Functional Dichotomy of Protein Kinase C (PKC) in Tumor Necrosis Factor-α (TNF-α) Signal Transduction in L929 Cells

TRANSLOCATION AND INACTIVATION OF PKC BY TNF-α*

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Tumor necrosis factor-α (TNF-α) is capable of inducing a variety of biologic responses through multiple signaling pathways. Because of the potential role of protein kinase C (PKC) in apoptosis, we examined the effects and mechanisms of TNF-α on PKC regulation, specifically on PKCa. In L929 murine fibroblasts, TNF-α (0.5–5 nM) caused potent inhibition of PKCa activity and induced translocation of PKCa from the cytosol to the membrane. Treatment of cells with TNF-α also induced dephosphorylation of PKCa as detected by a mobility shift on SDS-polyacrylamide gel and inhibition of PKC phosphorylation as probed by anti-phospho-PKC antibodies. Since PKC is activated directly by diacylglycerol and inactivated indirectly by ceramide, we next examined the roles of these lipid mediators in the regulation of PKCa. Addition of TNF-α led to accumulation of both ceramide and diacylglycerol. Fumonisin B1, an inhibitor of ceramide synthase, and glutathione, an inhibitor of neutral sphingomyelinase, both reversed the effect of TNF-α on PKCa activity, suggesting that ceramide production is necessary for the action of TNF-α. The diacylglycerol mimic phospholipid 12-myristate 13-acetate was sufficient to cause translocation of PKCa, but not the mobility shift. Okadaic acid at 2 nM, a potent protein phosphatase inhibitor, blocked the effects of TNF-α on PKCa activity, but not on PKCa translocation, thus demonstrating that dephosphorylation and translocation are independent processes. These results demonstrate that PKCa acts as a downstream target for TNF-α and that different lipid-mediated pathways in TNF-α signaling lead to opposing signals in the regulation of PKCa activity.

Tumor necrosis factor-α (TNF-α),1 a pleiotropic cytokine, mediates multiple biologic responses in different cell types through binding to either 55- or 75-kDa membrane receptors (1, 2). The biologic properties of TNF-α include inhibition of cell growth, induction of differentiation and apoptosis, modulation of gene transcription, and activation of protein phosphorylation cascades (3). In many cell systems, TNF-α receptor-mediated cellular responses are preceded by the elevation of intracellular ceramide levels through hydrolysis of sphingomyelin by sphingomyelinase (4, 5). Although the elevation of intracellular ceramide levels has been proposed to mediate at least some of the effects of TNF-α on cell growth and differentiation (6), the signaling mechanisms involved in transduction of biologic effects of TNF-α are not fully understood.

Ceramide-activated protein phosphatase (CAPP) is one of the cellular targets of ceramide (7, 8). CAPP belongs to the PP2A heterotrimeric subfamily of the serine/threonine protein phosphatases and is potently inhibited by okadaic acid, with an IC50 of 1–10 nM (7, 8). Activation of CAPP plays an important role in the induction of c-Myc down-regulation by ceramide in myeloid leukemia cells (9). The antiproliferative effect of ceramide and CAPP activity have also been detected in Saccharomyces cerevisiae (10), and a role of yeast CAPP in mediating growth suppressor effects of ceramide has been documented (11). We previously demonstrated that inhibition of PKCa activity by ceramide in Molt-4 leukemia cells was also through activation of a protein phosphatase of the PP2A subfamily, consistent with a role for CAPP (12). Other putative targets of ceramide include PKCa (13, 14) and ceramide-activated protein kinase, which is a kinase suppressor of Ras (5, 15) and which may function to transduce the effects of ceramide on Ras (15).

PKC is a family of serine/threonine kinases that plays an important role in modulating a variety of biologic responses ranging from regulation of cell growth to cell death. The involvement of PKC in signal transduction of TNF-α has been reported (16). It was shown that TNF-α activates PKC and induces PKC translocation in Jurkat, K562, and U937 cell lines. However, the specific subtypes of PKC activated by TNF-α and the underlying mechanisms for their role in TNF-α signaling remain to be elucidated (16). A recent study has also demonstrated that a atypical PKC isoform, PKCζ, may act as a molecular switch in transducing mitogenic and growth inhibitory signals of TNF-α (14). More recently, studies have pointed to specific effects of the PKCζ isoform in regulating apoptosis. One study has shown that inactivation of PKCζ is sufficient to induce apoptosis (17), whereas another study showed that activation of PKCζ protects from apoptosis (18). Therefore, given the emerging role of PKCζ in apoptosis, the role of ceramide in TNF-α signaling, and the effects of ceramide on PKCζ, we investigated the relationship of these regulators and the role of ceramide in this pathway.

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RESULTS

Inhibition of PKCα Activity by Ceramide—We had previously demonstrated that ceramide inhibits PKCα activity in Molt-4 cells (12). To investigate a possible role for ceramide in L929 cells, we determined the ability of exogenous ceramide to inhibit PKCα activity. L929 cells were treated with various concentrations of a synthetic and cell-permeable analog (C₁₆-ceramide) for 14 h. PKCα activity was determined using an immunoprecipitation/kinase assay as described previously (12). The lysates were immunoprecipitated with anti-PKCα antibody, and the immunocomplexes were collected. The calcium- and lipid-dependent PKCα activity was then determined in the absence and presence of calcium and lipid activators using histone H₁ as an exogenous substrate. Fig. 1 shows that immunocomplexes contained a basal level of histone H₁ kinase activity as defined by the presence of 10 mM EGTA. The histone H₁ kinase activity was significantly enhanced in the presence of the lipid activators phosphatidylycerine/diC₁₆:1. The maximal effects of ceramide on PKCα were detected at a concentration of 40 μM (Fig. 1A, upper panel). The structurally similar lipid molecule dihydro-C₁₆-ceramide did not have an appreciable effect on PKCα activity (Fig. 1A, upper panel). Densitometric analysis of kinase activity is shown in Fig. 1A (lower panel). In addition, the alteration of PKCα activity by ceramide was not due to the change in PKCα protein levels. Immunoprecipitation/Western blot analysis (Fig. 1B, upper panel) and densitometric analysis (lower panel) demonstrated that the amounts of protein in control and treated cells were similar. The intensity of the Coomassie Brilliant Blue staining indicated that the amounts of protein loaded in each lane were approximately equal (data not shown). These results are consistent with our previous finding that ceramide specifically inhibits PKCα activity in Molt-4 cells (12). To determine whether the effect of ceramide on PKC is isozyme-specific, we examined PKCβII, another major calcium-dependent isozyme present in significant amounts in L929 cells. It was found that PKCβII was also inhibited by C₁₆-ceramide (data not shown). For the purpose of this study, we focused on PKCα activity.

Inhibition of PKCα Activity by TNF-α—Since ceramide mediates some of the TNF-α effects on cellular growth regulation, we wondered whether TNF-α can affect PKCα activity. L929 cells were treated with various concentrations of TNF-α for 12–14 h. Treatment of cells with TNF-α (0.5–5 nM) led to a remarkable decrease in both basal and lipid-activated PKCα activity. L929 cells were treated with various concentrations of TNF-α for 12–14 h. Treatment of cells with TNF-α (0.5–5 nM) led to a remarkable decrease in both basal and lipid-activated PKCα activity. L929 cells were treated with various concentrations of TNF-α for 12–14 h. Treatment of cells with TNF-α (0.5–5 nM) led to a remarkable decrease in both basal and lipid-activated PKCα activity.
anti-PKCα antibody. TNF-α treatment did not cause a major change in the levels of PKC (Fig. 4). Interestingly, TNF-α treatment resulted in an appearance of a doublet of PKCα in SDS-PAGE in L929 cells (Fig. 4) as detected by Western blotting. The specific shift in the electrophoretic mobility of PKCα is indicated by the lower band of the doublet. This result suggests that TNF-α may lead to dephosphorylation of PKCα through activation of protein phosphatases since a similar shift has been detected in PKCα treated with the protein phosphatase PP2A (27, 28).

Mechanisms of TNF-α Action on PKCα Activity—To determine the mechanisms by which TNF-α regulates PKCα activity, we first examined the effects of TNF-α on the generation of ceramide in L929 cells. L929 cells were treated with various concentrations of C6-ceramide or dihydro-C6-ceramide (DHC6) for 14 h. A, the cleared lysates were immunoprecipitated with anti-PKCα antibody, and protein A-Sepharose beads were collected for kinase assays. The calcium-dependent PKCα activity was assayed in the presence of 1.2 mM calcium and the lipid activators phosphatidylserine (PS; 40 μg/ml) and dioleoylglycerol (DON; 3.3 mM) using histone H1 as the exogenous substrate in vitro. The basal activity was defined in the presence of 10 mM EGTA (upper panel), and densitometric analyses of the autoradiographs are shown (lower panel). B, shown are the results from the immunoprecipitation/Western blot analysis (upper panel) and densitometric analysis (lower panel) of lysates from control and treated samples. Results are representative of two separate experiments. Densitometric analyses of the autoradiographs are expressed as means ± S.D. of two or three experiments (lower panels).

Fig. 1. C6-ceramide inhibits PKCα activity in L929 cells. L929 cells were treated with various concentrations of C6-ceramide or dihydro-C6-ceramide (DHC6) for 14 h. A, the cleared lysates were immunoprecipitated with anti-PKCα antibody, and protein A-Sepharose beads were collected for kinase assays. The calcium-dependent PKCα activity was assayed in the presence of 1.2 mM calcium and the lipid activators phosphatidylserine (PS; 40 μg/ml) and dioleoylglycerol (DON; 3.3 mM) using histone H1 as the exogenous substrate in vitro. The basal activity was defined in the presence of 10 mM EGTA (upper panel), and densitometric analyses of the autoradiographs are shown (lower panel). B, shown are the results from the immunoprecipitation/Western blot analysis (upper panel) and densitometric analysis (lower panel) of lysates from control and treated samples. Results are representative of two separate experiments. Densitometric analyses of the autoradiographs are expressed as means ± S.D. of two or three experiments (lower panels).
Fig. 2. Inhibition of PKCα activity by TNF-α. L929 cells were treated with various concentrations of TNF-α for 12–14 h. A, the in vitro kinase assay (upper panel) and densitometric analysis (lower panel) were done as described under “Experimental Procedures” and in the legend to Fig. 1. B, the immunoprecipitation/Western blot analysis (upper panel) and data analysis (lower panel) were done as described under “Experimental Procedures.” Results are representative of three separate experiments. Densitometric analyses of the autoradiographs are expressed as means ± S.D. of two or three experiments. PS, phosphatidylserine; DON, dioleoylglycerol.

Fig. 3. Effects of TNF-α and ceramide on PKCα translocation. Cells were pretreated with 2 nM TNF-α (A) or 40 μM C6-ceramide (B) for 14 h, followed by PMA stimulation (0.1 μM for 20 min at 37 °C). Cytosolic (C, Cyt0) and membrane (M, Mem) fractions were analyzed for PKCα by Western blotting (upper panels). These results are representative of three independent experiments. Densitometric analyses of the autoradiographs are expressed as means ± S.D. of three experiments (lower panels).
Effects of TNF-α on the phosphorylation state of PKCα. We utilized anti-PKCα antibody as described under “Experimental Procedures.” Results are representative of three different experiments.

To determine whether activation of protein phosphatase and dephosphorylation of PKCα are responsible for the translocation process, we treated L929 cells with TNF-α in the absence and presence of okadaic acid (2–5 nM), followed by PMA stimulation. The membrane and cytosolic fractions were collected as described under “Experimental Procedures.” Clearly, PMA led to PKCα translocation in control and TNF-α-treated cells as seen before (Fig. 8). The addition of okadaic acid did not affect the induction of PKCα translocation by TNF-α. Furthermore, C6-ceramide had a minimal effect on the translocation of PKCα in the absence or presence of okadaic acid (data not shown). These results demonstrate that the dephosphorylation and translocation processes are two separate events and that translocation of PKCα by TNF-α is independent of ceramide.

**DISCUSSION**

This study demonstrates conflicting signals on PKCα activity by TNF-α. Specifically, TNF-α inactivates cellular PKCα, and TNF-α is also capable of inducing PKCα translocation from the cytosol to the membrane. In addition, this study on the mechanisms of TNF-α action illustrates the following. 1) TNF-α causes a concentration-dependent generation of ceramide and diacylglycerol; 2) activation of protein phosphatases (such as CAPP) appears to mediate some of the TNF effect on the alteration of PKCα activity; and 3) TNF-α-induced dephosphorylation and translocation of PKCα are independent processes. PKC activation is often initiated by direct translocation from the cytosol to the membrane, where it is activated by diacylglycerol. One potential mechanism by which TNF-α could exert its inhibitory effect on PKCα is through inhibition of its translocation. Our data demonstrate that this is not the case, as inhibition of PKCα activity by TNF-α is independent of enzyme translocation (Fig. 3). More important, TNF-α induced PKCα translocation on its own and independent of ceramide and activation of okadaic acid-inhibitable phosphatases (Fig. 8 and data not shown). These results suggest that the interactions between different signaling pathways may initiate a conflict in cell signaling. PKC is likely to be a downstream target of TNF-α signal transduction, and inhibition of PKCα activity may represent the end point of a series of different cellular signals. In fact, we found that the levels of diacylglycerol were increased, concomitantly with ceramide following TNF-α treatment (Fig. 5A). Thus, activation of diacylglycerol by TNF-α may lead to the induction of PKCα translocation. These results are in agreement with a previous study demonstrating cell type-specific activation and translocation of PKC by TNF-α (16). On the other hand, generation of ceramide by TNF-α may tilt the balance between PKC/diacylglycerol and ceramide/phosphatase interactions and lead to inhibition of PKCα activity.
Recently, PKC has been linked to the regulation of both cell growth and cell death. Numerous studies have demonstrated its involvement in stimulation of cell growth and transformation (34–38). Accumulating evidence also points to its negative regulation of cell growth and the initiation of apoptosis (39–42). In the prostate epithelial cell line LNCaP, activation of PKCα isozyme following treatment with 12-O-tetradecanoylphorbol-13-acetate led to apoptosis (43). A few other studies have also demonstrated that PKC activators inhibit DNA synthesis in certain cell types (44–47). These observations suggest that PKC isozymes play specific, differential roles in signal transduction. The differences among individual PKC isozymes with respect to their tissue distribution, substrate specificity, and mode of activation constitute the multifunctions of PKC under different circumstances. In support of this notion, it has been reported that C2-ceramide is able to induce translocation of PKCβ and PKCe to the cytosol, leading to apoptosis (48). Another recent study has also demonstrated that ionizing radiation induces PKCα translocation from the plasma membrane to the cytosol in the TF-1 human erythroleukemia cell line (18).

Elevation of ceramide has been implicated to be responsible in part for the regulation of cellular functions in TNF-α signal transduction pathways. Neutral sphingomyelinase is considered a key enzyme in the regulation of hydrolysis of sphingomyelin to form ceramide in response to a variety of extracellular agents (4, 5). Recent observations demonstrated that GSH inhibits neutral sphingomyelinase in vitro and provides a useful tool to further understand the regulatory mechanism of sphingolipid signaling (31). Besides activation of sphingomyelinases, another major pathway controlling cellular ceramide levels is through de novo synthesis. The realization of the importance of de novo synthesis has come from studies utilizing the myco-toxin fumonisin. Fumonisin B1, an inhibitor of ceramide synthase (30), has been widely used to block de novo synthesis of ceramide. Recent studies have also used fumonisin as a tool in ceramide-mediated apoptosis. Using fumonisin B1 and GSH, we were able to show that both fumonisin B1 and GSH reversed the effect of TNF-α on PKC activity (Fig. 5, B and C). These data suggest that ceramide may mediate some of the effects of TNF-α on cellular functions through either de novo biosynthesis or activation of neutral sphingomyelinase.

In addition to ceramide generation, TNF-α also leads to generation of arachidonic acid via activation of phospholipase A2 (49). Phospholipase A2 hydrolyzes phospholipids to generate free fatty acids and phospholipids. These fatty acids (including arachidonic acid) potentiate the diacylglycerol-dependent activation of PKC (50, 51). Therefore, generation of free fatty acids through activation of phospholipase A2 in TNF-α signal transduction (29) can lead to inhibition of PKCα activity. Thus, alteration of PKC activity reflects the existence of a complex array of cross-talk between signaling pathways. Tilting the balance of cell regulation by a variety of intracellular and extracellular agents including TNF-α will ultimately change cell growth processes.

Activation of PKC significantly influences cellular responses, including cell proliferation and differentiation (51). One important aspect of PKC activation relates to its phosphorylation state. Phosphorylation of PKC is a preliminary and necessary event for its subsequent activation. Initially, PKCα is synthesized as a dephosphorylated, membrane-bound, inactive protein of 74 kDa that is converted to an active and mature protein of 80 kDa by post-translational modification such as phospho-

![Diagram](image-url)
Functional Dichotomy of PKC in TNF-α Signal Transduction

A

Levels of Phosphorylated PKC Protein Detected by P500 Ab (arbitrary units)

control | TNF

B

Levels of Phosphorylated PKC Protein Detected by P500 Ab (arbitrary units)

p500 ave

c

C

Levels of Phosphorylated PKC Protein Detected by Ser660 Ab (arbitrary units)

control | TNF

D

Levels of Phosphorylated PKC Protein Detected by Ser660 Ab (arbitrary units)

Ser60 C6 ave

E

Levels of PKC Protein (arbitrary units)

control | TNF

total protein ave

F

Levels of PKC Protein (arbitrary units)

PKC C6 ave
ration (52, 53). Recent studies have identified three functionally distinct phosphorylation sites of protein kinase C (32). Transphosphorylation of threonine 500 on the activation loop yields catalytic competency of PKC autophasorylation. Autophosphorylation of threonine 641 at the carboxyl terminus maintains its competency of PKC autophasorylation. The membrane-bound enzyme is autophasorylated at position 660 at the carboxyl terminus. This autophosphorylation may play an important role in the regulation of the membrane affinity of the enzyme and its subcellular localization (32). Our results using anti-phosho-PKC antibodies demonstrated that treatment with TNF-α or C₆-ceramide altered the phosphorylation state of PKC at p500 and Ser⁶⁶⁰ (Fig. 7). Inhibition of PKC phosphorylation will ultimately lead to the inactivation of the enzyme. Another recent study (54) has shown that phosphorylation of PKC at Ser⁶⁶⁰ is important in controlling the accumulation of phosphate at other sites on PKCα and in maintaining its phosphatase-resistant conformation. PKCα with C-terminal mutations at Ser⁶⁶⁰ exhibited a doublet on SDS-PAGE. Dephosphorylation at Ser⁶⁶⁰ in PKCα could cause hypersensitivity of PKCα toward phosphatases, which in turn leads to a further alteration of the enzyme activity. It is conceivable that activation of CAPP by ceramide through TNF-α signal transduction may lead to dephosphorylation of PKCα at multiple sites, including Thr⁵⁰⁰, Ser⁶⁶⁰, and Ser⁶⁵⁷. Following the dephosphorylation at Ser⁶⁵⁷, the PKC isozyme becomes vulnerable to protein phosphatases and ultimately becomes inactivated. Clearly, the regulation of phosphorylation of PKCα has an essential role in cell signaling.

Following sustained activation of PKC by exposure to the metabolic activator 12-O-tetradecanoylphorbol-13-acetate, which leads to dephosphorylation of PKCα in COS cells (28), PKCα migrated as a doublet characteristic of phosphorylated and dephosphorylated PKCα species. Indeed, dephosphorylation of PKCα by 12-O-tetradecanoylphorbol-13-acetate correlates with the membrane-associated heterotrimeric PP2A. Consistent with this finding, our data have also demonstrated an appearance of a doublet of PKCα on SDS-PAGE upon TNF-α treatment (Fig. 4). Moreover, both TNF-α and ceramide were able to block PKC phosphorylation as detected by anti-phospho-PKC antibodies (Fig. 7). These results suggest that generation of ceramide following exposure to TNF-α may lead to dephosphorylation of PKCα through activation of a protein phosphatase such as CAPP and ultimately inactivation of the enzyme.

The elucidation of the role of phosphoinositide-dependent kinase-1 in phosphoinositide (PI) 3-kinase signaling unveiled a key regulatory pathway in the regulation of protein kinase B/Akt (55). Phosphoinositide-dependent kinase-1 binds to phosphatidylinositol 3,4,5-triphosphate and phosphorylates and activates PI 3-kinase targets, protein kinase B/Akt and p70⁷⁰⁶K (56, 57). Similarly, phosphoinositide-dependent kinase-1 regulates the phosphorylation of conventional PKC isozymes in vivo and in vitro (58). Phosphoinositide-dependent kinase-1 specifically phosphorylates the activation loop of both PKCα and PKCβII, thus providing a critical step in the regulation of PKC function. Given the fact that agents that activate the PI 3-kinase/Akt pathway trigger an anti-apoptotic signal (59) and that ceramide regulates phospholipase D and PKC (12, 60, 61) and leads to activation of apoptotic signaling, it is conceivable that TNF-α and/or ceramide could inhibit PKCα through regulation of phosphoinositide-dependent kinase-1. In fact, several recent studies have reported that TNF-α and/or ceramide activates PI 3-kinase (62, 63), whereas Zundel and Giaccia (64) have shown the inhibition of PI 3-kinase/Akt kinase by ceramide. A few other studies have demonstrated the inhibition of Akt kinase activity by ceramide, but the effect of ceramide on PI 3-kinase activity was not significant (65, 66). The differences between these studies may be due to the different experimental designs and the different model systems used in the studies. In attempt to delineate the mechanism by which ceramide regulates Akt kinase activity, Cuadrado and coworkers (66) found that down-regulation of the Akt kinase by ceramide is through dephosphorylation of Akt1 at Thr⁵³⁵ and Ser⁶⁷³ by activation of CAPP. These results are consistent with the finding that PP2A inactivates Akt kinase in vitro (67). However, the concentrations of okadaic acid used were relatively high and probably did not inhibit PP2A or CAPP specifically. Others did not observe a significant effect by okadaic acid (65, 68). Thus, the involvement of PP2A or CAPP in the regulation of phosphorylation of Akt kinase needs further clarification. Nonetheless, inhibition of Akt kinase by ceramide through modulation of phosphorylation provides one of the key mechanisms of its regulation. Whether disruption of the phosphoinositide-dependent kinase-1/PI 3-kinase/Akt signaling pathway has a direct impact on the regulation of PKCα activity by TNF-α and/or ceramide in L929 cells through activation of CAPP remains to be determined.

The underlying mechanism by which TNF-α regulates PKCα activity is still poorly understood. Evidently, the regulation of phosphorylation and translocation of PKC has a significant impact on its activity and signaling pathways. It remains unclear how phosphorylation is related to the translocation process in cell signaling. Our current studies demonstrate that activation of a protein phosphatase such as CAPP and dephosphorylation of PKCα by TNF-α are independent of PKCα translocation activated by TNF-α (Fig. 8). Thus, it is highly likely that a combination of a complex intracellular environment and extracellular influences dictate the balance among different signaling pathways (including PKC and TNF-α) that then direct the outcome of cellular responses. Studying the mechanisms by which PKC signaling pathways respond to TNF-α...
exposure may add to our knowledge of the basic biology of cell survival.

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