Protein Kinase Cε Activates Protein Kinase B/Akt via DNA-PK to Protect against Tumor Necrosis Factor-α-induced Cell Death*

Dongmei Lu, Jie Huang, and Alakananda Basu

From the Department of Molecular Biology & Immunology, University of North Texas Health Science Center, Fort Worth, Texas 76107

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We have previously shown that protein kinase Cε (PKCε) protects breast cancer cells from tumor necrosis factor-α (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 function. 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 small interfering RNA (siRNA) decreased TNF-induced Akt phosphorylation/activity and increased cell death. Introduction of constitutively active 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.

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). TNF-R1 is believed to be responsible for transduction of the death signals. 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 fibroblast, colonic, and prostatic epithelial cells (10, 11). We and others (9, 12, 13) have shown that PKCε acts as an antiapoptotic protein during receptor-initiated apoptosis. In addition, breast cancer cells containing a 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 serine/threonine kinases (15, 16). Akt is activated in a phosphoinositide 3-kinase (PI3K)-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, integrin-linked kinase or mitogen-activated protein kinase activated protein kinase 2 (15, 16, 19). Recent evidence suggests that phosphorylation of Akt at Ser473 may be mediated by the rictor-mammalian target of rapamycin (mTOR) complex or DNA-dependent protein kinase (DNA-PK) (20, 21).

DNA-PK, a member of the PI3K-related kinase 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 PI3K family members, including ataxia telangiectasia mutated gene, ataxia telangiectasia mutated gene-related, and p110 PI3K (22). However, DNA-PKcs is similar to PI3K family members, including ataxia telangiectasia mutated gene, ataxia telangiectasia mutated gene-related, and p110 PI3K (23). DNA-PKcs also 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...
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A.

|        | vector | CA-Akt |
|--------|--------|--------|
| TNF    | -      | +      |
| PARP   | 116 kD | 85 kD  |
| Akt    |        |        |
| HA     |        |        |

B. MCF-7/vector MCF-7/CA-Akt

- TNF

- Annexin V

Also co-localize with Akt on the cell membrane and phosphorylate Akt at Ser\(^ {473} \) in a PI3K-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 Ser\(^ {473} \) in murine fibrosarcoma WEHI-164 cells (28). There have been several reports that suggest that PKCe may regulate Akt activity (29). Inhibition of PKCe by dominant-negative PKCe was associated with inhibition of Akt phosphorylation by insulin, demonstrating that PKCe activity was required for Akt phosphorylation. However, wild-type PKCe had no effect on insulin-induced Akt activation (29). Akt has been reported to be a downstream effector of PKCe for ethanol-induced cardioprotection because ethanol consumption caused an increase in expression and activity of PKCe and Akt, and inhibition of PKCe prevented the increase in Akt activity (30). It is not known how PKCe activates Akt. Furthermore, it remains to be established whether the antiapoptotic function of PKCe is mediated by Akt. We made a novel observation that PKCe activates Akt by enhancing interaction between DNA-PK and Akt, resulting in phosphorylation of Akt at Ser\(^ {473} \). Thus, PKCe acts upstream of Akt to regulate antiapoptotic signaling in breast cancer cells. Furthermore, we report for the first time that DNA-PK can regulate the 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. Polyclonal antibody to Akt/PKB, phospho-Akt (Ser\(^ {473} \)) and Akt kinase assay kit were obtained from Cell Signaling (Beverly, MA). Polyclonal antibody against PKCe and DNA-PKcs were from Santa Cruz Biotechnology, Inc. (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 secondary antibodies were obtained from Jackson ImmunoResearch Laboratory Inc. (West Grove, PA). The polypeptidylidene difluoride membrane was from Millipore (Bedford, MA), and the enhanced chemiluminescence detection kit was from Amersham Biosciences. Anti-hemagglutinin 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 SMARTpool of non-targeting siRNA and siRNA specific for Akt1 (PKBα), PKCe, and DNA-PKcs were obtained from Dharmacon RNA Technologies (Lafayette, CO). PKCe and DNA-PKcs siRNA were also obtained from Santa Cruz Biotechnology, Inc.

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 °C with 95% air and 5% CO\(_2\). siRNA was transfected using Lipofectamine 2000 transfection reagent according to the manufacturer’s protocol (Invitrogen).

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

Coimmunoprecipitation—Cells were lysed in 20 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM dithiothreitol, 25 mM β-glycerophosphate, 10 mM NaF, 10 μg/ml phenylmethysulfonyl fluoride, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 4 μg/ml pepstatin. PKCe or Akt were immunoprecipitated with 1 μg of antibody and 30 μl of protein A/G-agarose. Immunocomplexes were washed four times in

FIGURE 1. Overexpression of 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. The total percentage of cell death, including early apoptotic cells stained as Annexin V-positive and late apoptotic cells as Annexin V- and PI-positive cells, is indicated. HA, Hemagglutinin.
lysis buffer and boiled in Laemmli sample buffer. The immunocomplexes were separated on SDS-PAGE and transferred to polyvinylidene difluoride membrane. The presence of Akt, PKC\textsubscript{ε}/H9280, 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 for 12 h and then treated with or without 1 nM TNF as indicated in the legend to Fig. 3. Akt was immunoprecipitated using immobi-

Annexin V/Propidium Iodide Binding Assay—Cells were treated with or without TNF as indicated in the legends to Figs. 1, 5, 6, and 9. At the end of the incubation, both detached and attached cells were collected and washed with phosphate-buffered saline. 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.

RESULTS

Activation of Akt Protects Breast Cancer MCF-7 Cells against TNF-induced Cell Death—We have previously shown that PKC\textsubscript{ε} acts as an antiapoptotic protein, but the level of PKC\textsubscript{ε} alone was not sufficient to explain its antiapoptotic function (9). Since Akt/PKB is an important antiapoptotic protein, we first wanted to determine whether 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 hemagglutinin-tagged constitutively active Akt

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

FIGURE 3. Overexpression of PKC\textsubscript{ε} increased TNF-induced Akt activation. MCF-7/Neo and MCF-7/PKC\textsubscript{ε} cells were serum-starved overnight and then treated with 1 nM TNF for the indicated time period. Akt kinase assay was performed as indicated under “Experimental Procedures.” A, Western blot analysis was performed following IP using phospho-GSK3\textsubscript{α/β} to monitor Akt activity. The level of Akt in total cell lysate used for IP was used to control for loading. B, intensity of phospho-GSK3\textsubscript{α/β} was determined by densitometry and standardized by loading. The data represent the fold increase in GSK3\textsubscript{α/β} phosphorylation compared with untreated MCF-7/Neo cells. Each bar represents the mean ± S.E. of two independent experiments. The open bar represents MCF-7/Neo cells; the solid bar represents MCF-7/PKC\textsubscript{ε} cells. *, \( p < 0.01 \) versus MCF-7/Neo cells using paired Student’s \( t \) test.

FIGURE 4. Knockdown of PKC\textsubscript{ε} by siRNA decreased TNF-induced Akt phosphorylation. MCF-7 cells were transfected with control (Con) siRNA, PKC\textsubscript{ε}-specific siRNA1 (PKC\textsubscript{ε}1) (Dharmacon SMARTpool), or siRNA2 (PKC\textsubscript{ε}2) (Santa Cruz Biotechnology, Inc.). Cells were serum-starved overnight and treated with 1 nM TNF for 30 min. Western blot analyses were performed with total cell lysates using indicated antibodies. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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(A) CA-Akt in MCF-7 cells using adenoviral vector. Fig. 1A shows that transduction of adenovirus containing CA-Akt resulted in an increase in Akt in MCF-7 cells as detected by Akt and hemagglutinin 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. We have calculated the total percentage of cell death that included both Annexin V-positive cells as well as PI- and Annexin V-positive cells since we cannot distinguish late apoptotic cells from necrotic cells (Fig. 1B). Treatment with TNF resulted in 45% cell death in MCF-7 cells infected with control vector compared with 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 with vector-infected MCF-7 cells.

PKCe Acts Upstream of Akt to Regulate TNF-induced Akt Activation—We have previously shown that overexpression of PKCe protects breast cancer MCF-7 cells from TNF-induced apoptosis (9). To determine whether PKCe has any effect on TNF-induced Akt activation, we compared the ability of TNF to induce Akt phosphorylation in MCF-7 cells transfected with either an empty vector (MCF-7/Neo) or vector containing wild-type PKCe (MCF-7/PKCe). As indicated in Fig. 2, TNF induced Akt phosphorylation at Ser473 in both MCF-7/Neo and MCF-7/PKCe cells in a time-dependent manner. The maximum increase in Akt phosphorylation was evident following treatment with TNF for 30 min. In contrast, TNF had little effect on the phosphorylation of Akt at Thr308 site. The extent of Akt phosphorylation was greater in MCF-7/PKCe cells compared with MCF-7/Neo cells during the entire time course.

To further evaluate the effect of TNF on Akt kinase activity, we immunoprecipitated Akt from MCF-7/Neo and MCF-7/PKCe cells with immobilized antibody against Akt and performed in vitro kinase assay using GSK3α/β fusion protein as the substrate (Fig. 3A). The extent of GSK3α/β phosphorylation was determined by densitometric scanning of immunoblots. Fig. 3B shows that TNF induced 1.7-fold stimulation in Akt phosphorylation of Akt at Ser473 in MCF-7/PKCe cells compared with MCF-7/Neo cells. A parallel reduction in GSK3α/β phosphorylation was also evident in MCF-7/PKCe cells compared with MCF-7/Neo cells. Thus, PKCe appears to act upstream of Akt to regulate TNF-induced Akt activation.

FIGURE 5. CA-Akt partially restored cell survival in PKCe-depleted cells. Control (Con) siRNA or PKCe-specific siRNA (Dharmacon SMARTpool) were transfected into MCF-7 cells, and cells were infected with control adenoviral vector or vector containing CA-Akt construct. Cells were treated with 1 ng 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. The open bar and solid bar represent without or with TNF treatment, respectively. *, p < 0.01 versus without TNF treatment. **, p < 0.01 versus control siRNA transfected MCF-7/Vector cells with TNF treatment. *** p < 0.01 versus PKCe siRNA transfected MCF-7/Vector cells with TNF treatment using one-way analysis of variance.
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 from Dharmacon (PKCε1) or Santa Cruz Biotechnology, Inc. (PKCε2). Fig. 4 shows that both siRNAs against PKCε caused substantial 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. 4). 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 whether Akt functions downstream of PKCε to mediate antiapoptotic signaling, we depleted PKCε by siRNA and monitored TNF-induced apoptosis in MCF-7 cells. Fig. 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 whether 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 Fig. 5B. Thus, CA-Akt partially restored cell survival in PKCε-depleted cells.

Knockdown of Akt Inhibits the Antiapoptotic Effect of PKCε—To further examine if Akt functions downstream of PKCε, we depleted Akt using Akt-specific siRNA in both
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MCF-7/Neo and MCF-7/PKCε cells. We have used either Dharmacon SMARTpool, which is a combination of four siRNAs (Fig. 6A) or individual siRNA from Dharmacon siRNA1(Akt1) or siRNA2(Akt2) (Fig. 6B). Fig. 6, A and B, show that depletion of Akt enhanced TNF-induced apoptosis as evident by the increase in PARP cleavage. While overexpression of PKCε inhibited PKCε-mediated PARP cleavage, knockdown of Akt restored sensitivity of MCF-7/PKCε cells to TNF. Dharmacon SMARTpool is a mixture of four different siRNAs targeted at distinct sites and has minimum off-target effect that may be associated with targeting of high concentrations of single siRNA to a specific site. Therefore, unless otherwise mentioned, we primarily used Dharmacon SMARTpool in our experiments. We also quantified cell death by Annexin V/PI dye binding assay. Fig. 6C shows that 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. 6C). 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 whether PKCε directly interacts with Akt, we performed coimmunoprecipitation. 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. Fig. 7 shows that PKCε was constitutively associated with Akt in PKCε-overexpressing cells, and TNF enhanced association of Akt with PKCε. We also examined whether DNA-PK, a recently identified PDK2, was involved in PKCε-mediated phosphorylation of Akt. Association of Akt with 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 modestly. 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 whether PKCε phosphorlases Akt via DNA-PK, we depleted DNA-PKcs using siRNA from Santa Cruz Biotechnology, Inc. (DNA-PK1) or Dharmacon (DNA-PK2). Fig. 8A shows that knockdown of DNA-PKcs depleted DNA-PKcs but did not affect the expression of another member of the PI3K family, PI3K110β in MCF-7 cells (Fig. 8A). Depletion of DNA-PK inhibited TNF-induced Akt phosphorylation in MCF-7 (Fig. 8A) and MCF-7/Neo and MCF-7/PKCε cells (Fig. 8B). 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. Fig. 9A shows that knockdown of DNA-PKcs enhanced TNF-induced PARP cleavage in MCF-7/Neo cells. While overexpression of PKCε inhibited TNF-induced PARP cleavage, depletion of DNA-PKcs 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.

**FIGURE 7.** Association of Akt 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 lysates were immunoprecipitated with Akt or PKCε antibodies. Western blot analyses were performed with immunocomplexes using the indicated antibodies.

**FIGURE 8.** Effect of DNA-PKcs depletion on Akt phosphorylation. A, MCF-7 cells were transfected with control siRNA, DNA-PKcs siRNA1 (DNA-PK1) (Santa Cruz Biotechnology, Inc.) or siRNA2 (DNA-PK2) (Dharmacon SMARTpool), 8, MCF-7/Neo and MCF-7/PKCε cells were transfected with control or DNA-PKcs siRNA (Dharmacon SMARTpool). 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.
tein during TNF-induced cell death (9). In the present study, we have investigated whether there is any cross-talk between these two important signaling molecules. We have made several important and novel observations. We have demonstrated that PKC acts upstream of Akt/PKB to exert its antiapoptotic function. First, overexpression of PKC increased Akt phosphorylation and activity in response to TNF. Second, depletion of PKC abrogated TNF-induced Akt phosphorylation and activation. Third, knockdown of Akt abolished the antiapoptotic effect of PKC. We also demonstrated that the activation of Akt by PKC is mediated by DNA-PK, and depletion of DNA-PKcs reversed the antiapoptotic function of PKC during TNF-induced apoptosis. The observation that inhibition of DNA-PK can reverse antiapoptotic signaling by Akt and PKC establishes a new role for DNA-PK in the extrinsic cell death pathway.

Several studies have reported that PKC acts upstream of Akt (16, 29, 30, 31–37, 39–42), but there are controversies as to whether PKC is a positive or negative regulator of Akt. For example, Doornbos et al. (32) have shown that Akt and PKC did not influence the activity of respective kinases, but growth factor-induced activation of Akt was abolished by PKC. In contrast, PKC and -z were shown to negatively regulate Akt phosphorylation/activity even though these isozymes are considered prosurvival proteins (33, 43). nPKC 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 PKC by dominant-negative PKC was associated with inhibition of Akt phosphorylation by three different stimuli, whereas other PKC isozymes had no effect, demonstrating that PKC 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 PKC attenuated TNF-induced apoptosis in MCF-7 breast cancer cells (9). However, the status of PKC alone could not explain TNF sensitivity/resistance (14). For example, although SKBR-3
and CAMA-1 breast cancer cells contained low levels of PKCα, 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 whether 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 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 increases 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, 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 caused a modest increase in basal and TNF-induced association between Akt and DNA-PKcs. At present, it is not clear how PKCε enhances interaction between DNA-PKcs 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 demonstrated (49). It has been reported that PKCδ associates with DNA-PKcs 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-PKcs 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 whether PKCε mediates its antiapoptotic function via Akt. Introduction of CA-Akt in MCF-7 cells conferred resistance to TNF. In addition, while knockdown of PKCε decreased Akt phosphorylation and enhanced TNF sensitivity, introduction of CA-Akt into PKCε-depleted MCF-7 cells partially restored cell survival, indicating that CA-Akt can counteract the effect of PKCε depletion. However, we cannot rule out the possibility that Akt may function through a PKCε-independent pathway. It has been reported recently that these two kinases may function independently on downstream targets (38). Nevertheless, knockdown of Akt in PKCε-overexpressing MCF-7 cells completely abrogated the antiapoptotic activity of PKCε, suggesting that the antiapoptotic function PKCε is dependent on the presence of Akt. These results provide strong evidence that PKCε activates Akt via DNA-PK to inhibit 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.

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