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Rottlerin induces apoptosis via death receptor 5 (DR5) upregulation through CHOP-dependent and PKC δ-independent mechanism in human malignant tumor cells

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Rottlerin has been shown to induce antiproliferation and apoptosis of human cancer cell lines. In this study, we demonstrate a novel mechanism of rottlerin-induced apoptosis via death receptor (DR) 5 upregulation. We found that treatment with rottlerin significantly induces DR5 expression both at its messenger RNA and protein levels. Downregulation of DR5 expression with small-interfering RNA (siRNA) efficiently attenuated rottlerin-induced apoptosis, showing that the critical role of DR5 in this cell death. Rottlerin-induced DR5 upregulation was accompanied by CCAAT/enhancer-binding protein–homologous protein (CHOP) protein expression and rottlerin-induced increase of DR5 promoter activity was diminished by mutation of a CHOP-binding site of DR5 promoter. Although rottlerin is known to be as an inhibitor of novel isoforms of protein kinase C (PKC), specifically PKC δ, not only suppression of PKC δ expression by siRNA but also over-expression of wild-type-PKC δ or dominant-negative-PKC δ did not affect the rottlerin-mediated induction of DR5 in our study. These results suggest that rottlerin induces upregulation of DR5 via PKC δ-independent pathway. Furthermore, subtoxic dose of rottlerin sensitizes human cancer cells, but not normal cells, to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis. Thus, DR5-mediated apoptosis, which is induced by rottlerin alone or by the combined treatment with rottlerin and TRAIL, may offer a new therapeutic strategy against cancer.

Introduction

Death receptor (DR) 5, which is a member of the tumor necrosis factor receptor family, is one of receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (1,2). TRAIL induces apoptosis through interacting with DR4 (TRAIL-R1) and DR5 (TRAIL-R2), leading to the formation of the death-inducing signal complex with the binding of caspase-8 (3). TRAIL has been shown to exhibit potent tumoricidal activity in a variety of human cancer cell lines *in vitro* and *in vivo* with little or no toxicity toward normal cells (4,5). Several reports have shown that the DR5 plays a crucial role in sensitizing tumor cells to apoptosis induced by TRAIL and chemotherapeutic agents (6–8). The transcriptional regulation of DR5 is complex and the multiple potential binding sites of several transcription factors, including a CCAAT/enhancer-binding protein–homologous protein (CHOP), p53 and nuclear factor-κB are present in the DR5 upstream region (9–13). The understanding of the regulatory mechanism of DR5 is an important issue at cancer therapy using combination of TRAIL and various sensitizing agents.

Rottlerin was originally identified as a specific inhibitor of the novel protein kinase C (PKC) isoform, PKC δ, and was shown to have anticarcinogenic properties (14). PKC δ activation and translocation are induced by a variety of apoptotic stimuli in different cellular systems (15). PKC δ translocates to and activates specific pathways in the plasma membrane, mitochondria and nucleus that eventually converge to the activation of caspase-3 and subsequent apoptosis (15). However, an increasing numbers of studies have demonstrated that rottlerin might not act directly on PKC δ, but can produce cellular changes that mimic those produced by the direct inhibition of PKC δ (16,17). Recently, rottlerin sensitized colon carcinoma cells and glioma cells to TRAIL-mediated apoptosis through uncoupling of the mitochondria and inhibition of Cdc2, respectively (18,19). However, the underlying mechanisms by which rottlerin-induced apoptosis and rottlerin sensitizes cancer cells to TRAIL-mediated apoptosis are not fully understood.

In this study, we elucidated for the first time that rottlerin-induced apoptosis is mediated through DR5 upregulation. Furthermore, rottlerin sensitized various cancer cells, but not normal cells, to TRAIL-mediated apoptosis, suggesting that the combined treatment with rottlerin and TRAIL may offer a safe and effective cancer therapy. We found that CHOP-mediated DR5 upregulation, which is independent of PKC δ activity, contributes to rottlerin-induced apoptosis.

Abbreviations: CHOP, CCAAT/enhancer-binding protein–homologous protein; DN, dominant negative; DR, death receptor; GFP, green fluorescent protein; HSC70, heat shock cognate protein 70; mRNA, messenger RNA; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; PKC, protein kinase C; ROS, reactive oxygen species; RT, reverse transcriptase; siRNA, small-interfering RNA; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; WT, wild-type.

Materials and methods

Cells and materials

HCT116, HT29, LNCap, PC-3, Hep3B, U2OS, SaOS2 and Caki cells were obtained from the American Type Culture Collection (Rockville, MD), whereas HCT116 p53(−/−) cells were kindly provided by Dr Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The human hepatoma cell line, Huh-7, was purchased from the Japan Health Research Resources Bank (Osaka, Japan). Primary culture of human mesangial cells (Cryo NHMC) and its corresponding growth medium (CC-3146 MsGM) were purchased from Clonetics (San Diego, CA). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium, containing 10% fetal calf serum, 20 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid buffer and 100 μg/ml gentamicin. Rottlerin was purchased from Biomol (Plymouth Meeting, PA) and dissolved in dimethyl sulfoxide and freshly diluted in culture media for all *in vitro* experiments. Recombinant human TRAIL/Apo2 ligand (the non-tagged 19 kDa protein, amino acid 114–281) was purchased from KOMA Biotech (Seoul, Korea). Anti-heat shock cognate protein 70 (HSC70), anti-poly(ADP-ribose) polymerase (PARP), anti-procaspase-3, anti-CHOP, anti-PKC δ, anti-DR4 and anti-DR5 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents were purchased from Sigma Chemical Co (St. Louis, MO).

Western blotting

Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer [137 mM NaCl, 15 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM 3-(*N*-morpholino)propane sulfonic acid, 0.5 mM phenylmethylsulfonyl fluoride and 20 μM leupeptin, adjusted to pH 7.2]. The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Detection of specific proteins was carried out with an electrochemiluminescence western blotting kit according to the manufacturer's instructions.

RNA isolation and reverse transcriptase–polymerase chain reaction

Total cellular RNA was extracted from cells using the TRIzol reagent (Life Technologies, Gaithersburg, MD). A complementary DNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (RT) (Gibco BRL, Gaithersburg, MD). The complementary DNA for DR5, DR4 and actin were amplified by

polymerase chain reaction (PCR) with specific primers: DR5 (sense) 5′-AAGACCCTTGTGCTCGTTGT-3′ and (antisense) 5′-GACACATTCGATGTCACTCCA-3′ and DR4 (sense) 5′-AGCTCAGCTGCAACCATCAA-3′ and (antisense) 5′-CACAACCCTGAGCCGATGCAA-3′. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Plasmids, transfection and luciferase assay

The plasmid encoding wild-type (WT) PKC δ and dominant-negative (DN)-PKC δ (PKC K376R) were generously gifted by Dr T.F. Mushinski, National Institutes of Health. The pDR5/SacI plasmid containing DR5 promoter sequence (−2500/+3) has been described previously (20). Other deletion mutants of human DR5 promoter were amplified from pDR5/SacI plasmid. Upstream oligonucleotide primers used for PCR of promoter region of DR5 gene contained a KpnI or SacI site and GL2 primer in pGL3-basic plasmid used for downstream primer. They are listed as follows: pDR5/−1036 5′-GACACGGTACCATGAAGGAATGAA-3′ (KpnI); pDR5/−717 5′-GTTCACCAATCTGAGCTCCAGAGA-3′ (SacI); pDR5/−534 5′-AAGGGGCAGGTACCCCTGGGAAG-3′ (KpnI); pDR5/−422 5′-CCCAGGTACCTCCCTCAACTCATT-3′ (KpnI); pDR5/−335 5′-CGCAGGTACCAGGGCGAAGGTTAG-3′ (KpnI); pDR5/−238 5′-GTCAGGTACCTGTGGTGGAATTTG-3′ (KpnI); pDR5/−135 5′-CAAGGGTACCTGGACACATAAATC-3′ (KpnI) and GL2 primer 5′-CTTTATGTTTTTGGCGTCTT-3′. The PCR products were digested with KpnI/NcoI or SacI/NcoI and cloned upstream of the firefly luciferase gene of pGL3-basic (Promega, Madison, WI). PCR products were confirmed by their size, as determined by electrophoresis and DNA sequencing. For transfection, in brief, cells were plated onto six-well plates at a density of 5×10^5 cells per well and grown overnight. Cells were cotransfected with 2 μg of various plasmid constructs and 1 μg of the pCMV-β-galactosidase plasmid for 5 h by the Lipofectamine method. After transfection, cells were cultured in 10% fetal calf serum medium with vehicle (dimethyl sulfoxide) or drugs for 24 h. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activity was normalized for β-galactosidase activity in cell lysates and expressed as an average of three independent experiments.

Flow cytometry analysis

Approximately, 1×10^6 HT29 cells were suspended in 100 μl of phosphate-buffered saline, and 200 μl of 95% ethanol was added while vortexing. The cells were incubated at 4°C for 1 h, washed with phosphate-buffered saline and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent-activated cell sorting on a FACScan flow cytometer for relative DNA content based on red fluorescence.

DNA fragmentation assay

After treatment with drugs, HT29 cells were lysed in buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10 000*g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 μg/ml ethidium bromide.

Asp-Glu-Val-Asp-ase activity assay

To evaluate Asp-Glu-Val-Asp-ase activity, cell lysates were prepared after their respective treatment with rottlerin. Assays were performed in 96-well microtiter plates by incubating 20 μg of cell lysates in 100 μl of reaction buffer (1% Nonidet P-40, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl and 10% glycerol) containing the caspases substrate (Asp-Glu-Val-Asp-chromophore *p*-nitroanilide) at 5 μM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

Small-interfering RNA

The 25 nucleotide small-interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen (Carlsbad, CA) and had the following sequences: CHOP, 5′-GAGCUCUGAUUGACCGAAUGGUGAA-3′ (470–494); DR5, 5′-AUCAGCAUCGUGUACAAGGUGUCCC-3′ (1073–1049); DR5m, 5′-AUCAGCAUCGUGUGCAAGGCGUCCC-3′ and green fluorescent protein (GFP), 5′-AAGACCCGCGCCGAGGUGAAG-3′. Cells were transfected with siRNA oligonucleotides using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations.

4′,6′-Diamidino-2-phenylindole staining for nuclei condensation and fragmentation

HT29 cells were treated with rottlerin for 24 h. The cells were fixed with 1% paraformaldehyde on slide glass for 30 min at room temperature. After washing with phosphate-buffered saline, 300 nM 4′,6′-diamidino-2-phenylindole (Roche, Mannheim, Germany) was added to the fixed cells for 5 min, after which they were examined by fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of nuclei. The 4′,6′-diamidino-2-phenylindole staining experiments were performed in duplicate.

Results

Rottlerin induces apoptosis in HT29 cells

To investigate the anticancer effect of rottlerin, human colon carcinoma HT29 cells were treated with various concentrations of rottlerin. Two established criteria were subsequently used to assess apoptosis in our system. First, apoptosis was determined using flow cytometric analysis. As shown in Figure 1A, treatment of HT29 cells with rottlerin resulted in a markedly increased accumulation of sub-G_1 phase in a dose-dependent manner of rottlerin. Second, we analyzed DNA fragmentation, which is another hallmark of apoptosis. Following agarose gel electrophoresis of DNAs from HT29 cells treated with rottlerin 24 h, a typical ladder pattern of internucleosomal fragmentation was observed (Figure 1B). In addition, treatment with rottlerin resulted in a markedly increased nuclear condensation and formation of apoptotic bodies in a dose-dependent manner of rottlerin (Figure 1C). Third, the involvement of caspases in rottlerin-induced cell death was examined. Pretreatment of HT29 cells with z-VAD-fmk (a pancaspase inhibitor) and z-IETD (caspase-8 inhibitor) significantly inhibited the rottlerin-induced apoptosis (Figure 1D), indicating that rottlerin-induced apoptosis is associated with activation of caspases.

Rottlerin upregulates expression of DR5 in several cancer cells and induction of DR5 is critical for rottlerin-induced apoptosis

Since caspase-8 inhibitor effectively inhibited apoptosis induced by rottlerin, we hypothesized that DR pathways might be involved in this apoptosis. We first investigated the effects of rottlerin on the expression of DRs (DR4 and DR5) using western blotting. As shown in Figure 2A, incubation with rottlerin demonstrated the induction of the protein levels of DR5 in HT29 cells, in a dose-dependent manner. However, the protein levels of DR4 were downregulated by rottlerin treatment. Next, to examine whether rottlerin-mediated change in DR5 or DR4 expression is controlled at the transcriptional level, we performed reverse transcriptase–PCR analysis. The basal messenger RNA (mRNA) levels of DR4 were much higher than those of DR5 in HT29 cells, the levels of DR4 mRNA were downregulated by treatment with rottlerin in a dose-dependent manner. Reverse transcriptase–PCR analysis demonstrated that rottlerin induced DR5 mRNA levels in a dose-dependent manner (Figure 2B). Upregulation of DR5 proteins by rottlerin was also observed in a variety of tumor cell types [(colon cancer cells; HCT116 and HCT116 (p53−/−), hepatocellular carcinoma (Huh-7 and Hep3B cells), prostate cancer cells (LNcap and PC-3), osteosarcoma cells (U2OS and SaOS2) and renal carcinoma cells (Caki)] (Figure 2C). As shown in Figure 2C, the protein levels of DR5, but not DR4, were significantly upregulated in all the rottlerin-treated cancer cell lines (expect LNcap cells), suggesting that DR5 upregulation is a common response to rottlerin in various cancer cells. To determine whether rottlerin-induced DR5 upregulation in several cancer cells is related to apoptosis, we also investigated the population in sub-G_1 phase using flow cytometric analysis. As shown in Figure 2D, rottlerin-induced DR5 upregulated cell lines [HCT116, HCT116 (p53−/−), PC-3, HT29 and SaOS2] were more sensitive to rottlerin-induced apoptosis. These results suggest that rottlerin-induced DR5 expression levels are associated with rottlerin-induced apoptosis rate.

Rottlerin activates the transcription from the DR5 promoter

To examine the mechanism by which rottlerin activates the DR5 promoter, we determined the DR5 promoter regions responsible for its transcriptional activation by rottlerin. A series of 5′-deletion constructs of the DR5 promoter were transiently transfected into HT29 cells, and luciferase activities were measured following rottlerin treatment. Deletion from −335 to −238 markedly reduced the

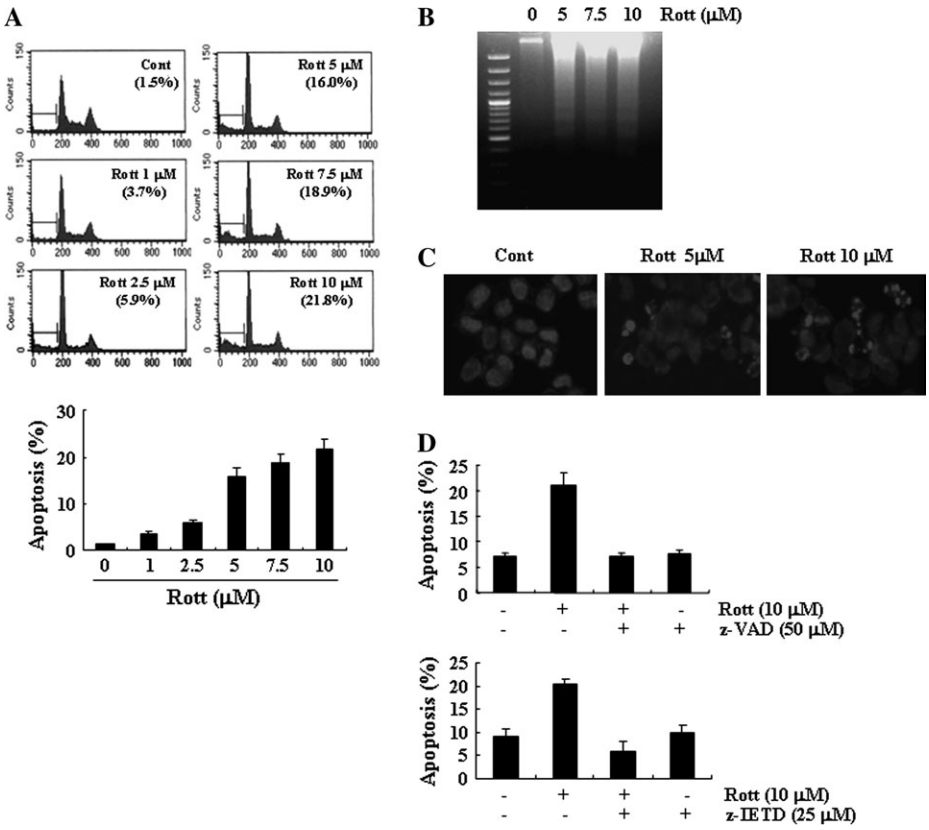

Fig. 1. Rottlerin induces apoptosis in HT29 cells. (**A**) HT29 cells were treated for 24 h with the indicated concentrations of rottlerin and then evaluated for DNA content after propidium iodide staining. The fraction of apoptotic cells is shown as indicated. Data are mean values obtained from three independent experiments and bars represent standard deviation. (**B**) Fragmentations of genomic DNA in HT29 cells treated for 24 h with the indicated concentrations of rottlerin. Fragmented DNA was extracted and analyzed on 2% agarose gel. (**C**) Detection of rottlerin-induced apoptosis in HT29 cells by 4′,6′-diamidino-2-phenylindole staining. HT29 cells were incubated for 24 h with the indicated concentrations of rottlerin. For 4′,6′-diamidino-2-phenylindole staining, cells were harvested and fixed in 1% paraformaldehyde for 30 min. The cells were stained with 300 nM 4′,6′-diamidino-2-phenylindole for 5 min and changes in nuclear morphology were observed by fluorescence microscopy. (**D**) Rottlerin-induced apoptosis is mediated by caspases. HT29 cells were incubated with 50 μM z-VAD (top), 25 μM z-IETD (bottom) or solvent for 1 h before treatment with 10 μM rottlerin. Cells were treated for 24 h and then evaluated for DNA content after propidium iodide staining. The fraction of apoptotic cells is shown as indicated. Data are mean values obtained from three independent experiments and bars represent standard deviation.

rottlerin-mediated activation as well as the basal promoter activity, compared with that of the control pGL2-DR5 (Figure 3A), suggesting that these regions play an important role in both the basal and rottlerin-mediated activation of the DR5 promoter.

Yamaguchi *et al.* (9) and other groups (10) recently reported that endoplasmic reticulum (ER) stress-mediated C/EBP homologous protein (CHOP) is a potential transcription factor for DR5. To determine whether the CHOP is associated with rottlerin-mediated transcriptional activation of DR5, we mutated the potential CHOP site (−281 to −261) of the DR5 gene (10). These constructs were transiently transfected into HT29 cells, and the response to rottlerin was determined by luciferase assay, in the presence or absence of rottlerin. As shown in Figure 3B, rottlerin increased the promoter activities of pDR5/605 in a dose-dependent manner. However, CHOP-mutated DR5 promoter was not activated by treatment with rottlerin, demonstrating that CHOP is involved in the rottlerin-mediated upregulation of DR5. Next, we further confirmed that rottlerin-induced DR5 upregulation is modulated by CHOP expression. Suppression of CHOP expression by CHOP siRNA inhibited rottlerin-induced expression of DR5 (Figure 3C). These results suggest that CHOP plays an important role in rottlerin-mediated DR5 upregulation.

DR5 siRNA attenuates rottlerin-induced apoptosis

We next examined whether upregulation of DR5 expression by rottlerin is directly associated with its effect on apoptosis in HT29 cells, employing siRNA duplex against DR5 mRNA. HT29 cells transfected with the control GFP, or DR5 siRNA, were treated with indicated concentrations of rottlerin for 24 h and examined its effect on PARP cleavage and apoptosis in response to rottlerin treatment. When we evaluated apoptosis by fluorescent-activated cell sorting analysis, GFP siRNA-transfected cells showed ∼24% apoptosis following treatment with rottlerin; however, DR5 siRNA-transfected cells decreased this to 11% (Figure 4A). As shown in Figure 4B, suppression of DR5 expression by transfection with its siRNA significantly inhibited the cleavage of PARP (Figure 4B). To confirm the effect of specificity of DR5 siRNA, we tested two nucleotides mutated DR5 siRNA (si-mDR5 siRNA). Treatment of HT29 cells with rottlerin after transfection with si-mDR5 siRNA or GFP siRNA did not affect DR5 expression levels, PARP cleavage and rottlerin-induced apoptosis (Figure 4C and D). Taken together, these results suggest that DR5 upregulation accounts, at least in part, for apoptosis induced by the rottlerin treatment of HT29 cells.

The effect of rottlerin on DR5 expression is independent of PKC δ inhibition

Because rottlerin is identified as a specific inhibitor of the novel PKC isoform, PKC δ, we examined whether rottlerin-induced DR5 upregulation was directly associated with inhibition of PKC δ activity. First, we tested whether overexpression of PKC δ WT, DN mutant PKC δ or control pcDNA3.1 affected rottlerin-induced apoptosis and

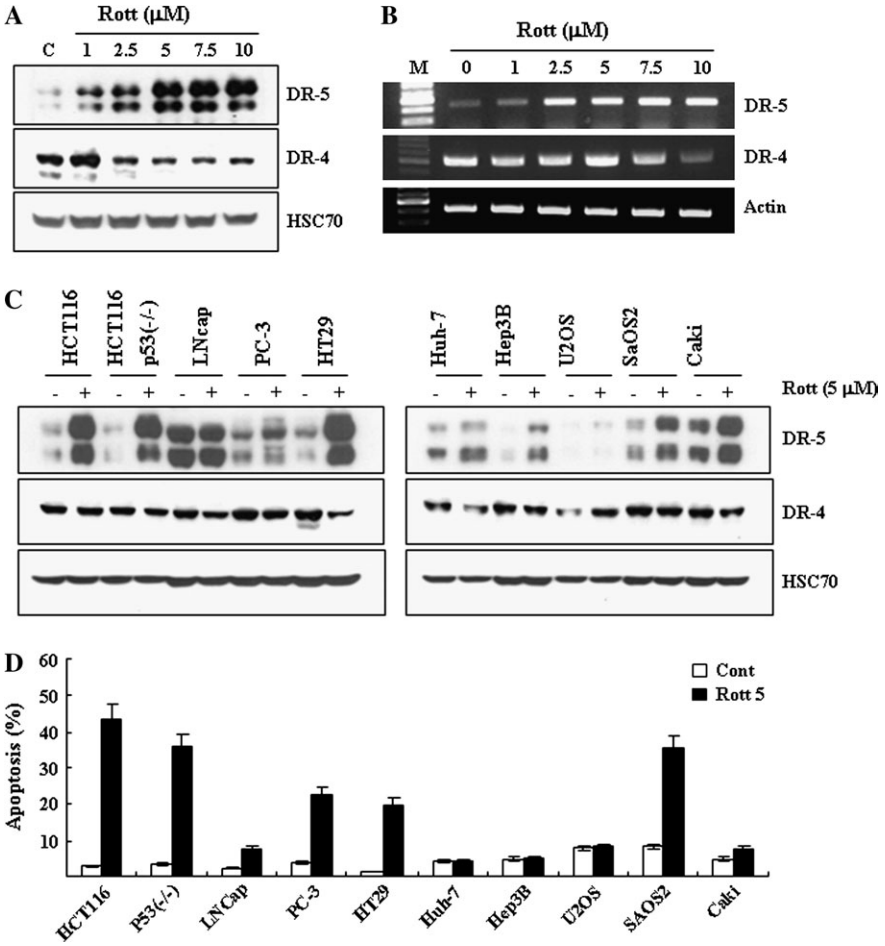

Fig. 2. The expression levels of the DR5 by treatment with rottlerin in various cancer cell lines. (**A**) HT29 cells were treated with the indicated concentrations of rottlerin for 24 h. Equal amounts of cell lysates (40 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with specific antibodies, anti-DR5, anti-DR4 or with anti-HSC70 antibody to serve as control for the loading of protein level. A representative study is shown; two additional experiments yielded similar results. (**B**) Total RNA was isolated and reverse transcriptase–PCR analysis was performed using DR5 gene- and DR4-specific primers and also the internal control gene, β-actin. A representative study is shown; two additional experiments yielded similar results. (**C**) HCT116, HCT116 ($p53^{-/-}$), LNcap, PC-3, HT29, Huh-7, Hep3B, U2OS, SaOS2 and Caki cells were treated with 5 μM rottlerin for 24 h. Expression levels of DR5 and DR4 were determined by western blot analysis. Anti-HSC70 antibodies serve as control for the loading of protein level. (**D**) Several cancer cells were treated with 5 μM rottlerin for 24 h and then evaluated for DNA content after propidium iodide staining. The fraction of apoptotic cells is shown as indicated. Data are mean values obtained from three independent experiments and bars represent standard deviation.

DR5 upregulation. As shown in Figure 5A, treatment of HT29 cells with rottlerin after transient transfection with plasmids encoding the WT-PKC δ, DN-PKC δ and control vector did not alter any significant difference in induction of apoptosis and DR5 upregulation (Figure 5A and B). To further confirm whether rottlerin-induced DR5 expression is dependent on PKC δ activity, we employed the siRNA duplex against PKC δ mRNA. HT29 cells transfected with the control GFP or PKC δ siRNA were treated with rottlerin for 24 h. Suppression of PKC δ expression by transfection with its siRNA failed to affect rottlerin-induced CHOP and DR5 upregulation in these cells (Figure 5C). Taken together, our results suggest that rottlerin-induced apoptosis or upregulation of DR5 is independent of its effect as a PKC δ inhibitor.

Rottlerin sensitizes TRAIL-mediated apoptosis

To investigate the effect of rottlerin on TRAIL-mediated apoptosis, HT29 cells were treated with rottlerin alone (2.5 μM), TRAIL alone (100 ng/ml) or combination of rottlerin and TRAIL. Two established criteria were subsequently used to assess apoptosis in our system. First, apoptosis in HT29 cells was determined using flow cytometric analysis to detect hypodiploid cell populations. As shown in Figure 6A, HT29 cells were resistant to TRAIL alone: <10% of these cells were apoptotic after treatment with 100 ng/ml TRAIL or rottlerin (Figure 6A). However, cotreatment of HT29 cells with rottlerin and TRAIL resulted in a markedly increased accumulation of sub-G_1 phase cells. Second, we analyzed whether cotreatment with rottlerin and TRAIL gave rise to the activation of caspases, key executioners of apoptosis. Cotreatment of HT29 cells with rottlerin and TRAIL strongly stimulated Asp-Glu-Val-Asp-ase activity (Figure 6B) and led to a reduction of the protein levels of 32 kDa precursor together with a concomitant cleavage of PARP, a substrate protein of caspases. Next, we investigated whether the combined treatment with rottlerin and TRAIL affects the induction of apoptosis in normal human mesangial cells or human skin fibroblast cells. The apoptotic characteristics, such as cell shrinkage, apoptotic bodies and detachment from the plate, were frequently observed in HT29 cells or Caki cells treated with rottlerin plus TRAIL (Figure 6C). However, the mesangial cells and human skin fibroblast cells were resistant to 2.5 μM rottlerin or 100 ng/ml TRAIL alone. Furthermore, their morphological changes and viability were not significantly affected by the combined treatment with 2.5 μM rottlerin and 100 ng/ml TRAIL, demonstrating that this combined treatment with rottlerin and TRAIL preferentially enhances TRAIL-induced apoptosis in cancer cells, sparing normal cells (Figure 6C).

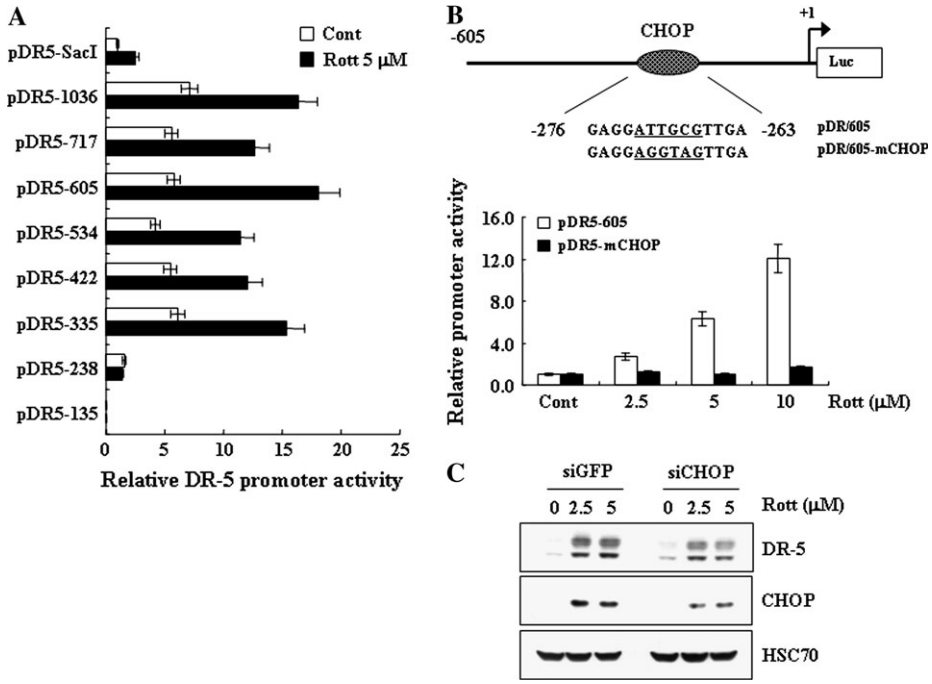

Fig. 3. CHOP mediates rottlerin-induced DR5 upregulation. (**A**) HT29 cells were transiently transfected with progressive 5′-deletion mutants of DR5 promoter. Twenty-four hours after transfection, cells were treated with rottlerin (10 μM) for 24 h. The cells were lysed and luciferase activity was measured. Data represent the mean ± SD of at least three independent experiments. (**B**) The effect of rottlerin on DR5 promoter activity. HT29 cells were transfected with pDR5/−605 or CHOP-mutated pDR5/−605 and then treated with the indicated concentrations of rottlerin. After 24 h, cell lysates are lysed and assayed for luciferase activity. Data represent the mean ± SD of at least three independent experiments. (**C**) HT29 cells were transfected with CHOP siRNA or GFP siRNA. Twenty-four hours after transfection, cells were treated with the indicated concentrations of rottlerin for 24 h. Equal amounts of cell lysates (40 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with specific antibodies, anti-DR5, anti-CHOP or with anti-HSC70 antibody to serve as control for the loading of protein level.

Discussion

Rottlerin, which is known as a PKC δ inhibitor, has been shown to induce apoptosis of hematopoietic cell lines (21). However, the molecular mechanisms responsible for the rottlerin-induced apoptosis have not been clearly established. In the present study, we provided for the first time the several observations that apoptosis induced by rottlerin is mediated by the upregulation of DR5: (i) rottlerin-induced apoptosis was closely correlated with DR5 upregulation (Figures 1 and 2); (ii) DR5 knockdown by siRNA duplexes effectively inhibited the cell death induced by rottlerin, demonstrating its critical role in this event (Figure 4) and (iii) Rottlerin sensitized cancer cells to TRAIL-mediated apoptosis, but not in normal cells (Figure 6).

Several papers provide evidence that DR5 upregulation may be a promising strategy for sensitizing tumor cells to TRAIL-induced apoptosis (6–8). DR5 is regulated by either a p53-dependent or -independent mechanism (11,12,22). Rottlerin induced the expression of DR5 in nine of the 10 tested cancer cell lines, regardless of p53 status {WT p53 [Caki and HCT116], mutant p53 [Hep3B and SaOS2] and p53 null [HCT116 (p53−/−) and PC-3]}, indicating that rottlerin upregulates DR5 expression via p53-indpendent manners. Our and other groups have reported about the mechanism in which various chemopreventive agents increased DR5 expression. Sulforaphane, curcumin and rosiglitazone upregulate DR5 expression through reactive oxygen species (ROS)-mediated pathway. Proteasome inhibitor (MG132) induced DR5 expression through ROS-dependent p53 activation (6,23–25). Therefore, we examined whether ROS generation could also be involved in rottlerin-induced DR5 upregulation. Pretreatement with antioxidants (*N*-acetylcysteine or glutathione) did not inhibit rottlerin-induced DR5-upregulation, CHOP expression and PARP cleavage (data not shown). These results suggest that rottlerin-induced DR5 expression is a ROS-independent pathway. In addition, several papers have reported that ER stress-mediated C/EBP homologous protein (CHOP) is a potential transcription factor for DR5 (7,9,10,26). Here, we provide evidence that the CHOP transcription factor is critical for rottlerin-induced DR5 upregulation. First, rottlerin induces CHOP expression. Second, rottlerin-induced activation of DR5 promoter activity was abrogated by a mutation in the CHOP-binding site of the DR5 promoter. Third, siRNA-mediated CHOP knockdown significantly blocks rottlerin-induced DR5 upregulation (Figure 3). Thus, rottlerin upregulates DR5 via ER stress-related CHOP induction in human colon carcinoma HT29 cells.

Rottlerin is a selective inhibitor of PKC δ commonly used in the analysis of the role of PKC δ to certain downstream signaling events. However, a variety of PKC δ-independent actions of rottlerin has been recently reported (16,17). Rottlerin was shown to act as an uncoupler of oxidative phosphorylation in a PKC δ-independent manner (14,16). In this study, the following data suggest that rottlerin induces DR5 upregulation through a PKC δ-independent mechanism: (i) exogenously expressed WT-PKC δ or DN-PKC δ did not alter rottlerin-induced DR5 expression levels (Figure 5B) and (ii) knockdown of PKC δ failed to enhance rottlerin-induced DR5 expression (Figure 5C). Recently, Tillman *et al.* (18) reported that treatment with rottlerin sensitizes colon carcinoma cells to TRAIL-mediated apoptosis through the PKC δ-independent uncoupling of the mitochondria. In addition, Kim *et al.* (19) reported that treatment with rottlerin sensitizes glioma cells to TRAIL-induced apoptosis via inhibition of cdc2 and the subsequent downregulation of survivin and X-linked inhibitor of apoptosis protein, but independently of PKC δ. One mechanism potentially involved in the regulation of TRAIL sensitivity is the expressional modulation of DR5. In the present study, rottlerin significantly upregulated DR5 protein levels in nine of the 10 tested cancer cell lines. Several studies have reported that DRs such as Fas and tumor necrosis factor-R can induce apoptosis in a ligand-independent manner (27–29). Because death domains function as oligomerization interfaces, overexpression of receptors that contain

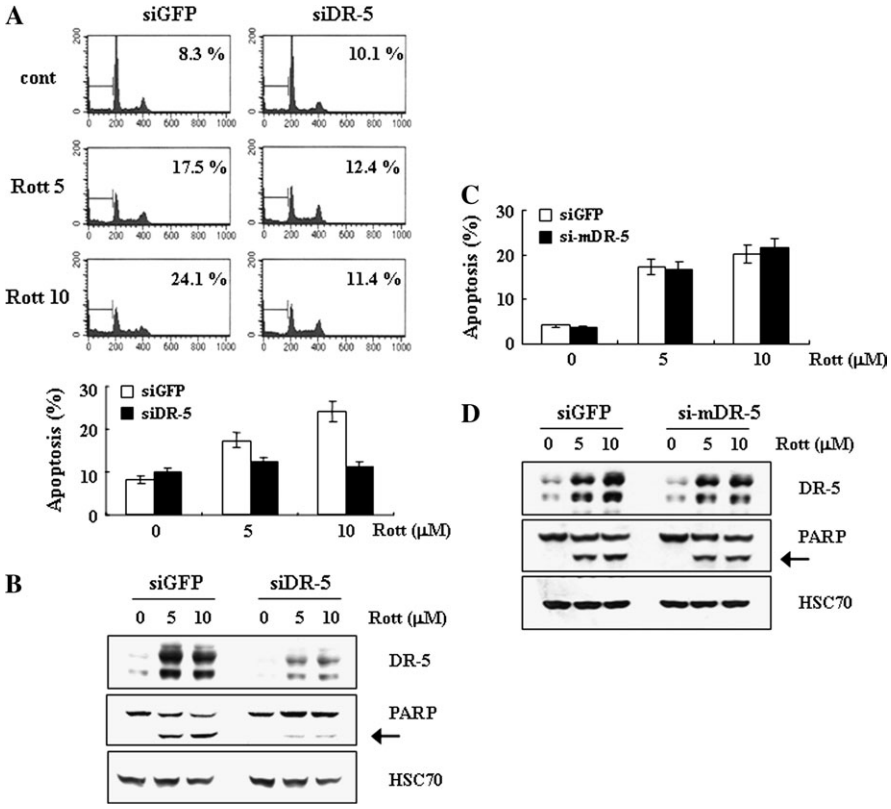

Fig. 4. Downregulation of DR5 attenuates rottlerin-induced apoptosis in HT29 cells. (**A**) HT29 cells were transfected with DR5 siRNA or GFP siRNA. Twenty-four hours after transfection, cells were treated with rottlerin for 24 h. Apoptosis was analyzed as a sub-G_1 fraction by fluorescent-activated cell sorting. Data are mean values obtained from three independent experiments and bars represent standard deviation. (**B**) Equal amounts of cell lysates (40 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with specific antibodies, anti-DR5, anti-PARP or with anti-HSC70 antibody to serve as control for the loading of protein level. (**C**) HT29 cells were transfected with mutated DR5 siRNA or GFP siRNA. Twenty-four hours after transfection, cells were treated with rottlerin for 24 h. Apoptosis was analyzed as a sub-G_1 fraction by fluorescent-activated cell sorting. Data are mean values obtained from three independent experiments and bars represent standard deviation. (**D**) Equal amounts of cell lysates (40 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with specific antibodies, anti-DR5, anti-PARP or with anti-HSC70 antibody to serve as control for the loading of protein level.

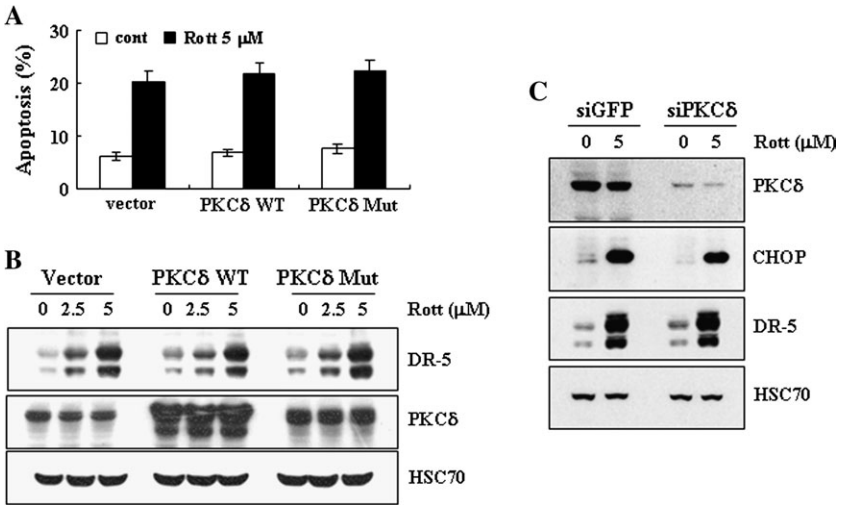

Fig. 5. Rottlerin-induced DR5 expression seems to be independent on the PKC δ activation. (**A**) HT29 cells were transfected with empty vector, WT-PKC δ and DN-PKC δ and then treated with or without rottlerin. Apoptosis was analyzed as a sub-G_1 fraction by fluorescent-activated cell sorting. Data are mean values obtained from three independent experiments and bars represent standard deviation. (**B**) HT29 cells were transfected with empty vector, WT-PKC δ and DN-PKC δ and then treated with or without rottlerin. The expression levels of PKC δ and DR5 were confirmed by immunoblotting. Western blotting of HSC70 levels was included to show that equivalent amounts of protein were loaded in each lane. (**C**) HT29 cells were transfected with PKC δ siRNA or GFP siRNA. Twenty-four hours after transfection, cells were treated with 5 μM rottlerin for 24 h. Equal amounts of cell lysates (40 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with specific antibodies, anti-PKC δ, anti-DR5, anti-CHOP or with anti-HSC70 antibody to serve as control for the loading of protein level.

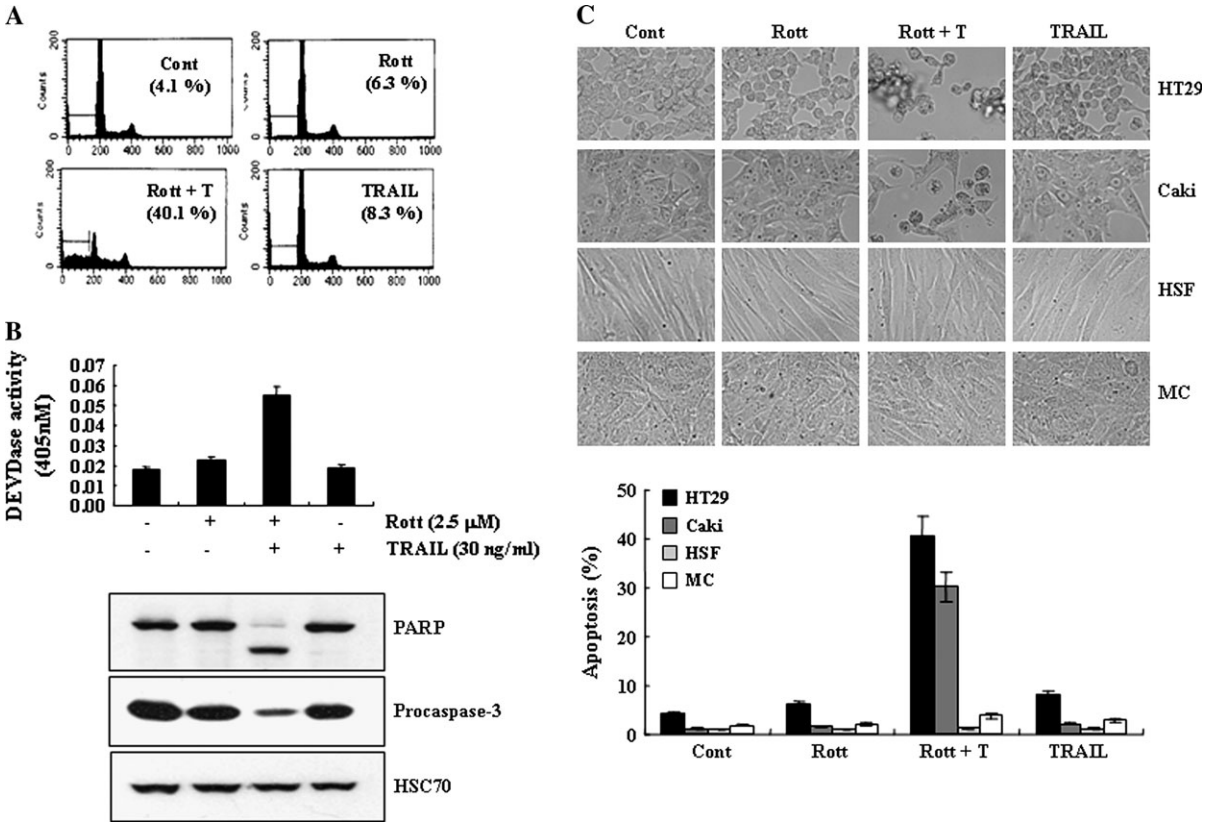

Fig. 6. Rottlerin sensitizes cancer cells to TRAIL-induced apoptosis. (**A**) Flow cytometric analysis of apoptotic cells. HT29 cells were treated with TRAIL (100 ng/ml) in either the absence or the presence of rottlerin (2.5 μM) for 24 h. Apoptosis was analyzed as a sub-G_1 fraction by fluorescent-activated cell sorting. (**B**) Activation of caspases in rottlerin-sensitized TRAIL-induced apoptosis. HT29 cells were treated with 2.5 μM rottlerin and TRAIL. The expression levels of apoptosis-related proteins in HT29 cells by treatment with rottlerin and TRAIL. Enzymatic activities of Asp-Glu-Val-Asp-ase were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrate (Asp-Glu-Val-Asp-*p*-nitroanilide) in a 100 μl assay buffer for 2 h at 37°C. The release of chromophore *p*-nitroanilide was monitored spectrophotometrically at 405 nm (upper panel). Equal amounts of cell lysates (40 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with specific antibodies, anti-caspase-3, anti-PARP or with anti-HSC70 antibody to serve as control for the loading of protein level (lower panel). A representative study is shown; two additional experiments yielded similar results. (**C**) Effect of rottlerin and/or TRAIL on normal cells. HT29, Caki, normal mesangial cells (MC) and normal skin fibroblast cells (HSF, human skin fibroblast) were treated with vehicle, 2.5 μM rottlerin alone, 100 ng/ml TRAIL and rottlerin (2.5 μM) plus TRAIL (100 ng/ml) for 24 h. The morphology of cells was determined by interference light microscopy (magnification, ×200) (upper panel). Apoptosis was analyzed as a sub-G_1 fraction by fluorescent-activated cell sorting. Data are mean values obtained from three independent experiments and bars represent standard deviation (lower panel).

such domains leads to activation of signaling in the absence of ligand (30). Moreover, DR5 overexpression induces ligand-independent apoptosis (31,32). Therefore, our data suggest a possibility that rottlerin-induced DR5 upregulation might induce apoptosis through ligand-independent manner. Although accumulating evidence suggest that rottlerin plays a PKC δ-dependent or -independent role in inducing apoptotic cell death, however, conflicting results regarding its antiapoptotic effect have been reported. Basu *et al.* (33) reported that rottlerin inhibit cisplatin-induced apoptosis through downregulation of caspase-2 expression. Rottlerin-induced downregulation of caspase-2 was triggered by proteasome-mediated pathway. Byun *et al.* (34) also reported that rottlerin inhibit tumor necrosis factor-induced necrosis through suppression of Nox1 nicotinamide adenine dinucleotide phosphate (reduced) oxidase activity. We were unable to clearly explain the underlying mechanism in rottlerin-induced proapoptosis or antiapoptosis.

Our studies have shown that combined treatment with rottlerin and TRAIL induces apoptosis in renal carcinoma cells and colon carcinoma cells, but not in primary cultures of normal cells. Although further animal studies will be required to support the clinical application, our data provide the first mechanistic evidence that rottlerin treatment results in CHOP-mediated DR5 upregulation, rendering cancer cells more sensitive to the cytotoxic activities of the TRAIL. Thus, in terms of a clinical perspective, the combination of rotterlin and TRAIL may be a novel strategy for the treatment of a variety of human cancers, which are resistant to chemotherapy.

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