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ARTICLES

PKCδ Protects Human Breast Tumor MCF-7 Cells Against Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Mediated Apoptosis

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Abstract Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induces apoptosis in a number of tumorogenic or transformed cells, yet is relatively non-toxic to most normal cells, therefore, it is a promising agent for cancer therapy. However, some cancer cell lines were resistant to TRAIL cytoxicity, including MCF-7 breast cancer cells. The mechanism is not clear. Here, we report that protein kinase C delta (PKCδ) protects MCF-7 cells from the recombinant soluble TRAIL (rsTRAIL)- mediated apoptosis. It was demonstrated that rottlerin, a PKCδ inhibitor, sensitized MCF-7 cells to rsTRAIL cytoxicity. Combination of rottlerin and rsTRAIL inhibited PKCδ translocation from the cytosol to membrane, and PKCδ kinase activity on the cell membrane was kept pace with the change of PKCδ expression. Moreover, inhibition of PKCδ by interference RNA could facilitate apoptosis of MCF-7 cells induced by rsTRAIL. Further experiments on the signal machinery showed that rottlerin increased the sensitivity of MCF-7 cells to rsTRAIL by suppressing the transcription activity of NF-κB, and enhancing the caspase-processing to generate executive apoptotic signals. These findings indicate that PKCδ functions as a survival factor protecting MCF-7 cells from the apoptosis induced by rsTRAIL. J. Cell. Biochem. 96: 522–532, 2005.

Key words: PKCδ; MCF-7; TRAIL; NF-κB; caspase; apoptosis

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a novel member of TNF family. It is a potential therapeutic agent since it is selectively toxic to the transformed and various cancer cells, but almost no toxic to most normal cells [Ashkenazi and Dixit, 1998]. Ligation of TRAIL with its receptors DR5 and DR4 leads to death-inducing signaling complex (DISC) formation including a rapid association with the adaptor protein Fas-associated death domain (FADD), which is a bifunctional molecule with an N-terminal death domain (DD) and a C-terminal death effector domain (DED). FADD then conjugates with the initiator caspase-8, mediating apoptosis either by the direct activation of downstream effectors caspase-3, -6, -7 cascade or cleavage of pro-apoptotic molecules, such as the Bcl-2 homolog Bcl-X_L and Bid in the mitochondria leading to activation of the Apaf-1/caspase 9 apoptosome complex [Herr and Debatin, 2001]. The caspases function to cleave cellular proteins critical for life, including poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, and lamin B. The cleavage of caspase substrates sets the stage for the morphological and biochemical changes that are hallmarks of apoptosis [Earnshaw et al., 1999]. It was reported recently that TRAIL receptor DR4 and DR5 not only transduced apoptotic signals from FADD to caspase family [Kischkel et al., 2000], but also activated nuclear factor-kappa B (NF-κB), which could prevent cells from undergoing

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apoptosis, most probably by up-regulating the expression of genes, whose products were critical for suppression of apoptosis [Karin, 1999].

Several serine/threonine protein kinases including the protein kinase C (PKC) isoforms, the mitogen-activated protein kinases (MAPKs), and the phosphoinositide 3-kinase (PI3K), are shown to regulate cell proliferation, differentiation, and apoptosis. There are 12 isoforms of PKC, which are classified as the conventional PKCs (α, βI, βII, and γ), which are Ca^{2+} and diacylglycerol (DAG)/phorbol ester-dependent, novel PKCs (δ, ε, η, μ, and θ), which are insensitive to Ca^{2+} but respond to DAG/phorbol esters, and the atypical PKCs (ζ and λ/ι), which are insensitive to both Ca^{2+} and DAG/phorbol esters [Martelli et al., 1999]. Recent studies have demonstrated that novel PKC isozymes, including PKCδ, -ε, -θ, and -μ are cleaved during apoptosis [Endo et al., 2000]. Additionally, PKCδ, -θ, and -μ have been identified as substrates of caspase-3 and the catalytic fragments of these isozymes have been shown to mediate apoptosis in certain cell types [Datta et al., 1997; Endo et al., 2000]. Overexpresson of PKCε in human breast cancer MCF-7 cells inhibited TNF-induced apoptosis [Basu et al., 2002]. Treatment with a PKC activator (phorbol-12, 13-dibutyrate, PDBu) inhibited TRAIL-mediated apoptosis in the MCF-7 cells [Sarker et al., 2002]. McCracken et al. [2003] analyzed the role of individual PKC isoforms in γ-radiation-induced cell death in MDA-MB-231 and MCF-7 breast cancer cell lines treated with oligonucleotide directed against the PKCδ isoform and showed that the antisense oligonucleotide suppressed cell survival. Furthermore, rottlerin (an inhibitor of novel isoforms of PKC, specifically for PKCδ) reduced MCF-7 and MDA-MB-231 cell survival, and MCF-7 cells transformed to express a dominant-negative mutant of PKCδ also decreased cell survival. However, little is known about the molecular mechanisms of PKCδ for the regulation of cell death induced by TRAIL in the MCF-7 cells. In the present study, we report the observation on the role of PKCδ in protecting MCF-7 cells from TRAIL-induced apoptosis.

MATERIALS AND METHODS

Reagents and Antibodies

The reagents of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carcoxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) were both purchased from Sigma Chemical Co. Ltd. Protease inhibitors were from Roche Molecular Biochemicals and myristoylated PKC peptide inhibitor from Promega (Madison, WI). PKCδ inhibitor rottlerin, classical PKC inhibitor Gö6976, MEK-1/2(mitogen-activated protein kinase kinase-1/2) inhibitor U0126, and PI3K inhibitor LY294002 were from Calbiochem (San Diego, CA). The various caspase inhibitors of fluormethyl ketone-derivatized peptides, Z-VAD-FMK, Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK were purchased from R&D Systems, Inc. (Minneapolis, MN). Horseradish peroxidase (HRP)-conjugated anti-rabbit, -goat, or -mouse IgG complex were purchased from PharMingen (San Diego, CA), monoclonal antibodies against caspase 8, NF-κBp65, β-actin, nPKCδ, cPKC, and polyclonal antibodies against ERK-1 were from Santa Cruz Biotechnology (Santa Cruz, CA), and the polyclonal antibodies against caspase-9 from Calbiochem.

Apoptosis Induction and Analysis

Human breast cancer MCF-7 cells from the American Type Culture Collection (Manassas, VA) were maintained and cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS, GIBCO-BRL) and 100 U/ml penicillin G, 100 μg/ml streptomycin, and 1 mM L-glutamine at 37°C in a humidified 5% CO_2. The cells in logarithmic phase were collected and distributed into the wells of 96-well flat-bottom microtitre plates (Costar, 5×10^3 cells/well in 100 μl medium) and pre-incubated with or without appreciable concentration of PKC isoform inhibitors or the tetrapeptide caspase inhibitors Z-VAD-FMK, Z-IETD-FMK, Z-LEHD-FMK, Z-DEVD-FMK for caspase family, caspase-8, -9, and -3 (Biomol Research Laboratories, Inc., Plymouth, PA), respectively, for 1 h and followed by the treatment with 1 μg/ml rsTRAIL (95 ~ 281 a.a., rsTRAIL) for 24 h. The preparation of rsTRAIL was as described previously [Liu et al., 1999]. The cell viability was determined by MTS assay as described in the manufacture's instruction manual of CellTiter 96^R Aqueous Non-radioactive Cell Proliferation Assay (Sigma Chemical Co. Ltd.). Briefly, 10 μl of reagent solution, or complete medium for control, were added into the wells of 96-well plates and then incubated at 37°C for required time. Then, 20 μl of the combined MTS/PMS

solution (containing 100 mg MTS and 2.3 mg PMS in 100 ml PBS) were added into each well and the cultures were incubated at 37°C for 1 ~ 4 h. The absorbance (A490) of each well was measured at 490 nm wavelength with the microculture plate reader (Wellscan MK3, Labsystems Dragon). Apoptotic rate of the cells was calculated according to the following formula:

$$\text{Apoptosis}\,\% = \left(1 - \frac{\text{A490 of treated cells}}{\text{A490 of control cells}}\right) \times 100\%$$

Apoptosis Evaluation

Annexin V staining of exposed membrane phospholipid phosphatidylserine was done by using the Annexin V assay kit (Baosai Company, Beijing, China). Briefly, 2×10^5 cells were harvested after treatment and washed twice with ice cold phosphate-buffered saline (PBS, pH 7.4), and re-suspended in 200 μl 1× binding buffer. Annexin V-FITC and propidium iodide (PI) were added to individual samples and incubated for 15 min in a low-light environment. The reaction was stopped by adding 300 μl 1× binding buffer. In the case of the apoptosis detected with flow cytometry, followed by fixing with methanol and incubated at 4°C for 30 min, the cells were analyzed with FACS Calibur Flow Cytometer (Becton Dickinson).

Subcellular Fractionation

The MCF-7 cells (2×10^7) were pretreated for 1 h with rottlerin (15 μM) followed by rsTRAIL (1 μg/ml) for 4 h, and then collected, washed twice with cold PBS, and scraped into cold homogenization buffer (HB buffer, 20 mM Tris-HCl, pH 7.4, 4 mM EDTA, 2 mM EGTA, 10% glycerol, 10 μg/ml leupeptin, and 1 mM PMSF). The cells were lysed via sonication 4×15 s intervals and complete lysis was monitored microscopically. The homogenate was ultracentrifuged at 4°C and 86,000*g* for 45 min, and the supernatant was designated as the cytosol fraction. The pellet was re-suspended in HB buffer containing 1% Triton-X 100 and incubated on ice for 30 min followed by centrifugation at 4°C and 14,000*g* for 20 min, where the supernatant was designated as the membrane fraction. Protein concentration was measured by lowry's method with bovine serum albumin as the standard. The 20 μl of the cell lysate containing 100 μg proteins were boiled and subjected to SDS–PAGE [Gauthier et al., 2003]. The translocation of PKC isoform was identified by Western blot analysis. The proteins in the gel were transferred onto PVDF membrane and probed with specific antibodies against PKCδ and classical PKC, respectively, and with the second antibody-enzyme complex followed by visualization with ECL system.

PKCδ Activity Assay

Cells were pretreated for 1 h with rottlerin (15 μM) followed by treatment with rsTRAIL (1 μg/ml) for 2 h, then lysed in phosphorylation lysis buffer (0.5% Triton X-100 or Nonidet P-40, 150 mM NaCl, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 50 mM HEPES, 1.5 mM magnesium chloride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). The cell lysates (300 μg/sample) were immunoprecipitated with anti-PKCδ antibody followed by centrifugation, and the pellets were washed three times with phosphorylation lysis buffer and two times with kinase buffer (25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 20 μg of phosphatidylserine, and 20 μM ATP) and re-suspended in 30 μl of kinase buffer containing 7.5 μg of histone H1 as an exogenous substrate, to which 20–30 μCi of [γ-^{32}P] ATP was added. The reaction was incubated at room temperature for 2 h and terminated by addition of SDS–PAGE sample buffer. Proteins were analyzed by SDS–PAGE, and phosphorylated histone H1 was detected by autoradiography.

Inhibition of PKCδ Expression by RNA Interference (RNAi)

The expression vector (siPKCδ/pAVU6 + 27) encoding PKCδ short interference RNA (siRNA) was constructed to contain orderly U6snRNA promoter cassettes, si-like RNA inserts, 19-nucleotide sense strand of 5′-AGT ACT TGG CAA AGG CAG C-3′ of PKCδ gene, followed by an UUCG tetra-loop sequence, the antisense strand, and an UUUU transcription terminator [Paul et al., 2002]. The MCF-7 cells were grown in 6-well dishes, transfected with siPKCδ/pAVU6 + 27 using Lipofectamine™ 2000 (Invitrogen) and incubated for 48 h. The cells were transferred into the medium containing G418 (800 μg/ml) and grown for 3 weeks, and then prepared for apoptosis analysis as described above or Western blot as below.

Western Blot

The 1×10^7 cells were lysed in the lysis buffer [1% Nonidet P-40, 20 mM HEPES, pH 7.5, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EGTA, 50 μM phenylarsine oxide, 1 μg/ml protease inhibitor mix of antipain, leupeptin, and pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM iodoacetamide]. The protein concentration was determined by Lowry's method. Twenty microliter cell lysate containing 100 μg proteins were isolated on SDS–PAGE (5% acrylamide for spacer gel and 12% for separation gel). The proteins in the gel were transferred onto the PVDF membrane (Millipore Co.) and probed with specific antibodies and horseradish peroxidase (HRP)-conjugated complexes followed by visualization with enhanced chemiluminescence system (ECL, Amersham Co.). Erk-1/2 was used as the internal control for the equal amount loading of the proteins in cell lysates.

NF-κB Activity Assay

The pGL_2 NF-κB-Luc reporter plasmid consisting three copies of NF-κB binding sequence and one copy of firefly luciferase gene was constructed as described by Shigeno et al. [2003]. The MCF-7 cells were grown in 6-well plates in triplicate and transfected with pGL_2NF-κB-Luc plasmid DNA and incubated for 24 h. The cells were then placed into 60 mm plates and cultured for another 24 h. The media were replaced with fresh media in the presence or absence of rottlerin (15 μM) and cultured for 1 h followed by treatment with rsTRAIL (1 μg/ml) for 2 h. Luciferase activity in the cells was determined by using the luciferase reporter assay system (Promega) on a luminometer (Analytical Luminescence Laboratory, San Diego, CA). The cells transfected with pCMV-Luc plasmid (Promega) harboring the CMV immediate-early enhancer/promoter and luciferase gene was used as positive control. The cells transfected with pGL_2 were used as negative control.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts (15 μg) of the MCF7 cells were incubated with ^{32}P-labeled double-stranded oligonucleotide 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ in buffer containing 50% glycerol, 100 mM HEPES (pH 7.9), 5 mM EDTA, 2.5 mM dithiothreitol, 250 mM KCl, 10 mM $MgCl_2$, 20% Ficoll 400, and 0.5 μg/μl Salmon DNA at room temperature for 30 min. The labeling mixture was then subjected to electrophoretic separation at room temperature on a non-denaturing 5% acrylamide gel at 120 V using 0.5× Tris borate/EDTA buffer. The gels were dried at 80°C for 1 h and exposed to radiography film for 6–18 h at −70°C with intensifying screens.

Statistical Analysis

All data presented in this report were of mean ± SD of nine replicates from three separate experiments. Statistical differences were evaluated by using Student's *t*-test and considered significant at the $P < 0.05$ level. Figures were obtained from three independent experiments at least, which gave similar patterns.

RESULTS

Rottlerin Sensitized the MCF-7 Cells to Apoptosis Induced by rsTRAIL

First of all, we analyzed the sensitivity of MCF-7 cells treated with the recombinant soluble TRAIL (95–281 a.a., rsTRAIL) made in our laboratory. As shown in Figure 1A, there were only less than 10% of the MCF-7 cells died by apoptosis after treatment with rsTRAIL (1 μg/ml) for 24 h. It was consistent with reports by Lee et al. [2004]. We hypothesized that the survival signaling pathways in MCF-7 cells might attenuate apoptosis induced by the rsTRAIL. To validate this hypothesis, we employed myristoylated PKC peptide inhibitor (an inhibitor for both classic and novel PKC isoforms), MEK-1/2 inhibitor U0126, and PI3K inhibitor Ly294002 to block the main survival signaling pathways, respectively, by which to explore the potential regulation mechanism in the MCF-7 cells against rsTRAIL. It was shown that there were about 30% cells died by the treatment with myristoylated PKC peptide inhibitor and followed by rsTRAIL, indicating that PKC might play an important role in the modulation of the sensitivity of MCF-7 cells to rsTRAIL-induced apoptosis. Whereas, the cell viabilities treated with MEK-1/2 inhibitor U0126 (15 μM) or PI3K inhibitor Ly294002 (20 μM) and then followed by rsTRAIL were not changed significantly, suggesting that MAPK and PI3K pathways did not influence the cell sensitivity to rsTRAIL (Fig. 1B). To further analyze the role of PKC isoforms in regulating

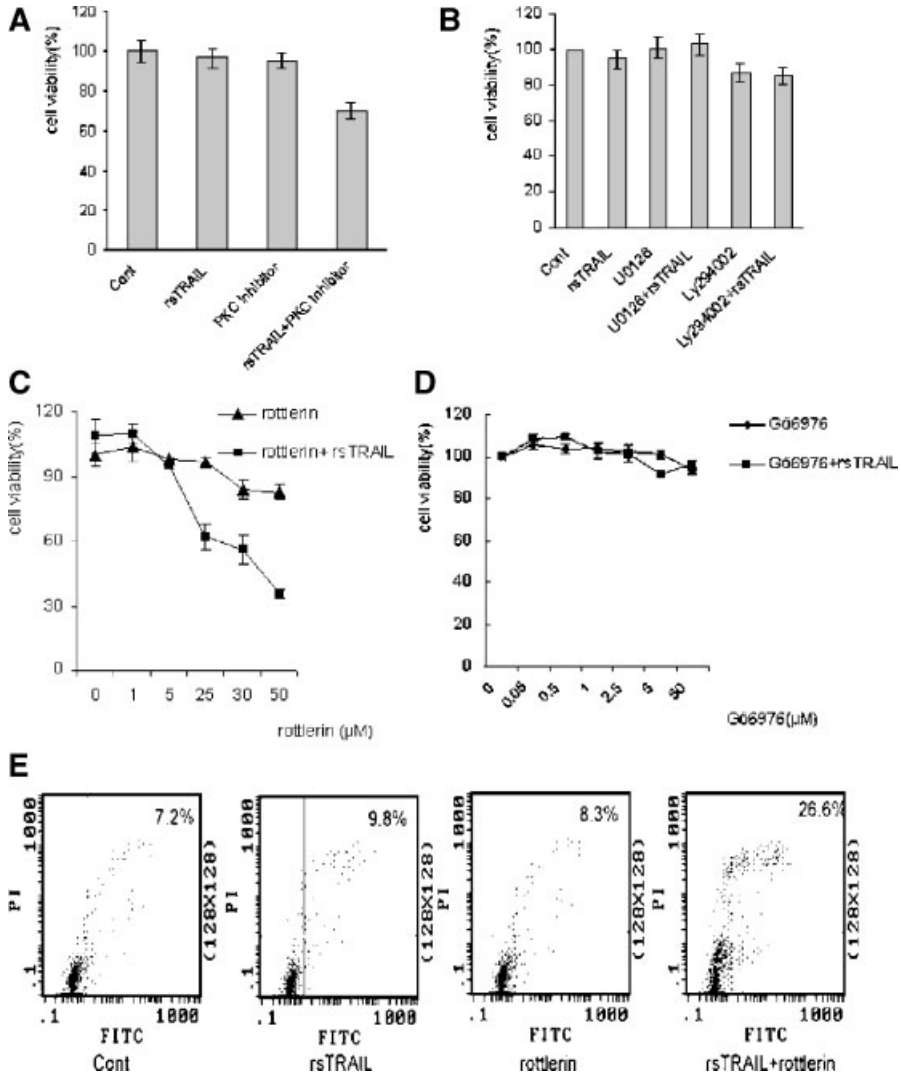

Fig. 1. Rottlerin sensitized MCF-7 cells to rsTRAIL-induced apoptosis. MCF-7 cells were pre-incubated with or without 50 μM myristoylated PKC peptide inhibitor (**A**) or rottlerin (**C**, 15–50 μM) or Gö6976 (**D**, 0.05–50 μM) for 1 h followed by treatment with rsTRAIL (1 μg/ml) for 24 h. Cont: control. **B**: MEK1/2 inhibitor U0126 or PI3K inhibitor Ly294002 had no effect on susceptibility of MCF-7 cells to rsTRAIL. Cell viability was determined by the MTS assay. The data shown are representative of at least three independent experiments ($P < 0.01$). **E**: The MCF-7 cells were pretreated with rottlerin (15 μM), then followed by rsTRAIL (1 μg/ml) for 14 h and stained with Annexin-V-FITC/PI. The dual color flow cytometric histograms display intensity of fluorescence staining with Annexin-V-FITC, detected at the FL4 channel versus PI staining detected at the FL3 channel.

the cell sensitivity to rsTRAIL, we treated the cells with Gö6976 (0.05–50 μM), which is an inhibitor of classic isoforms of PKC highly specific for Ca^{2+}-dependent PKC isoforms, including PKCα, but neither PKCδ nor PKCε [Hornia et al., 1999], and rottlerin (15–50 μM), which is an inhibitor of novel isoforms of PKC, specifically for PKCδ [Gschwendt et al., 1994; Iwabu et al., 2004] for 1 h, respectively, and followed by rsTRAIL (1 μg/ml) for 24 h. As shown in Figure 1C,D, the decrease of cell viability of MCF-7 at the present of rsTRAIL was enhanced dramatically by rottlerin. In contrast, Gö6976 was unable to augment the effect of rsTRAIL, suggesting that PKCδ rather than cPKCs was a critical element to the cell sensitivity to rsTRAIL-induced cell death. Annexin V staining followed by flow cytometry assay for the cell death by apoptosis further confirmed this observation. As shown in Figure 1E, the apoptotic percentages of the cells treated with rsTRAIL (1 μg/ml) or rottlerin (15 μM) alone were 9.8% and 8.3%, respectively, which were no significant difference from the control of 7.2%, while that of the cells treated with rottlerin and followed by rsTRAIL reached at 26.6% cell death.

Rottlerin and rsTRAIL Enhanced PKCδ Translocation

We further examined the effect of rottlerin on PKCδ translocation in MCF-7 cells from membrane to cytosol, which is known as a hallmark of PKC activation [Hug and Sarre, 1993]. The membrane and cytosol fractions of the MCF-7 cells treated with rottlerin and/or rsTRAIL were isolated and then subjected to SDS–PAGE and Western blot by using the specific antibody against PKCδ. As shown in Figure 2A, PKCδ expression was significantly increased in the cytosol and accordingly decreased on the membrane in the cells treated with rottlerin and rsTRAIL comparing with rottlerin or rsTRAIL alone. Analysis of the protein kinase activity (Fig. 2B) showed that PKCδ enzyme activity on the membrane was coincident with its protein expression. This phenomenon was not observed for the classical PKC isoforms. It suggests that rottlerin prohibits PKCδ translocation from cytosol to membrane specifically and suppresses PKCδ kinase activity in the MCF-7 cells. These results provide a novel explanation for the mechanisms by which rottlerin sensitizes MCF-7 cells to rsTRAIL-induced apoptosis.

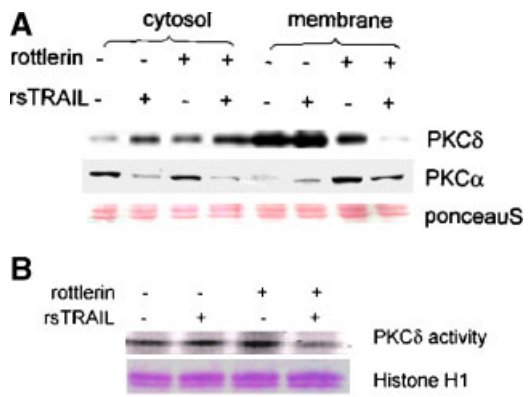

Fig. 2. The effects of rottlerin on the distribution of cPKC and PKCδ in the cells. The membrane and cytosol fraction were isolated from MCF-7 cells pretreated with rottlerin for 1 h and followed by rsTRAIL (1 μg/ml) for 4 h by using the methods described in the section of ''Materials and Methods.'' The fractions were subjected on SDS–PAGE and Western blot. The proteins in the gel were transferred on PVDF membrane followed by blotting with specific antibodies and second antibody–enzyme complex, and visualized by ECL system. Equal amount loading of the proteins was shown by ponceau S staining (**A**). For PKCδ activity assay, the cells were pretreated with rottlerin (15 μM) for 1 h, then rsTRAIL (1 μg/ml) for 4 h. The cells were lysed in phosphorylation lysis buffer. Cell membrane fractions were immunoprecipitated with anti-PKCδ antibody, and the immunoprecipitate was re-suspended in 30 μl kinase buffer containing 7.5 μg of histone H1 as an exogenous substrate. 20–30 μCi of γ-^{32}P-ATP was added in the solution and incubated for 2 h at room temperature. Proteins were identified by SDS–PAGE followed by autoradiography (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Inhibition of PKCδ by RNAi Facilitated Apoptosis of MCF-7 Cells in the Presence of rsTRAIL

To further confirm the ability of PKCδ to inhibit rsTRAIL-induced apoptosis, the MCF-7 cells were transfected with PKCδ(siRNA)/pAVU6 + 27, and then PKCδ expression and apoptosis of the cells-induced by rsTRAIL were evaluated. As shown in Figure 3A, PKCδ expression was down-regulated significantly by PKCδ(siRNA) and the rate of apoptosis of the transfected MCF-7 cells was remarkably increased to 50%–60% (Fig. 3B), indicating again that PKCδ definitely protects MCF-7 cells from rsTRAIL cytotoxicity.

Rottlerin Increased the Sensitivity of MCF-7 Cells to rsTRAIL by Suppressing the Activity of NF-κB

In view of the important role of NF-κB in the regulation of apoptosis [Harper et al., 2001; Ravi et al., 2001], we next investigated the NF-κB activity in the MCF-7 cells treated with rottlerin and/or rsTRAIL. The cells were transfected with pNF-κB-Luc plasmid DNA, which consist of three copies of the binding sequence of NF-κB and the reporter gene encoding for firefly luciferase, and then treated with rsTRAIL (1 μg/ml) for 2 h in the presence or absence of rottlerin (15 μM). NF-κB activation was represented by luciferase activity in the cells, which was determined by the luciferase reporter assay system on luminometer. It was shown that treatment with rsTRAIL alone increased the activity of NF-κB, whereas, treatment with rottlerin alone or combined with rsTRAIL significantly suppressed the activity of NF-κB (Fig. 4A), while the expression of NF-κB protein was not changed as revealed by Western blot assay (Fig. 4B). Electrophoretic mobility shift assay (EMSA) further confirmed that NF-κB binding activity with the cis-element was augmented in the cells treated with rsTRAIL alone or combined with rottlerin (Fig. 4C). These data suggest that rottlerin sensitizes MCF-7 cells to rsTRAIL cytotoxicity by suppressing the activity of NF-κB.

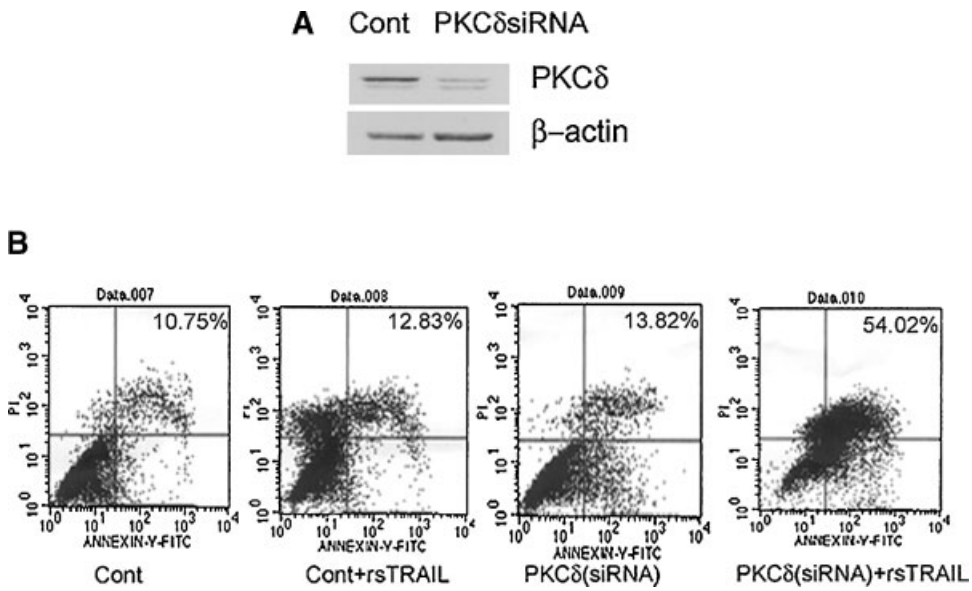

Fig. 3. Inhibition of PKCδ by RNAi increased the sensitivity of MCF-7 cells to rsTRAIL. MCF-7 cells transfected with PKCδ(siRNA)/pAVU6 + 27 and pAVU6 + 27 respectively and incubated for 36 h. **A**: The cells were lysed as described in ''Materials and Methods,'' then Western blots for PKCδ was performed. The β-actin was used as the internal control for the equal amount loading of the proteins in the cell lysate. **B**: The cells were treatment with rsTRAIL (1 μg/ml) for 22 h. Apoptotic cells were stained with Annexin-V-FITC/PI and determined by flow cytometry.

Caspase-9 was Activated in Rottlerin-Sensitized MCF-7 Cells

To test the effects of rottlerin on other intracellular pathways associated with apoptosis, we further assessed cleavage of key caspases, which is the hallmark of activation of the cell death machinery. Inhibitors of different caspases were used to block rottlerin and rsTRAIL-mediated apoptosis. As shown in Figure 5A, the viabilities of MCF-7 cells treated with rottlerin and rsTRAIL in the presence of caspase family inhibitor (Z-VAD-fmk), caspase-8 inhibitor (Z-IETD-fmk), or caspase-9 inhibitor (Z-LEHD-fmk) were enhanced significantly, but not affected by caspase-3 inhibitor (Z-DEVD-fmk), which was consistent with the fact that caspase-3 was defected in MCF-7 cells [Janicke et al., 1998]. To ascertain the activation of caspase-8 in rottlerin and rsTRAIL-induced cell death, Western blot was performed to show the cleavage of procaspase-8. As expected that rsTRAIL-induced caspase-8 activation was definitely facilitated by the treatment of rottlerin and rsTRAIL in the MCF-7 cells (Fig. 5B). Similarly, we analyzed whether the caspase-9 was also activated in the MCF-7 cells pretreated with or without rottlerin and followed with rsTRAIL by demonstrating the generation of 37 kDa proteolytic fragment of caspase-9. It confirmed that caspase-9 was cleaved in the MCF-7 cells treated with rottlerin or rottlerin plus rsTRAIL, but not rsTRAIL alone (Fig. 5B), suggesting that caspase-9 is activated and utilized to achieve the execution phase of cell death via mitochondrial pathway in the MCF-7 cells exposed to rottlerin and rsTRAIL.

Taken together, the above data suggest that PKCδ plays an important role in protection of MCF-7 cells from rsTRAIL-induced cell death. The combination of rottlerin and rsTRAIL suppresses the activity of NF-κB, and activates caspase-9 to execute apoptotic signal transduction.

DISCUSSION

TRAIL is a potential anti-cancer agent [Ashkenazi and Dixit, 1998]. Experiments from different laboratories including ours have confirmed in a number of in vivo studies that the rsTRAIL effectively reduces solid tumor formation and suppresses growth of human cancer cell xenografts [Griffith and Broghammer, 2001] without detectable toxicity to the host animals. The underlying mechanism of differential sensitivity of TRAIL in different cell models was initially attributed to the presence of decoy receptors on the surfaces of normal

cells. However, recent observation fails to correlate decoy receptor expression with TRAIL toxicity [Walczak and Krammer, 2000]. There are increasing evidences to demonstrate that the mechanism of TRAIL-resistance might be inside the cells. Many intracellular anti-apoptotic molecules can block the apoptotic signaling pathway or turn it to survival pathway. For instance, transcription factor NF-κB could upregulate many anti-apoptotic genes, such as c-FLIP, XIAP, cIAP-1, and cIAP-2, which can inhibit active caspases [Macfarlane, 2003].

In the present study, we have demonstrated that PKCδ acts as a pro-survival factor in the modulation of apoptosis of MCF-7 cells induced by rsTRAIL. It is known that MCF-7 cells are relatively resistant to TRAIL cytoxicity [Singh et al., 2003]. Since the survival signaling pathways may attenuate the cell death induced by TRAIL, we first employed Mek1/2 inhibitor U0126, PI3K inhibitor Ly294002, and myristoylated PKC peptide inhibitor (an inhibitor for both classic and novel PKC isoforms) to block the main survival pathways, respectively, by which to explore the potential regulation pathway in the MCF-7 cells against rsTRAIL. It was shown that the myristoylated PKC peptide inhibitor, but neither U0126 nor Ly294002, facilitated MCF-7 apoptosis significantly induced by rsTRAIL, suggesting that MAPK and PI3K pathways did not influence MCF-7 cell sensitivity to rsTRAIL. This result is discrepancy with the report by Sarker et al. [2002] that MAPK plays a crucial role for the associated survival effect in MCF-7 cells. This discrepancy might cause by the different experimental system utilized by our two

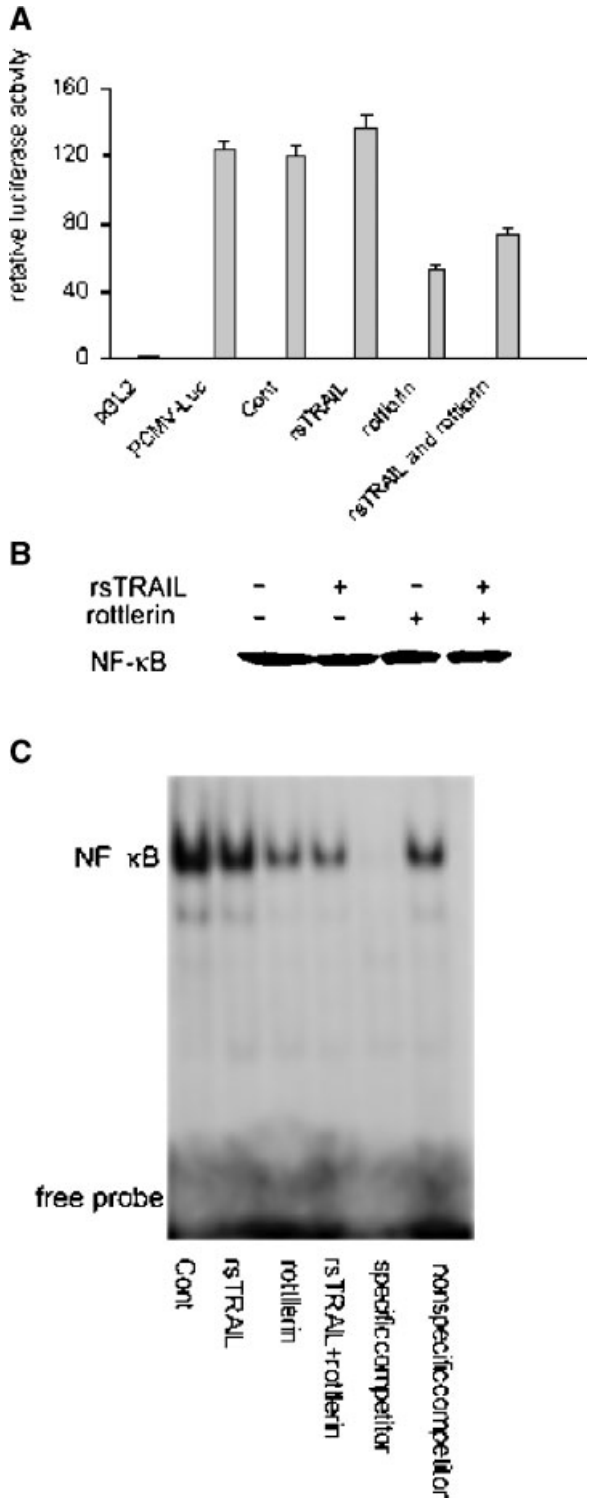

Fig. 4. Rottlerin inhibited rsTRAIL-induced activation of NF-κB in MCF-7 cells. The MCF-7 cells were grown in 6-well plates in triplicate and transfected with pNF-κB-Luc reporter plasmid DNA and incubated for 24 h. The cells were then placed into 60 mm plates and cultured for another 24 h. The media were replaced with fresh media in the presence or absence of rottlerin (15 μM) and cultured for 1 h, and rsTRAIL (1 μg/ml) was then added into the media and incubated for 2 h. **A**: Luciferase activity in the cells was determined by using the luciferase reporter assay system on luminometer. The cells transfected with pCMV-Luc plasmid harboring the CMV immediate-early enhancer/promoter and luciferase gene were used as positive control. The cells transfected with pGL_2 plasmid were used as negative control. **B**: Cell lysates were subjected to SDS-PAGE, followed by Western blot using NF-KB P65-specific antibody. **C**: Nuclear extracts were isolated from MCF-7 cells pretreated with rottlerin for 1 h and followed by rsTRAIL (1 μg/ml) for 4 h by using the methods described in the section of ''Materials and Methods.'' Nuclear extracts (15 μg) were used to determine NF-κB DNA binding activities by electrophoretic mobility shift assay (EMSA) using ^{32}P-labled oligonucleotide containing NF-κB cis-element. The unlabeled NF-κB consensus oligomer was used as the specific competitor and unlabeled SP1 consensus oligomer as the nonspecific competitor.

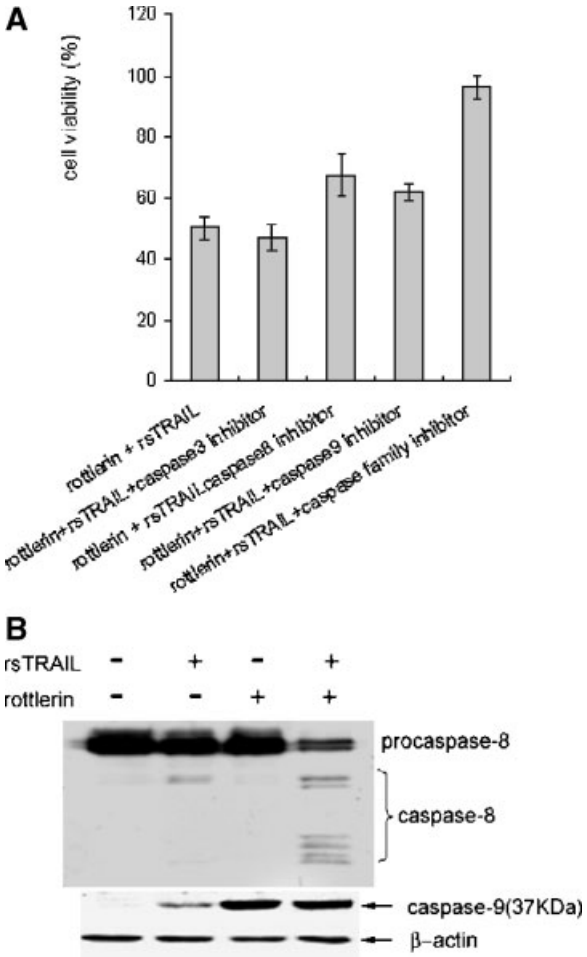

Fig. 5. Roles of the caspases in the MCF-7 apoptosis amplified by rottlerin and rsTRAIL. **A**: Caspase inhibitory peptides blocked apoptosis in MCF-7 cells induced by rottlerin and rsTRAIL. MCF-7 cells were placed on 96-well plates and cultured for 24 h. The cells were treated with 15 μM rottlerin and caspase inhibitors with different specificities for 1 h, then with 1 μg/ml rsTRAIL for 24 h. The cell viability was evaluated by MTS assay. **B**: Effect of rottlerin and rsTRAIL on the cleavage of procaspase-8 and -9. Western blot analysis was performed with the antibodies against caspase-8 and 37 kDa proteolytic fragment of caspase-9, respectively. β-actin was used as the internal control for the equal amount loading of the proteins in the cell lysate.

laboratories. In our experiments, MEK-1/2 inhibitor U0126 and TRAIL-resistant MCF-7 cells were used, whereas in Saker's experiments, ERK inhibitor PD098095 and TRAIL-sensitive MCF-7 cells were employed. Apparently, more specific and selective inhibitors for MAPK pathway are desired in further investigation.

Our further experiments demonstrated that it was rottlerin (an inhibitor of novel isoforms of PKC, specifically PKCδ), but not Gö6976 (an inhibitor of classic PKC isoforms), sensitized the cell death induced by rsTRAIL, and inhibited PKCδ translocation and kinase activity on the cell membrane. Moreover, inhibition PKCδ by RNAi also increased the cell death treated with rsTRAIL. We further transfected MCF-7 cells with PKC α expression vector, then treated with rotterlin and rsTRAIL, and observed TRAIL-induced apoptosis in the cells. The consequent results showed that PKC alpha over-expression in the MCF-7 cells did not affect on the cell death of the cells (data not shown). All these results indicate that PKCδ is responsible for the resistance of MCF-7 cells to rsTRAIL-induced cell death. There are contradictory reports in the literature on the role of PKCδ in cell survival and apoptosis [Brodie and Blumberg, 2003]. Carpenter et al. [2002] showed that inhibition of PKCδ protected rat INS-1 cells against interleukin-1 and streptozotocin-induced apoptosis. However, McCracken et al. [2003] reported that the human breast cancer MDA-MB-231 and MCF-7 cells treated with anti-sense oligonucleotide against PKCδ exhibited impaired survival in response to γ-radiation. These data indicate that PKCδ possesses distinct functions in the regulation of cell proliferation and apoptosis in various cell lines. Moreover, there exists an important controversy about the role of PKCδ in TRAIL-induced cell death. Tillman et al. reported that rottlerin affected mitochondrial function which was independent of PKCδ, thereby sensitizing cells to TRAIL. So they proposed that mitochondria constitutes an important target in overcoming inherent resistance to TRAIL in colon carcinomas [Tillman et al., 2003]. In our study, inhibition of PKCδ translocation by rottlerin or PKCδ activity by siRNA augmented the sensitivity of MCF-7 cells to rsTRAIL. This discrepancy may again result from different experimental models utilized. We reported previously that PKCδ was activated in some tumor cells that are sensitive to rsTRAIL, for instance, breast cancer MDA-MB-231 and Jurkat T lymphocyte cell lines, which are both sensitive to TRAIL [Zhang et al., 2004]. These data provide further evidences that PKCδ plays different roles in various cell species.

To understand molecular mechanism of rottlerin-sensitized TRAIL-mediated apoptosis in MCF-7 cells, we analyze the biochemical events, which modulates the cell sensitivity to TRAIL. NF-κB is an extensively described anti-apoptotic transcription factor because many of its target genes encode for anti-apoptotic molecules. Constitutive NF-κB activity has been

observed in a wide variety of cancer and is associated with resistance to apoptosis [Oya et al., 2001; Kim et al., 2002; Thomas et al., 2002]. In our experiments, TRAIL stimulation augmented endogenous NF-κB activity, whereas, rottlerin suppressed the NF-κB activity significantly in the MCF-7 cells. So that, rottlerin-dependent inhibition of NF-κB activation might sensitize MCF-7 cells to rsTRAIL-induced apoptosis. Whether TRAIL resistance could be restored in the rottlerin or RNAi treated cells by constitutively activating the NF-κB pathway remains to be investigated. NF-κB target genes include caspase inhibitors such as c-IAP1, c-IAP2, XIAP, and c-FLIP. So activation of caspases was detected in MCF-7 cells treated with rottlerin or rottlerin plus rsTRAIL. rsTRAIL-induced activation of caspase-8 was clearly enhanced by pretreatment with rottlerin in MCF-7 cells. More importantly, rottlerin alone could activate caspase-9. Activated caspase-9 may provide additional apoptotic signals to enhance disruption of mitochondrial functions. From these data we may conclude that signaling of apoptosis through the mitochondrial pathway may have important function in MCF-7 cells treated with rottlerin and rsTRAIL.

It had been reported that NF-κB plays a dominant role in TRAIL resistance in breast cancer cell lines [Keane et al., 2000]. In this study, we reported that rottlerin pretreatment sensitizes MCF-7 cells to rsTRAIL-induced apoptosis by suppressing the activity of NF-κB. On the basis of the present results, down-regulation PKCδ by siRNA approaches or rottlerin will likely be necessary for successful treatment of human breast cancer with TRAIL.

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