by the appearance of a hypodiploid peak using a flow cytometer. Figure 4 shows that when BT-20 cells were treated with TNF for 12 h and 24 h, the hypodiploid peak increased from 5.5% to 10.6% and 16.9%, respectively. Ly294002 alone had only a slight effect on the increase in hypodiploid peak but it potentiated TNF-induced apoptosis in a time- and concentration-dependent manner. In contrast to SKBR-3 cells, MCF-7 and BT-20 cells contained moderate amount of PKCε. Thus, the inability of Ly294002 to cause substantial amount of cell death in MCF-7 cells and BT-20 cells could be due to the presence of PKCε, which also acts as an antiapoptotic protein.

Comparison of the Effects of PKC and PKB Inhibitor on TNF-induced cell death

To determine if the status of both PKB and PKC is important for TNF-induced apoptosis, we compared the effects of pharmacological inhibitors of PKC and PKB on TNF-induced apoptosis in breast cancer cells. Figure 5 shows that inhibition of PKB by Ly294002 alone caused >25% cell death in SKBR-3 cells whereas the PKC specific inhibitor bisindolylmaleimide (BIM) had only a slight effect on cell death. These cells were relatively resistant to TNF and Ly294002 had only a modest effect on TNF induced apoptosis in SKBR-3 cells. In contrast, Ly294002 either alone or in combination with TNF had little effect on apoptosis in MDA-MB-231 cells, which did not contain any phospho-PKB. These cells expressed high level of PKCε (32) and the PKC inhibitor BIM increased cellular sensitivity to TNF markedly (Fig. 6A and 6B). TNF alone had only a modest effect on cell death in BT-20 cells (Fig. 7). While PKC and PKB inhibitor

by themselves increased cell death only slightly, they potentiated TNF-induced cell death substantially. Ly294002 was more effective than BIM in augmenting TNF-induced apoptosis. These results suggest that in the absence of PKC, PKB is the predominant pathway to determine cell survival; whereas if PKB is inactive, PKC may influence cell death. Thus, the status of both PKB and PKC is important for breast cancer cell survival.

Thus, the results of our study demonstrate that PKC cooperates with PKB to transduce antiapoptotic signaling in breast cancer cells. Future studies should explore if they trigger a linear, parallel or converging pathway to exert their pro-survival/antiapoptotic function.

Figure 1. Comparison of PKB and phospho-PKB levels in breast cancer cells.

Western blot analyses were performed with total cellular extracts using antibodies to PKB and phospho-PKB (S473).

Figure 1.

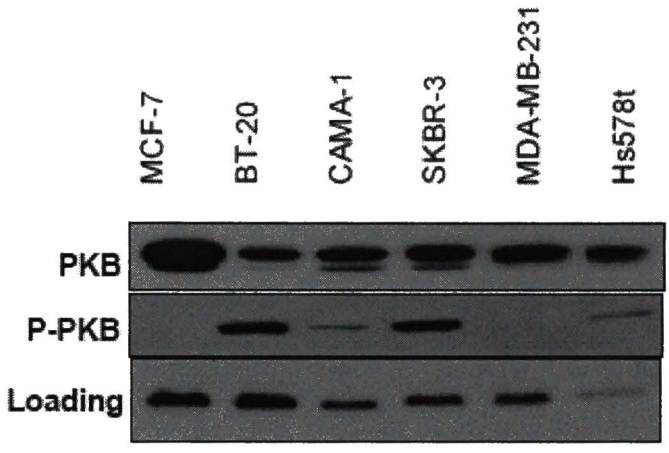


Figure 2. Effect of Ly294002 on PKB phosphorylation in MCF-7, SKBR-3 and BT-20 cells.

Cells were treated with indicated concentrations of Ly294002 and western blot analysis was performed with total cellular proteins using indicated antibodies.

Figure 2.

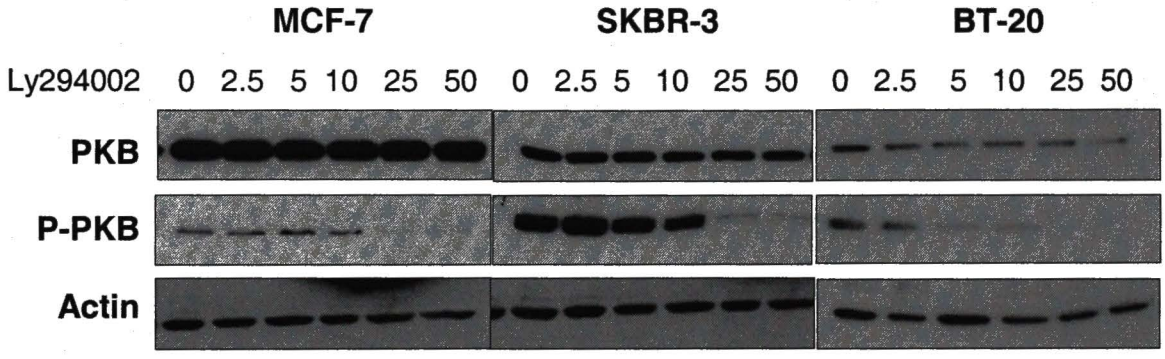

MCF-7
SKBR-3
BT-20
Ly294002 0 2.5 5 10 25 50 0 2.5 5 10 25 50 0 2.5 5 10 25 50
PKB
P-PKB
Actin

Figure 3. Effect of Ly294002 on PKB phosphorylation and PARP cleavage.

Cells were pretreated with Ly294002 for 30 min and then treated with 1nM TNF for 12h. Western blot analysis was performed with total cellular proteins using indicated antibodies. (A), SKBR-3 cells were treated with or without indicated concentration of Ly294002. (B and C), MCF-7 and BT-20 cells were pretreated with or without 25 μM Ly294002. The arrow indicates the processed form.

Figure 3.

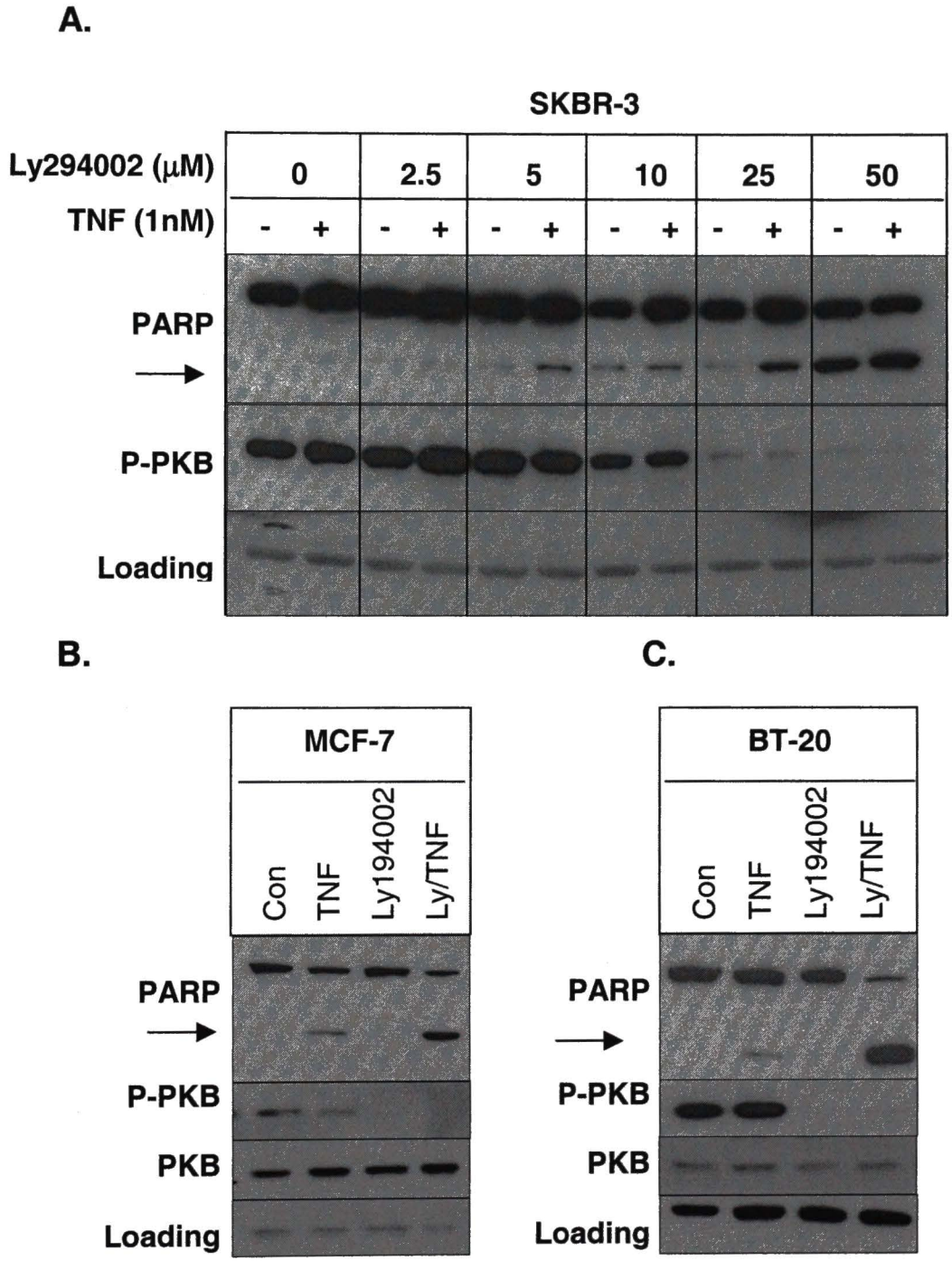

A.
SKBR-3
Ly294002 (μM)
0
2.5
5
10
25
50
TNF (1nM)
- + - + - + - + - + - +
PARP
P-PKB
Loading
B.
MCF-7
Con
TNF
Ly194002
Ly/TNF
PARP
P-PKB
PKB
Loading
C.
BT-20
Con
TNF
Ly194002
Ly/TNF
PARP
P-PKB
PKB
Loading

Figure 4. Effect of Ly294002 on TNF-induced apoptosis in BT-20 cells.

Cells were treated with or without 10 μM or 25 μM Ly294002 for 30 min and then treated with 1nM TNF for 12 h or 24 h. Cells were stained with propidium iodide and analyzed using a flow cytometer.

Figure 4.

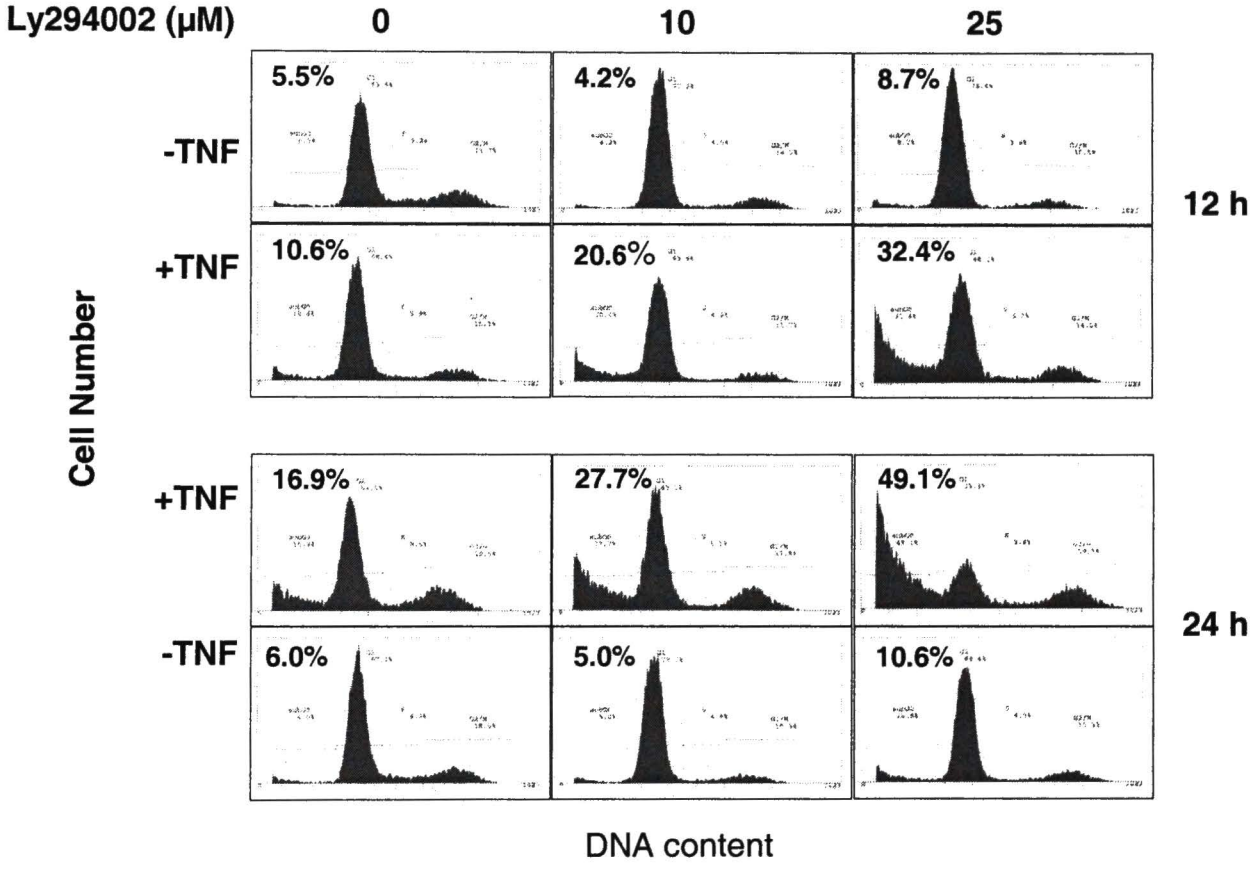

Ly294002 (μM)
0
10
25
-TNF
+TNF
12 h
5.5%
4.2%
8.7%
10.6%
20.6%
32.4%
Cell Number
+TNF
-TNF
24 h
16.9%
27.7%
49.1%
6.0%
5.0%
10.6%
DNA content

Figure 5. Effects of Ly294002 and BIM on TNF-induced apoptosis in SKBR-3 cells. Cells were treated with or without Ly294002 or BIM for 30 min and then treated with 1 nM TNF for 12 h. Cells were stained with propidium iodide and analyzed using a flow cytometer. Apoptosis was estimated by the fraction of cells in sub-G1 phase. Results are representative of three individual experiments. Open bar, -TNF; hatched bar, +TNF.

Figure 5.

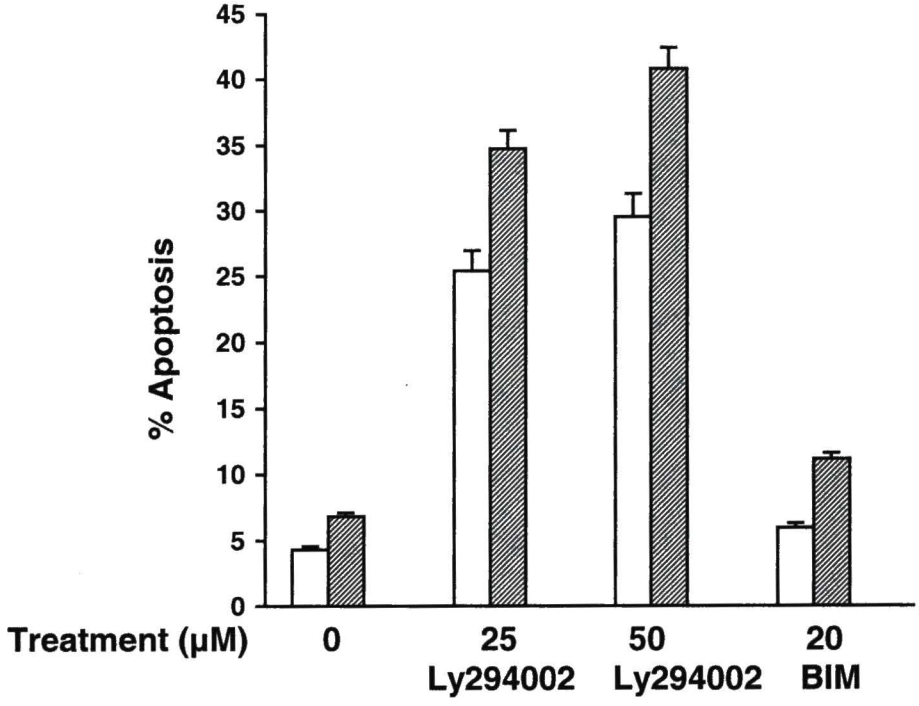

% Apoptosis
45
40
35
30
25
20
15
10
5
0
Treatment (μM)
0
25
Ly294002
50
Ly294002
20
BIM

Figure 6. Effect of Ly294002 and BIM on TNF induced apoptosis in MDB-MB-231 cells.

Cells were treated with or without indicated concentrations of Ly294002 or BIM for 30 min and then treated with 1nM TNF for 12 h. Apoptosis was estimated as described in the legend to Figure 6. (A), flow cytometric analysis; (B), Results are representative of three individual experiments. Open bar, -TNF; hatched bar, +TNF.

Figure 6.

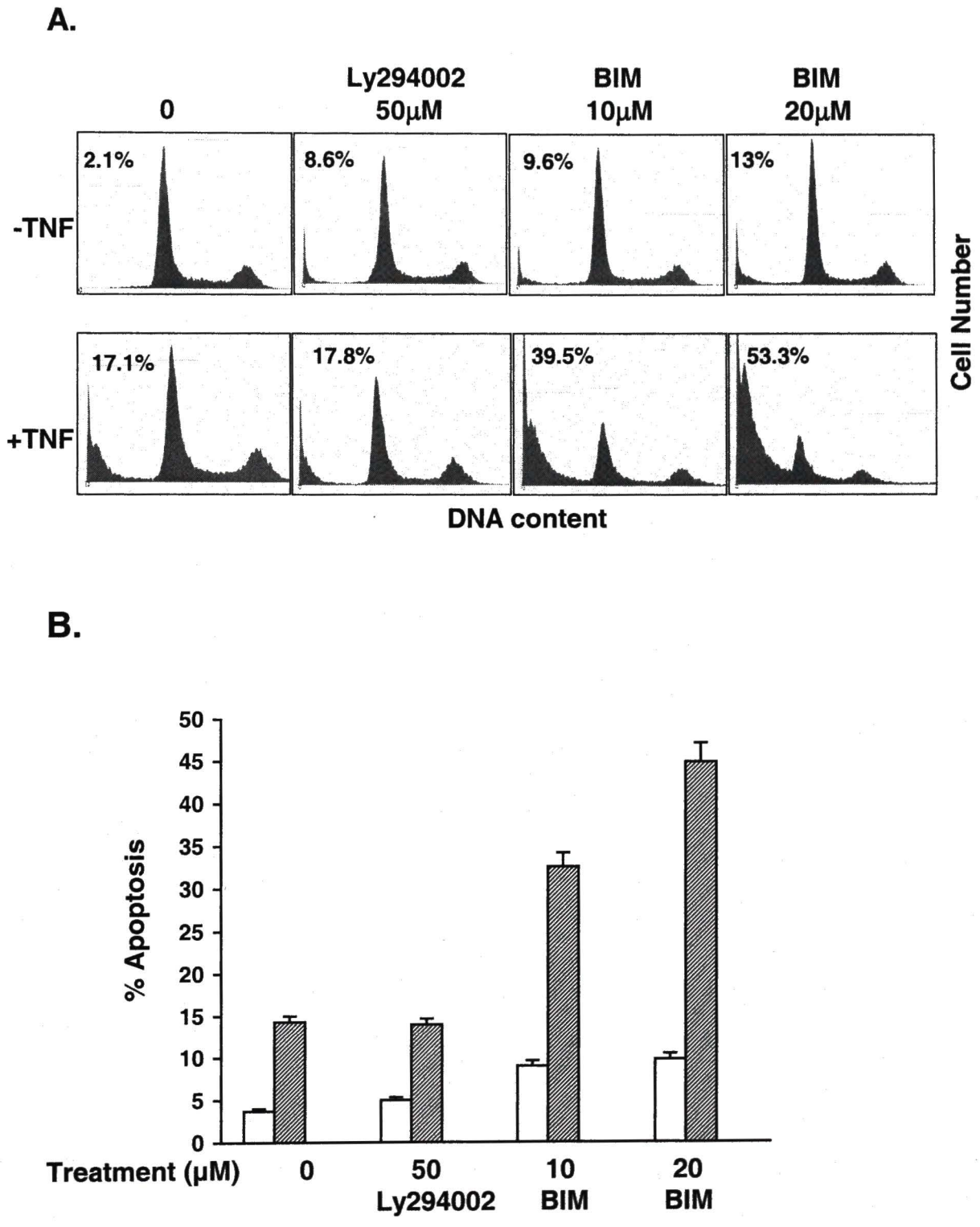
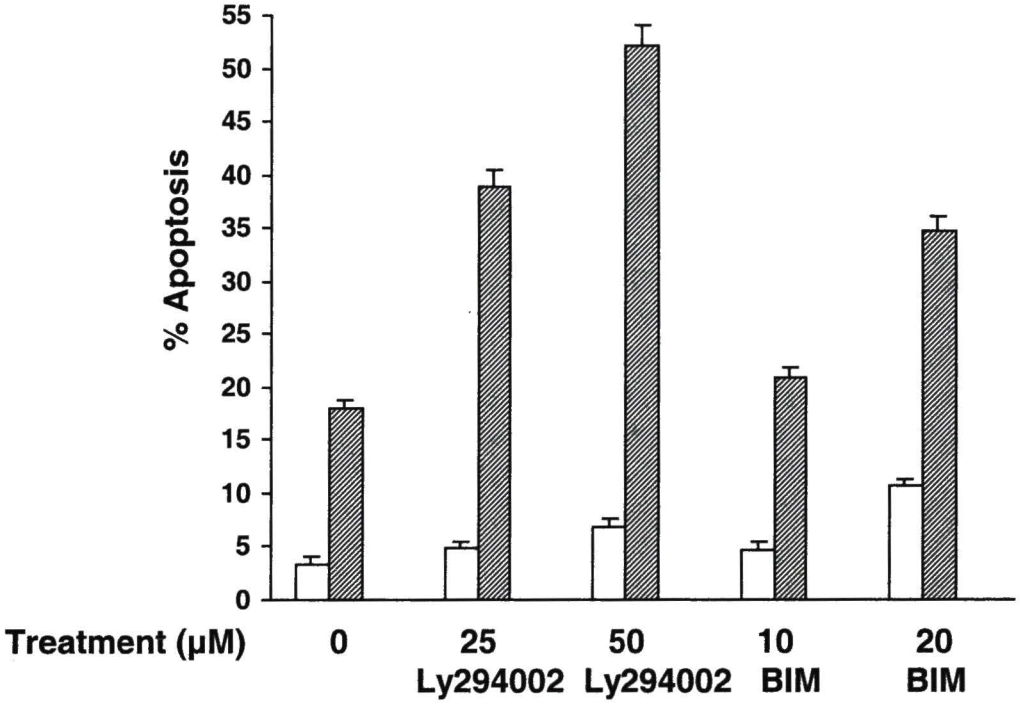

A.
0
Ly294002 50μM
BIM 10μM
BIM 20μM
-TNF
+TNF
2.1%
8.6%
9.6%
13%
17.1%
17.8%
39.5%
53.3%
Cell Number
DNA content
B.
% Apoptosis
50
45
40
35
30
25
20
15
10
5
0
Treatment (μM)
0
50
Ly294002
10
BIM
20
BIM

Figure 7. Effect of Ly294002 and BIM on TNF induced apoptosis in BT-20 cells. Cells were treated with or without indicated concentrations of Ly294002 or BIM for 30 min and then treated with 1 nM TNF for 12 h. Apoptosis was estimated as described in the legend to Figure 6. Results are representative of three individual experiments. Open bar, -TNF; hatched bar, +TNF.

Figure 7.


% Apoptosis
55
50
45
40
35
30
25
20
15
10
5
0
Treatment (μM)
0
25
Ly294002
50
Ly294002
10
BIM
20
BIM

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CHAPTER III

PROTEIN KINASE C-ε ACTIVATES PROTEIN KINASE B/AKT VIA DNA-PK TO PROTECT AGAINST TNF-INDUCED CELL DEATH

PREFACE

The first part of this dissertation showed that inhibition of PI3-K/Akt pathway sensitized breast cancer cells that overexpress Akt or contain constitutively-active Akt to TNF. Thus, both PKCε and Akt are important for the cellular response to TNF. The mechanism(s) by which PKCε activates Akt is not clear, and it remains to be established if PKCε plays its antiapoptotic effect via Akt. Studies in this chapter demonstrate that PKCε activates Akt via DNA-PK to exert its antiapoptotic effect in breast cancer cells.

CHAPTER III

PROTEIN KINASE C-ε ACTIVATES PROTEIN KINASE B/AKT VIA DNA-PK TO PROTECT AGAINST TNF-INDUCED CELL DEATH*

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The abbreviations used are: Akt, protein kinase B/(PKB)/Akt; ATM, ataxia telangiectasia mutated gene; ATR, ATM-related; DNA-PK, DNA-dependent protein kinase; GSK3α/β, glycogen synthase kinase-3α/β; ILK, intergrin-linked kinase; MAPKAP kinase-2, mitogen-activated protein kinase-activated protein kinase 2; NF-κB, nuclear factor kappa B; PDK1, Phosphoinositide-dependent protein kinase 1; PKCε, protein kinase C-ε; PI3-K, phosphoinositide 3-kinase; PIKK, PI3-kinase-related kinase; PI, propidium iodide; siRNA, short interfering RNA; TNF, tumor necrosis factor-α

SUMMARY

We have previously shown that PKCε protects breast cancer cells from TNF-induced cell death. In the present study, we have investigated if the antiapoptotic function of PKCε is mediated via Akt and the mechanism by which PKCε regulates Akt activity. TNF caused a transient increase in Akt phosphorylation at Ser473 in MCF-7 cells. Overexpression of PKCε in MCF-7 cells increased TNF-induced Akt phosphorylation at Ser473 resulting in its activation. Knockdown of PKCε by siRNA decreased TNF-induced Akt phosphorylation/activation and increased cell death. Introduction of constitutively active Akt (CA-Akt) protected breast cancer MCF-7 cells from TNF-mediated cell death and partially restored cell survival in PKCε-depleted cells. Depletion of Akt in MCF-7 cells abolished the antiapoptotic effect of PKCε on TNF-mediated cell death. Akt was constitutively associated with PKCε and DNA-dependent protein kinase (DNA-PK), and this association was increased by TNF treatment. Overexpression of PKCε enhanced the interaction between Akt and DNA-PK. Knockdown of DNA-PK by siRNA inhibited TNF-induced Akt phosphorylation and the antiapoptotic effect of Akt and PKCε. These results suggest that PKCε activates Akt via DNA-PK to mediate its antiapoptotic function. Furthermore, we report for the first time that DNA-PK can regulate receptor-initiated apoptosis via Akt.

INTRODUCTION

Tumor necrosis factor-α (TNF), a multifunctional cytokine, was originally characterized by its anti-tumor activity (1,2). It causes selective destruction of tumor tissues but has no effect on normal tissues (3). TNF exerts its effects by binding to its cell surface receptors, TNFR1 and TNFR2 (2). TNFR1 is believed to be responsible for the transduction of death signal. TNF triggers cell death through activation of a caspase cascade (4-6). Although TNF mediates apoptosis in breast cancer cells, some breast cancer cells are resistant to TNF. The presence of antiapoptotic proteins can counteract TNF-induced apoptosis.

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases that consist of at least 10 isozymes (7). PKC isozymes have distinct and in some cases opposing roles in cell growth and apoptosis (8,9). PKCε, a novel PKC, behaves as an oncogene when overexpressed in several fibroblast, colonic and prostatic epithelial cells (10,11). We and others have shown that PKCε acts as an antiapoptotic protein during receptor-initiated apoptosis (9,12,13). In addition, breast cancer cells containing high level of PKCε were sensitized to TNF by PKC inhibitor (14). However, the level of PKCε was not sufficient to explain breast cancer cell sensitivity to TNF (14).

Akt, also known as PKB, the cellular homologue of oncogene v-Akt, is a family of the serine/threonine kinases (15,16). Akt is activated in a phosphoinositide 3-kinase (PI3-K)-dependent manner and inhibited by phosphatase and tensin homologue tumor suppressor PTEN. Phosphorylation at both Thr308 in the activation loop and Ser473 in

the C-terminal domain of Akt is necessary for its complete activation (16). Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates Akt at Thr308 (17,18). The kinase that phosphorylates Ser473 of Akt has been tentatively designated PDK2. It has also been reported that phosphorylation of Akt at Ser473 may be mediated by PDK1, autophosphorylation, intergrin-linked kinase (ILK) or mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP kinase-2) (15,16,19). Recent evidence suggests that phosphorylation of Akt at Ser473 may be mediated by rictor-mTOR complex or DNA dependent protein kinase (DNA-PK) (20,21) .

DNA-PK, a member of the PI3-kinase-related kinase (PIKK) subfamily of protein kinases, is a nuclear serine/threonine protein kinase that is activated upon DNA damage (22). It is a three-protein complex consisting of a 470-kDa catalytic subunit (DNA-PKcs) and the regulatory DNA binding subunits, Ku heterodimer (Ku70 and Ku80) (22). The C-terminus of DNA-PKcs is similar to PI3-kinase family members, including ataxia telangiectasia mutated gene (ATM), ATM-related (ATR), and p110 PI3-K (23). However, DNA-PKcs acts as a protein kinase, not a lipid kinase (23). DNA-PK plays an important role in DNA repair and protects cells from apoptosis induced by DNA damaging agents, such as ionizing radiation, UV radiation and etoposide (24-26). A recent report suggests that DNA-PKcs can also colocalize with Akt on the cell membrane and phosphorylate Akt at Ser473 in a PI3-K dependent manner (21). Although Akt plays a critical role in cell survival, the involvement of DNA-PK in the antiapoptotic function of Akt has not been investigated.

Akt is constitutively active in many types of human cancers, including breast cancer (27). TNF has been shown to cause activation of Akt through phosphorylation at Ser473 in murine fibrosarcoma WEHI-164 cells (28). There have been several reports that suggest that PKCε may regulate Akt activity (29). Inhibition of PKCε by dominant-negative PKCε (DN-PKCε) was associated with inhibition of Akt phosphorylation by insulin, demonstrating that PKCε activity was required for Akt phosphorylation. However, wild type PKCε (WT-PKCε) had no effect on insulin-induced Akt activation (29). Akt has been reported to be a downstream effector of PKCε for ethanol-induced cardioprotection because ethanol consumption caused an increase in expression and activity of PKCε and Akt, and inhibition of PKCε prevented increase in Akt activity (30). It is not known how PKCε activates Akt. Furthermore, it remains to be established if the antiapoptotic function of PKCε is mediated by Akt. We made a novel observation that PKCε activates Akt by enhancing interaction between DNA-PK and Akt, resulting in phosphorylation of Akt at Ser473. Thus, PKCε acts upstream of Akt to regulate antiapoptotic signaling in breast cancer cells. Furthermore, we report for the first time that DNA-PK can regulate extrinsic or receptor-initiated cell death pathway via activation of Akt.

EXPERIMENTAL PROCEDURES

Materials—TNF was purchased from R&D Systems (Minneapolis, MN). Monoclonal antibody to PARP was obtained from Pharmingen (San Diego, CA). Polyclonal antibody to Akt/PKB, phospho-Akt (Ser473) and Akt kinase assay kit were obtained from Cell Signaling (Beverly, MA). Polyclonal antibody against PKCε and DNA-PK was from Santa Cruz (Santa Cruz, CA). Annexin V conjugated to Alexa Fluor 488 and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmuno Research Lab. Inc. (West Grove, PA). Poly (vinylidene difluoride) membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL). Anti-HA antibody was from Babco (Richmond, CA). Protein G Plus/Protein A Agarose suspension was from Oncogene Research Products (Boston, MA).

Adenovirus constructs and siRNA—Adenovirus containing constitutively-active Akt was a kind gift from Dr. Santosh DeMello (University of Texas, Dallas). Control non-targeting siRNA and siRNA specific for Akt1 and DNA-PKcs were obtained from Dharmacon RNA Technologies (Lafayette, CO). PKCε-specific siRNA and corresponding control siRNA were obtained from Santa Cruz (Santa Cruz, CA).

Cell Culture and Transfection—Breast cancer cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, and kept in a humidified incubator at 37^0C with 95% air and 5% CO_2. siRNA

was transfected using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Immunoblot Analysis—Equivalent amounts of total cellular extracts were electrophoresed by SDS-PAGE and transferred electrophoretically to poly (vinylidene difluoride) membrane. Immunoblot analyses were performed as described previously (14).

Co-immunoprecipitation—Cells were lysed in 20 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 1 mM DTT, 25 mM β-glycerophosphate, 10 mM NaF, 10 μg/ml phenymethylsulfonyl fluoride, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 4 μg/ml pepstatin. PKCε or Akt were immunoprecipitated with 1 μg antibody and 30 μl of protein A/G agarose. Immunocomplexes were washed four times in lysis buffer and boiled in Laemmlie sample buffer. The immunocomplexes were separated on SDS-PAGE and transferred to poly(vinylidene difluoride) membrane. The presence of Akt, PKCε or DNA-PK was detected using specific antibodies in Western blot.

Akt Kinase Assay—Akt kinase assay was performed using Akt kinase assay kit (Cell Signaling, Beverly, MA) according to manufacturer's protocol. Briefly, cells were serum-starved 12 h and then treated with or without 1 nM TNF as indicated in the text. Akt was immunoprecipitated using immobilized Akt antibody and immunokinase assay was performed using glycogen synthase kinase-3α/β (GSK3α/β) fusion protein as the substrate. The reaction mixture was separated by SDS-PAGE, and phosphorylation of GSK3α/β was determined by Western blot analysis using phospho-GSK3α/β antibody.

Annexin V/Propidium Iodide Binding Assay—Cells were treated with or without TNF as indicated in the text. At the end of the incubation, both detached and attached cells were collected and washed with PBS. Cells were then stained with Annexin V-Alexa 488 conjugate and PI according to the manufacturer's protocol and analyzed using a flow cytometer (Coulter Epics).

Statistical analysis—Data are presented as the mean ± S.E. and $n \geq 3$ unless otherwise specified. Statistical significance was determined using SigmaStat 2.03 (Systat Software, Inc. Point Richmond, CA). $P < 0.01$ was considered statistically significant.

Activation of Akt Protects Breast Cancer MCF-7 Cells Against TNF-Induced Cell Death—We have previously shown that PKCε acts as an antiapoptotic protein but the level of PKCε was not sufficient to explain its antiapoptotic function (9). Since Akt/PKB is an important antiapoptotic protein, we first wanted to determine if the status of Akt influences antiapoptotic function of TNF. MCF-7 cells overexpress Akt, but they contain low levels of constitutively-active phospho-Akt, presumably because these cells express PTEN. Therefore, we introduced HA-tagged constitutively-active Akt (CA-Akt) in MCF-7 cells using adenoviral vector. Figure 1A shows that transduction of adenovirus containing CA-Akt resulted in an increase in Akt in MCF-7 cells as detected by Akt and HA antibody. Overexpression of CA-Akt decreased TNF-induced apoptosis as evident by the cleavage of 116-kDa full-length PARP to an 85-kDa form. We quantified TNF-induced apoptosis using annexin V/PI dye binding assay. Treatment with TNF resulted in 45% cell death in MCF-7 cells infected with control vector compare to 13% cell death in MCF-7 cells overexpressing CA-Akt (Fig. 1B). Thus, the ability of TNF to induce cell death was compromised in CA-Akt overexpressing cells compared to vector-infected MCF-7 cells.

PKCε Acts Upstream of Akt to Regulate TNF-Induced Akt Activation—We have previously shown that overexpression of PKCε protects breast cancer MCF-7 cells from TNF induced apoptosis (9). To determine whether PKCε has any effect on TNF-induced Akt activation, we compared the ability of TNF to induce Akt phosphorylation at Ser473 in MCF-7 cells transfected with either an empty vector (MCF-7/Neo) or vector

containing wild-type PKCε (MCF-7/PKCε). As indicated in Figure 2, TNF induced Akt phosphorylation in both MCF-7/Neo and MCF-7/PKCε cells in a time-dependent manner. The maximum increase in Akt phosphorylation was evident following treatment with TNF for 30 min. It was difficult to estimate fold-stimulation since basal Akt phosphorylation in MCF-7/Neo cells was undetectable. The extent of Akt phosphorylation was greater in MCF-7/PKCε cells compared to MCF-7/Neo cells during the entire time course.

To further evaluate the effect of TNF on Akt kinase activity, we immnoprecipitated Akt from MCF-7/Neo and MCF-7/PKCε cells with immobilized antibody against Akt, and performed *in vitro* kinase assay using GSK3α/β fusion protein as the substrate. The extent of GSK3α/β phosphorylation was determined by densitometric scanning of immunoblots. Figure 3 shows that TNF induced 1.7-fold stimulation in Akt activity in MCF-7/Neo cells by 30 min. Overexpression of PKCε increased basal Akt activity and TNF caused a time-dependent increase in Akt activity; the maximum Akt activation (3-fold) was achieved following treatment with TNF for 30 min. These results indicate that PKCε may act upstream of Akt to enhance Akt activation by TNF.

To further examine whether PKCε is required for Akt phosphorylation/activation by TNF, we depleted endogenous PKCε using PKCε-specific siRNA. Figure 4A shows that PKCε siRNA caused approximately 60% decrease in PKCε content but had no effect on the levels of other PKC isozymes, such as PKCδ or PKCα. Knockdown of PKCε decreased TNF induced Akt activation but did not affect Akt protein content (Fig. 4B). These results suggest that PKCε activity was required for TNF-induced Akt activation.

Expression of Constitutively-Active Akt Restores Cell Survival in PKCε-Depleted Cells—To examine if Akt functions downstream of PKCε to mediate antiapoptotic signaling, we depleted PKCε by siRNA and monitored TNF-induced apoptosis in MCF-7 cells. Figure 5A shows that knockdown of PKCε alone caused an increase in cell death from 12% to 21%, and it enhanced TNF-induced apoptosis from 45% to 54%. We then examined if overexpression of CA-Akt prevents TNF-induced apoptosis in PKCε-depleted cells. CA-Akt attenuated TNF-induced apoptosis to 24% in control siRNA transfected cells and to 35% in PKCε–depleted cells (Fig. 5B). The average of several independent experiments is shown in Figure 5B. Thus, CA-Akt partially restored cell survival in PKCε-depleted cells.

Knockdown of Akt Inhibits Antiapoptotic Effect of PKCε—To further examine if Akt functions downstream of PKCε, we depleted Akt using Akt-specific siRNA in both MCF-7/Neo and MCF-7/PKCε cells. Figure 6A shows that depletion of Akt enhanced TNF-induced apoptosis as evident by the increase in PARP cleavage. While overexpression of PKCε inhibited TNF-induced PARP cleavage, knockdown of Akt restored sensitivity of MCF-7/PKCε cells to TNF. We also quantified cell death by Annexin V/PI dye binding assay. Figure 5B shows knockdown of Akt alone caused appearance of apoptotic cells in both MCF-7/Neo and MCF-7/PKCε cells. When cells were transfected with control siRNA, TNF caused 55% cell death in MCF-7/Neo cells, and overexpression of PKCε attenuated TNF-induced apoptosis to 12.6%. However, TNF-induced apoptosis was equivalent in both MCF-7/Neo and MCF-7/PKCε cells when Akt was depleted with siRNA (Fig 6B). Thus, depletion of Akt abrogated the

antiapoptotic effect of PKCε, suggesting that Akt acts downstream of PKCε.

Akt Associates With PKCε and DNA-PK—To examine if PKCε directly interacts with Akt, we performed co-immunoprecipitation. MCF-7/Neo and MCF-7/PKCε cells treated with 1 nM TNF were immunoprecipitated with either Akt or PKCε antibody and then Western blot analyses were performed using PKCε or Akt antibody, respectively. Figure 7 shows that PKCε was constitutively associated with Akt in PKCε-overexpressing cells, and TNF enhanced association of Akt with PKCε. We also examined if DNA-PK, a recently identified PDK2, was involved in PKCε-mediated phosphorylation of Akt. Association of Akt and DNA-PK was detected in both control and PKCε-overexpressing cells (Fig. 7). PKCε did not directly interact with DNA-PK but it increased the association between Akt and DNA-PK. These results suggest that PKCε may activate Akt by enhancing the interaction between DNA-PK and Akt.

Depletion of DNA-PKcs Inhibits Akt Phosphorylation and Antiapoptotic Function of Akt and PKCε—To determine if PKCε phosphorylates Akt via DNA-PK, we depleted DNA-PKcs using siRNA and examined the ability of PKCε to phosphorylate Akt. Figure 8 shows that knockdown of DNA-PKcs inhibited TNF-induced Akt phosphorylation in both MCF-7/Neo and MCF-7/PKCε cells. To determine the functional significance of DNA-PK-mediated phosphorylation of Akt on its antiapoptotic function, we compared TNF-induced apoptosis in cells transfected with either control siRNA or siRNA targeted against DNA-PKcs. Figure 9A shows that knockdown of DNA-PK enhanced TNF-induced PARP cleavage in MCF-7/Neo cells. While overexpression of PKCε inhibited TNF-induced PARP cleavage, depletion of DNA-PK partially restored TNF sensitivity in

PKCε-overexpressing cells. Similar results were obtained when we monitored apoptosis using Annexin V/PI dye binding assay (Fig. 9B). These results suggest that PKCε may mediate its antiapoptotic function by regulating TNF-induced Akt phosphorylation via DNA-PK.

PKCε-overexpressing cells. Similar results were obtained when we monitored apoptosis using Annexin V/PI dye binding assay (Fig. 9B). These results suggest that PKCε may mediate its antiapoptotic function by regulating TNF-induced Akt phosphorylation via DNA-PK.

DISCUSSION

The ability of apoptotic stimuli to induce cell death is counteracted by the presence of antiapoptotic proteins. PI3-K/Akt pathway plays an important role in cell survival. We have shown before that a member of the novel PKC family, namely PKCε, acts as an antiapoptotic protein during TNF-induced cell death (9). In the present study, we have investigated if there is any cross-talk between these two important signaling molecules. We have made several important and novel observations. We have demonstrated that PKCε acts upstream of Akt/PKB to exert its antiapoptotic function. First, overexpression of PKCε increased Akt phosphorylation and activity in response to TNF. Second, depletion of PKCε abrogated TNF-induced Akt phosphorylation/activation. Third, knockdown of Akt abolished the antiapoptotic effect of PKCε. We also demonstrated that the activation of Akt by PKCε is mediated by DNA-PK, and depletion of DNA-PKcs reversed the antiapoptotic function of PKCε during TNF-induced apoptosis. The observation that inhibition of DNA-PK can reverse anti-apoptotic signaling by Akt and PKCε establishes a new role for DNA-PK during extrinsic cell death pathway.

Several studies have reported that PKC acts upstream of Akt (16,29,31-42) but there are controversies whether PKC is a positive or a negative regulator of Akt. For example, Doornbos et al. have shown that Akt and PKC did not influence the activity of respective kinases, but growth factor-induced activation of Akt was abolished by PKCζ (32). In contrast, PKCα and -ζ were shown to negatively regulate Akt phosphorylation/activity even though these isozymes are considered prosurvival proteins (33,43). nPKCθ has also

been shown to interact with Akt but this interaction had no effect on the phosphorylation of PKC or Akt (34,44). Inhibition of PKCε by DN-PKCε was associated with inhibition of Akt phosphorylation by three different stimuli whereas other PKC isozymes had no effect, demonstrating that PKCε activity was required for Akt phosphorylation (29). Thus, how Akt and PKC cooperate with each other depends on the extracellular stimulus as well as on the cell type.

We have previously shown that overexpression of PKCε attenuated TNF-induced apoptosis in MCF-7 breast cancer cells (9). However, the status of PKCε alone could not explain TNF sensitivity/resistance (14). For example, although SKBR-3 and CAMA-1 breast cancer cells contained low levels of PKCε, they were highly resistant to TNF (14). We reasoned that multiple signaling pathways that exist in a cell type might decide the final outcome of cell death or survival. Cells that were resistant to TNF contained constitutively active Akt (45). Therefore, we examined if PKCε and Akt trigger parallel survival pathways or if PKCε acts upstream of Akt or *vice versa*. It was difficult to genetically manipulate SKBR-3 and CAMA-1 cells. Since MCF-7 cells express both Akt and PKCε, we manipulated both these kinases at the molecular level to directly demonstrate how these two signaling pathways interact with each other.

We have shown that activation of Akt is an early event following binding of TNF to its cell surface receptors. Complete activation of Akt requires phosphorylation at Thr308 and Ser473 by PDK1 and PDK2, respectively. We have shown that TNF specifically increased phosphorylation of Akt at Ser473 site as has been reported earlier (28). Overexpression of PKCε increased both basal and TNF-induced Akt phosphorylation.

We also directly determined Akt activity using GSK3α/β as the substrate. Akt activity measured in response to TNF was also increased by PKCε overexpression. Furthermore, knockdown of PKCε by siRNA abolished TNF-induced Akt phosphorylation/activation. These results provide direct evidence that PKCε acts upstream of Akt to regulate its activity.

Although there have been several studies that reported Akt may be regulated by PKCε, it is not clear how it regulates Akt activity. It has been reported that PKCε may serve as a substrate for PDK1, and the mechanism by which kinase-dead PKCε inhibited insulin-induced Akt activation was via PDK1 (29). TNF had no effect on Thr308 phosphorylation in MCF-7 cells (data not shown), presumably because PDK1 was constitutively active in these cells as has been reported earlier (17,46). Functional proteomics analysis demonstrated that PKCε directly interacts with Akt in cardiomyocytes (47). We also found that PKCε interacts with Akt in MCF-7 cells. Since phosphorylation of Akt at Ser473 is mediated by PDK2 and DNA-PK has been recently identified as PDK2 (21), we examined the association of Akt and PKCε with DNA-PKcs. Although we were unable to detect any direct interaction between PKCε and DNA-PKcs, overexpression of PKCε enhanced both basal and TNF-induced association between Akt and DNA-PKcs. At present, it is not clear how PKCε enhances interaction between DNA-PK and Akt. However, increased association of DNA-PKcs with Akt could explain how PKCε enhanced Akt activity following treatment of breast cancer cells with TNF.

The involvement of DNA-PK during DNA damage-induced apoptosis is well established. DNA-PK is activated in response to DNA damage (22) and

autophosphorylation of DNA-PKcs has been shown to inhibit DNA-PK activity (48). A link between novel PKCδ and DNA-PK during DNA damage-induced apoptosis has also been established (49). It has been reported that PKCδ associates with DNA-PK and phosphorylation of DNA-PKcs by PKCδ catalytic fragment inhibits the function of DNA-PKcs to form complexes with DNA (49). We have now shown that PKCε can activate Akt/PKB via DNA-PK. Furthermore, depletion of DNA-PK by siRNA not only inhibited the ability of PKCε to enhance TNF-induced Akt phosphorylation at Ser473, it also reversed the antiapoptotic function of PKCε. These results suggest that DNA-PK may also play a critical role in receptor-initiated apoptosis via activation of Akt/PKB.

We also determined if PKCε mediates its antiapoptotic function via Akt. Introduction of constitutively-active Akt in MCF-7 cells conferred resistance to TNF. In addition, while knockdown of PKCε enhanced TNF sensitivity, PKCε-depleted MCF-7 cells expressing CA-Akt were less sensitive to TNF compared to cells that did not express CA-Akt. Furthermore, knockdown of Akt in PKCε-overexpressing MCF-7 cells completely abrogated the antiapoptotic activity of PKCε. These results provide strong evidence that Akt acts downstream of PKCε to mediate TNF-induced apoptosis in breast cancer cells. Thus, a cross-talk between multiple signaling pathways is an important determinant of cell survival and cell death. Furthermore, although the involvement of DNA-PK during DNA damage-induced apoptosis is well known, we have established a new role for DNA-PK during receptor-initiated apoptosis.

FIGURE 1. **Overexpression of constitutively active Akt (CA-Akt) protected MCF-7 cells from TNF-induced cell death.** MCF-7 cells were infected with control adenovirus vector or the vector containing CA-Akt construct. *A*, Western blot analyses were performed with total cell lysates using the indicated antibodies. *B,* Cells were treated with or without 1 nM TNF for 16 h and then stained with annexin V-Alexa 488 conjugate and PI and analyzed using a flow cytometer.

Figure 1.

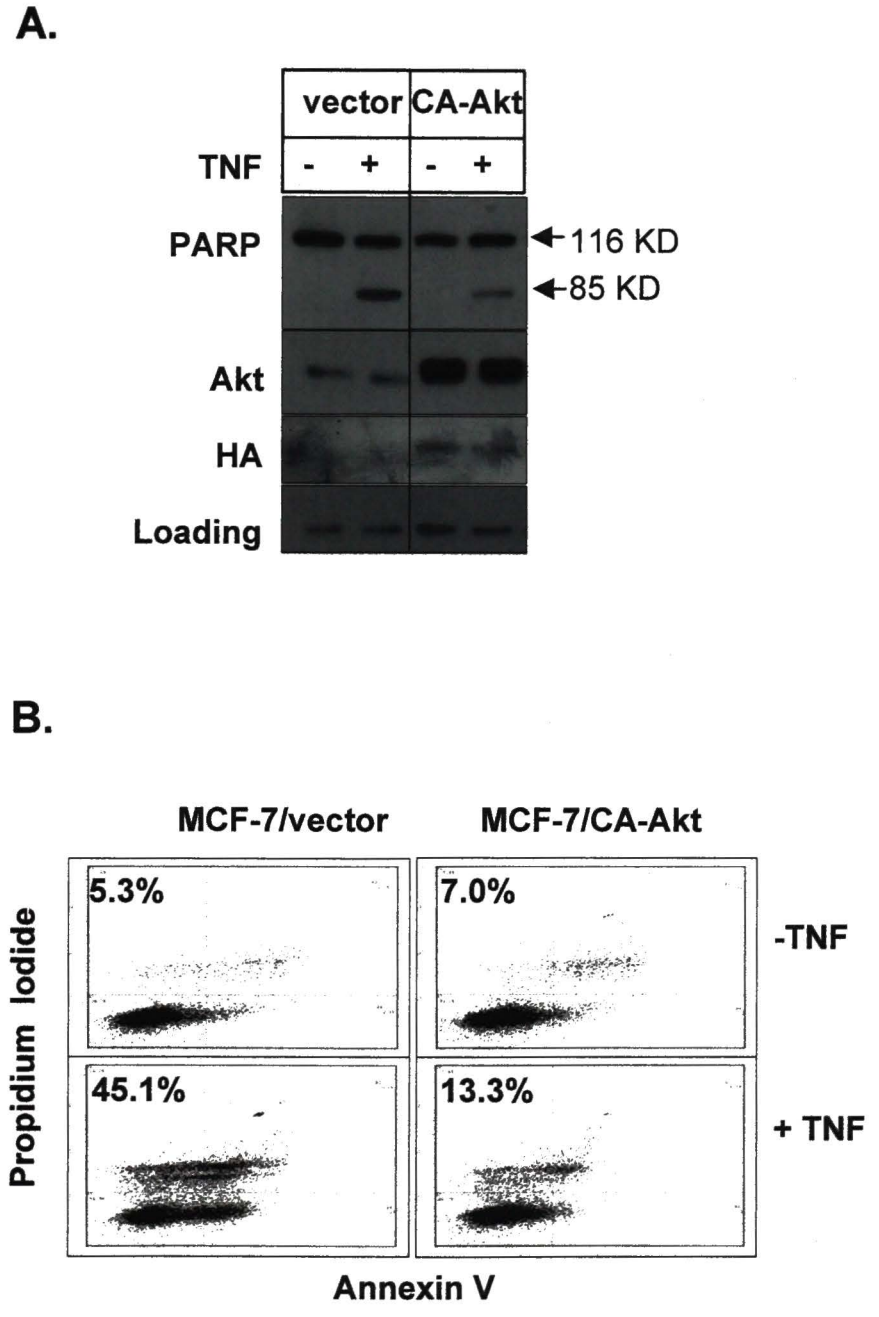

A.
vector
CA-Akt
TNF
- + - +
PARP
116 KD
85 KD
Akt
HA
Loading
B.
MCF-7/vector
MCF-7/CA-Akt
5.3%
7.0%
45.1%
13.3%
-TNF
+ TNF
Propidium Iodide
Annexin V

FIGURE 2. **PKCε overexpression enhanced TNF-induced Akt phosphorylation.** MCF-7 cells were stably transfected with pcDNA3 (MCF-7/Neo) and the vector containing PKCε (MCF-7/PKCε). Cells were serum starved overnight, then treated with 1 nM TNF for the indicated time period. Western blot analyses were performed with total cell extracts using indicated antibodies.

Figure 2.

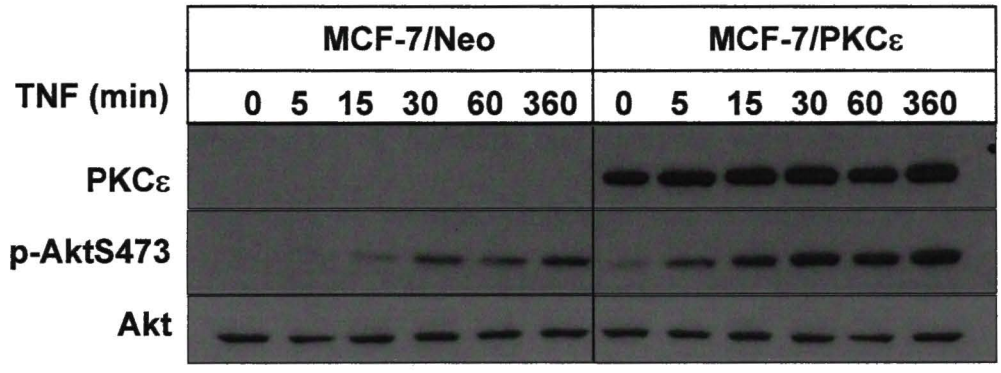
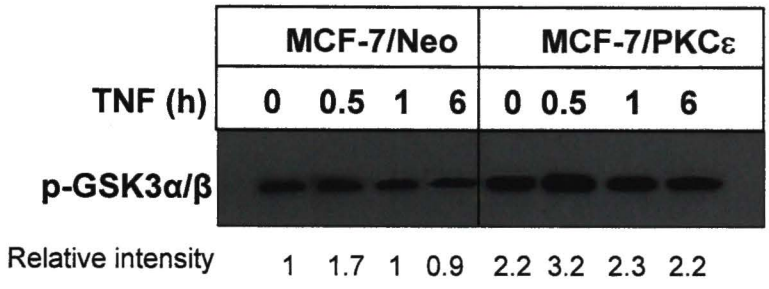

MCF-7/Neo
MCF-7/PKCε
TNF (min)
0 5 15 30 60 360
0 5 15 30 60 360
PKCε
p-AktS473
Akt

FIGURE 3. **Overexpression of PKCε increased TNF-induced Akt activation.** MCF-7/Neo and MCF-7/PKCε cells were serum starved overnight, then treated with 1 nM TNF for the indicated time period. Akt kinase assay was performed as indicated in "experimental procedures". *A,* Western blot analyses were performed using indicated antibodies. *B,* Intensity of phospho-GSK3α/β was determined by densitometry and standardized by loading. The data represent the fold increase in GSK3α/β phosphorylation. Each *bar* represents the mean ± S.E. of two independent experiments. Open bar represents MCF-7/Neo cells; Hatched bar represents MCF-7/ PKCε cells. *, $P<0.01$ versus MCF-7/Neo cells using paired student's t-test.

Figure 3.

A.


MCF-7/Neo
MCF-7/PKCε
TNF (h)
0 0.5 1 6 0 0.5 1 6
p-GSK3α/β
Relative intensity
1 1.7 1 0.9 2.2 3.2 2.3 2.2

B.

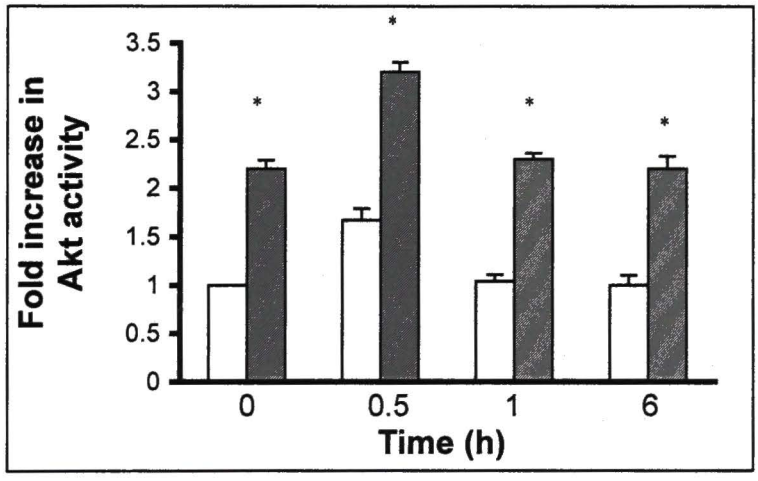

Fold increase in Akt activity
3.5
3
2.5
2
1.5
1
0.5
0
*
0
0.5
1
6
Time (h)

FIGURE 4. **Knockdown of PKCε by siRNA decreased TNF-induced Akt phosphorylation.** MCF-7 cells were transfected with control siRNA or PKCε specific siRNA. Then cells were serum starved overnight and treated with 1 nM TNF for 30 min. *A & B,* Western blot analyses were performed with total cell lysates using indicated antibodies.

Figure 4.

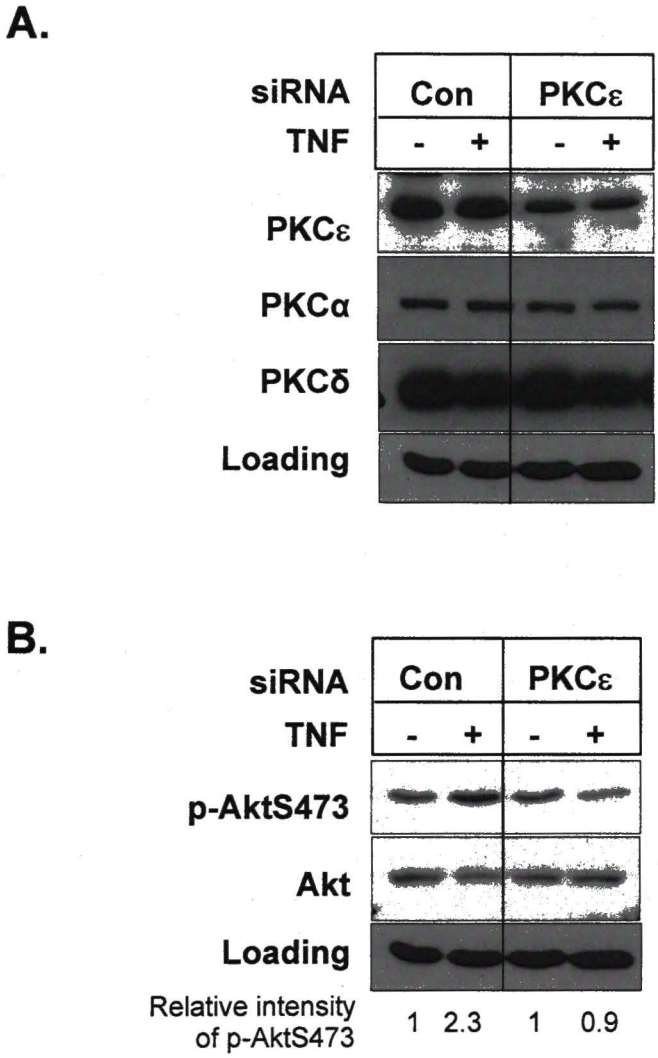

A.
siRNA
Con
PKCε
TNF
- + - +
PKCε
PKCα
PKCδ
Loading
B.
siRNA
Con
PKCε
TNF
- + - +
p-AktS473
Akt
Loading
Relative intensity of p-AktS473
1 2.3 1 0.9

FIGURE 5. **CA-Akt partially restored cell survival in PKCε-depleted cells.** Control siRNA or PKCε specific siRNA were transfected into MCF-7 cells, and adenovirus vector containing either control or CA-Akt constructs were introduced into both cells. Cells were treated with 1 nM TNF for 16 h. *A,* Cells were stained with annexin V-Alexa 488 conjugate and PI and analyzed using a flow cytometer. *B,* The data represent the percentage of apoptosis. Each *bar* represents the mean ± S.E. of two independent experiments. Open bar and hatched bar represent without or with TNF treatment respectively. *, $P<0.01$ versus without TNF treatment. **, $P<0.01$ versus control siRNA transfected MCF-7/Neo cells with TNF treatment. ***, $P<0.01$ versus PKCε siRNA transfected MCF-7/Neo cells with TNF treatment using one way ANOVA.

Figure 5.

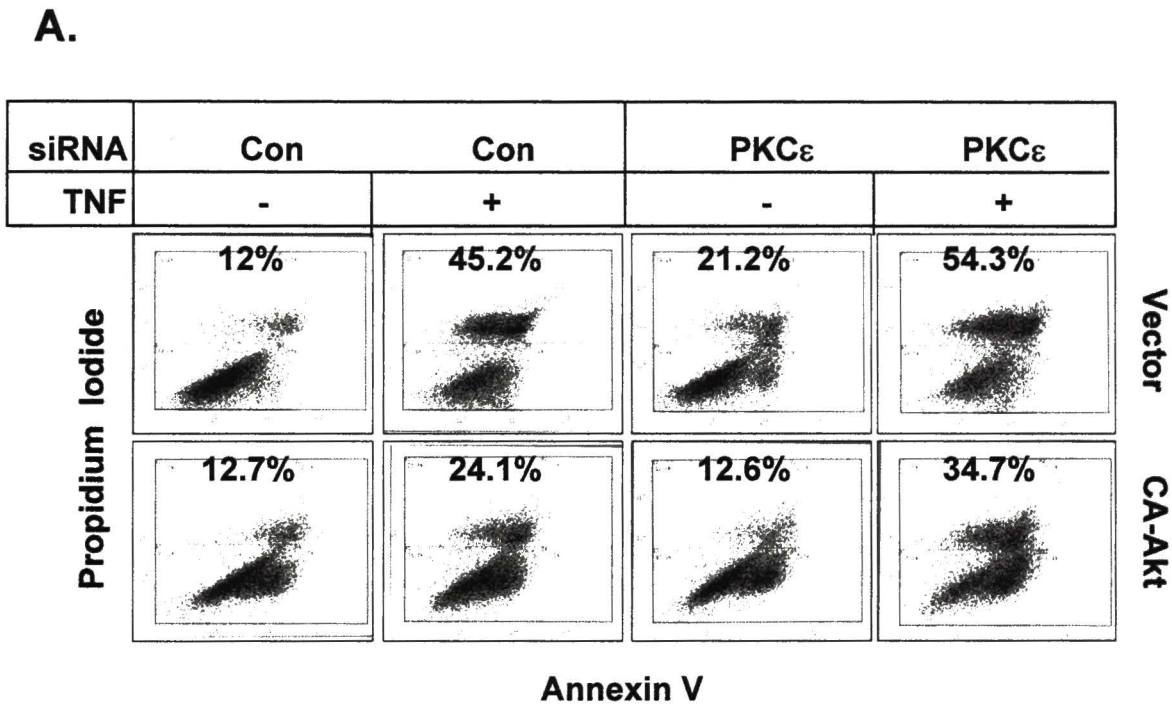

A.
siRNA
Con
Con
PKCε
PKCε
TNF
-
+
-
+
12%
45.2%
21.2%
54.3%
12.7%
24.1%
12.6%
34.7%
Propidium Iodide
Annexin V
Vector
CA-Akt

Figure 5.

B.

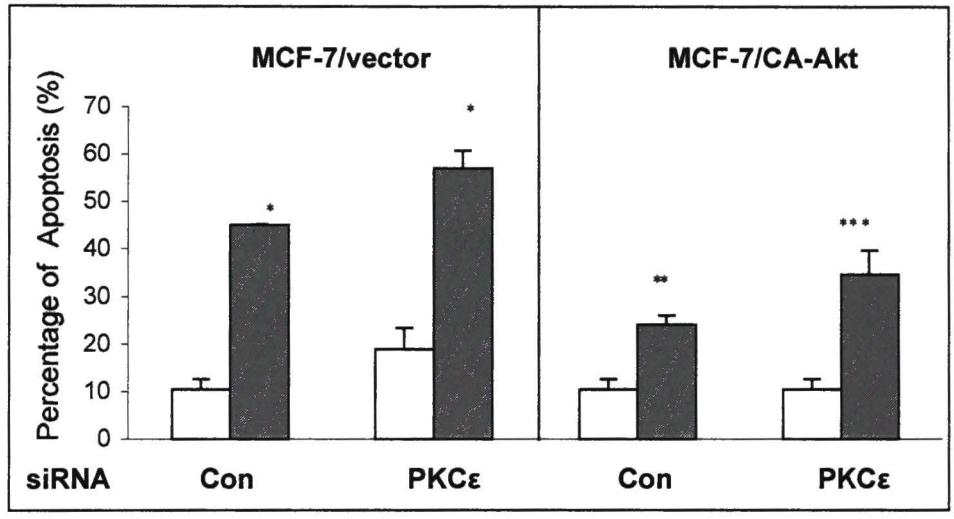

MCF-7/vector
MCF-7/CA-Akt
Percentage of Apoptosis (%)
70
60
50
40
30
20
10
0
*
*
**
***
siRNA
Con
PKCε
Con
PKCε

FIGURE 6. **Depletion of Akt abolished antiapoptotic effect of PKCε.** Control siRNA or Akt specific siRNA were transfected into MCF-7/Neo and MCF-7/PKCε cells. Cells were treated with 1 nM TNF for 16 h. *A,* Western blot analyses were performed with total cell lysates using indicated antibodies. *B,* Cells were stained with annexin V-Alexa 488 conjugate and PI and analyzed using a flow cytometer.

Figure 6.

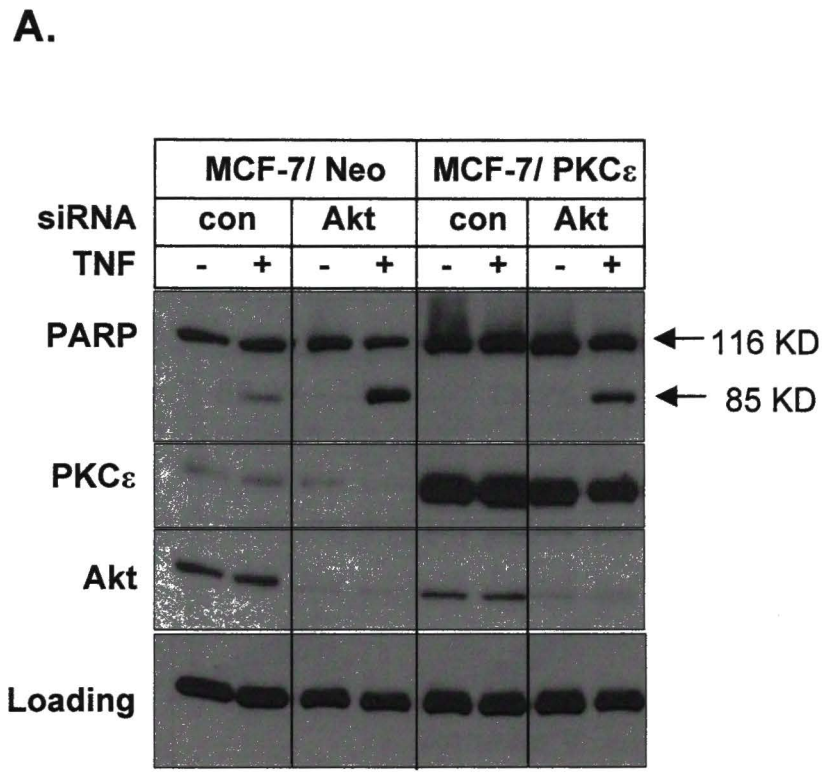

A.
MCF-7/ Neo
MCF-7/ PKCε
siRNA
con
Akt
con
Akt
TNF
- + - + - + - +
PARP
116 KD
85 KD
PKCε
Akt
Loading

Figure 6.

B.

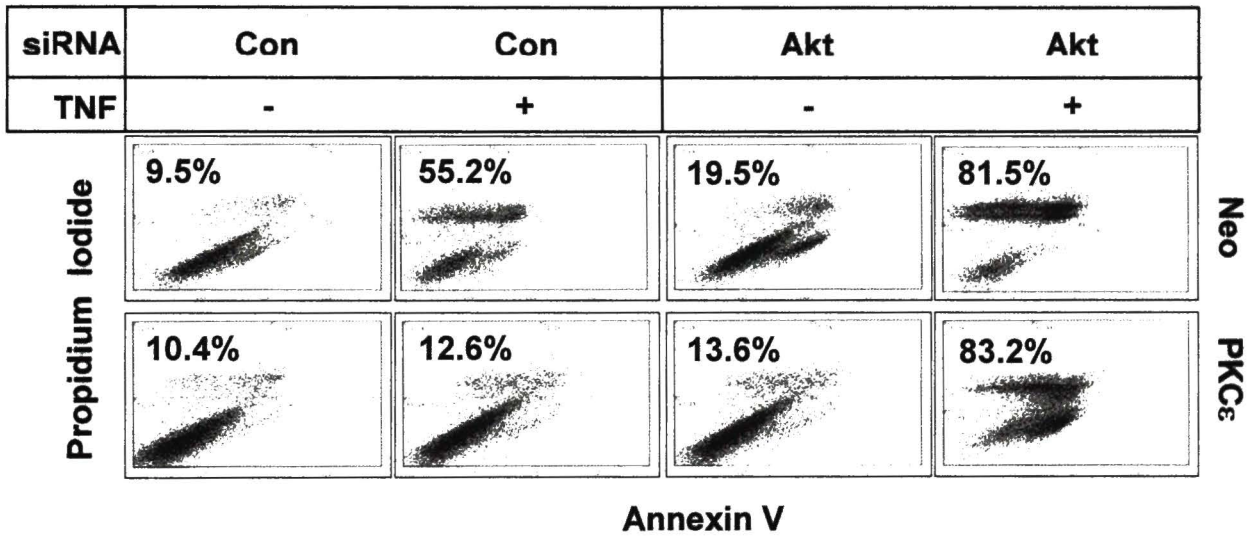

siRNA
Con
Con
Akt
Akt
TNF
-
+
-
+
9.5%
55.2%
19.5%
81.5%
10.4%
12.6%
13.6%
83.2%
Propidium Iodide
Annexin V
Neo
PKCε

FIGURE 7. **Akt Association with PKCε and DNA-PK.** MCF-7/Neo and MCF-7/PKCε cells were serum-starved overnight, and then treated with 1 nM TNF for 30 min. Total cell lyates were immunoprecipitated with Akt or PKCε antibodies. Western blot analyses were performed with total cell lysates and immunocomplexes using the indicated antibodies.

Figure 7.

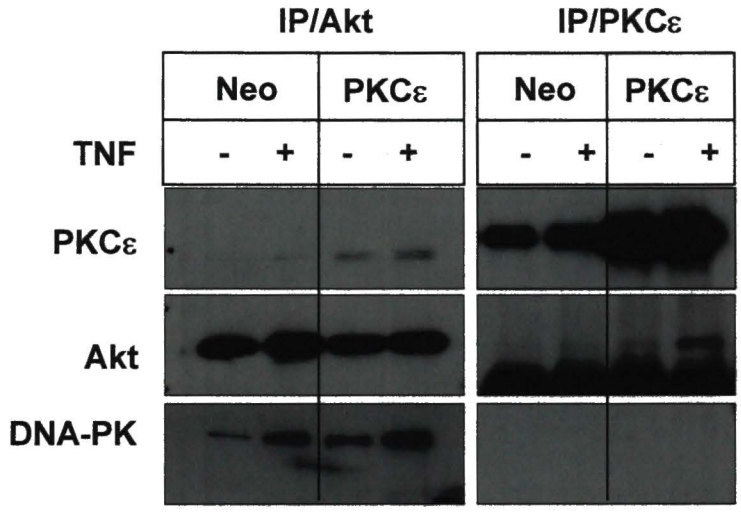


FIGURE 8. **Effect of DNA-PKcs depletion on Akt phosphorylation.** MCF-7/Neo and MCF-7/PKCε cells were transfected with control or DNA-PKcs siRNA. Cells were then serum-starved for 4 h and treated with or without 1 nM TNF for 30 min. Western blot analyses were performed with total cell extracts using indicated antibodies.

Figure 8.

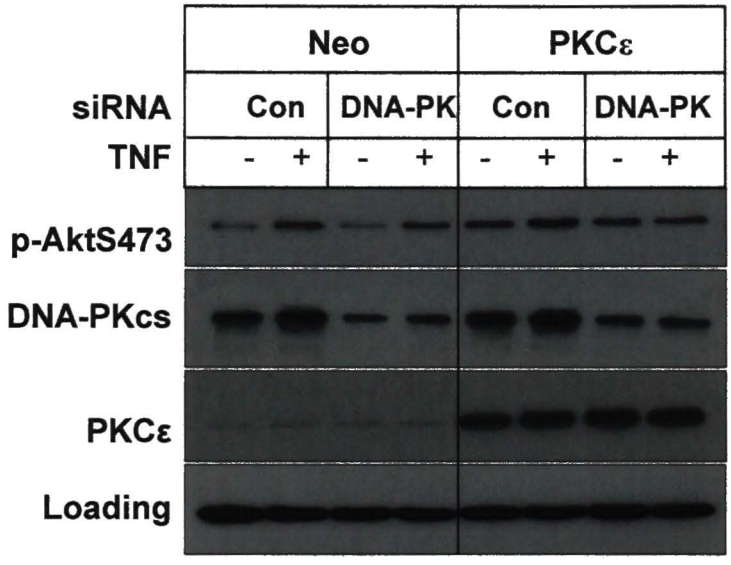
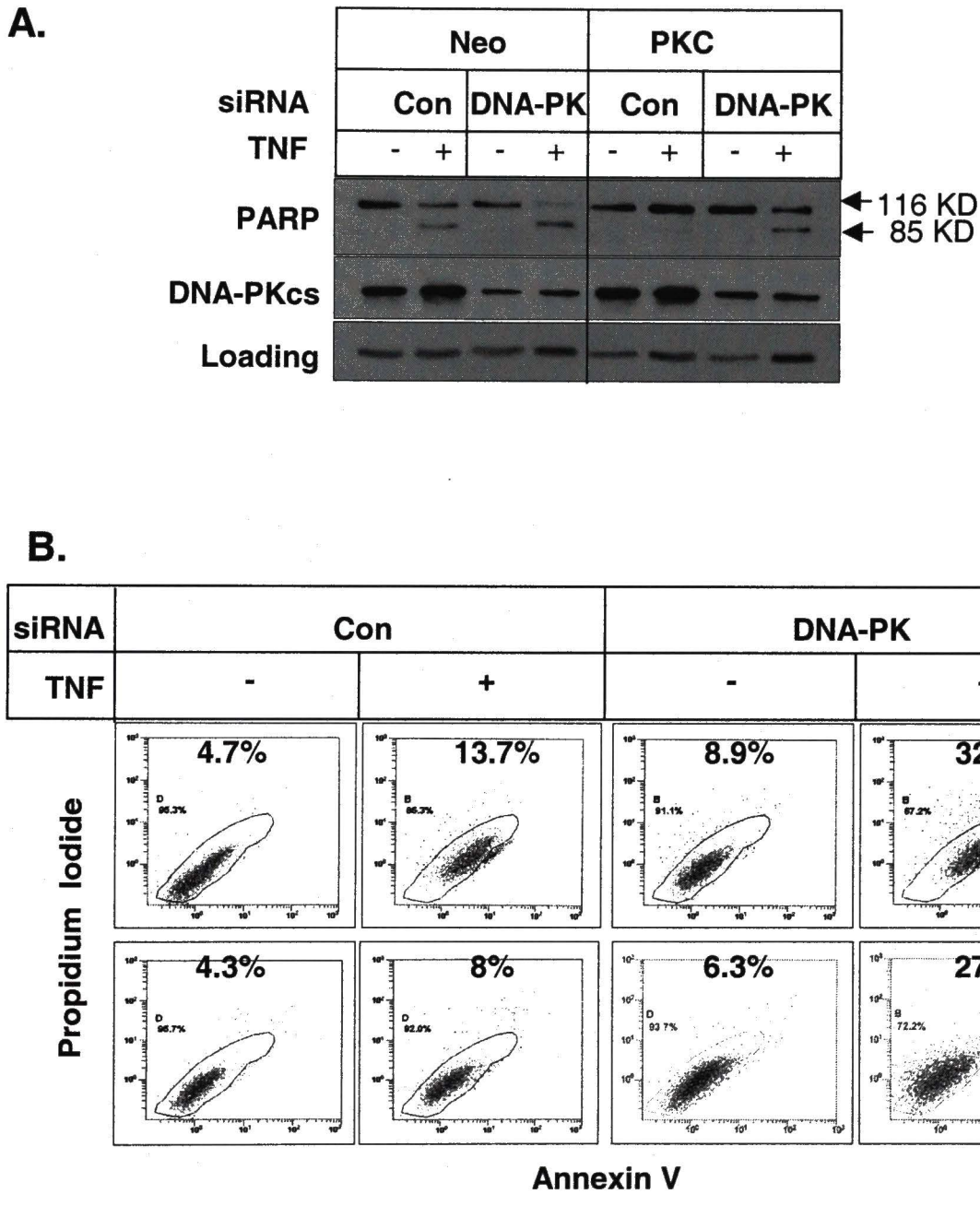

Neo
PKCε
siRNA
Con
DNA-PK
Con
DNA-PK
TNF
- + - + - + - +
p-AktS473
DNA-PKcs
PKCε
Loading

FIGURE 9. **Effect of DNA-PKcs depletion on TNF-mediated cell death.** MCF-7/Neo and MCF-7/PKCε cells were transfected with control or DNA-PKcs siRNA, and treated with or without 1 nM TNF for 10 h. *A,* Western blot analyses were performed with total cell extracts using indicated antibodies. *B,* Cells were stained with annexin V-Alexa 488 conjugate and PI and analyzed using a flow cytometer. Marked region are viable cells. The numbers show the percentage of dead cells including early and late apoptotic cells.

Figure 9.


A.
Neo
PKC
siRNA
Con
DNA-PK
Con
DNA-PK
TNF
- + - + - + - +
PARP
116 KD
85 KD
DNA-PKcs
Loading
B.
siRNA
Con
DNA-PK
TNF
-
+
-
+
4.7%
13.7%
8.9%
32.8%
MCF-7/Neo
4.3%
8%
6.3%
27.8%
MCF-7PKCε
Propidium Iodide
Annexin V

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CHAPTER IV

PROTEIN KINASE C-ε PROTECTS MCF-7 CELLS FROM TNF-MEDIATED CELL DEATH BY INHIBITING BAX TRANSLOCATION

PREFACE

Studies in the previous chapter demonstrated that PKCε acts upstream of Akt to play its antiapoptotic effect in breast cancer cells. TNF-mediated cell death signals can be amplified by intrinsic pathway that is critically regulated by Bcl-2 family members. Mitochondrial depolarization was reported as an early event during TNF treatment in MCF-7 cells. Akt has been shown to phosphorylate and inhibit the proapoptotic function of Bax. However, it is not clear if PKCε also regulates Bax function and further inhibits mitochondrial cell death pathway. Studies in the following section represent unpublished observations that focused on the hypothesis that PKCε inhibits Bax to attenuate the intrinsic cell death signals.

CHAPTER IV

PROTEIN KINASE C-ε PROTECTS MCF-7 CELLS FROM TNF-MEDIATED CELL DEATH BY INHIBITING BAX TRANSLOCATION

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Running Title: antiapoptotic signaling by PKC

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ABSTRACT

Previous studies from our research group have shown that overexpression of PKCε protected breast cancer cells from TNF-induced cell death. However, the mechanism by which PKCε plays an antiapoptotic role is not clear. Bax, a proapoptotic member of Bcl-2 family, is critical for the mitochondria-mediated intrinsic cell death pathway. Translocation of Bax to the mitochondria is important for its pro-apoptotic function. In this paper, we investigated whether Bax was the downstream target of PKCε to regulate TNF-mediated cell death. Overexpression of wild-type PKCε (WT-PKCε) but not dominant-negative PKCε (DN-PKCε) protected MCF-7 cells from TNF-mediated mitochondrial depolarization. Depletion of Bax using siRNA protected MCF-7 cells from TNF-induced cell death. PKCε overexpression decreased the dimerization and traslocation of Bax to mitochondria in MCF-7 cells compared to the vector-transfected cells. Knockdown of PKCε increased Bax dimerization and translocation to mitochondria. Bax was associated with PKCε in MCF-7 cells overexpressing PKCε. Together, there observations suggest that interaction of PKCε and Bax is important for the antiapoptotic role of PKCε in breast cancer cells.

INTRODUCTION

TNF, a multifunctional cytokine, was originally characterized by its anti-tumor activity (1, 2). TNF has two distinct receptors, TNFR1 (CD120a; p55/60) and TNFR2 (CD120b; p75/80) (3-5), through which TNF plays its biological function. TNFR1 is the major receptor to mediate cell death signaling. TNF induces cell death primarily by binding to TNFR1 followed by recruitment of caspase-8 in the death-inducing signal complex (DISC), resulting in activation of caspase cascade (2). In addition, TNFR1-mediated cell death pathway is amplified by activation of mitochondria-mediated intrinsic pathway through the caspase-mediated cleavage of Bid, a pro-apoptotic member of Bcl-2 family member (1, 2). Although TNF induces apoptosis in cancer cells including breast cancer, some cancer cells are resistant to TNF.

PKC is a family of phospholipid-dependent serine/threonine kinases that consist of at least 10 isozymes that have distinct, and in some cases, opposing roles in cell survival and apoptosis (6-8) . The PKCs are divided into three groups based on their structural and activation characteristics: conventional or classical PKCs (cPKCs: α, βI, βII and γ), novel PKCs (nPKCs: δ, ε, η and θ) and atypical PKCs (aPKCs: ζ and λ/ι) (6-8). While activation of cPKCs requires Ca^{2+} and diacylglycerol (DAG)/phorbol esters, nPKCs are Ca^{2+}-independent, but still need DAG/phorbol esters for activation; aPKCs are not sensitive to either Ca^{2+} or DAG/phorbol esters.

PKCε plays an important role in apoptosis and tumorigenesis (9-13). PKCε has oncogenic effects when overexpressed in several fibroblast and colonic and prostatic

epithelial cells (14-16). Inhibition of PKCε can suppress tumor promotion (17). PKCε has been reported to be cleaved by caspase-3/-7 or calpain during apoptosis (18, 19). Our laboratory has shown that overexpression of PKCε attenuated TNF-induced apoptosis in breast cancer MCF-7 cells (18). In addition, breast cancer cells containing high level of PKCε were sensitized to TNF by PKC inhibitor (20). However, the mechanism by which PKCε plays an antiapoptotic role is not clear.

Bcl-2 family members are key regulators of apoptosis (21). This family consists of about 20 homologues of important pro- and antiapoptotic regulators of programmed cell death. The established mode of function of the individual members is to either preserve or disturb mitochondrial integrity, thereby inducing or preventing release of apoptogenic factors like cytochrome *c (Cyt c*) from mitochondria.

Bax, a proapoptotic member of the Bcl_2 family is a monomeric cytosolic protein in healthy primary tissue cells, which upon apoptotic stimulus, changes its conformation and translocates to the mitochondria (21). Bax then integrates into the mitochondria where it undergoes oligomerization (21). Whether such mitochondrial Bax oligomers form Bax pores or influence existing mitochondrial channels is not completely clear. In either case, release of apoptogenic factors, like *Cyt c,* from the mitochondria sets the stage for the apoptosomal amplification loop causing activation of caspase-9 (22).

Recent evidence implicates Bax in death receptor-mediated apoptosis (23). Down-regulation or inactivation of pro-apoptotic Bax-like death factors is observed in several human cancers (24). Interestingly, Bax was reported to be associated with PKCε and this interaction promoted cell survival in prostate cancer cells (25). In the current study, we

found that PKCε inhibits TNF-mediated cell death through its interaction with Bax in breast cancer cells.

Materials

TNF was purchased from R&D Systems (Minneapolis, MN). Monoclonal antibody to PARP was obtained from Pharmingen (San Diego, CA). Polyclonal antibody against PKCε and Bax was from Santa Cruz (Santa Cruz, CA). JC-1 (5,5', 6, 6',-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide), Mitotracker red CMXRos, anti-rabbit secondary antibody conjugated to Alexa-568, Annexin V conjugated to Alexa Fluor 488 and propidium iodide were purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmuno Research Lab. Inc. (West Grove, PA). Poly(vinylidene difluoride) membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL). Protein G Plus/Protein A Agarose suspension was from Oncogene Research Products (Boston, MA). Control short interfering RNA (siRNA) and Bax-specific siRNA were obtained from Dharmacon RNA Technologies (Lafayette, CO). PKCε specific siRNA was from Santa Cruz (Santa Cruz, CA).

Cell Culture and Transfection

Breast cancer cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine, and kept in a humidified incubator at 37^0C with 95% air and 5% CO2. Cells were transfected with plasmids using FuGENE 6 transfection reagent (Boehringer Manheim) according to the manufacturer's

protocol and selected using geneticin (Life Technologies). siRNA was transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Immunoblot Analysis

Equivalent amounts of total cellular extracts were electrophoresed by SDS-PAGE and transferred electrophoretically to poly(vinylidene difluoride) membrane. Immunoblot analyses were performed as described previously (26).

Co-immunoprecipitation

Equivalent amounts of total cellular lysates were incubated with the precipitating antibody against PKCε or Bax overnight, followed by 1 hr incubation with 30 μl of protein G Plus/Protein A agarose suspension. Immune complexes on beads were then washed three times in wash buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM DTT, 50 mM β-glycerophosphate, 50 mM NaF, 10 μg/ml phenymethylsulfonyl fluoride, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 4 μg/ml pepstatin [pH 7.4]) followed by centrifugation at 2,000 rpm for 5 minutes. All incubations were performed at 4°C with rocking. Samples were boiled in 2x sample buffer for 5 minutes. The immunocomplex was separated on SDS-PAGE and transfer to PVDF membrane. The presence of Bax or PKCε was detected using specific antibodies in Western blot.

Assessment of Apoptosis by Annexin V/Propidium Iodide Binding Assay

Cells were treated with or without TNF for 16 h. At the end of the incubation, both detached cells and attached cells were collected and washed with PBS. Cells were then

stained with Annexin V-Alexa 488 conjugate and propidium iodide according to the manufacturer's protocol and analyzed using a flow cytometer (Coulter Epics).

Measurement of Mitochondrial Potential

Mitochondrial potential was assessed by using the fluorescent potentiometric dye JC-1 (5,5', 6, 6',-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) according to Manufacture's protocol (Molecular Probes). Briefly, the cells were trypsinized and centrifuged for 8 min at 450 × *g* at room temperature and resuspended in 1 ml of staining solution (PBS containing 10% FBS and 5 μg/ml JC-1). Cells were stained for 15 min in a 37°C incubator (5% CO_2). After staining, cells were collected at room temperature and washed once with PBS. The cell pellet was then resuspended in PBS (prewarmed to 37°C), and JC-1 fluorescence was quantified by using a flow cytometer (Coulter Epics).

Subcellular Fractionation

Cells were trypsinized, pooled together with media and washes containing floating cells, and pelleted by centrifugation at 500 × *g* for 3 min at 4 °C. Pellets were resuspended with 3 volumes of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine, 1 mM dithiothreitol, 250 mM sucrose, plus protease and phosphatase inhibitors), lysed with a Dounce homogenizer, and fractionated by differential centrifugation. Briefly, homogenates were centrifuged twice at 500 × *g* for 5 min at 4 °C, and the nuclear pellet was resuspended in buffer A, sonicated, and stored at -80 °C in multiple aliquots. The supernatants were combined and further centrifuged at 10,000 × *g* for 30 min at 4 °C, and the resultant mitochondrial pellets were resuspended in buffer A, sonicated, and stored at -80 °C in

multiple aliquots. The supernatant from the 10,000 × *g* spin was further centrifuged at 100,000 × *g* for 15 min at 4 °C. The resulting supernatant was designated cytosolic fraction and stored at -80 °C in multiple aliquots. Protein concentrations were adjusted to 1 mg/ml of protein (Bradford), and equal amounts of protein were loaded for immunoblotting.

Immunocytochemistry

MCF-7 cells grown on microscope cover glass (Fisher Scientific) were treated with TNF. 200 nM Mitotracker Red CMXRos was added into the cells and incubated for 45 min before harvesting. The cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Slides were washed three times for 5 min each time with PBS and the cells were then permeabilized and blocked for 30 min at room temperature with PBS plus 2% BSA containing 1% NP-40. The slides were then incubated with Bax polycolonal antibody (N-20; Santa Cruz), diluted 1:100 in blocking buffer overnight at 4 °C in a humidified chamber. Slides were washed four times for 5 min each time with PBS, followed by incubation for 1 h at room temperature with anti-rabbit secondary antibody conjugated to Alexa-568 (Molecular Probes, Eugene, OR) diluted 1:800 in blocking buffer. Slides were washed three times for 5 min with PBS, mounted, and visualized on Zeiss LSM410 confocal microscope with a krypton/argon laser.

Statistical analysis

Data are presented as the mean ± S.E. and $n \geq 3$ unless otherwise specified. Statistical significance was determined using SigmaStat 2.03 (Systat Software, Inc. Point Richmond, CA). $P < 0.01$ was considered statistically significant.

Overexpression of WT-PKCε but not DN-PKCε protected MCF-7 cells from TNF-induced mitochondrial depolarization.

Mitochondria are critical organelles for cell survival, and decreased mitochondrial membrane potential triggers the release of the proapoptotic molecules, such as *Cyt c,* into the cyotsol to mediate apoptosome formation. Overexpression of PKCε protected MCF-7 cells from TNF-mediated cell death (18). We asked whether PKCε overexpression regulated mitochondrial function. JC-1, the mitochondrial membrane potential sensor was employed to stain the MCF-7 cells stably transfected with pcDNA3, WT-PKCε or DN-PKCε. JC-1 is a cationic mitochondrial potential sensitive dye that exhibits a fluorescence emission shift from green (525 nm) to red (590 nm) upon depolarization of mitochondria. As seen in Figure 1, TNF treatment induced mitochondrial depolarization in 17.2% of MCF-7/Neo cells compared to 0.04% of MCF-7/PKCε cells and 20% in MCF-7/DN-PKCε cells. These data suggest that PKCε protected mitochondria from depolarization during TNF treatment, and PKCε activity is critical for the protective effect.

Knockdown of Bax decreased TNF-mediated cell death

To examine whether Bax is required for TNF-mediated cell death in MCF-7 cells, Bax-specific siRNA and control siRNA were transfected into the cells. Figure 2A shows that transfection of MCF-7 cells with Bax siRNA but not control siRNA caused 90% depletion of Bax. Depletion of Bax did not change the level of other Bcl-2 family

members such as Bcl-2, Bcl-X_L, and Bid. TNF-mediated cell death was inhibited by Bax depletion as evident by a decrease in PARP cleavage shown in Figure 2A. Annexin V/PI binding assay (Figure 2B) showed that the incidence of cell death was decreased to 17% in Bax-depleted cells compared to 56% in control siRNA transfected cells following treatment with TNF. These results indicate that Bax is required for TNF-mediated cell death in MCF-7 breast cancer cells.

Effect of PKCε overexpression on Bax dimerization and translocation

Oligomerization of Bax and translocation to mitochondria are essential for its pro-apoptotic function. We asked if PKCε regulated the dimerization of Bax. Figure 3 showed that Bax formed a dimer upon TNF treatment, and the dimerization was more evident in MCF-7/Neo cells than in MCF-7/PKCε cells. To determine the translocation of Bax, cytosolic and mitochondrial fractions were isolated after 1 nM TNF treatments for 8 h. Western blot was performed to detect Bax level in the subcellular fractions. Figure 4 showed that Bax was significantly translocated to the mitochondria in MCF-7/Neo cells but not in MCF-7/PKCε cells with TNF treatment. Furthermore, immunostaining data showed that Bax was evenly distributed in the cytoplasm and translocated to the mitochondria in MCF-7/Neo cells with TNF treatment in a time-dependent manner, but not in MCF-7/PKCε cells as indicated in Figure 5A and 5B. These results suggest that PKCε regulates TNF-mediated cell death in MCF-7 cells by regulating Bax dimerization and translocation to mitochondria.

Effect of PKCε depletion on Bax dimerization and translocation

To further confirm the role of PKCε in the regulation of Bax conformational change, PKCε was knocked down using siRNA against PKCε. Figure 6 shows that Bax formed a dimer following TNF treatment, and the extent of dimerization was greater in PKCε-depleted cells than in control siRNA transfected cells. To determine the translocation of Bax, immunostaining was performed to determine the colocalization of Bax with mitochondria. Figures 7A and B show that colocalization of Bax with mitochondria was increased with TNF treatment. Bax translocation to mitochondria was increased to approximately 50% in PKCε-depleted cells compared to 20% in control siRNA transfected cells. These results suggest that PKCε is essential to protect TNF-mediated Bax dimerization and translocation to mitochondria.

Association of PKCε and Bax

To investigate if PKCε and Bax were associated in breast cancer MCF-7 cells, co-immunoprecipitation was performed. Interaction of PKCε and Bax was detected in MCF-7/PKCε cells but not in MCF-7/Neo cells as indicated in Figure 8. The association between these two proteins was increased after TNF treatment in MCF-7/PKCε cells.

DISCUSSION

PKCε plays antiapoptotic role in TNF-mediated cell death in breast cancer cells (18). TNF has been shown to stimulate cell death not only through receptor-initiated pathway, but it can also amplify death signal through mitochondrial pathway (3-5). Bax is a critical proapoptotic molecule to regulate the mitochondria-mediated intrinsic cell death pathway (21). Here we report that PKCε protects TNF-mediated mitochondrial depolarization by inhibiting Bax. This is substantiated by the following observations. First, overexpression of WT-PKCε, but not DN-PKCε abolished TNF-mediated mitochondrial depolarization. Second, depletion of Bax protected MCF-7 cells from TNF-mediated cell death. Third, PKCε overexpression diminished TNF-induced Bax dimerization as well as translocation to mitochondria. Knockdown of PKCε increased dimerization and translocation of Bax to mitochondria. Finally, interaction between Bax and PKCε was detected by co-immunoprecipitation.

Protein kinase C family members play important role in regulating cell survival and cell death. PKCε has tumorigenic potential when overexpressed in some epithelial cells and inhibition of PKCε may suppress tumor promotion (14-16). Previous studies showed that PKC inhibitor sensitized breast cancer MCF-7 cells to TNF-mediated cell death and overexpression of WT-PKCε protected MCF-7 cells from apoptosis (18, 20). Introduction of DN-PKCε into MCF-7 cells induced apoptosis and depletion of PKCε sensitized breast cancer cells to TNF (18), suggesting that PKCε is critical for the survival of MCF-7 cells. In this study, we investigated the molecular mechanism by which PKCε plays antiapoptotic role in MCF-7 cells.

PKCε was shown to stimulate cell survival by various signaling pathways: including Raf-1/MEK/ERK kinase cascade, NIK/IKK/NF-kappaB activation (27). In addition, stress-activated kinases p46/p54 JNKs, are activated by PKCε (28). The colocalization of PKCε and three MAPKs, ERKs, JNKs and p38MAPK with mitochondria was reported in cardiomyocytes (28). However, the mechanism by which PKCε inhibits TNF-mediated cell death in breast cancer cells is not clear.

In the present study, TNF-mediated mitochondrial depolarization was abolished by overexpression of WT-PKCε but not by DN-PKCε, suggesting that PKCε regulates its antiapoptotic signal at the level of mitochondria, and the activity of PKCε is critical for this regulation.

Mitochondria play a central role in commitment of cells to apoptosis via increased permeability of outer mitochondial membrane and decreased transmembrane potential followed by release of *Cyt c* and apoptosis-inducing factors. Antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-X_L can block these mitochondrial events, whereas proapoptotic members, including Bax can trigger these changes (29). It has been shown that Bax resides largely in cytosol in healthy cells. Apoptotic stimuli induce a conformational change of the Bax protein, resulting in exposure of its N-and C-termini that appears to be required for its insertion into mitochondrial membranes (30). Once integrated into membranes, Bax can form dimers and larger oligomers, causing *Cyt c* release and apoptotic cell death (30).

In the present study, depletion of proapoptotic protein Bax protected MCF-7 cells from TNF-induced cell death, suggesting that Bax was required for cell death mediated

by TNF. To determine if Bax function is regulated by PKCε, Bax dimerization and translocation were determined following TNF treatment in MCF-7/Neo and MCF-7/PKCε cells. TNF induced dimerization of Bax and its translocation to the mitochondria. Bax dimmerization was decreased in PKCε-overexpressing cells compared to vector-transfected cells. Immunostaining and subcellular distribution studies showed that TNF-induced translocation of Bax from the cytosol to mitochondria was prevented by PKCε overexpression. Furthermore, Bax dimerization and translocation was increased by depletion of PKCε using siRNA.

The mechanisms regulating Bax conformational change and oligomerization are not clear. Bax contains a C-terminal conserved hydrophobic motif, which appears to be critical for mitochondrial targeting and proapoptotic function. The C-terminal α9 helix of Bax, which contains the BH3 domain, forms stable interface with the hydrophobic BH3 binding pocket of itself (31). This interface may prevent dimer formation in the cytosol. Alternatively, helical packing/bundling at the interface of Bax and its binding partners also influence oligomerization (31).

Co-immunoprecipitation results showed that PKCε interacts with Bax in the MCF-7/PKCε cells but not in MCF-7/Neo cells. The results indicated that the decrease in cell death induced by TNF in PKCε-overexpressing cells might be due to the association of PKCε with Bax. Whether the interaction between Bax and PKCε is direct or indirect needs to be studied further. Based on the current literature, the interaction of Bax with PKCε appears to be indirect and may be mediated by other intermediate molecules. PKCε does not contain BH3 domain or BH3 binding pocket. The eight-stranded, anti-

parallel β-sandwich structure of the C2-like domain of PKCε, which is important for the interaction with other molecules (32), does not appear to be suited for the helical/bundling with Bax. The present study suggests that PKCε may protect MCF-7 cells from TNF-mediated cell death by inhibiting Bax conformational rearrangement that is important for Bax oligomerization, mitochondrial integration and function. The data presented in this study provide valuable insight about the mechanism by which PKCε inhibits the TNF-mediated cell death pathway.

Figure 1. Effect of PKCε overexpression on mitochondrial depolarization. MCF-7 cells were stably transfected with pcDNA3 (MCF-7/Neo), vector containing wild-type PKCε (WT-PKCε) or dominant-negative PKCε (DN-PKCε). Cells were treated with 1 nM TNF for 8 h. Cells then were harvested for JC-1 staining and analyzed using a flow cytometer.

Figure 1.

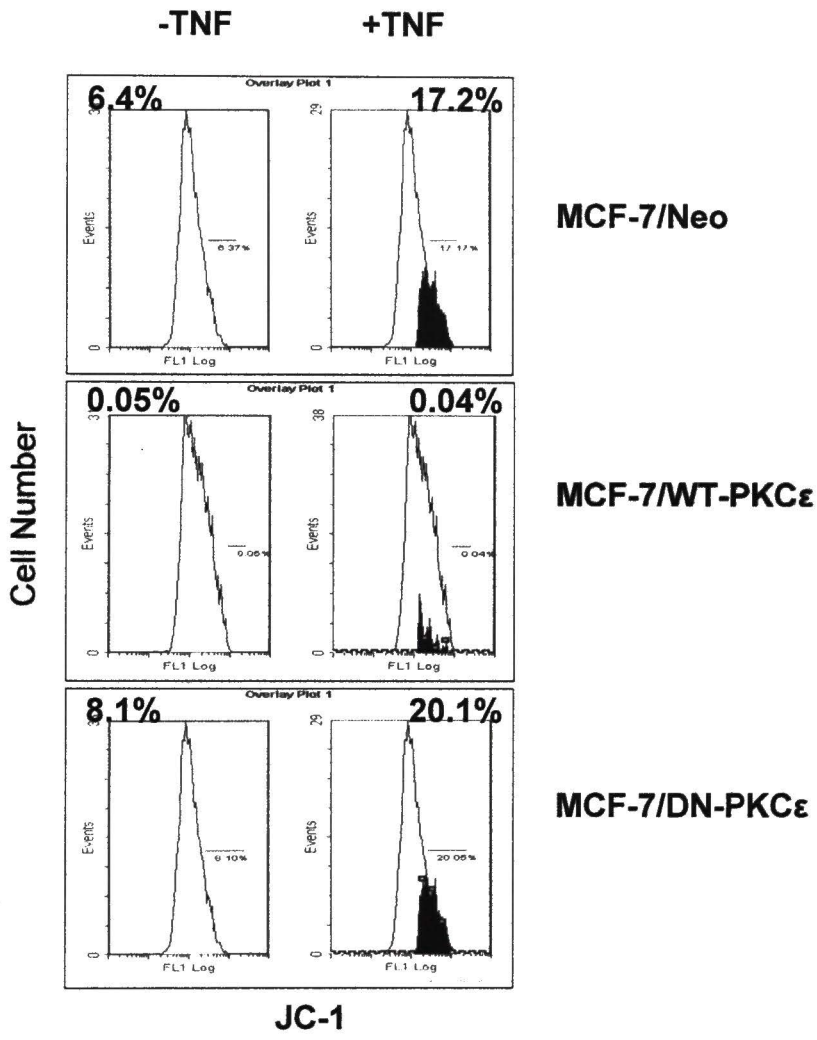

-TNF
+TNF
6.4%
17.2%
0.05%
0.04%
8.1%
20.1%
Overlay Plot 1
Events
FL1 Log
MCF-7/Neo
MCF-7/WT-PKCε
MCF-7/DN-PKCε
Cell Number
JC-1

Figure 2. Effect of Bax depletion on TNF-mediated cell death. MCF-7 cells were transfected with control siRNA or Bax-specific siRNA. The cells were treated with 1 nM TNF for 16 h. A. Western blot analysis was performed using indicated antibodies. B. Cell death was determined using Annexin/PI binding assay and detected using a flow cytometer.

Figure 2.

A.

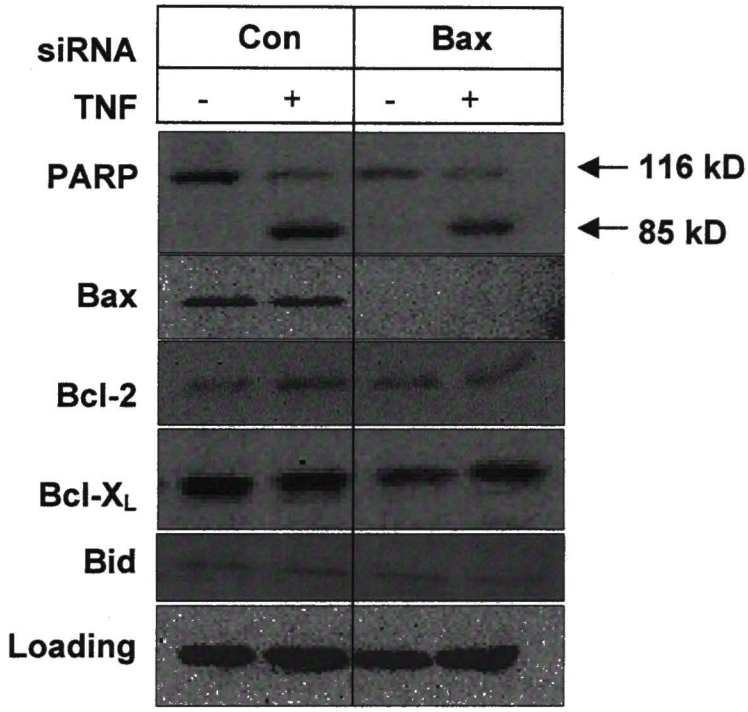


Figure 2.

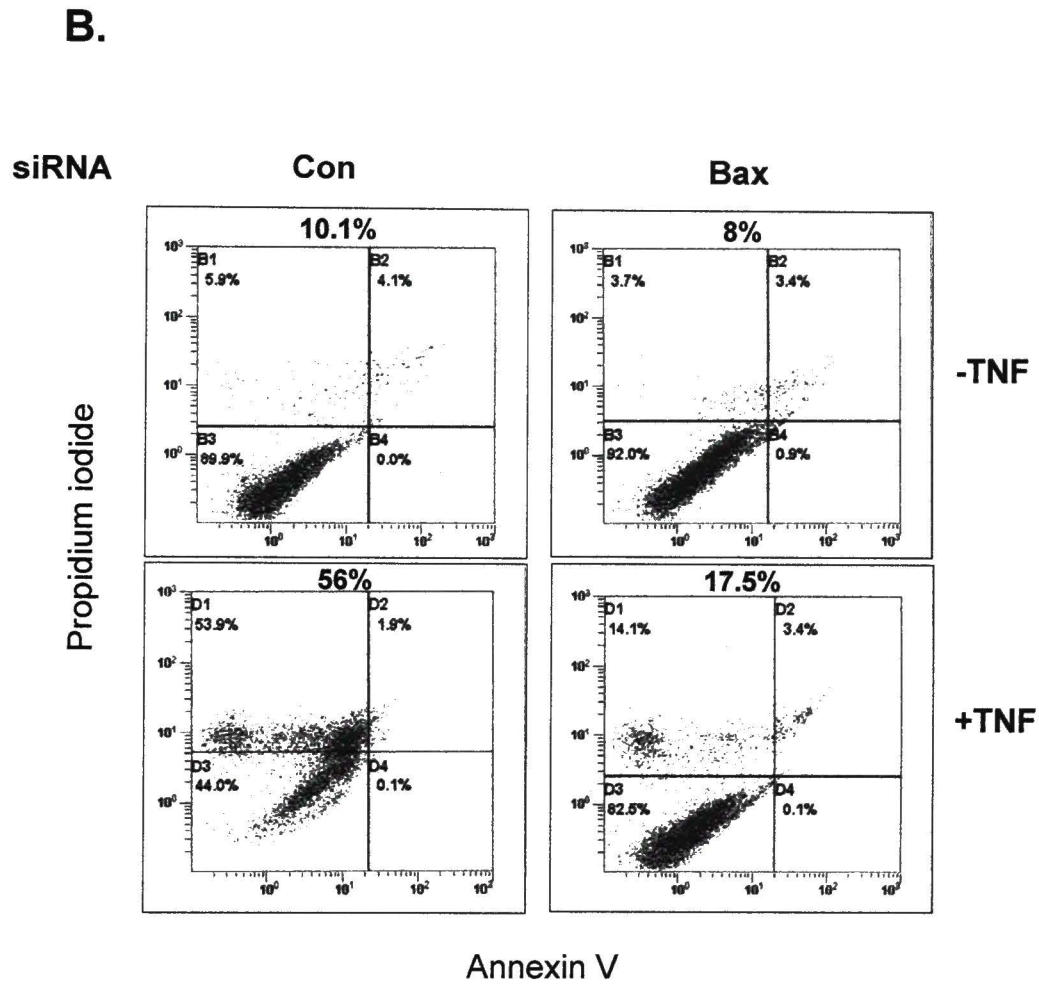

B.
siRNA
Con
Bax
10.1%
8%
B1
5.9%
B2
4.1%
B3
89.9%
B4
0.0%
B1
3.7%
B2
3.4%
B3
92.0%
B4
0.9%
-TNF
56%
17.5%
D1
53.9%
D2
1.9%
D3
44.0%
D4
0.1%
D1
14.1%
D2
3.4%
D3
82.5%
D4
0.1%
+TNF
Propidium iodide
Annexin V

Figure 3. Effect of PKCε overexpression on Bax dimerization. MCF-7/Neo and MCF-7/PKCε cells were treated with 1 nM TNF for 16 h. Western blot analysis was performed with total cell extracts using the indicated antibodies.

Figure 3.

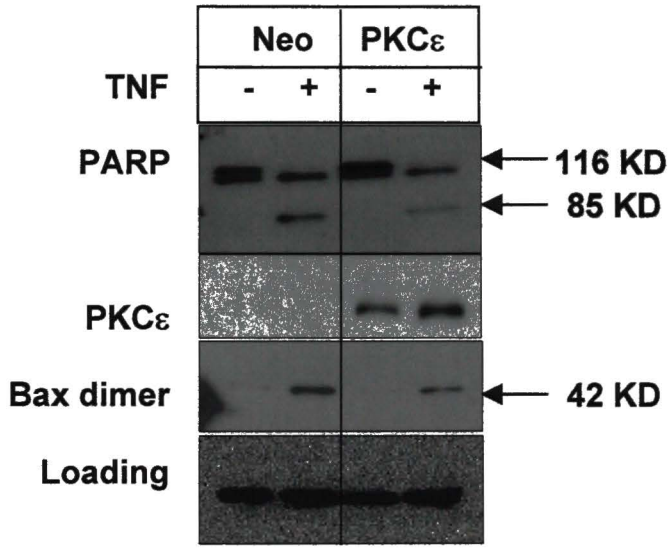


Figure 4. Effect of PKCε overexpression on Bax subcellular distribution. MCF-7/Neo and MCF-7/PKCε cells were treated with 1 nM TNF for 8 h prior to subcellular fractionation. The cytosolic and mitochondrial fractions were normalized for protein content and subjected to Western blot analysis with indicated antibodies. Hsp60 was used as a loading control for mitochondrial fraction.

Figure 4

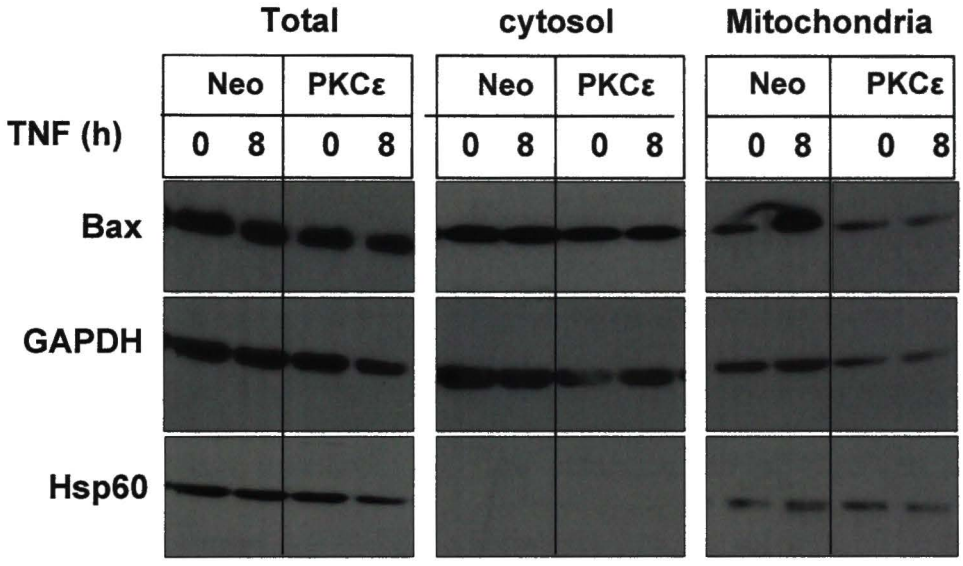

Total
cytosol
Mitochondria
Neo
PKCε
Neo
PKCε
Neo
PKCε
TNF (h)
0 8 0 8
0 8 0 8
0 8 0 8
Bax
GAPDH
Hsp60

Figure 5. Effect of PKCε overexpression on Bax translocation. A. MCF-7/Neo and MCF-7/PKCε cells were treated with 1 nM TNF for 8 h. Cells were treated with 200 nM Mitotracker red CMXRos (Mito) to stain for mitochondria and Alexa 568-conjugated secondary antibody to detect Bax and then analyzed by confocal microscopy, and the two images were overlaid (overlay). B. The colocalization of Bax and mitochondria following TNF treatment was quantified using the Image-J software. The data represent the percentage of colocalization of Bax and mitochondria. Each bar represents the mean ± S.E. of three independent fields. *, $P<0.01$ versus MCF-7/Neo cells using paired student's t-test.

Figure 5.

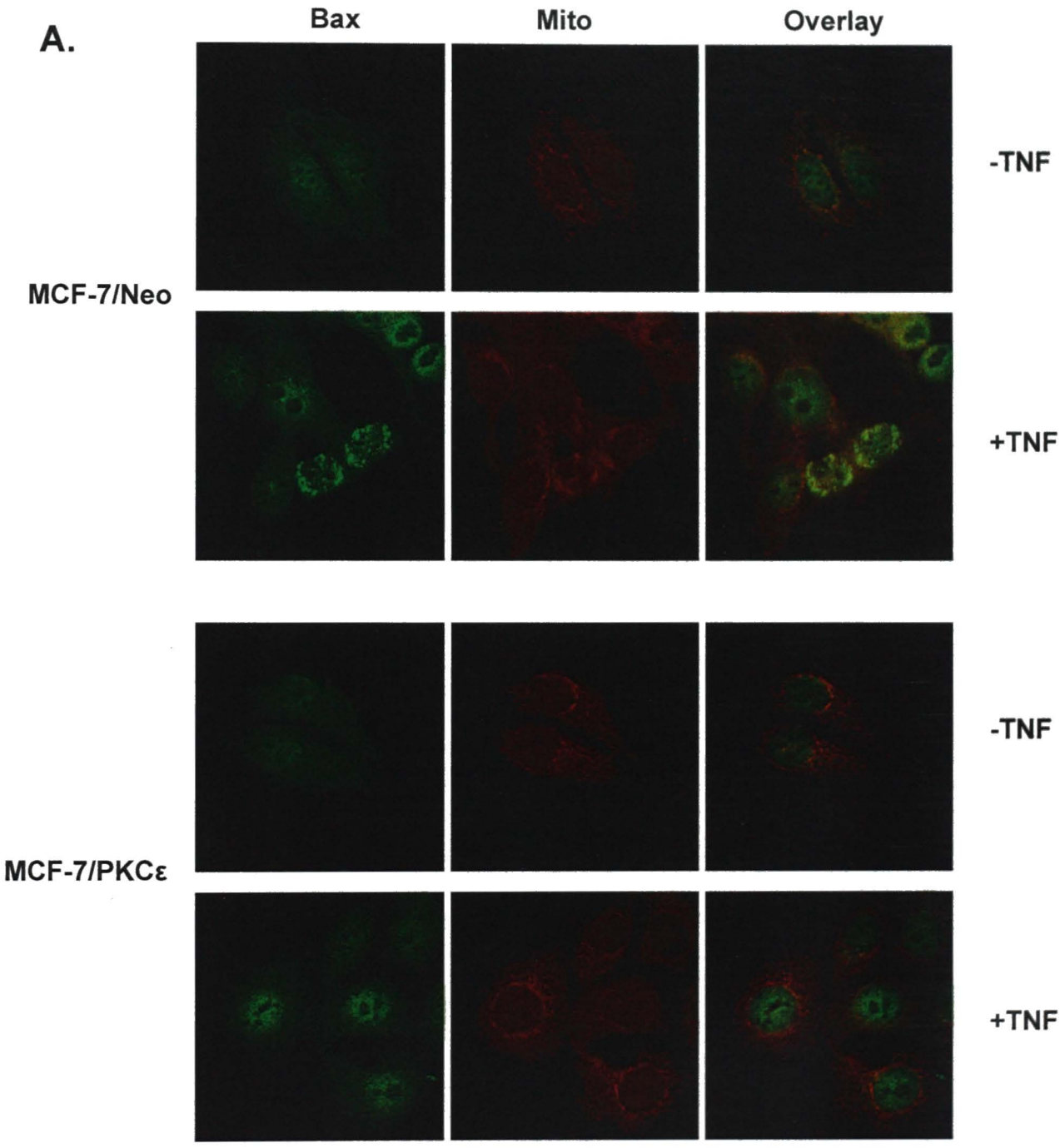


Figure 5.

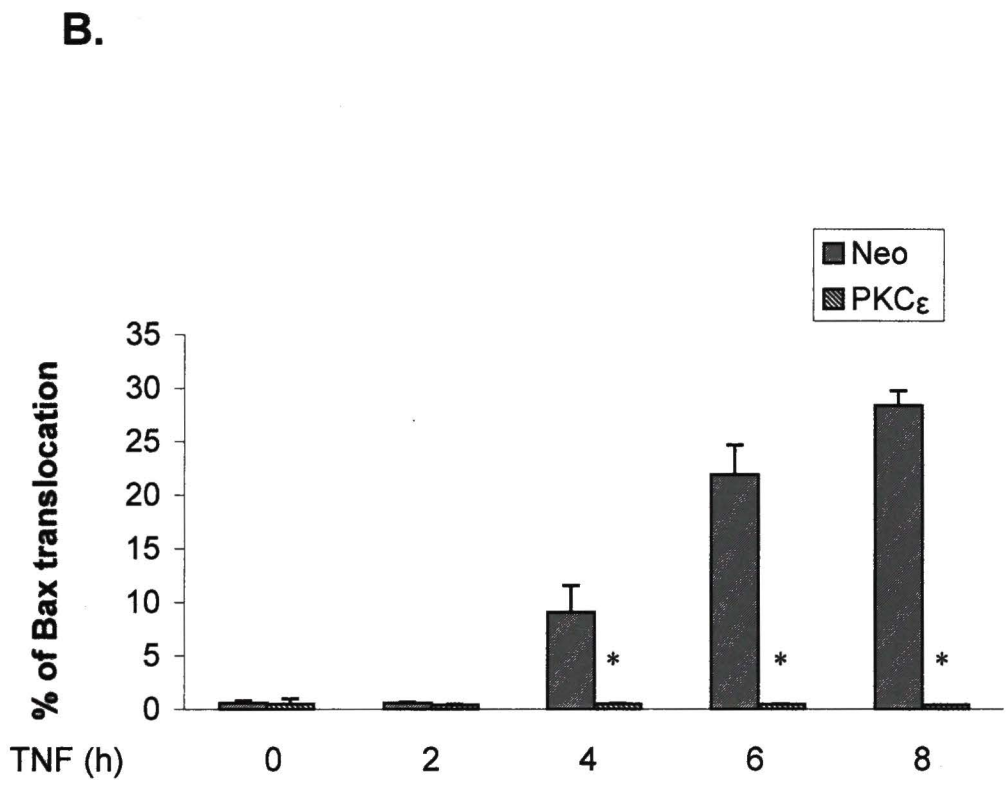


Figure 6. Effect of PKCε depletion on Bax dimerization. MCF-7 cells were transfected with control or PKCε siRNA. Cells were treated with 1 nM TNF for 16 h. Western blot analysis was performed with total cell extracts using the indicated antibodies.

Figure 6

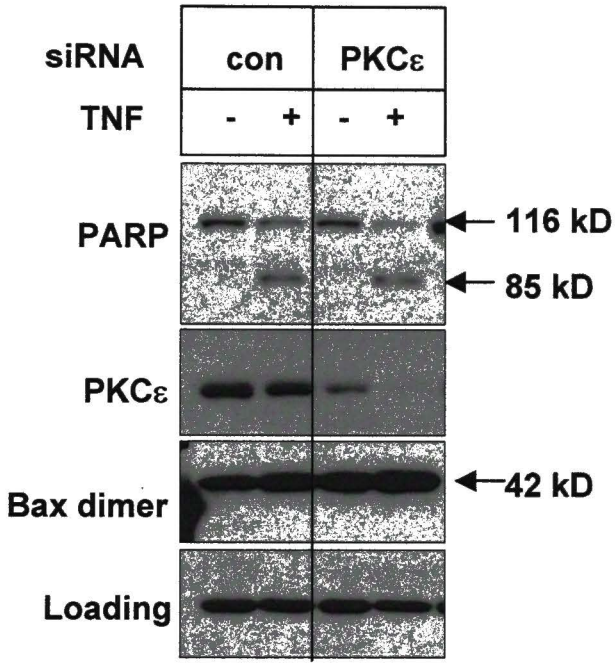

siRNA
con
PKCε
TNF
- + - +
PARP
116 kD
85 kD
PKCε
Bax dimer
42 kD
Loading

Figure 7. Effect of PKCε depletion on Bax translocation. MCF-7 cells were transfected with control or PKCε siRNA. Cells were then treated with 1 nM TNF for 8 h. A. Cells were treated with 200 nM Mitotracker red CMXRos (Mito) to stain for mitochondria and Alexa 568-conjugated secondary antibody to detect Bax and then analyzed by confocal microscopy, and the two images were overlaid (overlay). B. The colocalization of Bax and mitochondria following TNF treatment was quantified using the Image-J software. The data represent the percentage of colocalization of Bax and mitochondria. Each bar represents the mean ± S.E. of three independent fields. *, $P<0.01$ versus control siRNA transfected cells using paired student's t-test.

Figure 7.

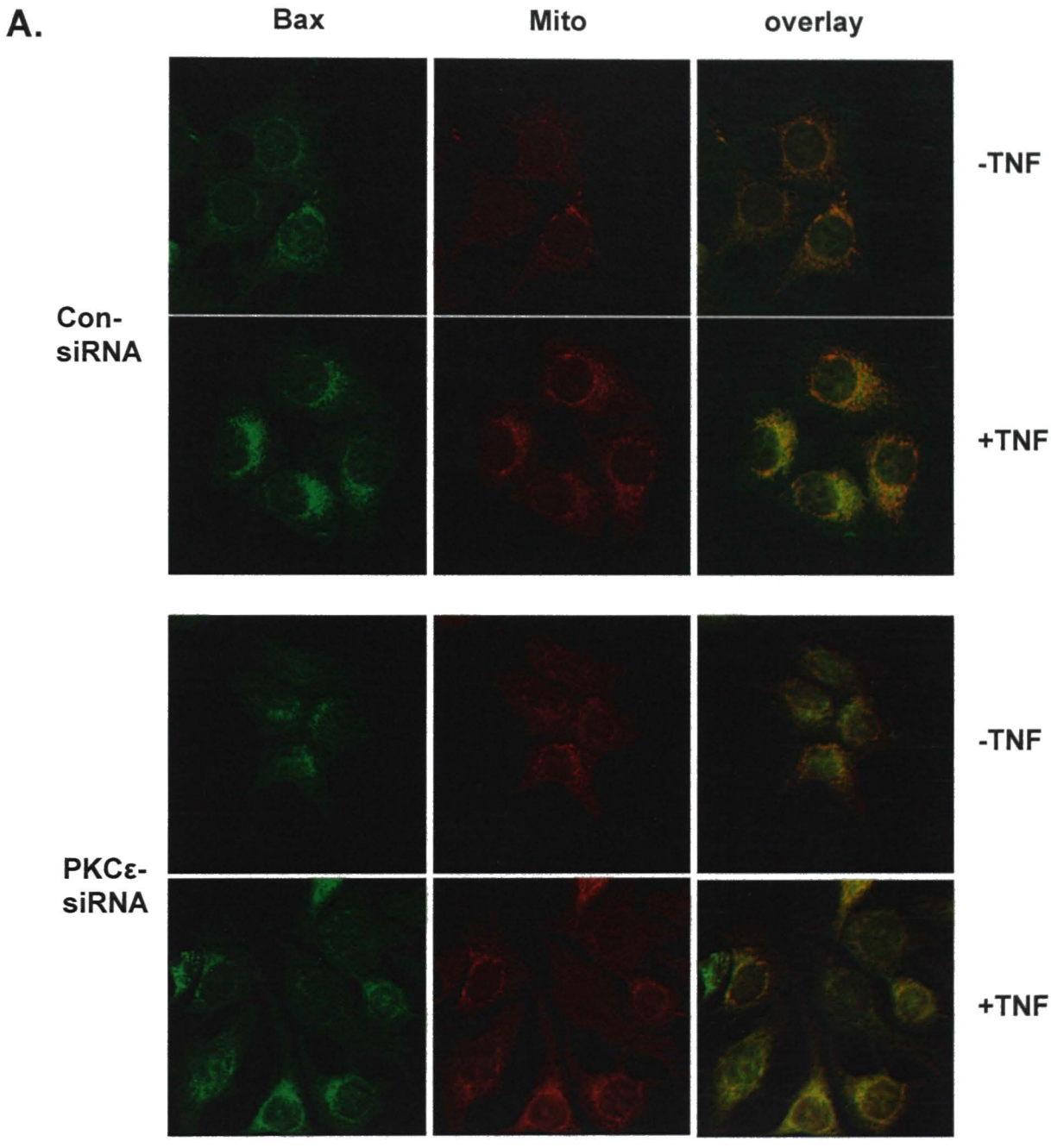


Figure 7.

B.

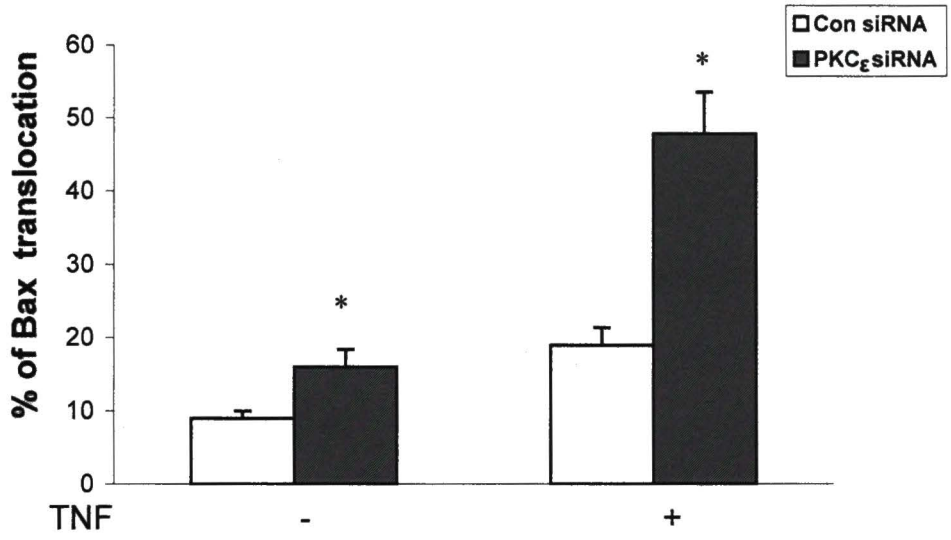
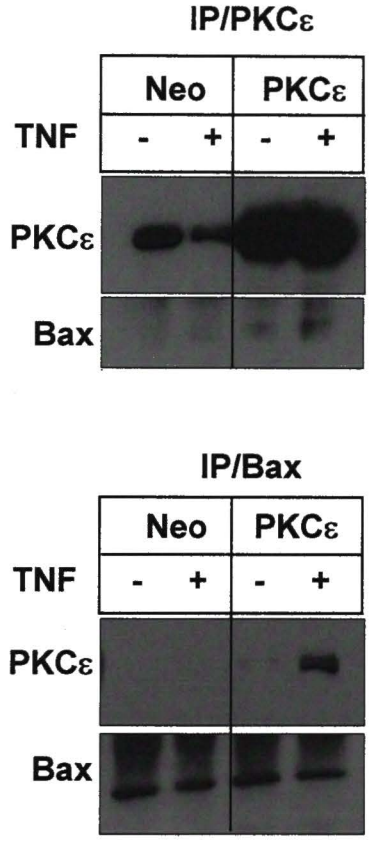

Con siRNA
PKCε siRNA
% of Bax translocation
60
50
40
30
20
10
0
*
*
TNF
-
+

Figure 8. Association of Bax and PKCε. MCF-7/Neo and MCF-7/PKCε cells were treated with 1 nM TNF for 12 h. PKCε or Bax were immunopricipitated from cell lysate using appropriate antibodies. Immunocomplex was then subjected to Western blot analysis using the indicated antibodies.

Figure 8.



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CHAPTER V

CONCLUSIONS

TNF causes selective destruction of tumor cells but many malignant cells are resistant to TNF. The purpose of this dissertation is to investigate the molecular mechanism(s) by which breast cancer cells evade apoptosis. We particularly focused on how the PKCε signaling pathway confers resistance to TNF cytotoxicity. Data described in this dissertation demonstrate that PKCε activates Akt via DNA-PK to mediate antiapoptotic signals. In addition, PKCε attenuates the mitochondrial cell death pathway by inhibiting Bax translocation.

PKC isoforms play an important role in the regulation of cell death. Previous studies in our laboratory have shown that the PKC inhibitor bisindolylmaleimide (BIM) sensitized breast cancer cells that express high level of PKCε to TNF (1). Overexpression of WT-PKCε protected MCF-7 cells from apoptosis while introduction of DN-PKCε enhanced cell death (2), suggesting that PKCε acts as an antiapoptotic protein. However, the protein level of PKCε alone was not sufficient to explain TNF resistance in breast cancer cells (2). For example, SKBR-3 and CAMA-1 cells contain low level of PKCε, and these cells are highly resistant to TNF. Thus, there must be some other cell survival signals that counteract TNF cytotoxicity in these cells.

Akt has been shown to be overexpressed or activated in breast cancer cells (3). Therefore, the first part of this dissertation focused on the hypothesis that activation of Akt causes resistance to TNF in breast cancer cells. Data described in the chapter II demonstrated that the activation status of Akt is another critical determinant of TNF sensitivity in breast cancer cells. Breast cancer SKBR-3 cells contained constitutively-active Akt. PI3-K/Akt inhibitor Ly294002 alone induced cell death in these cells and it further enhanced TNF-mediated cell death, suggesting that PI3-K/Akt pathway is responsible for the resistance of these breast cancer cells to TNF. In contrast, MDA-MB-231 cells, which contain a high level of PKCε but no phospho-Akt, were sensitized to TNF by the PKC inhibitor BIM, but not by Ly294002, indicating that PKCε is the predominant antiapoptotic molecule to mediate cell survival in MDA-MB-231 cells. Both PKC and PI3-K/Akt inhibitors sensitized MCF-7 and BT-20 cells to TNF, indicating that both PKC and Akt signal transduction pathways are critical in deciding the cellular response to TNF in these cells. Therefore, multiple signaling pathways determine the final outcome of cell death or survival in breast cancer cells. Excisional biopsies, which are obtained from patients before they are treated with chemotherapies or radiation can be screened for PKCε and Akt expression using immunohisochemistry. Patients containing high level of PKCε but no phospho-Akt can be treated with specific PKCε inhibitors or siRNA against PKCε in combination with TNF. Akt inhibitors or Akt-specific siRNA can be used in combination with TNF to treat breast cancers that contain constitutively-active Akt but low level of PKCε. As for the cells that contain both PKCε and phospho-Akt, the antiapoptotic signaling is more complex. Therefore, an

understanding of how PKCε and Akt cooperate with each other in the antiapoptotic signaling network is important for developing effective cancer therapies.

Breast cancer MCF-7 cells express both PKCε and Akt, and the cells were sensitized to TNF by inhibiting either PI3-K/Akt or PKC pathways. Therefore, MCF-7 cells were used as a model system to examine if PKCε and Akt trigger parallel survival pathways or if PKCε acts upstream of Akt or *vice versa.*

Although MCF-7 cells overexpress Akt, phospho-Akt level is low due to the presence of wild type PTEN. Introduction of constitutively-active Akt in MCF-7 cells inhibited TNF-induced apoptosis, indicating that Akt activation is required for its antiapoptotic function. Complete activation of Akt requires phosphorylation at Thr308 and Ser473 by PDK1 and PDK2, respectively (4, 5). In the present study, TNF induced Akt activation in MCF-7 cells by increasing phosphorylation at Ser473 but not at Thr308, presumably because these cells express constitutively-active PDK1 (6, 7). Overexpression of PKCε increased both basal and TNF-induced Akt phosphorylation/activation. Knockdown of PKCε by siRNA inhibited Akt activation by TNF, indicating that PKCε acts upstream of Akt and is required for Akt phosphorylation/activation in MCF-7 cells. PKCε depletion by siRNA increased TNF-mediated cell death, while introduction of CA-Akt partially restored cell survival in PKCε-depleted MCF-7 cells. On the other hand, knockdown of Akt by siRNA in PKCε-overexpressing MCF-7 cells abolished the antiapoptotic effect of PKCε. These results suggest that one mechanism by which PKCε plays its antiapoptotic function is through activation of Akt.

There are several potential mechanisms by which PKCε may interact with Akt. Constitutively-active PKCε has been reported to upregulate the gene expression of heat shock protein 90 (HSP90) (8), which binds to Akt and protects it from PP2A-mediated dephosphorylation (9). However, HSP90 level was not changed by either PKCε overexpression or depletion in my preliminary studies. Another study showed that PKCε might be activated by PDK1 and may further cooperate with PDK1 to phosphorylate Akt on Ser473 (10). We reported in this dissertation that Akt was associated with PKCε as well as a newly identified PDK2 (DNA-PK) in MCF-7 cells, and PKCε overexpression increased the interaction between Akt and DNA-PK. DNA-PK has been reported to be colocalized and associated with Akt at the plasma membrane and to phosphorylate Akt at Ser473 in the hydrophobic motif (11). Our data suggest that introduction of exogenous PKCε facilitates interaction between Akt and DNA-PK and results in an increase in Akt phosphorylation/activation. Depletion of DNA-PK by siRNA abolished TNF-mediated Akt activation and attenuated the antiapoptotic effect of PKCε. Therefore, we made a novel finding that PKCε activates Akt via DNA-PK to mediate its antiapoptotic effect in breast cancer cells. These results also indicate that DNA-PK not only plays an important role in DNA damage-induced apoptosis, but also regulates the receptor-mediated or extrinsic cell death pathway through Akt.

Receptor-initiated cell death can be amplified via the mitochondrial or intrinsic cell death pathway. There is evidence that mitochondria serve as an early target during TNF-induced cytotoxicity (12). The results presented in this dissertation demonstrate that PKCε protected TNF-mediated mitochondrial depolarization by inhibiting Bax

translocation. Overexpression of WT-PKCε, but not DN-PKCε abolished mitochondrial depolarization mediated by TNF, indicating that PKCε regulates the intrinsic cell death pathway. Depletion of Bax inhibited TNF-mediated cell death in MCF-7 cells, suggesting that Bax is required for TNF-initiated cell death. PKCε overexpression diminished TNF-induced Bax dimerization as well as translocation to mitochondria. In contrast, depletion of PKCε increased Bax dimerization and translocation. Furthermore, PKCε was shown to interact with Bax by coimmunoprecipitation. These results suggest that interaction of PKCε with Bax inhibits the conformational change of Bax upon TNF stimulation, thus preventing Bax translocation and thereby attenuating the mitochondrial cell death pathway. The results in chapter III showed that PKCε associated with Akt in breast cancer cells. Akt has been reported to phosphorylate Bax at Ser184 and inhibit Bax proapoptotic function (13). It is possible that Akt mediates the interaction between PKCε and Bax. If PKCε regulates Bax function independent of Akt, Bax would be a common target for both PKCε and Akt. Therefore, PKCε may cooperate with Akt and inhibit Bax conformational change by direct phoshphorylation of Bax at a site other than Ser184.

In summary, as depicted in Figure 1, this dissertation demonstrated that PKCε activates Akt via DNA-PK to mediate its antiapoptotic signals. Furthermore, PKCε inhibits the mitochondrial cell death pathway by inhibition of proapoptotic Bcl-2 family member Bax. This dissertation provides novel information regarding the mechanisms by which PKCε inhibits TNF-mediated cell death, and it provides valuable insights that may be exploited for therapeutic benefit.

Future Directions

We demonstrated that PKCε activated Akt phosphorylation/activation via DNA-PK in breast cancer cells. However, the mechanism by which PKCε regulates DNA-PK remains to be established. We have shown direct association of Akt with DNA-PK and PKCε, but we could not detect direct interaction between DNA-PK and PKCε using coimmunoprecipitation method. Yeast or mammalian two-hybrid assay can be employed in the future to determine if DNA-PK is directly associated with PKCε. DNA-PK kinase activity has been shown to be regulated by phosphorylation (14, 15). *In vitro* immunokinase assay can be performed in the future to determine if PKCε directly phosphorylates DNA-PK. It has been reported that PKCδ and c-Abl interact and phosphorylate DNA-PK to inhibit its activity (16), and there is cross-talk between PKCε and PKCδ (17). Therefore, it is reasonable to speculate that PKCε regulates DNA-PK via PKCδ. The role of PKCδ in the regulation of DNA-PK by PKCε can be investigated in the future.

PKCε was shown to interact with Bax to regulate the dimerization and the translocation of Bax to regulate mitochondrial function. In my preliminary study, phosphorylation of Bax was not detectable in an *in vitro* kinase assay using active recombinant PKCε, but this result needs to be confirmed further. Akt has been shown to phosphorylate Bax (13). Future experiments can be performed using active recombinant Akt as well as active recombinant PKCε to perform *in vitro* kinase assay. Coimmunoprecipitation results in chapter IV showed that the interaction between PKCε and Bax was only detectable in PKCε-overexpressing cells. To examine if PKCε directly

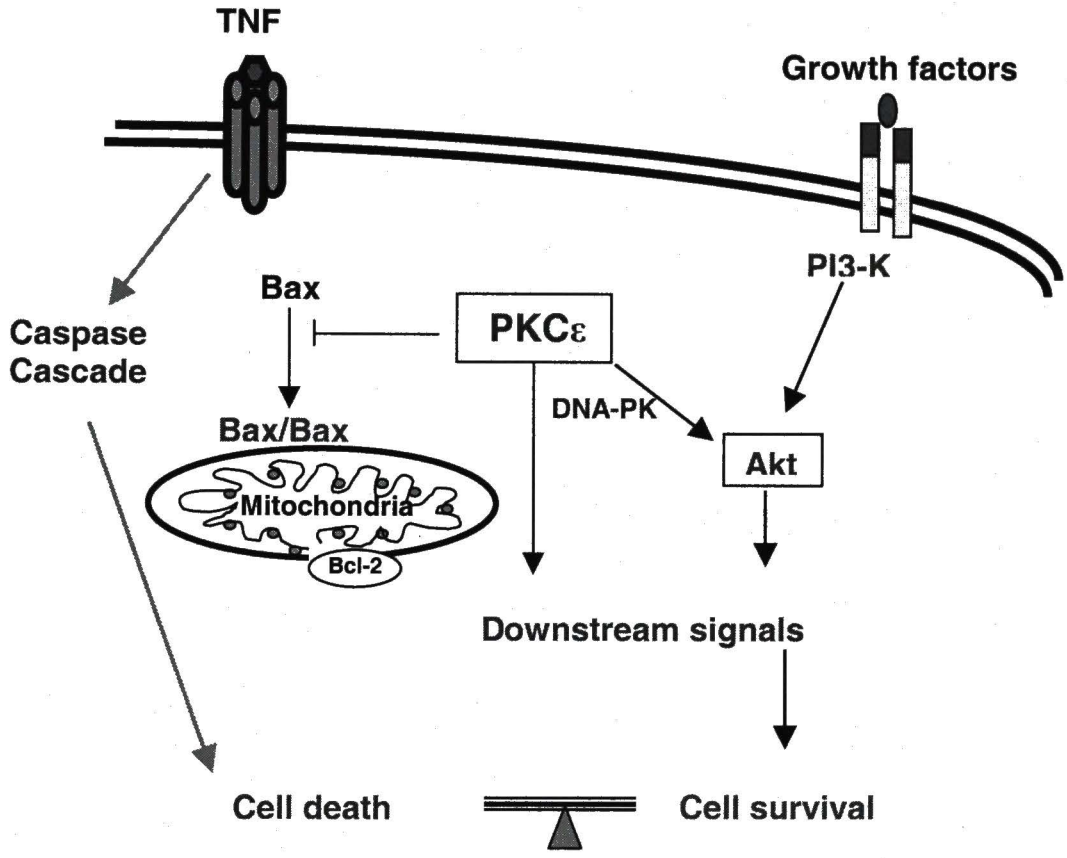
interacts with Bax, GFP-tagged Bax can be transfected into human embryonic kidney (HEK) cells as well as breast cancer MCF-7 cells. Fluorescence resonance energy transfer (FRET) can be performed to determine direct interaction between these molecules. FRET is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon (18). FRET is a powerful technique for investigating a variety of biological phenomena that produce changes in molecular proximity and direct interaction between two molecules (19). Additionally, the interacting sites need to be characterized in the future by mutagenesis studies.

Proteomic study in cardiomyocytes has revealed interaction of PKCε with many different molecules (20), but the specific binding partners of PKCε in breast cancer cells may be different from cardiomyocytes. Therefore, 2-dimensional (2D) gel electrophoresis and mass spectrometry may be performed in the future to identify novel downstream targets of PKCε in breast cancer cells.

The work presented in this dissertation is limited to established breast cancer cell lines. Future study with primary cultures from breast cancer patients should corroborate these findings.

Figure 1. Antiapoptotic signaling of PKCε in breast cancer cells. TNF mediates apoptosis mainly through activation of a caspase cascade, and the mitochondrial cell death pathway amplifies TNF cytotoxicity. PKCε counteracts TNF-induced cell death in breast cancer cells by activation of Akt via DNA-PK. Additionally, PKCε inhibits proapopototic Bax to attenuate the mitochondrial cell death pathway.

Figure 1.


TNF
Growth factors
PI3-K
Bax
PKCε
Caspase
Cascade
DNA-PK
Akt
Bax/Bax
Mitochondria
Bcl-2
Downstream signals
Cell death
Cell survival

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