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# Overexpression of Protein Kinase C-$\eta$ Attenuates Caspase Activation and Tumor Necrosis Factor-$\alpha$-Induced Cell Death

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**The protein kinase C (PKC) signal transduction pathway regulates cell death by tumor necrosis factor-$\alpha$ (TNF). We previously showed that the induction of novel PKC$\eta$ isozyme by PKC activators correlated with their ability to protect MCF-7 breast cancer cells against TNF cytotoxicity. In the present study, we have transfected PKC$\eta$ in MCF-7 cells to directly examine its involvement in cell death by TNF. Overexpression of PKC$\eta$ delayed TNF-induced cell death in MCF-7 cells. TNF caused a rapid activation of caspase-8 and -7 in cells transfected with a vector. The activation of these caspases was potentiated by the PKC inhibitor bisindolylmaleimide (BIM) which downregulates PKC$\eta$ and sensitizes cells to TNF. Overexpression of PKC$\eta$ delayed the activation of caspase-8 and -7 by both TNF and the combination of BIM and TNF. These results suggest that PKC$\eta$ protects MCF-7 cells against TNF-induced cell death by preventing the activation of caspases.** © 2000 Academic Press

***Key Words:*** **PKC$\eta$; TNF; caspases; MCF-7 cells.**

Tumor necrosis factor-$\alpha$ (TNF) is a macrophage-derived cytokine that can induce inflammatory responses as well as cell death (1). TNF binds to at least two distinct receptors (55-kDa TNFRI and 75-kDa TNFRII), which belong to the TNF receptor superfamily and includes other transmembrane glycoproteins, including Fas and CD40 (2). Several intracellular signal transduction pathways are activated upon binding of TNF to its receptors, and activation of caspases, a family of cysteine proteases, is important for cell death mediated by TNF (3, 4). Caspases exist as an inactive zymogen that undergoes autoproteolysis to generate an active fragment that can cleave itself or other caspases (5). Ligand binding to TNFR1 induces receptor trimerization and association with adaptor proteins TRADD and FADD followed by local clustering and activation of the initiator caspase-8 (3, 4, 6, 7). Caspase activation is a coordinated event and activation of a proteolytic cascade triggers activation of a series of downstream caspases (8). For example, initiator caspase-8 causes activation of downstream effector caspase-3 and -7, which cleave critical cellular proteins, including poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase and lamin B. The cleavage of caspase substrates sets the stage for the morphological and biochemical changes that are hallmarks of apoptosis.

Several studies have implicated protein kinase C (PKC) in the cell death pathway (9–15). PKC consists of a family of phospholipid-dependent serine/threonine kinases that include eleven different isozymes (16–18). These isozymes are classified as the conventional PKCs ($\alpha$, $\beta$I, $\beta$II, and $\gamma$), which are $Ca^{2+}$- and DAG/phorbol ester-dependent, novel PKCs ($\delta$, $\epsilon$, $\eta$, and $\theta$), which are insensitive to $Ca^{2+}$ but respond to DAG/phorbol esters and the atypical PKCs ($\zeta$ and $\lambda/\iota$), which are insensitive to both $Ca^{2+}$ and DAG/phorbol esters. In addition, PKC$\mu$ resembles nPKCs structurally but aPKCs functionally (19). All isozymes share a basic structural relatedness that includes a catalytic domain and a regulatory domain separated by a hinge region. The separation of the regulatory domain from the catalytic domain, either by PKC activator-induced conformational change or by cleavage of the holoenzyme at the hinge region, results in activation of PKC (18). Recent studies have demonstrated that novel PKC isozymes, including PKC$\delta$, -$\epsilon$, -$\theta$, and -$\mu$ are cleaved during apoptosis (9, 10, 12, 15). Additionally, PKC$\delta$, -$\theta$, and -$\mu$ have been identified as substrates for caspase-3 and the catalytic fragments of these isozymes have been

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shown to induce apoptosis in certain cell types (9, 10, 12). We have shown that PKC can also regulate the activation of caspases and cell death induced by the DNA damaging agent cisplatin (15).

Although it is well-established that PKC regulates cell death by TNF (20–26), little is known about the PKC isozyme(s) important in TNF-induced cell death and how it regulates cell death by TNF. We have previously shown that PKC activators cause induction of PKC$\eta$ in MCF-7 cells and that there was a good correlation between PKC$\eta$ up-regulation and protection against TNF cytotoxicity by PKC activators (26). A recent report also implicated PKC$\eta$ as a substrate for caspase-3 and the proteolytic activation of PKC$\eta$ was associated with apoptotic induction in B-cells (27). MCF-7 cells, however, do not express caspase-3 (28). Therefore, to unequivocally demonstrate the importance of PKC$\eta$ in MCF-7 cell death, we overexpressed PKC$\eta$ by transfection. Our results show that overexpression of PKC$\eta$ in MCF-7 cells attenuates caspase activation and cell death by TNF.

## MATERIALS AND METHODS

*Materials.* TNF and caspase-8 antibody were purchased from R&D systems (Minneapolis, MN). BIM was obtained from CalBiochem (San Diego, CA) and MTT was purchased from Sigma (St. Louis, MO). Monoclonal antibodies to caspase-2 and caspase-7 were purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibody to PKC$\delta$ and -$\eta$ were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from JacksonImmuno Research (West Grove, PA). Enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

*Cell culture.* MCF-7 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% $CO_2$. Cells were transfected using FuGENE 6 transfection reagent (Boehringer Mannheim) using the manufacturer's protocol and selected using geneticin (Life Technologies).

*Immunoblot analysis.* Cells were treated with or without TNF and/or PKC inhibitor as described in the text. At the end of the incubation, cells were harvested and washed with cold PBS. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membrane. Immunoblot analyses were performed with different antibodies as described previously (29). The blots were visualized using the enhanced chemiluminescence detection reagents and the manufacturer's protocol. Intensities of immunoreactive proteins were quantified by laser densitometry (Molecular Dynamics). The same blot was probed with several different antibodies, including one that does not change with different treatments to control for equal loading.

## RESULTS AND DISCUSSION

The interaction of TNF with its receptors is not sufficient to trigger the cell death pathway (1). TNF induces several distinct intracellular signal transduction pathways that include not only the activation of caspases but also activation of cellular protective proteins, such as transcription factor NF-$\kappa$B, to counteract the cytotoxic effect of TNF (1). Additionally, the activation of caspases is highly regulated and several antiapoptotic proteins, such as Bcl-2 family proteins, Akt/protein kinase B and mitogen-activated protein kinase that inhibit activation of caspases, need to be inactivated for cell death to occur (7).

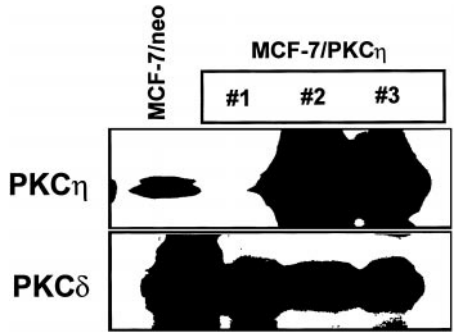


**FIG. 1.** Overexpression of PKC$\eta$ in MCF-7 cells. MCF-7 cells were transfected with an expression vector encoding PKC$\eta$ and Western blot analysis was performed with cell lysates using antibodies against PKC$\eta$ or -$\delta$ as described under Materials and Methods.

The PKC signal transduction pathway also exerts an antiapoptotic effect on TNF-induced cell death; activators of PKC protected cells against TNF cytotoxicity whereas BIM, a PKC-specific inhibitor, potentiated TNF-induced cell death (26). High concentrations of BIM ($\geq$10 $\mu$M) were required to inhibit cell death by TNF whereas low concentrations of BIM that inhibits cPKCs had little effect on TNF sensitivity. In addition, Gö 6976, a specific inhibitor of cPKCs, failed to sensitize MCF-7 cells to TNF. Since PKC inhibitors that sensitized cells to TNF caused downregulation of PKC$\eta$ and PKC activators that protected cells against TNF cytotoxicity induced upregulation of PKC$\eta$, we speculated that PKC$\eta$ might exhibit antiapoptotic activity against cell death by TNF. To further explore the role of PKC$\eta$ in TNF-induced cell death, we overexpressed PKC$\eta$ in MCF-7 breast carcinoma cells by transfection of a mammalian expression vector pcDNA3 encoding a full-length PKC$\eta$ cDNA and neomycin resistance marker into MCF-7 cells (MCF-7/PKC$\eta$). Control cells were transfected with the pcDNA3 vector without the PKC$\eta$ cDNA construct (MCF-7/neo). Figure 1 shows that MCF-7 cells transfected with 1 $\mu$g (#2) and 3 $\mu$g of PKC$\eta$ cDNA (#3), respectively, highly overexpressed PKC$\eta$ compared to vector-transfected cells but the level of another novel PKC isozyme PKC$\delta$ remained unchanged. The high level of PKC$\eta$ expression seen 1 week posttransfection rapidly declined and after several months of propagation, the PKC$\eta$ expression was comparable to vector-transfected cells (#1). Therefore, we restricted our experiments to early time points following transfection and used a pool of G418 resistant cells instead of se-

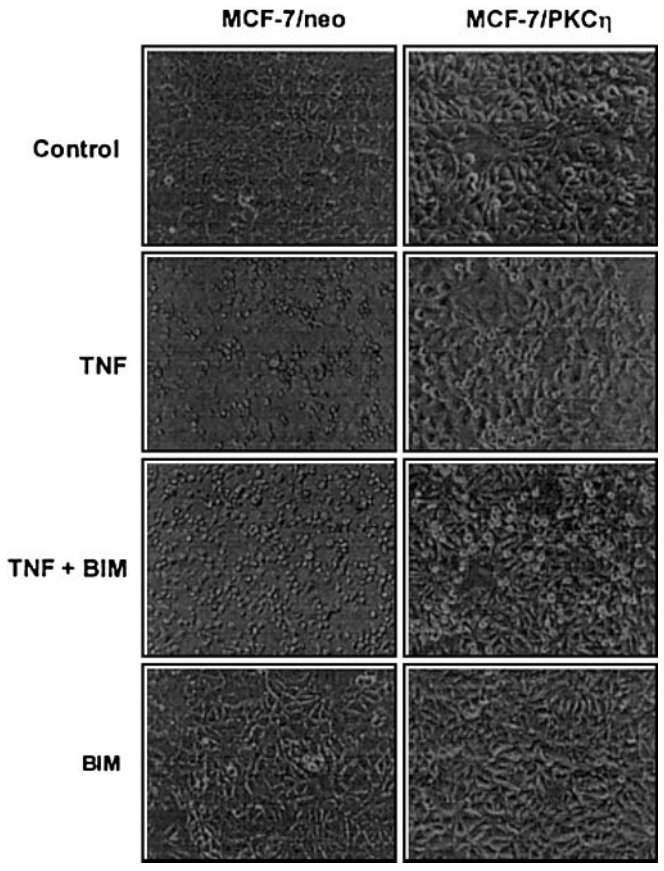


**FIG. 2.** Effect of PKC$\eta$ overexpression on TNF-induced apoptotic morphology. MCF-7 cells were treated with or without 10 $\mu$M BIM for 30 min and then with or without 1 nM TNF for 10 h. The cellular morphology was examined under a microscope.

lecting individual clone. We also monitored PKC$\eta$ overexpression by Western blot analysis.

To determine whether PKC$\eta$ overexpression influences TNF-induced cell death, we compared the morphology of vector-transfected and PKC$\eta$ overexpressing MCF-7 cells following exposure to TNF. We were unable to demonstrate DNA fragmentation or appearance of sub-G1 peak in a flow cytometer following exposure of MCF-7 cells to TNF. It is consistent with an earlier report that caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis and MCF-7 cells do not express caspase-3 (28). When cells undergo apoptosis they round-up and detach from the tissue culture dish. Treatment of vector-transfected MCF-7 cells with 1 nM TNF for 12 h induced morphological changes consistent with apoptosis and the number of cells with apoptotic morphology decreased substantially in cells overexpressing PKC$\eta$ (Fig. 2). When cells were pretreated with 10 $\mu$M BIM prior to exposure to TNF, the number of floating cells increased substantially in vector-transfected MCF-7 cells compared to cells treated with TNF alone but the effect of BIM was decreased in cells overexpressing PKC$\eta$. Neither BIM nor PKC$\eta$ overexpression had any effect on the morphology of MCF-7 cells. When cells were exposed for a prolonged period ($\geq$18 h) with TNF, the ability of PKC$\eta$ to block cell death decreased (data not shown). These results suggest that overexpression of PKC$\eta$ attenuates cell death by TNF.

Proteolytic cleavage of several novel PKCs by caspases has been associated with apoptotic cell death (9–13). Therefore, we examined the effect of PKC$\eta$ overexpression on TNF-induced activation of caspases. Since most of the commercially available caspase substrates have overlapping specificity, we monitored caspase activation by the decrease in proform to processed form. Figure 3 shows that TNF induced a time-dependent activation of the initiator caspase-8 as evident by the decrease in the proform near 55-kDa region and an increase in the 10-kDa active fragment. Due to low binding of small molecular proteins on nitrocellulose, it was difficult to detect the smaller fragments. In addition, we have consistently seen that when cells are exposed to TNF for a prolonged period, the decrease in procaspase does not correspond to the increase in processed forms presumably due to degradation of the active fragments by some other proteases. TNF also induced activation of effector caspase-7. Overexpression of PKC$\eta$ delayed the activation of both caspase-8 and -7 by TNF; the active fragments were visible in PKC$\eta$ overexpressing MCF-7 cells only when cells

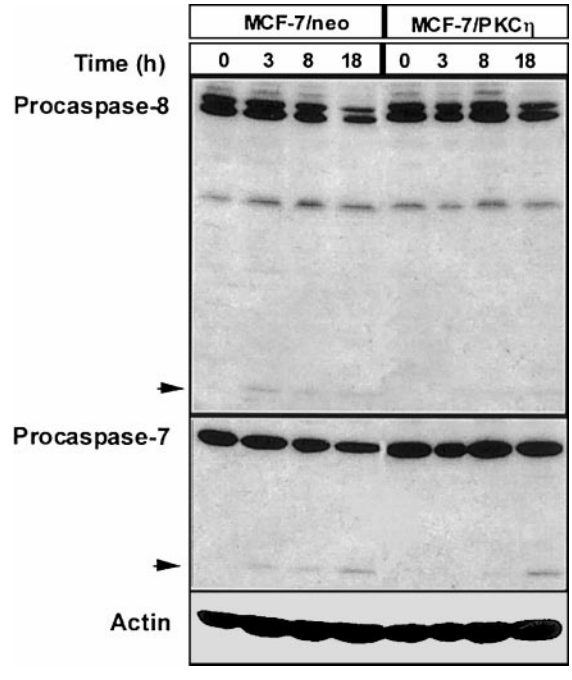


**FIG. 3.** Effect of PKC$\eta$ overexpression on caspase activation. Cells transfected with the control vector (pcDNA3) or the vector containing the PKC$\eta$ construct were treated with 1 nM TNF for various periods of time and Western blot analyses were performed with antibodies to caspase-8, -7 and actin. Arrows indicate the processed forms.

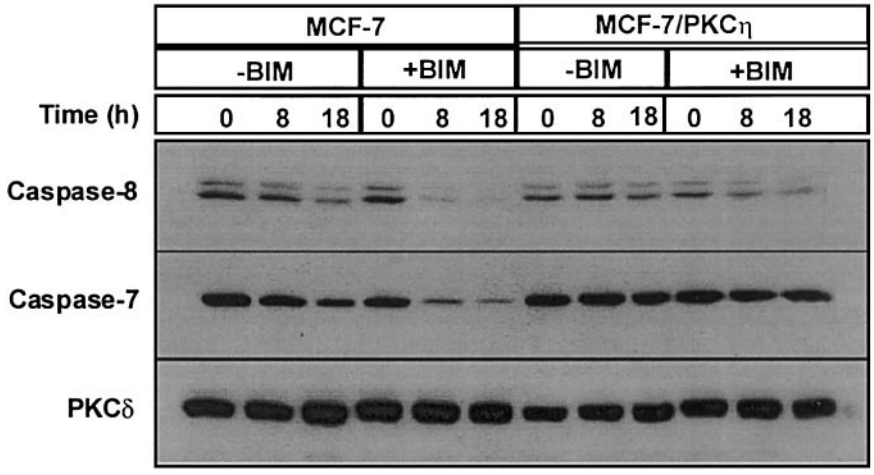


**FIG. 4.** Effect of PKC$\eta$ overexpression and BIM on TNF-induced caspase activation. Cells were treated with or without 10 $\mu$M BIM for 1 h followed by 1 nM TNF for the indicated time periods. Western blot analyses were performed with total cell lysates using antibodies against caspase-8, -7 and PKC$\delta$.

were exposed to TNF for 18 h. Thus, PKC$\eta$ overexpression delayed TNF-induced caspase activation.

We have previously shown that the PKC-specific inhibitor BIM potentiated sensitivity of MCF-7 cells to TNF (26). Therefore, we examined the effect of BIM on TNF-induced caspase activation. Figure 4 shows that while an 8 h exposure to 1 nM TNF caused only a modest activation of caspase-8 and -7 in MCF-7 cells, pretreatment of MCF-7 cells with 10 $\mu$M BIM enhanced TNF-induced activation of both caspase-8 and -7 substantially. For example, 1 nM TNF induced a 35% cleavage of procaspase-7 by 8 h whereas more than 85% of procaspase-7 was converted to the active form when cells were pretreated with 10 $\mu$M BIM prior to the exposure to TNF for 8 h. Overexpression of PKC$\eta$ decreased TNF-induced activation of caspase-8 and -7 in cells treated with TNF or the combination of TNF and BIM. TNF alone had no effect on the processing of caspase-8 or -7 by 8 h and caused only a 25% cleavage of procaspase-7 in cells pretreated with BIM. Since PKC$\delta$ is not cleaved in MCF-7 cells due to lack of caspase-3, we included PKC$\delta$ to demonstrate that a decrease in procaspase-8 or -7 cannot be explained by loading variations. Thus, PKC$\eta$ overexpression blocked the potentiation of TNF-induced caspase activation by BIM.

Although MCF-7 cells do not express caspase-3 (28), our results suggest that activation of effector caspase-7 by TNF was influenced by PKC$\eta$ overexpression. Because catalytic fragments of several novel PKCs, including PKC$\eta$, have been implicated in apoptotic cell death (9–12, 27), we examined whether TNF induces proteolytic activation of PKC$\eta$ in MCF-7 cells. Since the level of PKC$\eta$ was low in MCF-7 cells we used PKC$\eta$ overexpressing MCF-7 cells for this study (MCF-7/PKC$\eta$). Although we could detect a band near the 50-kDa region, the expected size of PKC$\eta$ catalytic fragment, the intensity of the 50-kDa protein did not alter following TNF treatment (Fig. 5). TNF caused a time-dependent increase in PKC$\eta$ level in these cells. Based on densitometric scanning, TNF led to an 18% and 47% increase in PKC$\eta$ content by 8 h and 18 h, respectively. Although BIM induced down-regulation of PKC$\eta$, long-term exposure to the combination of TNF and BIM counteracted the effect of BIM on PKC$\eta$ down-regulation. We have reported earlier that PKC activators caused induction of PKC$\eta$ in MCF-7 cells (26), although the magnitude of PKC$\eta$ induction by

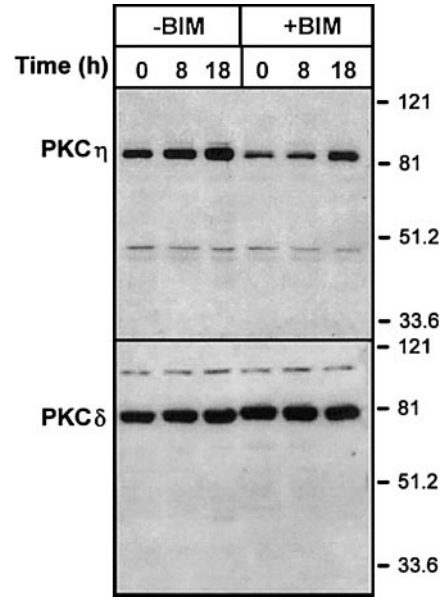


**FIG. 5.** Effect of BIM and TNF on the proteolytic activation of PKC$\eta$ and -$\delta$. MCF-7/PKC$\eta$ Cells were treated with or without 10 $\mu$M BIM for 1 h followed by 1 nM TNF for the indicated periods of time. Western blot analyses were performed with total cell lysates using antibodies against PKC$\delta$ and -$\eta$.

TNF was much less compared to PKC activators and required prolonged cellular exposure to TNF. This is consistent with earlier reports that TNF can mimic the effects of PKC activators (22, 30, 31). Induction of PKC$\eta$ could be one of the cellular protective responses triggered by TNF. TNF had no effect on the proteolytic activation of PKC$\delta$, suggesting that PKC$\delta$ is cleaved specifically by caspase-3 and not by caspase-7.

Most of the studies thus far have implicated the catalytic fragments of novel PKCs in apoptotic cell death (9–12). Although PKC$\eta$ has also been reported to be a substrate for caspase-3-like proteases (27), we were unable to cleave *in vitro* translated PKC$\eta$ by recombinant caspase-3 (A. Basu, unpublished observation). We have previously shown that PKC can act upstream of caspases to regulate cell death by a DNA damaging agent (15). When novel PKCs are cleaved by caspase-3, it is difficult to establish the relative contribution of catalytic fragment versus the holoenzyme during apoptosis. Since MCF-7 cells are devoid of caspase-3 and TNF did not induce cleavage of PKC$\eta$, these results suggest that PKC$\eta$ holoenzyme was important in regulating cell death by TNF. We believe that novel PKC$\eta$ can act as an antiapoptotic protein by inhibiting TNF-induced activation of caspases.

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