The Involvement of Novel Protein Kinase C Isozymes in Influencing Sensitivity of Breast Cancer MCF-7 Cells to Tumor Necrosis Factor-α

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ABSTRACT
Protein kinase C (PKC) has been implicated in tumor necrosis factor-α (TNF) signaling. Structurally and functionally distinct PKC activators and selective inhibitors of PKC were used to investigate the involvement of PKC isoforms in influencing TNF sensitivity in MCF-7 cells. Activators of PKC, such as phorbol-12,13-dibutyrate (PDBu) (1.0 μM), indolactam V (10 μM), and bryostatin 1 (1.0 μM), decreased the sensitivity of MCF-7 cells to TNF by 5-, 10-, and 1.7-fold, respectively. The PKC-specific sensitivity in MCF-7 cells. Activators of PKC, such as phorbol-12,13-dibutyrate (PDBu) (1.0 μM), indolactam V (10 μM), and bryostatin 1 (1.0 μM) decreased the sensitivity of MCF-7 cells to TNF by 5-, 10-, and 1.7-fold, respectively. The PKC-specific inhibitor bisindolylmaleimide II (BIM) (≥1 μM) antagonized the effect of PDBu in protecting MCF-7 cells against TNF cytotoxicity. High concentrations of BIM (≥10 μM) also significantly enhanced the sensitivity of MCF-7 cells to TNF. In contrast, Gö 6976, a specific inhibitor of cPKCs, did not potentiate TNF sensitivity and failed to reverse the effect of PDBu. In addition, BIM but not Gö 6976 blocked PDBu-mediated down-regulation of TNF receptors. There was no correlation between down-regulation of PKCα, -δ, and -ε, and protection against TNF cytotoxicity by PKC activators. A 6-hr exposure to 1.0 μM PDBu, 10 μM indolactam V, and 1.0 μM bryostatin 1 caused a 1.8-, 3.5-, and 1.2-fold induction, respectively, of nPKCη in MCF-7 cells. Similar exposure to BIM but not Gö 6976 led to a significant down-regulation of nPKCη. This novel regulation of PKCη implicates this isozyme in PDBu-mediated protection of MCF-7 cells against TNF cytotoxicity.

TNF, a pleotropic cytokine, exhibits cytotoxic/cytolytic activity against several tumors (Beyaert and Fiers, 1994; Heller and Kronke, 1994). TNF mediates its action by binding to its cell surface receptors, and two receptors, with molecular masses of 55–60 kDa (TNFRI) and 70–80 kDa (TNFRII), have been identified (Lewis et al., 1991; Schall et al., 1990; Smith et al., 1990). Most cells, including TNF-resistant cells, express TNFRI, which is believed to be the major mediator of TNF cytotoxicity (Lewis et al., 1991; Schall et al., 1990; Smith et al., 1990). The binding of TNF to its receptors is necessary but not sufficient for its cytotoxic action (Cressey et al., 1987; Lewis et al., 1991).

Unlike growth factor receptors, TNF receptors do not possess any intrinsic kinase activity (Schall et al., 1990; Smith et al., 1990). Nevertheless, the interaction of TNF with its receptors induces phosphorylation of several proteins, and inhibitors of protein kinases influence TNF sensitivity significantly, suggesting that protein phosphorylation plays a critical role in TNF signaling (Beyaert and Fiers, 1994; Vilcek and Lee, 1991). Several protein kinases, including PKC, have been implicated in mediating TNF responses (Galeotti et al., 1993; Hamamoto et al., 1990; Johnson and Baglioni, 1988; Sampson et al., 1993; Schutze et al., 1990; Zhang et al., 1994). It has been demonstrated that TNF can resemble a PKC activator. It can cause rapid production of DAG, activation of PKC, and phosphorylation of proteins (Krone et al., 1992; Pusztai et al., 1993; Sampson et al., 1993; Schutze et al., 1990). TNF-stimulated protein phosphorylation could be blocked by PKC inhibitors (Sampson et al., 1993). In addition, PKC-dependent protein phosphorylation induced resistance to TNF-mediated cytotoxicity, and inhibition of PKC potentiated the cytotoxicity of TNF (Sampson et al., 1993). The regulation of TNF sensitivity by PKC, however, varied significantly among cell types. TNF caused translocation of PKC in some but not all cells (Schutze et al., 1990). TNF-induced DAG production was not accompanied by an increase in cellular calcium (Heller and Kronke, 1994). Nonspecific PKC inhibitors, such as staurosporine, did not influence TNF sensitivity at concentrations required to block PKC activity in vitro and in intact cells (Beyaert et al., 1993). Finally, inhibition and/or down-regulation of PKC by 12-O-tetradeca-

ABBREVIATIONS: TNF, tumor necrosis factor; PKC, protein kinase C; DAG, diacylglycerol; pPKC, atypical protein kinase C; cPKC, conventional protein kinase C; nPKC, novel protein kinase C; NF-κB, nuclear factor κB; PDBu, phorbol-12,13-dibutyrate; ILV, indolactam V; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′,N′′,N′′-tetraacetic acid; BIM, bisindolylmaleimide II; Me2SO, dimethyl sulfoxide; PKA, cAMP-dependent protein kinase.

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noyolphosphol-13-acetate failed to inhibit TNF-mediated activities (Beyaert et al., 1993; Pusztai et al., 1993).

These apparent anomalies can be attributable, in part, to the differential expression, complex regulation, and distinct functions of PKC isozymes, a family of 12 closely related proteins [reviewed in Basu (1993)]. Based on structural variations and biochemical properties, the PKC isozymes can be categorized into three groups: group A or cPKC (α, βI, βII, and γ); group B or nPKC (δ, ε, η, θ, and μ); and group C or aPKC (ξ and λ) (Basu, 1993; Johannes et al., 1994). The isozymes differ in biochemical properties, tissue specific distribution, and intracellular localization. Whereas cPKCs and nPKCs can be activated by DAG and tumor-promoting phorbol esters, whereas aPKCs are insensitive to phorbol ester/phorbol ester/calcium- and phospholipid-dependent, nPKCs and aPKCs do not require any calcium for their activities. Both cPKCs and nPKCs can be activated by DAG and tumor-promoting phorbol esters, whereas aPKCs are insensitive to phorbol ester/DAG. The expression and regulation of PKC isozymes vary significantly with cell types.

Several studies have suggested a role for phorbol ester-insensitive PKC, namely aPKC, in TNF signaling. First, interaction of TNF with its receptors generates second messengers, ceramide, and arachidonic acid that can regulate PKCζ (Muller et al., 1995). Second, overexpression of PKCζ in fibroblasts activated NF-κB, a critical mediator of TNF signaling (Diaz-Meco et al., 1993). Third, the expression of a dominant negative mutant of PKCζ inhibited NF-κB activation (Diaz-Meco et al., 1993). Another group was, however, unable to demonstrate NF-κB activation by overexpression of PKCζ in NIH 3T3 cells (Montaner et al., 1995), thus questioning the importance of PKCζ in NF-κB activation. In addition, although the observations that TNF can generate DAG in the absence of an increase in cellular calcium and that tumor promoting phorbol esters influence TNF sensitivity suggest a strong role for DAG/phorbol ester-dependent but calcium-independent PKC in TNF signaling, there have been no reports on the involvement of nPKCs in TNF signaling. In the present study, I have used several activators and inhibitors of PKC to examine the role of PKC isozymes in influencing TNF sensitivity in breast cancer MCF-7 cells.

Experimental Procedures

Materials. TNF was purchased from R & D Systems (Minneapolis, MN). PDBu and ILV were from LC Service Corporation (Woburn, MA) and protein kinase inhibitors from CalBiochem (San Diego, CA). MTT was purchased from Sigma (St. Louis, MO) and Alamar Blue from GIBCO-BRL (Grand Island, NY), and PKCζ and -ε were from Santa Cruz Biootechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson Immuno Research (West Grove, PA). Enhanced chemiluminescence detection kit was from Amersharm (Arlington Heights, IL). 125I-TNF (specific activity, 44.6 μCi/μg) was from DuPont-New England Nuclear (Wilmington, DE).

Cell culture. 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° with 95% air and 5% CO₂.

Assessment of cell viability. Exponentially growing cells were plated in microtiter plates and incubated at 37° in 5% CO₂. The following day, cells were pretreated without or with protein kinase modulators and then with different concentrations of TNF. After 6–15 hr, the medium was replaced with fresh medium containing 10% fetal bovine serum. The number of viable cells was determined after 48–96 hr using the dye MTT as described previously (Basu et al., 1990). Recently, I have adopted an Alamar Blue assay instead of MTT assay because of its simplicity. Very similar results were obtained using either method. In the Alamar Blue assay, cells in the microtiter plate were incubated with 20 μl per well (0.1 of the volume of the culture medium) dye at 37° for 4–6 hr, and fluorescence was determined using a Cytofluor II fluorescence plate reader (PerSeptive Biosystems, Cambridge, MA) using an excitation wavelength of 530 nm and emission wavelength of 590 nm.

Immunoblot analysis. Cells were treated with TNF or PKC modulators as described in Results and in the legend to Fig. 1. At the end of the incubation, cells were harvested and washed with cold phosphate-buffered saline. Briefly, cells were homogenized in buffer A (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin and aprotinin) and centrifuged at 100,000 × g for 1 hr, and the pellet was homogenized in buffer A. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to a polyvinylidene difluoride membrane. Immunoblot analyses were performed with PKC isozyme-specific antibodies as described previously (Basu et al., 1996). The blots were visualized using the enhanced chemiluminescence detection reagents and the manufacturer’s protocol. Intensities of immunoreactive proteins were quantified by laser densitometry. Because the abundance of PKCζ and -ε was not altered by any of the treatments, the level of these isozymes was used as an internal control to account for any variability associated with the amount of protein loaded in each lane during electrophoresis.

Binding assay. Cells (1 × 10⁵) were treated with or without PKC activators and/or inhibitors as described in the text, washed, and then incubated with different concentrations of 125I-TNF in complete medium at 4° for 2 hr. Cells were then washed extensively with phosphate-buffered saline containing 0.1% BSA, solubilized in 0.25 N NaOH, and counted in a γ-counter. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled TNF. Specific binding was defined as the difference between total binding and nonspecific binding. The maximum binding sites (B_max) and binding affinity (K_d) were calculated from the nonlinear regression analysis of saturation binding isotherms using the Prism computer program (GraphPad Software, San Diego, CA).

Results

Effects of protein kinase modulators on the sensitivity of MCF-7 cells to TNF. I have compared the ability of structurally and functionally distinct PKC activators, namely PDBu, ILV, and bryostatin 1, to influence the sensitivity of MCF-7 cells to TNF. Continuous exposure to TNF for several hours was necessary for the cytotoxic action of TNF. Because PKC modulators by themselves may affect cell growth, I pretreated cells with PKC modulators for 1 hr, exposed them to TNF for an additional 6 hr, and then incubated them in fresh medium for 2–4 days. Under that condition, contribution of PKC activators and inhibitors on cell growth was significantly reduced. Fig. 1 shows that 1 μM PDBu and 10 μM ILV decreased TNF sensitivity by approximately 5- and 9-fold, respectively. One micromolar ILV was slightly less effective than 1 μM PDBu in protecting cells against TNF cytotoxicity (data not shown). In contrast, 1 μM bryostatin 1 had only a modest effect (1.7-fold) on TNF sensitivity.

Fig. 2 shows the effects of three PKC inhibitors on the sensitivity of MCF-7 cells to TNF. It has been shown before that the nonspecific PKC inhibitor staurosporine potentiates
25 nM staurosporine. Higher concentrations of staurosporine in TNF sensitivity; a 4-fold sensitization was achieved with 2, staurosporine caused a concentration-dependent increase in TNF sensitivity even at 10 μM concentrations. Fig. 3 shows that cells treated with 10 μM BIM (Fig. 3B) retained morphology similar to untreated control cells (Fig. 3A), whereas treatment with 1.0 nM TNF (Fig. 3C) or combination of TNF and BIM (Fig. 3D) induced chromatin condensation indicative of cell death by apoptosis.

To further evaluate the involvement of PKC, I tested the ability of PKC inhibitors to reverse PDBu-mediated protection against TNF cytotoxicity. As shown in Fig. 4, both staurosporine and 10 μM BIM not only sensitized MCF-7 cells to TNF but also antagonized the effect of PDBu. Because the IC_{50} values of 10 μM BIM-treated cells were extremely low (0.005 nM and 0.007 nM in the absence and presence of PDBu, respectively), it was difficult to see the columns. Although 1 μM BIM did not sensitize cells to TNF, it was able to counteract the effect of PDBu in a statistically significant manner (p < 0.005). In contrast, the effect of Gö 6976 on TNF sensitivity was statistically insignificant in both the presence and the absence of PDBu.

Effects of PKC modulators on PKC isozyme expression. To investigate the possible involvement of a PKC isozyme in influencing TNF sensitivity, I monitored the level of PKC isozymes after treatment with TNF or PKC modulators (Fig. 5). The intensity of PKC isozymes was quantified by scanning immunoblots with a laser densitometer. A 6-hr exposure to 1.0 nM TNF had little effect on the expression of any of the PKC isozymes. In addition, the expression of PKCμ, -γ (Fig. 5A), and -ε (data not shown) was not altered significantly by any of the treatments. Fig. 5B shows the changes in expression of PKCα, -δ, -ε, and -γ by PKC modulators. A 6-hr exposure to 1 μM PDBu and 1 μM bryostatin 1 led to a 25 and 60% down-regulation of PKCα, respectively. They also caused approximately 50% down-regulation of PKCε. In contrast, 10 μM ILV had no effect on the expression of PKCα and -ε. All three PKC activators caused a substan-

t cells to TNF by approximately 100-fold, whereas 1 μM BIM had no effect (Fig. 2). In contrast, the cPKC inhibitor Gö 6976 caused only a 2-fold increase in TNF sensitivity even at 10 μM concentrations. Fig. 3 shows that cells treated with 10 μM BIM (Fig. 3B) retained morphology similar to untreated control cells (Fig. 3A), whereas treatment with 1.0 nM TNF (Fig. 3C) or combination of TNF and BIM (Fig. 3D) induced chromatin condensation indicative of cell death by apoptosis.

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TNF cytotoxicity in several tumor cells (Beyaert et al., 1993; Hamamoto et al., 1990; Zhang et al., 1994). As shown in Fig. 2, staurosporine caused a concentration-dependent increase in TNF sensitivity; a 4-fold sensitization was achieved with 25 nM staurosporine. Higher concentrations of staurosporine (≥100 nM) were extremely toxic to MCF-7 cells, causing more than 50% cell kill, presumably due to inhibition of several kinases. I also investigated the effects of two PKC inhibitors that are specific for PKC but exhibit distinct selectivity toward PKC isozymes. BIM inhibits all PKC isozymes, whereas Gö 6976 inhibits only cPKCa and -β1 (Martiny-Baron et al., 1993; Toullec et al., 1991). BIM (10 μM) by itself had little effect on cell growth (<20%), but it enhanced the sensitivity of MCF-7 cells to TNF by approximately 100-fold, whereas 1 μM BIM had no effect (Fig. 2). In contrast, the cPKC inhibitor Gö 6976 caused only a 2-fold increase in TNF sensitivity even at 10 μM concentrations.
the means of PKC activity in several cell lines (Schutze, 1990), I compared the effects of BIM on the sensitivity of MCF-7 cells to TNF and on the expression of PKC isozymes. Although PDBu and ILV caused a significant protection against TNF cytotoxicity, bryostatin 1 had little effect. PKC activators had no major effect on the protection against TNF cytotoxicity by itself also blocked TNF receptor down-regulation by PDBu completely. Go 6976 at 10 μM did not influence 125I-TNF binding to cell surface receptors either alone or in combination with PDBu.

**Discussion**

Binding of TNF to its receptors triggers two competing signals—activation of cell death and induction of cellular protective responses to abrogate its own cytotoxicity. The ultimate effect is a balance between these two pathways. PKC activators have been shown to protect cells against TNF cytotoxicity but the isozyme of PKC that is responsible for the protection against TNF cytotoxicity has not been documented. The results of our present study indicate that nPKCs but not aPKCs are important in regulating sensitivity of breast cancer MCF-7 cells to TNF.

I have compared the effects of three PKC activators, PDBu, ILV, and bryostatin 1 that are structurally and functionally distinct, on the sensitivity of MCF-7 cells to TNF and on the expression of PKC isozymes. Although PDBu and ILV caused a significant protection against TNF cytotoxicity, bryostatin 1 had little effect. PKC activators had no major effect on the abundance of PKCα, -ζ, or -η. In addition, there was no correlation between down-regulation of PKCα, -ζ, and -η, and protection against TNF cytotoxicity by PKC activators. Interestingly, both PDBu and ILV caused a significant induction of PKCζ, whereas bryostatin 1 had only a little effect. Similar up-regulation of PKCζ by PDBu was also noted in EL4 mouse thymoma cells (Resnick et al., 1997). There was a good correlation between PKCζ up-regulation and fold-protection by PKC activators (Fig. 1). In contrast, Gö 6976 did not influence the subcellular distribution of aPKCζ.

A 6-hr exposure to 1.0 nM TNF had little effect on the distribution of any of the PKC isozymes.

**Effects of PDBu and BIM on binding of TNF to its receptor.** PDBu has been shown to cause down-regulation of TNF receptors (Aggarwal and Eessalu, 1987; Johnson and Baglioni, 1988). Fig. 7 shows that pretreatment of MCF-7 cells with 1 μM PDBu for 1 hr significantly reduced binding of 125I-TNF to its receptors. PDBu caused a significant decrease in maximum binding sites (Bmax) with little change in binding affinity (Kd); Bmax was decreased from 3681 ± 151 to 608 ± 33 cpm and Kd from 1.48 ± 0.11 to 1.0 ± 0.11 nM. To examine whether the effect of PDBu on TNF binding was mediated by cPKCs or nPKCs, I compared the effects of BIM and Gö 6976 on PDBu-mediated receptor down-regulation (Fig. 7). Although incubation of MCF-7 cells with 10 μM BIM alone for 2 hr had little effect on TNF binding, it abolished the decrease in TNF binding elicited by PDBu. In addition, 1 μM BIM that had no effect on TNF cytotoxicity by itself also blocked TNF receptor down-regulation by PDBu completely. Gö 6976 at 10 μM did not influence 125I-TNF binding to cell surface receptors either alone or in combination with PDBu.
selectively inhibits cPKCα and β1 (IC50 < 10 nM) but has little effect on Ca2+-independent PKCs at micromolar levels (Martiny-Baron et al., 1993).

The studies with PKC inhibitors also support the notion that nPKCs are important in TNF signaling. For example, the broad PKC inhibitors BIM and staurosporine influenced TNF sensitivity, whereas cPKC inhibitor Gö 6976 had no effect (Fig. 4). Although low concentrations of BIM (1 μM) and staurosporine (10 nM) had little effect on TNF sensitivity by themselves, they reversed the effect of PDBu in protecting cells against TNF cytotoxicity (Figs. 2 and 4). Because aPKCs are PDBu-insensitive, these results also indicate that nPKCs influence the protection against TNF cytotoxicity by PDBu.

Interestingly, both BIM and staurosporine caused significant down-regulation of nPKCγ, suggesting that nPKCγ level may be regulated by a phosphorylation-dephosphorylation mechanism. Based on this novel regulation of PKCγ by PKC activators and inhibitors, it is tempting to speculate that this isozyme was responsible for protection of MCF-7 cells against TNF cytotoxicity by PKC activators. Similar results were observed with another breast cancer BT-20 cell line, which also expresses nPKCγ (A. Basu, unpublished observation). The generality of this observation, however, must await study in transfected model systems and a wider spectrum of PKCγ-expressing nontransfected cells.

Our results suggest that PKC inhibitors act at more than
one step in the TNF signal transduction pathway. BIM completely blocked TNF receptor down-regulation by PDBu, suggesting that receptor down-regulation can partly explain the mechanism of protection by PDBu. In contrast, the cPKC inhibitor Gö 6976 had no effect on PDBu-mediated reduction in TNF binding. Although both 1 and 10 μM BIM blocked receptor down-regulation, only 10 μM BIM enhanced TNF cytotoxicity, suggesting that inhibition of receptor down-regulation was not responsible for TNF sensitization. It has been reported earlier that TNF cytotoxicity does not correlate with receptor number and/or affinity (Creasey et al., 1987; Lewis et al., 1991; Sugarman et al., 1985). The differential effects of 1 and 10 μM BIM on TNF cytotoxicity, in fact, dissociates receptor binding from TNF sensitization and emphasizes that the postreceptor signaling event was responsible for potentiation of TNF cytotoxicity by high concentrations of BIM. In addition, inasmuch as 1 μM BIM caused significant down-regulation of nPKCζ but did not sensitize cells to TNF, it is unlikely that down-regulation of PKCζ was associated with the enhancement of TNF cytotoxicity. Thus, high concentrations of BIM may trigger a parallel pathway that may involve some as yet unidentified kinase or nPKCζ, which has also been implicated in TNF signaling (Diaz-Meco et al., 1993; Muller et al., 1995). Furthermore, the regulation of PKC isozymes depends on the cellular context. Cytosolic translocation of nPKCδ and -e has been associated with ceramide-induced apoptosis (Sawai et al., 1997). I have shown that overexpression of nPKCζ in rat fibroblasts prevented apoptosis induced by the chemotherapeutic drug cisplatin (Basu and Cline, 1995). Future studies are needed to determine the contribution of these isozymes in TNF signaling.

Recent studies suggest that the activation of NF-xB provides protection against TNF cytotoxicity and inhibition of NF-xB sensitizes cells to TNF (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996). BIM by itself had no effect on NF-xB activation (A. Basu, unpublished observation). Although both TNF and PDBu caused induction of NF-xB in MCF-7 cells, BIM inhibited NF-xB induction by PDBu but not by TNF. In fact, like staurosporine (Beyaert et al., 1993; Hofmann et al., 1992), BIM enhanced TNF-mediated NF-xB induction by almost 2-fold, suggesting that the mechanism of TNF sensitization may not involve inhibition of NF-xB. Because many tumor cells are resistant to TNF, an understanding of the mechanism(s) by which BIM enhances TNF cytotoxicity will greatly facilitate development of novel approaches to cancer therapy.

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