

Phorbol 12-Myristate 13-Acetate Protects against Tumor Necrosis Factor (TNF)-Induced Necrotic Cell Death by Modulating the Recruitment of TNF Receptor 1-Associated Death Domain and Receptor-Interacting Protein into the TNF Receptor 1 Signaling Complex: Implication for the Regulatory Role of Protein Kinase C

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ABSTRACT

Protein kinase C (PKC) triggers cellular signals that regulate proliferation or death in a cell- and stimulus-specific manner. Although previous studies have demonstrated that activation of PKC with phorbol 12-myristate 13-acetate (PMA) protects cells from apoptosis induced by a number of mechanisms, including death receptor ligation, little is known about the effect or mechanism of PMA in the necrotic cell death. Here, we demonstrate that PMA-mediated activation of PKC protects against tumor necrosis factor (TNF)-induced necrosis by disrupting formation of the TNF receptor (TNFR)1 signaling complex. Pretreatment with PMA protected L929 cells from TNF-induced necrotic cell death in a PKC-dependent manner, but it did not protect against DNA-damaging agents, including doxorubicin (Adriamycin) and camptothecin. Analysis of the upstream signaling events affected by PMA revealed that it markedly inhibited the TNF-induced recruitment of TNFR1-associated death domain

protein (TRADD) and receptor-interacting protein (RIP) to TNFR1, subsequently inhibiting TNF-induced activation of nuclear factor- κ B and c-Jun NH₂-terminal kinase (JNK). However, JNK inhibitors do not significantly affect TNF-induced necrosis, suggesting that the inhibition of JNK activation by PMA is not part of the antinecrotic mechanism. In addition, PMA acted as an antagonist of TNF-induced reactive oxygen species (ROS) production, thereby suppressing activation of ROS-mediated poly(ADP-ribose)polymerase (PARP), and thus inhibiting necrotic cell death. Furthermore, during TNF-induced necrosis, PARP was significantly activated in wild-type mouse embryonic fibroblast (MEF) cells but not in RIP^{-/-} or TNFR-associated factor 2^{-/-} MEF cells. Taken together, these results suggest that PKC activation ensures effective shutdown of the death receptor-mediated necrotic cell death pathway by modulating formation of the death receptor signaling complex.

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Members of the tumor necrosis factor (TNF) superfamily are critical regulators of the balance between opposing signals that can trigger either cell survival or death, depending upon the predominating signaling pathway in the particular

ABBREVIATIONS: TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRADD, tumor necrosis factor receptor 1-associated death domain; FADD, FAS-associated death domain; TRAF, tumor necrosis factor receptor-associated factor; RIP, receptor-interacting protein; NF- κ B, nuclear factor- κ B; NCD, necrotic cell death; FasL, Fas ligand; ROS, reactive oxygen species; MEF, mouse embryonic fibroblast; PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; ERK, extracellular signal-regulated kinase; PARP, poly(ADP-ribose)polymerase; GFP, green fluorescent protein; PAR, poly(ADP-ribose); JNK, c-Jun NH₂-terminal kinase; p-, phospho-; CHX, cycloheximide; BHA, butylated hydroxyanisole; BAY-11, BAY 11-7082; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; MEK, mitogen-activated protein kinase kinase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; CM-H₂ DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HEK, human embryonic kidney; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; NP-40, Nonidet P-40; FITC, fluorescein isothiocyanate; PI, propidium iodide; DCF-DA, 2,7-dichlorofluorescein diacetate; Ro 31-8220, 3-[1-[3-(amidinothio)propyl]-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimide (bisindolylmaleimide IX); 3-AB, 3-aminobenzamide; DPQ, 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1-(2H)-isoquinolinone; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one; z-VAD-FMK, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

cell type (Aggarwal, 2003). These responses are elicited by the TNF-induced trimerization of two distinct cell surface receptors, TNF receptor (TNFR)1 (p55) and TNFR2 (p75), at least one of which is present in almost all cell types (Tartaglia and Goeddel, 1992; Vandenabeele et al., 1995). Although most of the biological activities of TNF seem to be transduced by TNFR1, many can also be mediated by TNFR2 (Tartaglia and Goeddel, 1992; Vandenabeele et al., 1995). Extensive research has identified a number of molecules that are involved in the upstream signaling mechanism of the TNFRs. Upon binding of TNF to TNFR1, the death domain in the cytoplasmic tail of the receptor is able to recruit TNFR1-associated death domain protein (TRADD) (Baud and Karin, 2001). TRADD then serves as a platform for the recruitment of other adaptor proteins, such as FAS-associated death domain protein (FADD), TNFR-associated factor (TRAF)2, and receptor-interacting protein (RIP), to build a receptor complex. Apoptotic responses to TNF can be mediated by the recruitment of FADD and the subsequent activation of caspase-8, and the antiapoptotic responses can be mediated by the activation of the NF- κ B pathway by RIP and TRAF2 (Liu et al., 1996; Ting et al., 1996; Kelliher et al., 1998).

Although caspases are critical for the mediation of apoptosis, they do not seem to be obligatory for all forms of cell death. Indeed, TNF can cause cell death by both apoptotic and necrotic (nonapoptotic) mechanisms, depending on the specific cell type and treatment conditions involved (Deanecker et al., 2001; Vanden Berghe et al., 2004). Interestingly, blockade of caspases can increase the sensitivity of the necrotic cell death (NCD) response to FasL and TNF (Vercaemmen et al., 1998; Los et al., 2002), and reactive oxygen species (ROS) were found to be essential for TNF-induced NCD in L929 cells and mouse embryonic fibroblast (MEF) cells (Los et al., 2002; Lin et al., 2004). It has been suggested that RIP and/or TRAF2 are the key mediators of NCD induced by FasL, TRAIL, TNF, or oxidative stress (Chan et al., 2003; Lin et al., 2004; Shen et al., 2004). MEF cells deficient in RIP or TRAF2 have been shown to be resistant to TNF-induced NCD. This resistance arises from a failure to accumulate ROS in response to TNF (Lin et al., 2004), indicating that RIP- and/or TRAF2-mediated ROS accumulation plays a pivotal role in TNF-induced NCD.

The protein kinase C (PKC) family is responsible for transducing many cellular signals during a variety of cellular processes, including cell proliferation, differentiation, and death. PKCs have been classified into three groups based on their structure and response mechanisms to regulatory factors. Conventional PKCs (α , β I, β II, and γ) are Ca^{2+} -dependent and are activated by diacylglycerol (DAG) and phorbol 12-myristate 13-acetate (PMA), a synthetic phorbol ester. The novel isotypes (δ , ϵ , θ , and η) do not respond to Ca^{2+} but are activated by DAG and PMA, and the atypical PKCs (λ , ξ , and ι) are insensitive to both Ca^{2+} and DAG (Newton, 1995). The results from a number of previous studies have shown that activation of PKC pathways by PMA protects cells from apoptosis induced by FasL, TRAIL, and TNF (Sordet et al., 1999; Gomez-Angelats and Cidlowski, 2001; Basu et al., 2002; Herrant et al., 2002). Activation of the phosphatidylinositol-3-kinase and ERK, or the stabilization of X-linked inhibitor of apoptosis have been implicated in the protective mechanism of PKC against FasL- and TRAIL-induced apoptosis (Holmstrom et al., 1998, 2002; Shi et al., 2005). More-

over, PKC has also been reported to be essential for functional suppression of apoptosis through the phosphorylation of Bcl-2 (Ito et al., 1997; Ruvoletto et al., 1998), which may explain the ability of PKC to block death receptor-mediated apoptosis.

Although recent studies have greatly advanced our understanding of the regulatory mechanisms of PKC in death receptor-mediated apoptosis, little is known about the effects or regulatory mechanisms of PKC in NCD. In this study, our data demonstrate that PMA-induced activation of PKC suppresses TNF-induced NCD by disrupting the recruitment of TRADD and RIP to the TNFR1 complex. Moreover, our results indicate that PMA inhibits TNF-induced ROS production, thereby suppressing activation of ROS-mediated poly(ADP-ribose) polymerase (PARP) leading to the inhibition of NCD.

Materials and Methods

Reagents. Anti-RIP antibody was purchased from BD Transduction Laboratories (Lexington, KY). Anti-I κ B- α , anti-TRAF2, anti-TRADD, and anti-GFP antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-I κ B kinase complex- β , anti-PAR, and anti-PARP antibodies were purchased from BD Biosciences PharmMingen (San Diego, CA). Anti-p-JNK, anti-JNK, anti-p-ERK, anti-ERK, anti-p-p38, and anti-p38 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti- β -actin and anti-FLAG antibody, cycloheximide (CHX), and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich (St. Louis, MO). PMA, doxorubicin (Adriamycin), camptothecin, the caspase inhibitor z-VAD-FMK, the NF- κ B inhibitor BAY-11, the p38 inhibitor SB203580, the JNK inhibitor SP600125, the MEK inhibitor U0126, the PARP inhibitors 3-aminobenzamide and 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone, and peroxidase-conjugated secondary antibodies were purchased from Calbiochem (San Diego, CA). 5(6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂ DCFDA) was purchased from Invitrogen (Carlsbad, CA). Anti-TNF receptor 1 antibody and recombinant mouse TNF- α were purchased from R&D Systems (Minneapolis, MN). Protein A- and G-Sepharose were purchased from GE Healthcare (Piscataway, NJ).

Cell Culture and Transfection. L929 cells, HeLa cells, HEK293 cells, and MEF cells, including wild-type, RIP $^{-/-}$, TRAF2 $^{-/-}$, and JNK1 $^{-/-}$ cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. For transient transfection, 2×10^7 HEK293 cells were seeded on 100-mm plates. Twenty-four hours later, cells were transfected with GFP-tagged TNFR1 and FLAG-tagged TRADD expression plasmids using Lipofectamine Plus reagent according to the manufacturer's instructions (Invitrogen). Coimmunoprecipitation after transient transfection was performed as described previously (Ting et al., 1996).

Subcellular Fractionation. HEK293 cells were fractionated into membrane and cytosolic fractions, according to the procedures described previously (Tapia et al., 2003). In brief, after treatment with TNF or PMA as described in the figure legends, cells were resuspended in 1 ml of ice-cold KCl containing lysis buffer [50 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 0.5 mM dithiothreitol, 0.25 M sucrose, 2.5 μ g/ml pepstatin, 2.5 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], and homogenized using a glass homogenizer with a Teflon pestle. Homogenates were first centrifuged at low speed (2000g) for 15 min at 4°C to precipitate nuclei and debris. The supernatant was centrifuged for 1 h at 100,000g at 4°C to separate the membrane fraction (pellet) and cytosolic fraction (supernatant). The membrane fraction was washed with phosphate-buffered saline, resuspended in lysis buffer containing 0.5% NP-40,

and sonicated for 5 s at 4°C. Protein concentration was estimated using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA), and an equal amount of proteins per sample of each fraction was further analyzed by SDS-PAGE and Western blotting.

Western Blot Analysis. After treatment with different reagents as described in the figure legends, cells were collected and lysed in M2 buffer (20 mM Tris, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM PMSF, 20 mM β -glycerol phosphate, 1 mM sodium vanadate, and 1 μ g/ml leupeptin). Fifty micrograms of the cell lysates was fractionated by 10% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membrane. After blocking with 5% skim milk in PBS containing 0.05% Tween 20, the membrane was probed with the relevant antibodies and visualized by enhanced chemiluminescence, according to the manufacturer's instructions (GE Healthcare).

Immunoprecipitation Assay. For immunoprecipitation assays, 5×10^7 L929 cells were treated with 15 ng/ml TNF for 10 or 45 min in the absence or presence of 50 nM PMA, as described in the figure legends. Cells were then collected and lysed in a buffer containing 50 mM HEPES, pH 7.6, 0.1% NP-40, 150 mM NaCl, 5 mM EDTA, 0.5 mM PMSF, and 1 μ g/ml each aprotinin, leupeptin, and pepstatin. The lysates were mixed and incubated for 4 h to overnight with 1 μ g of anti-TNFR1 antibody and protein G-agarose beads to immunoprecipitate TNFR1-associated proteins. Immunoprecipitate was washed four times with lysis buffer and boiled in SDS-PAGE sample buffer. The banded proteins were resolved in 10% SDS-polyacrylamide gels and detected by Western blot analysis.

Determination of Cell Death. Cells were seeded in six-well plates and treated with the indicated concentrations of reagents as described in the figure legends. Cell death, as observed by microscopy, was characterized by rounding up and detaching of the cells from the plates. Cell death was quantified by trypsinization, followed by staining with trypan blue (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) and counting with a hemacytometer (American Optical, New York, NY). The stained cells (blue) were counted as dead cells and were expressed as a percentage of total cells. For each treatment, triplicate experiments were performed three times. For measurement of early apoptotic or necrotic/late apoptotic cell death, L929 cells were treated with TNF in the absence or presence of PMA for the indicated times as described in the figure legends. Approximately, 1×10^5 cells were stained for 10 min at room temperature with 10 μ M fluorescein isothiocyanate (FITC)-labeled annexin V (BD Biosciences PharMingen), and propidium iodide (PI; BD Biosciences PharMingen), in a Ca^{2+} -enriched binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl_2), and analyzed by two-color flow cytometry. Annexin V and PI emissions were detected in the FL1 and FL2 channels of a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), using emission filters of 488 and 532 nm, respectively. The annexin V⁻/PI⁻ population was regarded as normal healthy cells, whereas annexin V⁺/PI⁻ cells were taken as a measure of early apoptosis and annexin V⁺/PI⁺ as necrosis/late apoptosis. At least 10,000 cells were analyzed in each of three independent experiments.

Determination of Intracellular ROS Production. Production of intracellular ROS was measured using the fluorescent dye 27-dichlorofluorescein diacetate (DCF-DA). DCF-DA is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed to 27-dichlorofluorescein, a nonfluorescent polar compound that is trapped within cells. In the presence of an oxidizing compound, DCF-DA is converted into highly fluorescent 27-dichlorofluorescein. For assays, cells in 12-well plates were cultured in phenol red-free medium and treated with TNF, BHA, and PMA for the indicated times as described in the figure legends. CM-H₂-DCF-DA (1 μ M) was added 30 min before collecting cells. The stained cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences), and data were processed with the CellQuest software (BD Biosciences).

Statistical Analysis. Data are expressed as the mean \pm S.E. from at least three separate experiments performed triplicate. The

differences between groups were analyzed using a Student's *t* test, and *P* < 0.05 is considered statistically significant. Statistical analyses were carried out using SPSS software (ver. 11.0; SPSS Inc., Chicago, IL.).

Results

PMA Inhibits TNF-Induced NCD in L929 Cells in a PKC-Dependent Manner. We studied the effect of PMA on death receptor-mediated NCD in L929 murine fibrosarcoma cells. NCD in this cell line can be efficiently triggered without the use of CHX, an inhibitor of de novo protein synthesis (Hehner et al., 1998). To quantify the modes of cell death induced by TNF, cell death was assessed by exposure of phosphatidylserine, as indicated by annexin V-FITC staining, and loss of plasma membrane integrity, as indicated by PI staining, and then analyzed by flow cytometry. As shown in Fig. 1A, treatment of cells with TNF resulted in a time-dependent increase in both necrotic and late phase apoptotic cells, whereas very few cells were stained exclusively with annexin V (early phase apoptosis). These results indicate that TNF predominantly triggers necrotic, rather than apoptotic, cell death in L929 cells, which is consistent with previous reports (Vercammen et al., 1998; Los et al., 2002). To elucidate the role of PKC on TNF-induced NCD, we treated L929 cells with the PKC activator PMA and assessed its effects. Treatment with PMA alone was not cytotoxic to L929 cells at the concentration used. Pretreatment of L929 cells with 50 nM PMA before TNF exposure dramatically abrogated TNF-induced NCD, as measured by trypan blue exclusion assays (Fig. 1B). In contrast, PMA failed to protect the cells from death caused by DNA-damaging agents such as doxorubicin and camptothecin. These results suggest that, under necrotic conditions, PMA negatively and specifically regulates death receptor pathways rather than inhibiting the mitochondrial pathway. Based on the ability of PMA to protect against TNF-induced NCD, we evaluated the possibility that inhibiting PKC would restore the necrotic effects of TNF, despite the presence of PMA. Pretreatment of L929 cells with either Ro 31-8220 or bisindolylmaleimide I, both of which are PKC-specific inhibitors, significantly decreased the protective effect of PMA against TNF-induced NCD (Fig. 1C). This result suggests that the protective mechanism of PMA is PKC-dependent in these cells.

Activation of PKC by PMA Disrupts Formation of the TNFR1 Signaling Complex and Subsequently Abolishes TNF-Induced Activation of NF- κ B and JNK. Recent studies have established that death domain adaptor molecules, including TRADD, RIP, and TRAF2, are required for TNF-induced NCD (Chan et al., 2003; Lin et al., 2004). Because our results clearly demonstrated that PMA-mediated activation of PKC inhibits TNF-induced NCD, we next examined the effect of PMA on the formation of the TNFR1 signaling complex in response to TNF. Immunoprecipitation experiments revealed that treatment of L929 cells with TNF led to the immediate recruitment of TRADD, RIP, and TRAF2 to TNFR1 (Fig. 2A). Only RIP molecules recruited to TNFR1 were modified, as expected; indeed, such modifications have been noted previously (Lee et al., 2004) and may be characteristic of polyubiquitination. When TNFR1 was immunoprecipitated from cells pretreated with PMA, TNF-induced recruitment of TRADD to TNFR1 was found to be

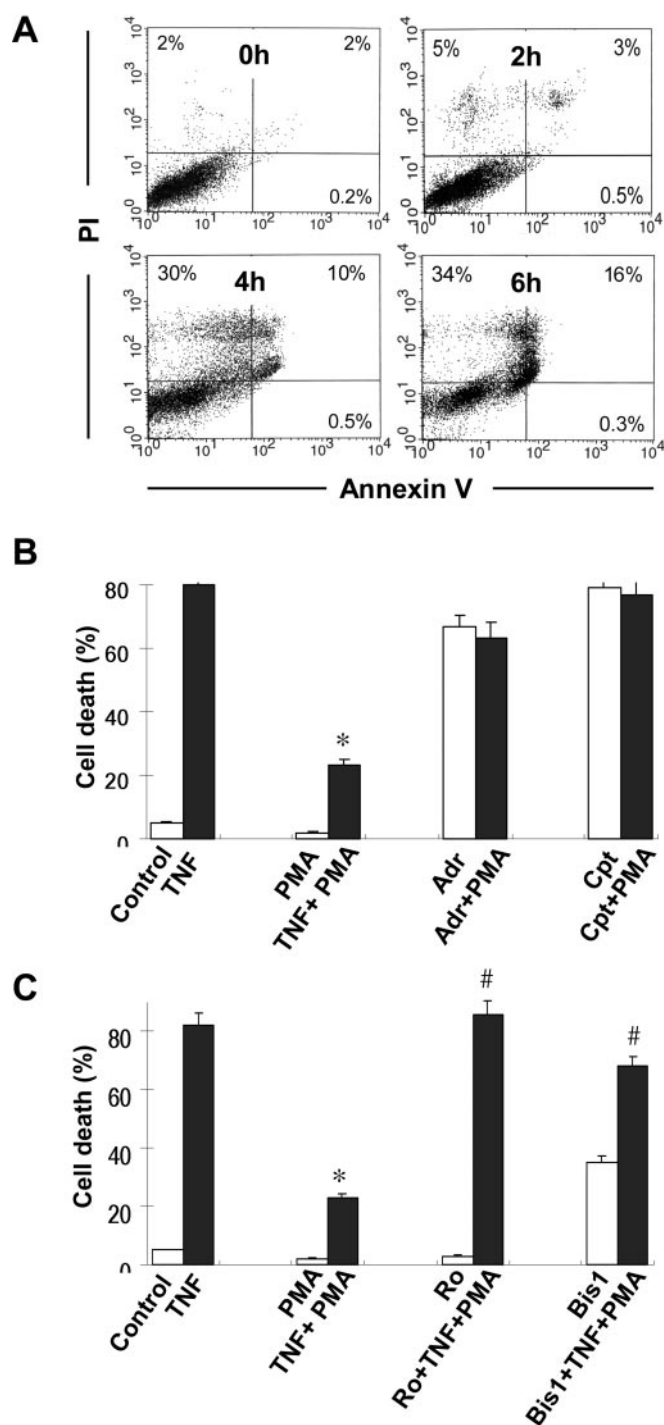


Fig. 1. PMA protects L929 cells against induction of NCD by TNF but not by DNA damage, in a PKC-dependent manner. **A**, assessment of mode of cell death induced by TNF. L929 cells were treated with 15 ng/ml TNF for various times as indicated and then stained with FITC-labeled annexin V and PI and analyzed by flow cytometry as described under *Materials and Methods*. **B**, L929 cells were treated with 15 ng/ml TNF for 8 h, or with 10 μ M doxorubicin or 100 μ M camptothecin for 24 h, in the absence or presence of 50 nM PMA. Cells were trypsinized and collected in PBS, and cell death was quantified by trypan blue exclusion assay. Data are normalized to the rate of spontaneous cell death occurring in untreated cells. **C**, after 10-min pretreatment with a general PKC inhibitor (10 μ M Ro 31-8220) or with 10 μ M bisindolylmaleimide I, L929 cells were treated with 15 ng/ml TNF for 8 h in the absence or presence of 50 nM PMA. Cell death was quantified using triplicate trypan blue exclusion assays. Each column shows mean \pm S.E. of at least of three independent experiments. *, $P < 0.05$, compared with TNF-treated group. #, $P < 0.05$, compared with TNF plus PMA-treated group.

dramatically abolished, and recruitment of RIP and TRAF2 to TNFR1 was also inhibited, suggesting that PMA can regulate the formation of the TNFR1 signaling complex through disrupting the recruitment of adaptor molecules. To ensure that the same amount of TNFR1 was precipitated in each sample, TNFR1 levels in the immunoprecipitate were measured (Fig. 2A, bottom). Furthermore, when cells were pretreated with the PKC-specific inhibitor Ro 31-8220, before the addition of PMA, binding of RIP and TRAF2 to TNFR1 was restored (Fig. 2B), indicating that PMA-induced disruption on the binding of these adaptor molecules to TNFR1 is dependent upon activation of PKC.

Because PMA-induced activation of PKC has been reported to down-regulate TNF responsiveness by reducing the number of cell surface receptors available for TNF binding (Aggarwal and Eessalu, 1987), we directly investigated the possibility that PMA causes a change in cell surface TNF binding capacity by using cell fractionation experiments. As expected, the expression of TNFR1 occurred principally in the membrane fraction, whereas no signals were detected in the cytosolic fraction (Fig. 2C, top). It is noteworthy that TNFR1 expression levels on the membrane fraction were unaffected by treatment with PMA or PMA plus TNF. In contrast, PKC- δ was predominantly present in the cytosolic fraction before PMA treatment, and PMA caused translocation of PKC- δ from the cytosolic fraction to the membrane fraction (Fig. 2C, middle). To further support the negative role of PKC on the formation of TNFR1 signaling complex, we examined the effect of PMA on the interactions of TNFR1 and TRADD in HEK293 cells, where each protein was overexpressed (Fig. 2D). TRADD was found to interact consistently with TNFR1, and this interaction was enhanced by TNF treatment, as consistent with a previous report (Hsu et al., 1996). Furthermore, PMA treatment abolished the interaction between TNFR1 and TRADD in cell extracts from non-stimulated and TNF treated cells, despite the fact that comparable amounts of TNFR1 were precipitated in each sample. The results of these binding experiments further support the hypothesis that PKC activation disrupts the recruitment of death domain adaptor molecules to the TNFR1 signaling complex.

If PMA inhibits the recruitment of RIP and TRAF2 into the TNFR1 complex, then PMA would be predicted to impair NF- κ B and JNK activation, because RIP and TRAF2 are required for the TNF-induced NF- κ B and JNK activation. Treatment of L929 cells with TNF induced NF- κ B and JNK activation within 10 min, as assessed by the levels of I κ B- α degradation and JNK phosphorylation (Fig. 2E). PMA pretreatment significantly inhibited these TNF-induced effects, which is consistent with our observation that PMA inhibited the recruitment of RIP and TRAF2 to the TNFR1 complex. Significantly, pretreatment with Ro 31-8220 restored the effects of TNF on I κ B- α degradation and JNK activation in the presence of PMA treatment (data not shown).

The Protective Mechanism of PMA against TNF-Induced NCD Does Not Involve the PMA-Induced Decrease in JNK Activation. Results of recent studies have indicated that JNK can potentiate TNF-induced NCD (Ventura et al., 2004). Here, we show that PMA pretreatment inhibits TNF-induced JNK activation in L929 cells. Therefore, we next examined the possibility that PMA might be acting by inhibiting this signaling pathway in TNF-induced

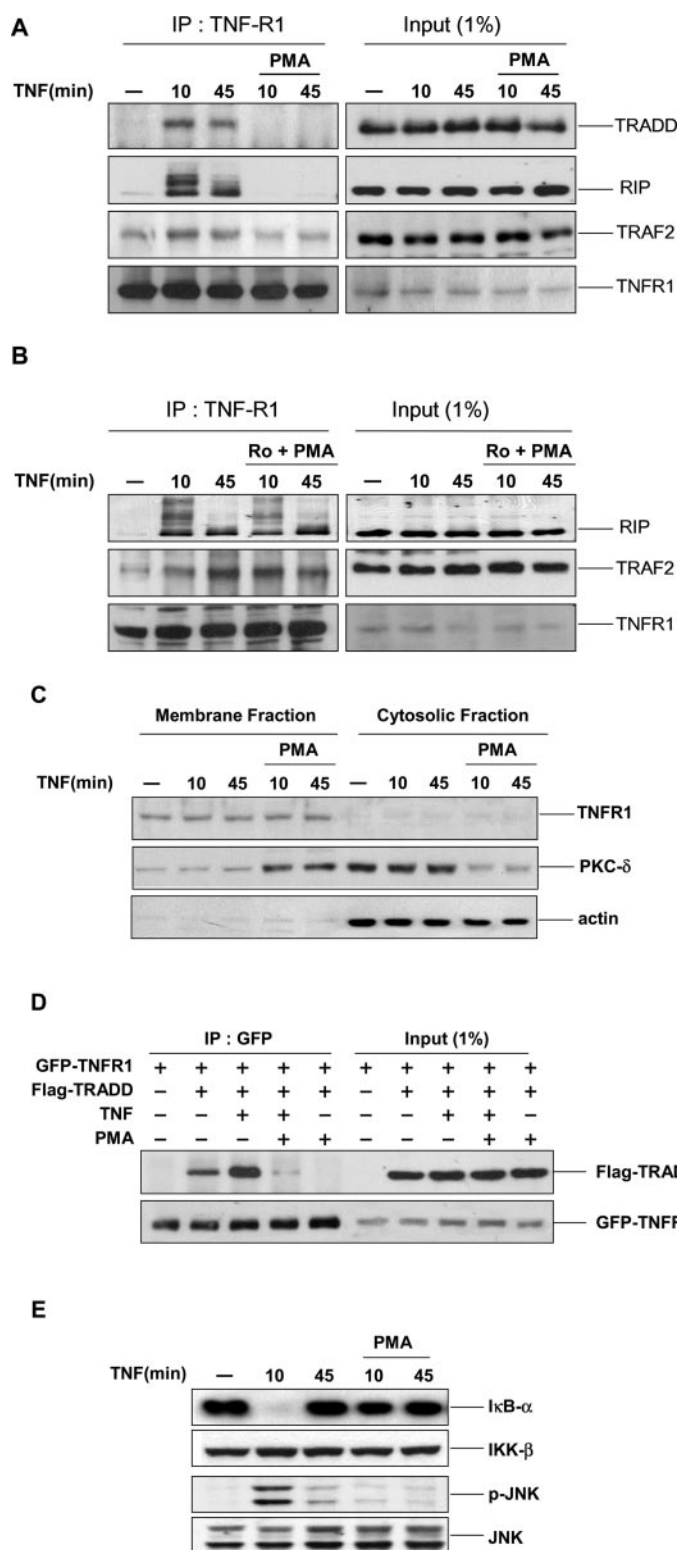


Fig. 2. Activation of PKC disrupts recruitment of death-domain adaptor molecules into TNFR1 and subsequently inhibits TNF-induced NF- κ B and JNK activation. **A**, L929 cells were treated with 15 ng/ml TNF for 10 or 45 min in the absence or presence of 50 nM PMA, and cell extracts were prepared as described under *Materials and Methods*. Cell extracts from each sample were subjected to immunoprecipitation with anti-TNFR1 antibody. Immunoprecipitate was analyzed by SDS-PAGE and Western blotting with antibodies against TRADD, RIP, TRAF2, and TNFR1. To control protein input, 1% of the cell extract volume from each sample was analyzed. **B**, after 10-min pretreatment with 10 μ M Ro 31-8220, L929 cells were treated with 15 ng/ml TNF for 10 or 45 min in the absence or

NCD. Unexpectedly, there was relatively little protection from TNF-induced NCD by pretreatment with SP600125, a specific JNK inhibitor (Fig. 3A), even though SP600125 efficiently blocked JNK activation, as measured by JNK phosphorylation (Fig. 3B, top). Pretreatment with SB203580, a p38 inhibitor, or U0126, an MEK inhibitor, also did not block TNF-induced NCD, indicating it is not mediated via activation of these mitogen-activated protein kinases. In addition, the NF- κ B inhibitor BAY-11 did not block TNF-induced NCD (Fig. 3A). This result is consistent with a previous report asserting that TNF-induced NCD and NF- κ B activation are not coupled in L929 cells (Hehner et al., 1998).

Because TNF induces NCD under caspase-inhibited conditions in MEFs (Lin et al., 2004), we further examined whether TNF-induced JNK activation plays a role in TNF-induced NCD by comparing the responses of JNK1-knockout and wild-type MEF cells. As shown in a previous report (Lin et al., 2004), NCD was induced by pretreatment with z-VAD-FMK, a membrane-permeable pan-caspase inhibitor, followed by treatment with TNF and CHX in both wild-type and JNK1 $^{-/-}$ MEF cells (data not shown). Surprisingly, under these conditions, the extent of cell death was higher in JNK1 $^{-/-}$ cells than in wild-type cells, although PMA pretreatment significantly inhibited TNF-induced NCD in both cell types (Fig. 3C). It is noteworthy that the NCD response of JNK $^{-/-}$ cells to TNF was the opposite of the response to H₂O₂, which is consistent with a previous report (Shen et al., 2004). These results confirmed that JNK activation is not required for TNF-induced NCD, and, therefore, that the protection conferred by PMA is unlikely to be a result of decreased JNK activation. To ensure that JNK signaling is indeed defective in JNK1 $^{-/-}$ cells, we confirmed that TNF-induced JNK activation occurred in wild-type cells but not in JNK1 $^{-/-}$ cells (Fig. 3D).

TNF-Induced ROS Production Is Essential for NCD-Enhancing Activation of PARP. Recent reports have indicated that TNF-induced accumulation of cellular ROS is required for TNF-induced NCD in L929 cells as well as in MEF cells deficient in NF- κ B subunit p50 or TRAF2/TRAF5 (Vercammen et al., 1998; Sakon et al., 2003). Activation of PARP and depletion of ATP have also been proposed to play a role in TNF-induced NCD (Los et al., 2002). However, the relationships between each of these signaling components have not been well addressed. To determine whether PARP activation plays a role in TNF-induced NCD, we compared

presence of 50 nM PMA. Cell extracts from each sample were immunoprecipitated and immunoblotted as described in A. C, HEK293 cells were treated with 15 ng/ml TNF for 10 or 45 min in the absence or presence of 50 nM PMA and then lysed. Cytosolic and membrane fractions were obtained as described under *Materials and Methods*, and lysates were analyzed by SDS-PAGE followed by Western blotting with antibodies against TNFR1, PKC- δ and actin. D, HEK293 cells were transfected with the GFP-tagged TNFR1 and FLAG-tagged TRADD expression plasmids, treated with 15 ng/ml TNF for 10 min in the absence or presence of 50 nM PMA, and subjected to immunoprecipitation with anti-GFP antibody. Immunoprecipitate was analyzed by Western blotting with anti-FLAG and anti-GFP antibodies, and the efficiency of immunoprecipitation was measured with anti-GFP antibody. E, L929 cells were treated with 15 ng/ml TNF for 10 or 45 min in the absence or presence of 50 nM PMA. Cell extracts were analyzed by SDS-PAGE followed by Western blotting with anti-I κ B- α antibody and with phosphospecific antibody that recognize only the phosphorylated forms of JNK. As a protein loading control, the same amounts of extracts were applied to SDS-PAGE for Western blotting with anti-I κ B kinase complex- β and anti-JNK antibodies.

the extent of poly(ADP-ribosyl)ation in L929 cells under necrotic conditions and in HeLa cells under typical TNF-induced apoptotic conditions. Immunoblotting with anti-PAR antibody showed that TNF treatment of L929 cells caused an increase in poly(ADP-ribosyl)ation by 3 h, after which the level gradually continued to increase (Fig. 4A, left). More importantly, the time course of PARP activation in L929 cells correlated well with the onset of the ROS production, as measured using the cell-permeable fluorescent dye CM-H₂DCFDA (Fig. 4B). In contrast, PARP was not activated in HeLa cells under apoptotic conditions induced by TNF plus CHX (Fig. 4A, right), indicating that PARP activation may play a role in TNF-induced NCD but not in apoptotic cell death. Furthermore, we also observed that the cleavage form of PARP was detected in L929 cells at a much lower level and later in the time course than in apoptotic HeLa cells, suggesting that the presence of noncleavable form of PARP augments the NCD phenomenon. This conjecture is consistent with a recent result (Los et al., 2002) showing that cells in which PARP cleavage is blocked by treatment with a combination of TNF and z-VAD-FMK exhibit more pronounced

NCD than do cells treated with TNF alone. Because the kinetics of TNF-induced PARP activation and ROS production correlated well in L929 cells, we next examined whether TNF-induced ROS production is required for PARP activation. When the cells were pretreated with BHA, an ROS scavenger, TNF-induced ROS production was eliminated, and PARP activation was efficiently suppressed (Fig. 4, C and D). This result indicates that TNF-induced ROS production is essential for PARP activation under necrotic conditions.

We next examined whether PARP activation by TNF-triggered ROS production is responsible for NCD. Pretreatment with either of the PARP inhibitors 3-aminobenzamide (3-AB) or 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1-(2H)-isoquinoline (DPQ) significantly suppressed TNF-induced NCD (Fig. 4E) and completely inhibited TNF-induced PARP activation (Fig. 4F). However, the PARP inhibitors suppressed TNF-induced NCD to a lesser extent than did BHA. These results suggest that, although ROS-dependent PARP activation is not essential for TNF-induced NCD, it may contribute to this

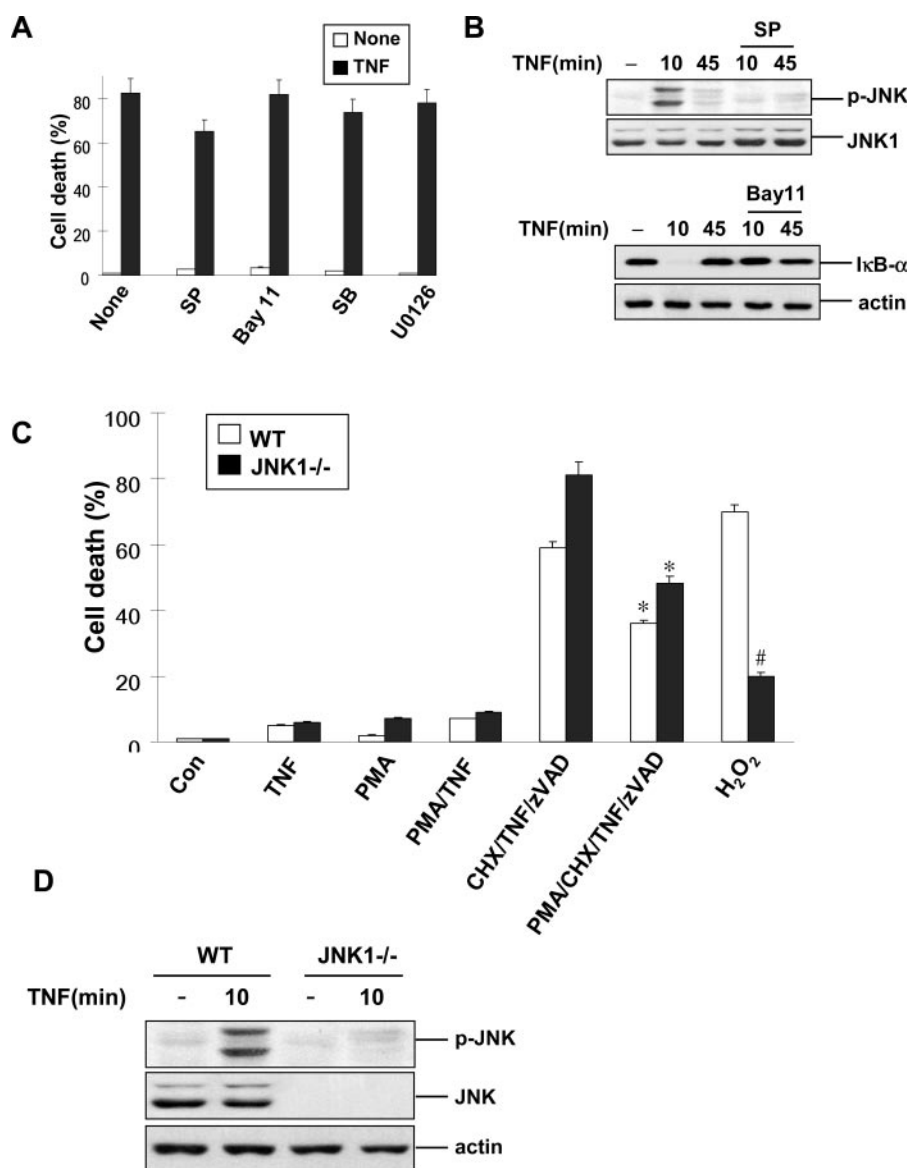


Fig. 3. Decreased JNK activation is not part of the mechanism responsible for the protective effect of PMA against TNF-induced NCD. **A**, L929 cells were pretreated with the JNK inhibitor, 20 μ M SP600125 (SP); the NF- κ B inhibitor, 5 μ M BAY-11; the p38 inhibitor, 20 μ M SB203580 (SB); or the MEK inhibitor, 10 μ M U0126 for 30 min and then treated with 15 ng/ml TNF for 8 h. Cell death was quantified using a trypan blue exclusion assay. Each column represents the average of three independent experiments. **B**, L929 cells were pretreated with 20 μ M SP or 5 μ M BAY-11 and then treated with 15 ng/ml TNF for the indicated times. Cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies against JNK, phospho-JNK, I κ B- α , and actin. **C**, wild-type and JNK-/- MEF cells were pretreated with 50 μ M z-VAD-FMK for 30 min and then treated with 15 ng/ml TNF, 15 ng/ml TNF plus 10 μ g/ml CHX, or 200 μ M H₂O₂ for 8 h, and cell death was quantified by trypan blue exclusion assay. Each column shows mean \pm S.E. of at least of three independent experiments. *, $P < 0.05$, compared with TNF/CHX/z-VAD-FMK-treated group. #, $P < 0.05$, compared with H₂O₂-treated wild-type MEF cells. **D**, wild-type and JNK-/- MEF cells were treated with 15 ng/ml TNF for 10 min, and cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies against JNK, phospho-JNK, and actin.

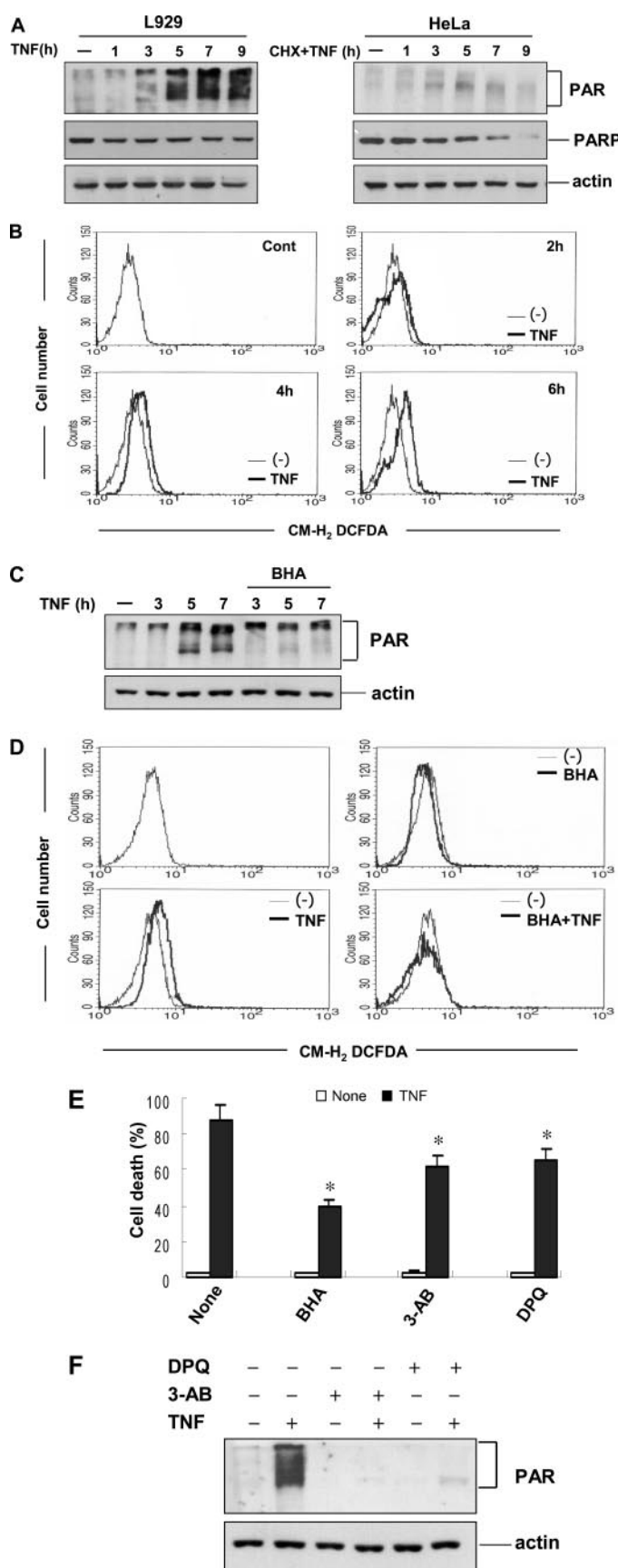


Fig. 4. TNF-induced ROS production plays an essential role in PARP activation contributing to NCD. **A**, L929 and HeLa cells were treated with TNF in the absence or presence of 10 μ g/ml CHX for various times,

process. Other factors, in addition to PARP activation, may participate in the TNF-induced NCD pathway.

PMA Antagonizes PARP Activation through the Suppression of TNF-Induced ROS Production. Because TNF treatment under necrotic conditions led to a time-dependent accumulation of intracellular ROS, concomitant with PARP activation (Fig. 4, A and B), we next examined the possibility that PMA protects against TNF-induced NCD by decreasing intracellular ROS or ROS-dependent PARP activation. As shown in Fig. 5A, staining of cells with cell-permeable fluorescence dye CM-H₂DCFDA showed that pretreatment with PMA completely blocked TNF-induced ROS production. Because these results suggested that PMA pretreatment inhibits signaling events downstream of TNF, we next examined the effects of PMA on TNF-induced PARP activation. We found that PMA pretreatment blocked TNF-induced PARP activation in a time-dependent manner (Fig. 5B). These results strongly suggest that the protective mechanism of PMA against TNF-induced NCD involves decreased ROS production and the subsequent inhibition of PARP activation.

In a recent study, TNF treatment of RIP^{-/-} and TRAF2^{-/-} MEF cells caused no detectable increase in ROS production or NCD (Lin et al., 2004), establishing that RIP and TRAF2 are essential for TNF- or ROS-induced NCD. To determine whether these adaptor proteins are required for TNF-induced PARP activation, we compared the extent of poly(ADP-ribosylation) under necrotic conditions of RIP^{-/-}, TRAF2^{-/-}, and wild-type MEF cells. As expected, when wild-type cells were treated with a combination of TNF, CHX, and z-VAD-FMK to induce necrosis, the amount of poly(ADP-ribosylation) gradually increased in a time-dependent manner (Fig. 6, left), as observed for L929 cells. In RIP^{-/-} and TRAF2^{-/-} cells; however, PARP activity did not increase, and the amount of the cleaved form of PARP was greatly decreased (Fig. 6, middle and right). These data provide additional evidence that activation of PARP via RIP and TRAF2 contributes to TNF-induced NCD.

Discussion

Although previous studies have demonstrated that the activation of PKC with PMA can protect cells from death induced by various apoptotic stimuli, including FasL-, TRAIL-, or TNF-mediated signaling (Sordet et al., 1999; Gomez-Angelats and Cidlowski, 2001; Basu et al., 2002), little is known about the effect of PMA on NCD or the regulatory mecha-

as indicated. Cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies against PAR, PARP, and actin. **B**, L929 cells were treated with 15 ng/ml TNF for various times, as indicated. CM-H₂DCFDA (1 μ M) was added 30 min before the end of treatment. ROS were detected with a flow cytometer (FACSCalibur; BD Biosciences), and data were processed with the CellQuest software (BD Biosciences). **C**, L929 cells were treated with 15 ng/ml TNF in the absence or presence of 100 μ M BHA for various times, as indicated, and PARP activity was measured as described in **A**. **D**, L929 cells were treated with 15 ng/ml TNF for 6 h in the absence or presence of 100 μ M BHA, and ROS production was measured as described in **B**. **E**, after pretreatment with an antioxidant 100 μ M BHA or with PARP inhibitors (1 mM 3-AB or 30 μ M DPQ), L929 cells were treated with 15 ng/ml TNF for 8 h. Cell death was quantified by trypan blue exclusion assay, as described in the legend of Fig. 1B. Each column shows mean \pm S.E. of at least of three independent experiments. *, $P < 0.05$, compared with TNF-treated group. **F**, L929 cells were treated with 15 ng/ml TNF for 7 h in the absence or presence of 1 mM 3-AB or 30 μ M DPQ, and PARP activity was measured as described in **A**.

nisms associated with its effect. In this study, we demonstrated for the first time that activation of PKC with PMA leads to inhibition of TNF-induced NCD by disrupting the formation of the TNFR1 signaling complex. In addition, although PMA suppresses TNF-induced NF- κ B and JNK activation, these signaling cascades seem to be dispensable for the effect of PMA on TNF-induced NCD. Moreover, PMA treatment antagonizes TNF-induced ROS production, subsequently suppressing ROS-mediated PARP activation and thereby inhibiting NCD. These results constitute direct evidence that PKC activation may modulate death receptor-mediated cell death under necrotic conditions.

The participation of PKC in cell death has been generally understood to occur at a point downstream of caspases, consistent with the fact that PKC isotypes are cleaved in a caspase-dependent manner during apoptosis (Datta et al., 1997). Some reports suggested that PKC activation inhibited Fas- or TRAIL-induced apoptosis through the caspase-8-dependent cleavage of BID or stabilization of antiapoptotic

proteins (Scaffidi et al., 1999; Holmstrom et al., 2002; Shi et al., 2005). Several recent reports, however, have shown that PMA inhibits FADD recruitment or the formation of the TRAIL death-inducing signaling complex by FasL or TRAIL (Meng et al., 2002; Harper et al., 2003), suggesting that PKC activation modulates the formation of the death receptor signaling complex and that PKC acts, at multiple steps, in suppressing apoptosis. However, previous studies have not determined fully whether PKC also modulates death receptor-mediated NCD. In this study, we found that activation of PKC by PMA protects against TNF-induced NCD but not against cell death by DNA-damaging agents such as doxorubicin and camptothecin (Fig. 1B). Therefore, PMA is unlikely to act on the mitochondrial pathway, but may act directly on the death receptor pathway. This interpretation is supported by our finding that the PMA inhibits the formation of the TNFR1 signaling complex by disrupting the recruitment of the death domain adaptor molecules TRADD, RIP, and TRAF2. Therefore, the primary target of PKC activation with

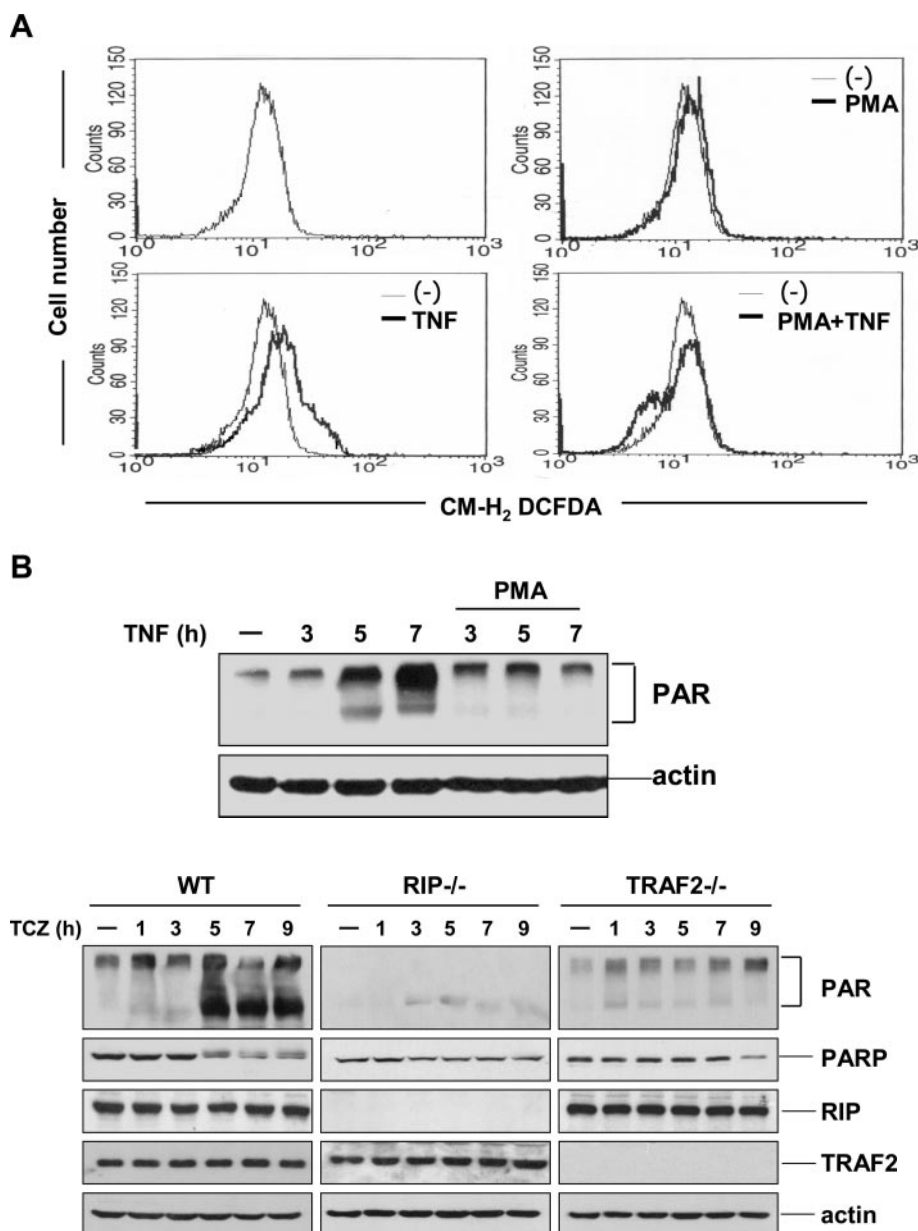


Fig. 5. PMA antagonizes TNF-induced ROS production and subsequently inhibits PARP activation (A) L929 cells were treated with 15 ng/ml TNF for 6 h in the absence or presence of 50 nM PMA, and ROS production was measured as described in the legend of Fig. 4B. B, L929 cells were treated with 15 ng/ml TNF in the absence or presence of 50 nM PMA for various times, as indicated. Cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies against PAR and actin.

Fig. 6. Activation of PARP is impaired during the NCD process in RIP^{-/-} and TRAF2^{-/-} cells. Wild-type, RIP^{-/-}, and TRAF2^{-/-} cells were pretreated with 50 μ M z-VAD-FMK for 30 min and then treated with 15 ng/ml TNF plus 10 μ g/ml CHX for various times, as indicated. Cell extracts were analyzed by SDS-PAGE and Western blotting with antibodies against PAR, PARP, RIP, TRAF2, and actin.

PMA in the inhibition of TNF-induced NCD seems to be the blocking of the interaction between the death domain molecules and TNFR1. Consistent with this idea, we also found that the interaction of TNFR1 and TRADD in a coimmunoprecipitation assay was abolished by PMA pretreatment (Fig. 2D). Although it has been proposed that a possible regulatory site for PMA is at the level of TNFR1 (Aggarwal and Eessalu, 1987), it is still unclear how PMA has a negative role in the formation of TNFR1 signaling complex. Based on our finding that TNFR1 expression on the cell surface was unaffected after PMA treatment (Fig. 2C), we speculated that the receptor oligomerization required for the formation of the TNFR1 signaling complex was hindered by PMA treatment. However, PMA treatment did not seem to affect the formation of TNFR1 aggregates (data not shown). Because PMA can activate several conventional and novel PKCs (Newton, 1995), identification of the PKC isotypes that might function in TNFR1 signaling is critical to increasing our understanding of the role of PKC in TNF-induced NCD. To address this issue, we examined the effects of specific PKC inhibitors on the recruitment of the adaptor molecules, including TRADD and RIP, into the TNFR1 complex upon treatment with TNF or TNF plus PMA. However, pretreatment of cells with Gö6976 (an inhibitor of classic PKCs) or rottlerin (a specific inhibitor of PKC- δ) did not seem to affect the PMA-induced disruption of binding between these adaptor molecules and TNFR1, suggesting that several PKC isotypes may act cooperatively in disrupting the formation of the TNFR1 signaling complex.

Although the signaling cascade of TNF-induced NCD pathway has not yet been completely characterized, it has recently been established that RIP and TRAF2 are essential components of this pathway (Lin et al., 2004). Furthermore, ROS production is essential for death receptor-mediated necrosis in L929 cells as well as in MEF cells deficient in NF- κ B subunit p65- or TRAF2/TRAF5 (Garg and Aggarwal, 2002; Sakon et al., 2003). In our study, ROS accumulated during TNF-induced NCD in L929 cells and MEF cells, and the blocking of this ROS accumulation inhibited necrosis, suggesting a crucial role for ROS in this type of cell death. Furthermore, we found that PMA completely blocks TNF-induced ROS production under necrotic conditions (Fig. 5A), which is consistent with a previous report that ROS production is impaired in RIP-/- and TRAF2-/- MEF cells (Lin et al., 2004). These results suggest that these adaptor molecules function within the TNFR1 signaling complex that initiates ROS production as an upstream component.

Despite these insights, the mechanism that accounts for ROS-mediated execution of NCD remains to be completely elucidated. Given that overactivation of PARP after cellular insults consumes large amounts of NAD, which may cause massive ATP depletion in the effort to resynthesize NAD, and thus shift the mode of cell death toward necrosis (Szabo and Dawson, 1998; Los et al., 2002). Consistent with this conclusion, cells from PARP-deficient mice are reportedly protected against ATP depletion and NCD but not against apoptotic cell death (Ha and Snyder, 1999). In our study, prevention of PARP cleavage by the caspase inhibitor z-VAD-FMK led to increased TNF-induced NCD (data not shown). Interestingly, we found a significant increase in PARP activation after TNF treatment in L929 cells under necrotic conditions, whereas no change in PARP activity was observed in HeLa cells under

apoptotic conditions (Fig. 4A). Furthermore, not only did the lipophilic antioxidant BHA efficiently protect against TNF-induced NCD but also it prevented poly(ADP-ribosyl)ation (Fig. 4, C and E). These results strongly suggest that intracellular ROS accumulation upon TNF treatment triggers the induction of PARP activity under necrotic, but not apoptotic, conditions. However, treatment with specific PARP inhibitors did not reduce TNF-induced NCD as much as did BHA (Fig. 4E), suggesting that other factors, in addition to PARP, mediate the TNF-induced NCD pathway. Therefore, further studies will be required to elucidate the ROS signaling mechanisms involved in NCD.

Taken together, our results indicate that, in TNF-induced NCD, a primary function of PKC is inhibition of the TNFR1 signaling complex via disruption of the interaction between the TNFR1 and its adaptor molecules. In addition, PMA inhibits TNF-induced production of ROS and subsequently suppressing PARP activation that may lead to energy depletion, an important factor driving cells toward NCD. Interestingly, cell death in vivo can be mediated by both apoptotic and necrotic signaling mechanisms in response to the same stimulus. Because both types of cell death are represented in pathophysiological conditions such as brain ischemia, reperfusion injury of cardiomyocytes, and inflammation (Eliasson et al., 1997; Thiemermann et al., 1997), an understanding of the regulatory role of PKC during cell death process will be of great value in developing novel therapeutic approaches for their relevant diseases.

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