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Caspase-dependent generation of reactive oxygen species in human astrocytoma cells contributes to resistance to TRAIL-mediated apoptosis

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family of cytokines, causes apoptosis by caspase activation in various cell types, particularly in transformed cells. Numerous types of tumors are relatively resistant to TRAIL-induced cytotoxicity; however, the reasons for this are not yet fully understood. We report here a new signal transduction pathway involving protein kinase Cδ (PKCδ), NADPH oxidase 4 (NOX4) and reactive oxygen species (ROS), that inhibits caspase-dependent cell death induced by TRAIL ligation in human malignant astrocytoma cells. In our experiments, TRAIL ligation-induced generation of intracellular ROS through caspase-dependent proteolytic activation of PKCδ and subsequent activation of the NOX4 complex. Suppression of intracellular ROS induction using various pharmacological inhibitors or PKCδ- or NOX4-specific RNA interference enhanced the enzymatic activity of caspase-3 by blocking the oxidative modification of its catalytic cysteine residue, resulting in marked augmentation of TRAIL-mediated cell death. These results collectively indicate that TRAIL-induced activation of PKCδ and NOX4 can modulate TRAIL-mediated apoptosis by promoting oxidative modification of active caspase-3 in a negative-feedback manner.
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Along with other members of the tumor necrosis factor (TNF) family of cytokines, TNF-related apoptosis-inducing ligand (TRAIL) transduces apoptotic signals through direct protein–protein interactions, such as those between caspases and adapter proteins such as Fas-associated protein with death domain (FADD). Although TRAIL is of interest to oncologists and cancer biologists because it is more cytotoxic to transformed cells than to their normal counterparts, numerous types of malignancies are reported to be resistant to TRAIL-induced toxicity. The postulated molecular mechanisms underlying this resistance to death-receptor signaling include downregulation of agonistic receptors, overexpression of decoy receptors, lack of death-inducing machinery, and abundance of endogenous inhibitors of apoptosis.[1–4]

TRAIL has recently been shown to induce not only caspase-dependent apoptosis but also the expression of pro-inflammatory genes such as CXCL8.[5] TRAIL-induced expression of CXCL8 is also mediated by upstream caspase-8 and the subsequent activation of the transcription factors NF-κB and AP-1.[5] Resistance of cells to death receptor-mediated cell death might enhance their ability to transduce proliferative and pro-inflammatory responses.[6] Therefore, it is important to delineate the molecular mechanisms responsible for relative resistance to TRAIL-mediated apoptotic processes.

Reactive oxygen species (ROS), such as superoxide anions, H_2O_2, and hydroxyl radicals, are generated by the mitochondrial electron-transport chain during normal cellular respiration. Under normal conditions, antioxidant defense systems involving catalase, superoxide dismutase, and glutathione peroxidase regulate intracellular ROS levels to maintain physiological homeostasis. Recently, the production of these ROS has been suggested to be an integral component of receptor-mediated signal transduction.[7] Receptor-generated ROS function as second messengers that modulate the activity of protein kinases, protein phosphatases, transcription factors, phospholipases, ion channels, and G proteins.[8] Although ROS are also generated by various enzymes, including lipoxygenase, xanthine oxidase, and cytochrome P450, NADPH oxidase (NOX) is regarded as the most essential source of receptor-mediated ROS in both phagocytic and non-phagocytic cells.

Although the exact molecular mechanisms responsible for receptor-mediated ROS generation and the role of ROS in receptor-mediated apoptosis remain unclear, several lines of evidence implicate ROS in apoptotic signaling through death receptors. ROS have been shown to be generated via the mitochondrial electron-transport chain and to have an important role in TNF-induced cytotoxicity.[9] ROS have also been shown to enhance Fas-mediated apoptosis in a number

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Keywords: apoptosis; ROS; caspase; oxidative modification
Abbreviations: ROS, reactive oxygen species; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; hrTRAIL, human recombinant TRAIL; NOX, NADPH oxidase; siRNA, small-interfering RNA; PKC, protein kinase C; NAC, N-acetyl cysteine; EGCG, epigallocatenin gallate; BIAM, biotin-conjugated iodoacetamide; DPI, diphenyl iodonium; DCF-DA, 2-7-dichlorofluorescin-diacetate
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of different cell types, such as B-cell lymphomas, T cells, and monocytes.[10,11] On the other hand, H_2O_2 attenuates Fas-mediated apoptosis by inhibiting caspase-3 activity[12,13] Glutathione depletion suppresses Fas-mediated caspase-8 activation and apoptosis induction, thereby suggesting that pro-oxidative conditions are cytoprotective under certain circumstances.[14] TRAIL has been reported to induce ROS potentiation of receptor-mediated activation of caspases and apoptotic cell death in HeLa human adenocarcinoma cells.[15] As ROS have been postulated to act as second messengers in signaling through various receptors, we investigated whether intracellular ROS also have roles in TRAIL-mediated signaling as molecular modifiers.

Results

TRAIL-induced generation of intracellular ROS is the causative mechanism of TRAIL-mediated apoptosis. To study the role of ROS in TRAIL-mediated signaling, we first examined whether TRAIL ligation induces an increase in intracellular ROS levels. Treatment of CRT-MG human astrocytoma cells with human recombinant TRAIL increased intracellular ROS levels in a time-dependent manner, and the maximal ROS level, which was reached after 30 min of exposure, was comparable to the level obtained upon exogenous administration of H_2O_2 (Figure 1a). We also observed dose-dependent increases in intracellular ROS levels upon TRAIL ligation (Supplementary Figure 1a). TRAIL ligation also induced increases in intracellular ROS levels in other human astrocytoma cells, including U87-MG and U251-MG cells (data not shown). Pretreatment with various ROS scavengers, including N-acetyl cysteine (NAC), epigallocatechin gallate (EGCG), and melatonin, abrogated the TRAIL-induced ROS increase (Figure 1b).

As caspases have a key role in TRAIL-mediated signaling events such as apoptosis and gene induction,[5] we examined the involvement of caspases in the TRAIL-mediated generation of ROS. Preincubation with broad-spectrum caspase inhibitors abrogated the induction of ROS by TRAIL but not by TNF-α (Figure 1c). Caspase-3- and caspase-8-specific inhibitors also significantly suppressed TRAIL-mediated ROS induction; the caspase-3 inhibitor was much more potent than the caspase-8 inhibitor.

We next investigated the physiological function of this new pathway in TRAIL-mediated apoptosis. As TRAIL-mediated ROS generation is involved in receptor-mediated activation of caspases and subsequent apoptotic cell death,[16] we also expected that this pathway might have a pro-apoptotic role in TRAIL-induced apoptosis of human astrocytoma cells. However, contrary to our expectation, we observed that TRAIL-induced apoptosis increased dramatically in the presence of various ROS scavengers, such as NAC, EGCG, and melatonin, whereas treatment with these inhibitors alone had little effect on cell viability (Figure 1d and e). Pre-incubation with these inhibitors also potentiated TRAIL-mediated loss of the mitochondrial transmembrane potential ($\Delta\Psi$m) (Supplementary Figure 1b). Pre-incubation with caspase inhibitors abrogated the enhancement of TRAIL-induced cell death by ROS scavengers (Figure 1f). Similar results were obtained in U87-MG and U251-MG astrocytoma cells (data not shown). These results clearly demonstrate that suppression of ROS levels sensitizes cells to TRAIL-mediated apoptosis in a caspase-dependent manner.

TRAIL-induced ROS affect the activity but not the amount of active caspase-3. Next, we attempted to discover the molecular mechanisms responsible for the anti-apoptotic properties of ROS in TRAIL-induced cell death. As we found that the enhancement of TRAIL-induced cell death by ROS scavengers was mediated by caspases, we hypothesized that caspases might be the target of the ROS generated by TRAIL ligation. However, we observed a moderate increase in the proteolytic cleavage of caspase-8 or caspase-3 when cells were pretreated with NAC (Figure 2a) or other ROS scavengers (data not shown), whereas the same treatment significantly enhanced the TRAIL-induced *in vitro* enzymatic activity of caspase-3 (Figure 2b and c). Previously, we reported that proteasomal inhibition by MG-132 or lactacystin enhanced TRAIL-induced apoptosis, mainly by stabilizing the active caspase-3 fragments.[17] Although the effects of ROS scavengers and proteasome inhibitors on TRAIL-mediated cell death were comparable, proteasomal inhibition dramatically increased the level of active caspases, compared with the effect of NAC treatment (Figure 2d). The effect of NAC and MG-132 on caspase-3 activity was confirmed by increased cleavage of poly(ADP-ribose) polymerase (PARP), a bona-fide caspase-3 substrate (Figure 2e).

Next, we examined the enzymatic activity of caspase-3 *in vivo* using a cell-permeable fluorogenic substrate. Treatment with TRAIL alone for 2 h induced only a minimal increase in DEVDase activity inside the cells, but pre-incubation with NAC significantly increased the enzymatic activity of caspase-3 (Figure 2e). Consistent with the *in vitro* results, intracellular staining using an antibody specific for active caspase-3 demonstrated that NAC pretreatment only minimally altered the amount of active caspase-3. On the other hand, pretreatment with MG-132, a proteasomal inhibitor, increased both the amount and the *in vivo* activity of active caspase-3, in agreement with our previous report.[17] These results collectively indicate that intracellular ROS inhibit TRAIL-induced cell death primarily by modulating the enzymatic activity of caspases as new ROS-sensitive targets in this setting.

Oxidative modification of p17/p22 fragments of caspase-3. The proteolytic activity of caspase proteases is dependent on a catalytic cysteine residue in the active site. Thus, oxidative modification of the catalytic cysteine residue of caspase-3 is expected to impair or eliminate its proteolytic activity. Biotin-conjugated iodoacetamide (BIAM) selectively labels reduced cysteine residues, but oxidized cysteine residues are not susceptible to BIAM labeling. The extent of BIAM labeling can be monitored by streptavidin blot analysis.[18] As compared with treatment with TRAIL alone, pretreatment with NAC increased BIAM labeling of protein bands and spots corresponding to active fragments (p22/p17) of caspase-3 (Figure 3a and b).

Caspase-3 has eight cysteine residues, including a cysteine in the active catalytic site. To examine whether the catalytic

cysteine of caspse-3 is the cysteine that is subject to oxidation, we generated a caspase mutant, C6A-caspase-3, with only two cysteine residues, one in the catalytic site and one at the forty-seventh residue from the N-terminus (Supplementary Figure 2a), and tested whether this C6A mutant was subject to oxidation with BIAM labeling (Figure 3c and Supplementary Figure 2b). The replacement of six cysteine residues with alanine in the C6A mutant did not affect the BIAM labeling of active caspase-3 fragments, compared with the labeling of wild-type fragments. Collectively, these results support the hypothesis that TRAIL-induced H_2O_2 contributes to the specific oxidative modification of the catalytic cysteine residue of caspase-3, thus inactivating caspase-3 in a negative-feedback manner.

NOX4 and protein kinase Cδ (PKCδ) are the molecular links between caspase-3 and ROS. As NOXs are thought to be involved in ROS generation induced by a variety of biological agonists, we next investigated whether the NOX system is also responsible for TRAIL-induced ROS generation. First, we used diphenyl iodonium (DPI), a flavoprotein inhibitor that has been widely used to inhibit the NOX system. Incubation with DPI significantly suppressed ROS induction by TRAIL ligation, but not those induced by exogenous administration of H_2O_2 (Figure 4a).

Although the mechanism by which various agents activate the NOX complex remains unclear, PKC is the best-known signal transducer that has been shown to induce phosphorylation and subsequent translocation of various

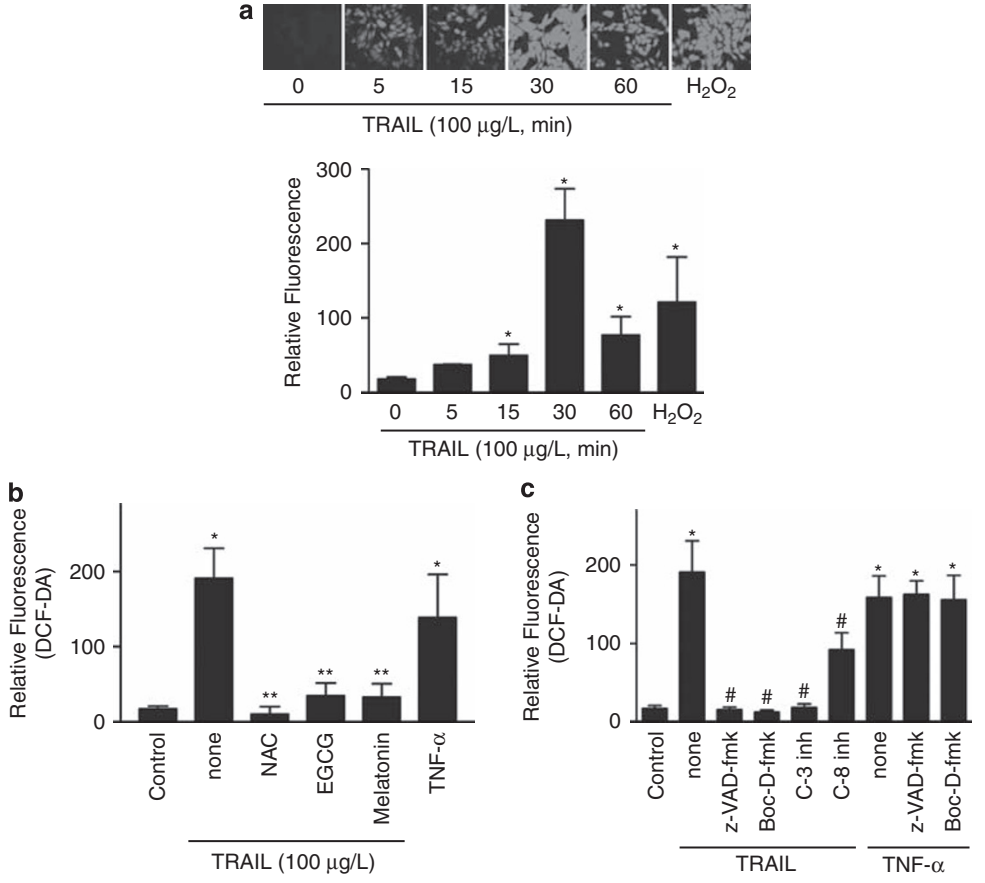
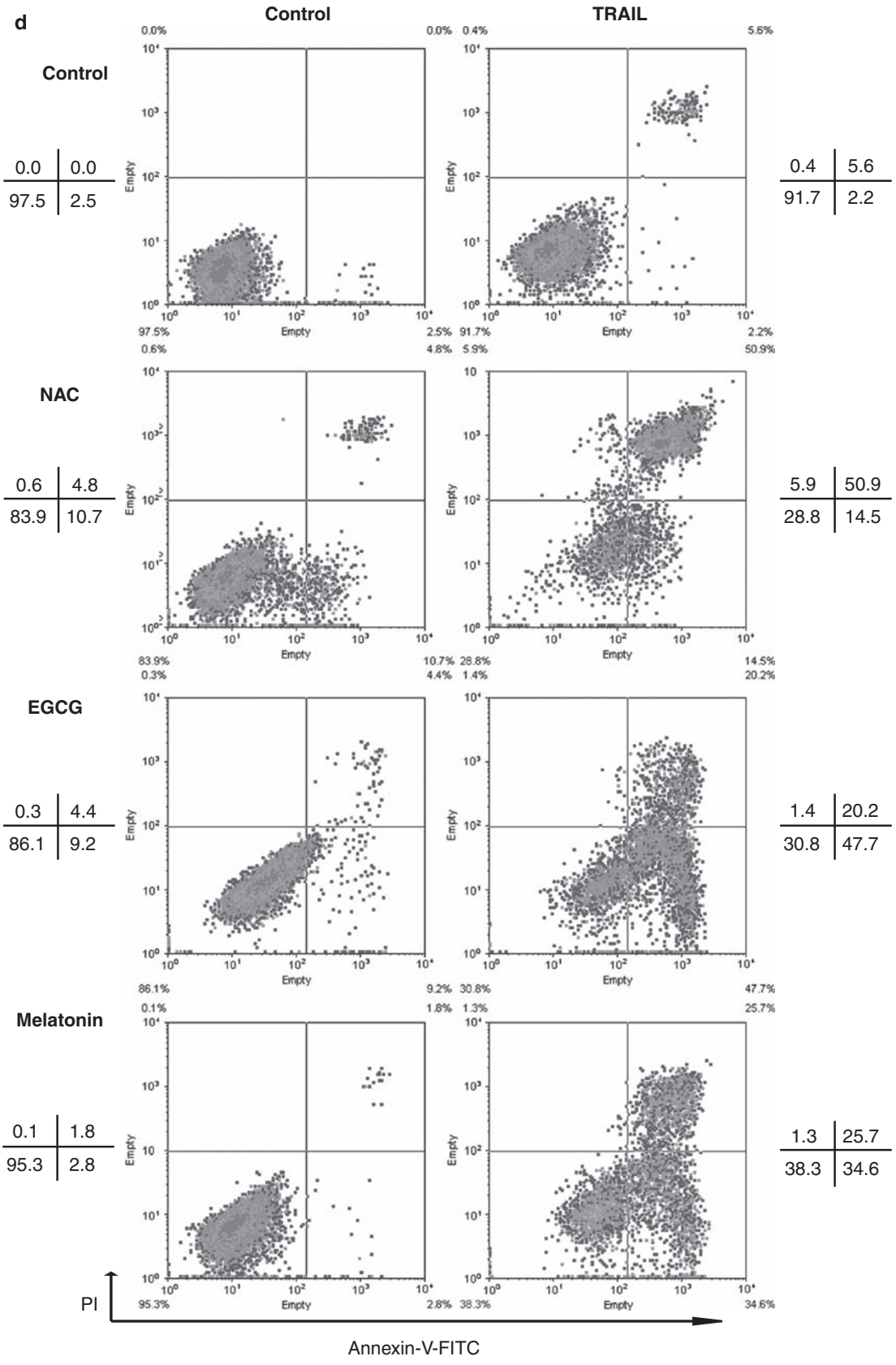


Figure 1 Suppression of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) induction of reactive oxygen species (ROS) augments caspase-dependent cell death. (**a**) Human astrocytoma CRT-MG cells were incubated with human recombinant (hr)TRAIL (100 μg/l) for varying lengths of time (0–60 min), and intracellular ROS levels were determined after staining with 2-7-dichlorofluorescin-diacetate (DCF-DA) (2.5 μmol/l). Cells treated with H_2O_2 (1 mmol/l) for 1 min were used as a positive control. Data are representative of five independent experiments. *$P \leqslant 0.01$ compared with the unstimulated samples. (**b**) Cells were incubated in the absence or presence of various ROS scavengers (20 mmol/l of N-acetyl cysteine (NAC), 100 μmol/l of epigallocatechin gallate (EGCG) or melatonin) for 1 h and treated with hrTRAIL (100 μg/l) for an additional 30 min. Intracellular ROS levels were then determined. Cells treated with TNF-α (10 μg/l) were analyzed for intracellular ROS levels as a positive control. *$P \leqslant 0.001$ compared with the unstimulated sample; **$P \leqslant 0.001$ compared with the sample treated with hrTRAIL alone without any pretreatment. Data are representative of three independent experiments. (**c**) CRT-MG cells were incubated in the absence or presence (10 μmol/l) of broad-spectrum caspase inhibitors (z-VAD-fmk and Boc-D-fmk), a caspase-3-specific inhibitor (z-DEVD-fmk), or a caspase-8-specific inhibitor (z-IETD-fmk) for 1 h and treated with hrTRAIL or TNF-α for 30 min. Intracellular ROS levels were then determined. *$P \leqslant 0.001$ compared with the unstimulated samples. #$P \leqslant 0.001$ compared with the sample treated with hrTRAIL alone without any pretreatment. (**d**) CRT-MG cells were incubated in the absence or presence of various ROS scavengers (NAC, EGCG, and melatonin) for 1 h and treated with hrTRAIL for an additional 6 h. Cell death was measured after staining with Annexin V-FITC and propidium iodide. Data are representative of four independent experiments. (**e**) Cells were incubated in the presence of varying doses of NAC for 1 h and treated with increasing doses of hrTRAIL for an additional 24 h. Cell death was measured by MTT analysis ($n = 4$). (**f**) Cells were incubated in the absence or presence of z-VAD-fmk, a caspase-3-specific inhibitor, a caspase-8-specific inhibitor, and/or NAC for 1 h and treated with hrTRAIL for an additional 6 h. Cell death was measured after staining with Annexin V-FITC and propidium iodide



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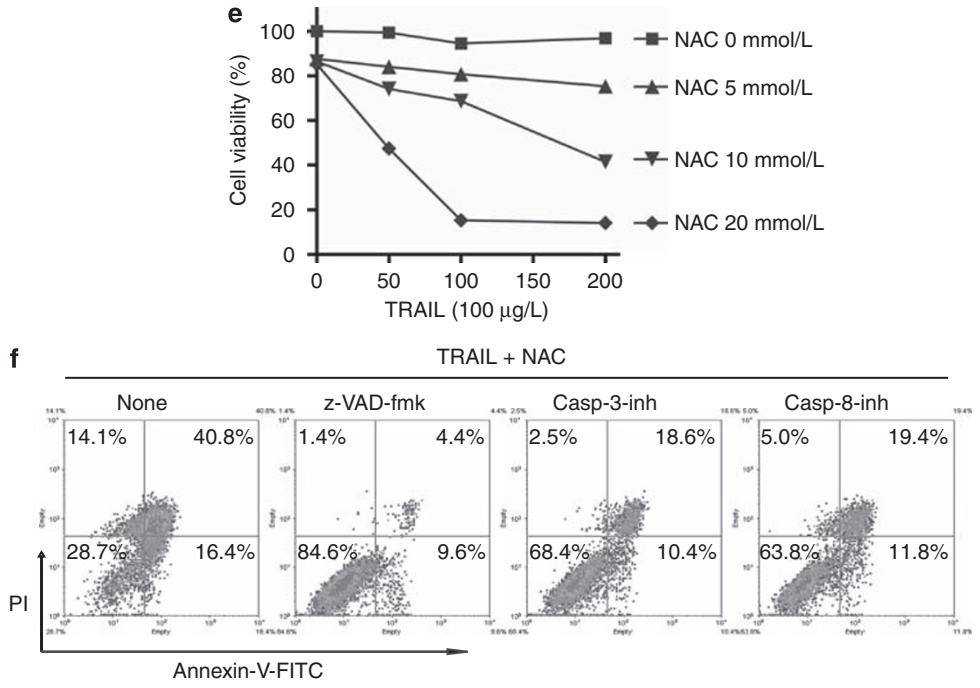

Figure 1 Continued

NOX components to the plasma membrane or mitochondria for full activation of the complex.[19] The PKC isotype PKCδ is cleaved by caspases and is involved in receptor-mediated apoptosis.[20] To test the involvement of PKCs and the PKCδ isotype, in particular in TRAIL-induced ROS generation, we used the broad-spectrum PKC inhibitor GF109203X and the PKCδ-specific inhibitor rottlerin. Pre-incubation with these PKC inhibitors abrogated TRAIL-induced ROS generation (Figure 4b), whereas the same treatment had no effect on H_2O_2-induced increases in ROS levels (data not shown). Consistent with these results, pretreatment with DPI or with PKC inhibitors significantly enhanced TRAIL-induced cell death (Figure 4c and d).

To confirm the involvement of NOX and PKCδ in TRAIL-mediated ROS generation and subsequent apoptotic cell death, we used an RNA interference technique to knock down the expression of NOX4 and PKCδ. Of the five different NOX family members, human astrocytoma cells predominantly express NOX4, as confirmed by quantitative real-time RT-PCR (data not shown). Transient transfection with small-interfering RNA (siRNA) specific for either PKCδ or NOX4 abrogated TRAIL-mediated ROS generation, whereas transient transfection with a control siRNA had no effect (Figure 4e). Consistent with these results, transfection with PKCδ- or NOX4-specific siRNA, but not control siRNA, increased TRAIL-induced cell death and caspase-3-like activity (Figure 4f and g, Supplementary figure 3). Transient knockdown of PKCδ or NOX4 protein also induced a marked loss of the mitochondrial transmembrane potential ($\Delta\Psi$m) after TRAIL treatment (data not shown). We then tested whether treatment with H_2O_2 could reverse the sensitizing effect of NOX4 or PKCδ knockdown on TRAIL-induced apoptosis (Figure 4h). Cells transfected with either NOX4 or PKCδ siRNA showed enhanced TRAIL-induced cytotoxicity, and this was significantly suppressed by pretreatment with increasing doses (0–100 μmol/l) of H_2O_2. No cytotoxic effects were observed with H_2O_2 alone, even at the highest concentration. These results clearly indicate that PKCδ and NOX4 are involved as anti-apoptotic signaling mediators in TRAIL-mediated ROS generation.

As PKCδ is proteolytically activated by caspases,[20,21] we examined the effect of TRAIL ligation on PKCδ in the absence or presence of the caspase inhibitor z-VAD-fmk. Using immunoblot analysis, we found that TRAIL ligation induced a time-dependent proteolytic cleavage of PKCδ to produce a short carboxy-terminus fragment (41 kDa) in a caspase-dependent manner (Figure 5a). We confirmed the caspase-dependent cleavage of PKCδ by introducing a PKCδ^{D329A} construct, which has a mutation in the caspase cleavage site (Figure 5b).

Proteolytic activation of PKCδ cleaves the regulatory domain from the catalytic domain and generates an active kinase domain.[21] To determine whether proteolytic cleavage of PKCδ by caspase-3 increases its enzyme activity, we assayed the *in vitro* histone kinase activity of PKCδ immunoprecipitated from cells treated with TRAIL in the absence or presence of z-VAD-fmk. The TRAIL-induced kinase activity of PKCδ was suppressed by preincubation with z-VAD-fmk, whereas the kinase activity induced by phorbol 12-myristate 13-acetate (PMA), a broad-spectrum PKC activator, was not affected by z-VAD-fmk (Figure 5c).

Next, we transiently transfected cells with a carboxy-terminal myc-tagged PKCδ cDNA construct, and the kinase activity of PKCδ was determined after immunoprecipitation with an anti-myc antibody. Kinase activity of this overexpressed PKCδ was also induced by TRAIL treatment in a caspase-

dependent manner (Figure 5d). To confirm that the kinase activity of PKCδ is caspase dependent, we tested the *in vitro* kinase activity after introduction of PKCδ^{D329A}. Although PMA could still activate the *in vitro* kinase activity of the mutated PKCδ^{D329A}, TRAIL failed to activate PKCδ^{D329A} (Figure 5e).

To confirm the involvement of PKCδ in the TRAIL-mediated signaling pathway, we generated a truncation mutant representing the caspase-cleaved form of PKCδ, so-called PKCδ-CF (Figure 6a). We introduced this construct into cells that had been transfected with either control or NOX4-specific siRNA and measured the intracellular ROS levels (Figure 6b). The introduction of PKCδ-CF significantly increased intracellular ROS, and the knockdown of NOX4 abrogated the PKCδ-CF-induced ROS generation. The introduction of PKCδ-CF also significantly suppressed TRAIL-induced cell death in PKCδ-knockdown cells, but not in NOX4-knockdown cells (Figure 6c). These results clearly demonstrate that the catalytic fragment of PKCδ processed by active caspase-3 can increase the intracellular ROS level, subsequently protecting human astrocytoma cells against TRAIL-induced apoptosis, in a NOX4-dependent manner.

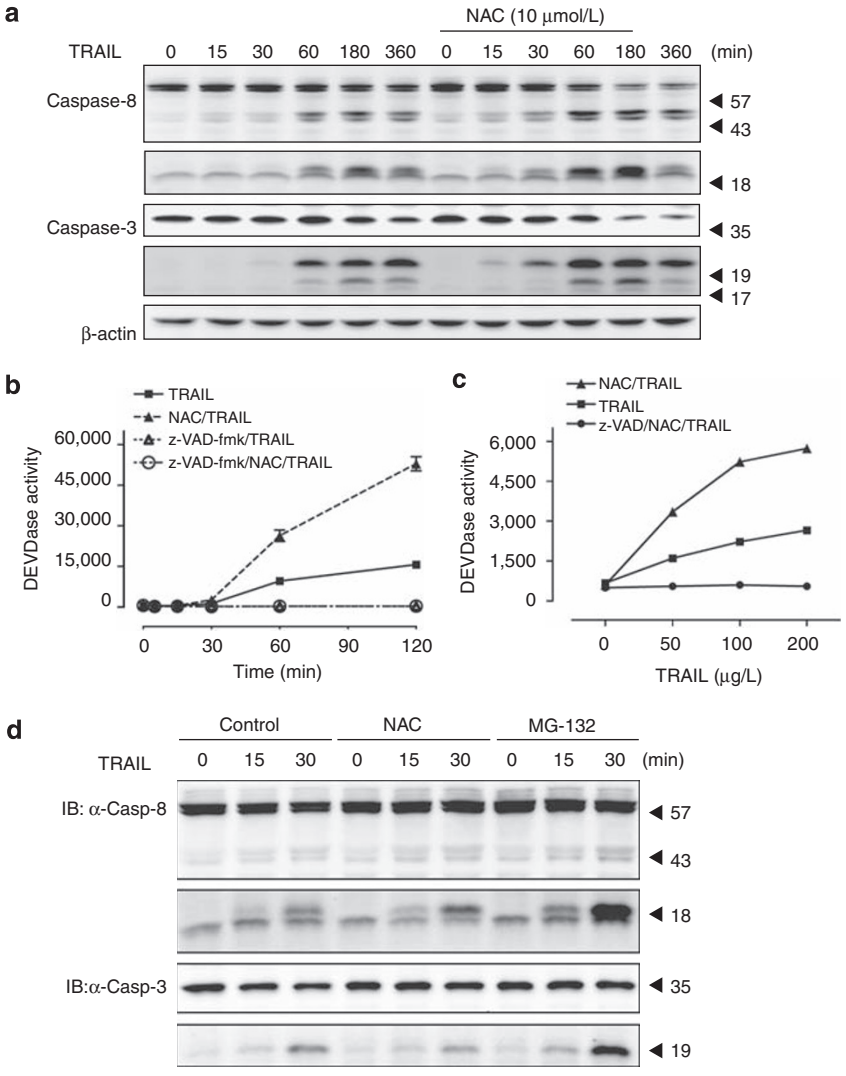

Figure 2 Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated reactive oxygen species (ROS) affect the enzymatic activity of active caspase-3. (**a**) CRT-MG cells were incubated in the absence or presence of N-acetyl cysteine (NAC) for 1 h and treated with human recombinant (hr)TRAIL for varying lengths of time (0–6 h). Soluble lysates (50 μg total protein/aliquot) were analyzed for caspase-8 and caspase-3 levels by immunoblotting. Arrowheads indicate precursors and cleaved fragments of caspase-8 and caspase-3. Data are representative of three independent experiments. (**b**) Cells were incubated in the absence or presence of z-VAD-fmk or NAC for 1 h and then treated with hrTRAIL (100 μg/l) for varying lengths of time (0–2 h). Soluble lysates were assayed for *in vitro* DEVDase activity using a fluorogenic caspase-3 substrate. (**c**) Cells were incubated in the absence or presence of z-VAD-fmk or NAC for 1 h and then treated with various doses of hrTRAIL for 2 h. (**d**) Cells were incubated in the absence or presence of NAC or MG-132 for 1 h and treated with hrTRAIL for various time periods. Cell lysates were subjected to immunoblot analysis for caspase-3 and -8. (**e**) Cells were incubated in the absence or presence of NAC or MG-132 along with z-VAD-fmk for 1 h and treated with hrTRAIL for 3 h. Cell lysates were subjected to immunoblot analysis for poly(ADP-ribose) polymerase (PARP). (**f**) Cells were incubated in the absence or presence of NAC or MG-132 for 1 h and treated with hrTRAIL for an additional 3 h. They were examined for *in vivo* DEVDase activity using a cell-permeable caspase-3 substrate to detect the amount of active caspase-3 after intracellular staining with an FITC-conjugated antibody specific for the cleaved caspase-3 by FACS analysis

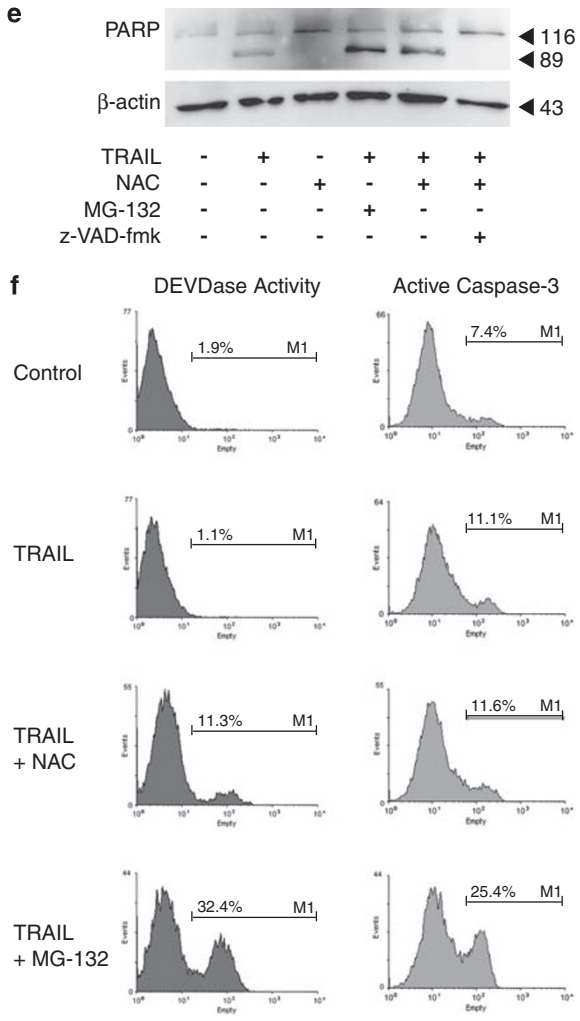

Figure 2 Continued

Discussion

It has been postulated that ROS act as endogenous inhibitors of caspase-dependent apoptosis;[22] however, our results clearly demonstrate that caspase-mediated signal transduction generates ROS, which in turn inhibit receptor-induced apoptosis. Furthermore, the caspase-dependent proteolytic activation of PKCδ and the subsequent activation of NOX4 may be molecular links constituting a new negative-feedback loop against TRAIL-mediated apoptosis (Figure 7).

Although the redox state of a cell is considered to be an important determinant of its susceptibility to various apoptotic stimuli, the mechanism by which the redox state contributes to death receptor-mediated apoptosis remains unclear. Numerous reports have revealed a role for ROS as mediators of the death program, but other reports have shown that oxidative stress inhibits apoptotic signaling[13,23–25] In this study, we have demonstrated that suppression of TRAIL-induced ROS levels sensitizes cells to TRAIL-mediated apoptosis in a caspase-dependent manner, and that this effect occurs before changes in TRAIL-induced apoptosis can be detected. These results imply that ROS can act as anti-apoptotic mediators in TRAIL-mediated signal transduction and can render tumor cells resistant to TRAIL-induced apoptosis, depending on the cellular context. Similarly, growth factor-induced ROS produced by NOX protect pancreatic cancer cells from apoptosis.[26]

Consistent with our *in vitro* result (Supplementary Figure 4) and previous reports[12,27] we have demonstrated that caspases are a major target of intracellular ROS for modulation of apoptotic programs. ROS can affect the signaling pathway by oxidizing reactive cysteine residues in target proteins, thus altering protein–protein interactions and enzymatic function. The proteolytic activity of the caspase family depends on the presence of a catalytic cysteine residue; oxidative modifications of this reduced cysteine would result in the loss of enzymatic activity. The formation of a sulfenic ion in an oxidized reactive cysteine of caspase reduces its enzymatic activity. Moreover, the sulfenic acid (S-OH) formed during oxidative stress can reversibly be reduced back to cysteine thiolate anion ($Cys\text{-}S^-$).[22] Other types of post-translational modification, such as phosphorylation, S-nitrosylation, and ubiquitination, can also regulate activation of caspase. Nitric oxide reversibly inhibits caspase activity through S-nitrosylation of the active site cysteine.[28] Caspases can also be directly phosphorylated by Akt and mitogen-activated protein kinases, resulting in loss of enzymatic activity.[29,30] Recently, it was reported that active caspase-3 and caspase-8 may be critical targets for ubiquitination and subsequent proteasomal degradation.[17,31]

Although the precise molecular mechanisms responsible for receptor-mediated ROS generation are not yet fully understood, NOX seems to be the major source of ligand-induced H_2O_2. It has been proposed that growth factor-induced H_2O_2 production is mediated by the sequential activation of phosphatidylinositol 3-kinase, Rac1, and NOX in non-phagocytic cells.[32] PKC is responsible for the phosphorylation and translocation of cytosolic NOX components to the plasma membrane in phagocytic and non-phagocytic cells and tissues.[19] Several studies have revealed that H_2O_2 production occurs downstream of caspase activation, although the exact molecular mechanisms were not determined.[33] Our results clearly indicate that caspase-8 and -3 are involved in TRAIL-mediated ROS generation. Although our data suggest that the pharmacological inhibition of caspase-3 has a more potent effect on TRAIL-induced ROS generation compared to that of caspase-8, the specificity of caspase inhibitors for a given caspase used in our study is limited.[34] Presumably, the caspase-8 inhibitor did not completely inhibit caspase-8 function, as caspase-3 function leading to ROS generation is dependent on it.

Our results indicate that PKCδ acts as a molecular link between caspases and NOX4. To confirm the specificity of PKCδ in TRAIL-mediated signaling, we first checked the expression levels of the PKC isoforms PKCα, PKCβ, and PKCδ (Supplementary Figure 5). The PKCβ isoform was not expressed in human astrocytoma cells, whereas the expression of PKCα and PKCδ was confirmed by western blot analysis. We further confirmed the specificity of siRNAs against the different isoforms. As expected, transfection with either PKCα- or PKCβ-specific siRNA had no effect on TRAIL-induced cell death (data not shown). PKCδ is a member of a novel PKC family and is activated by caspase-3-mediated

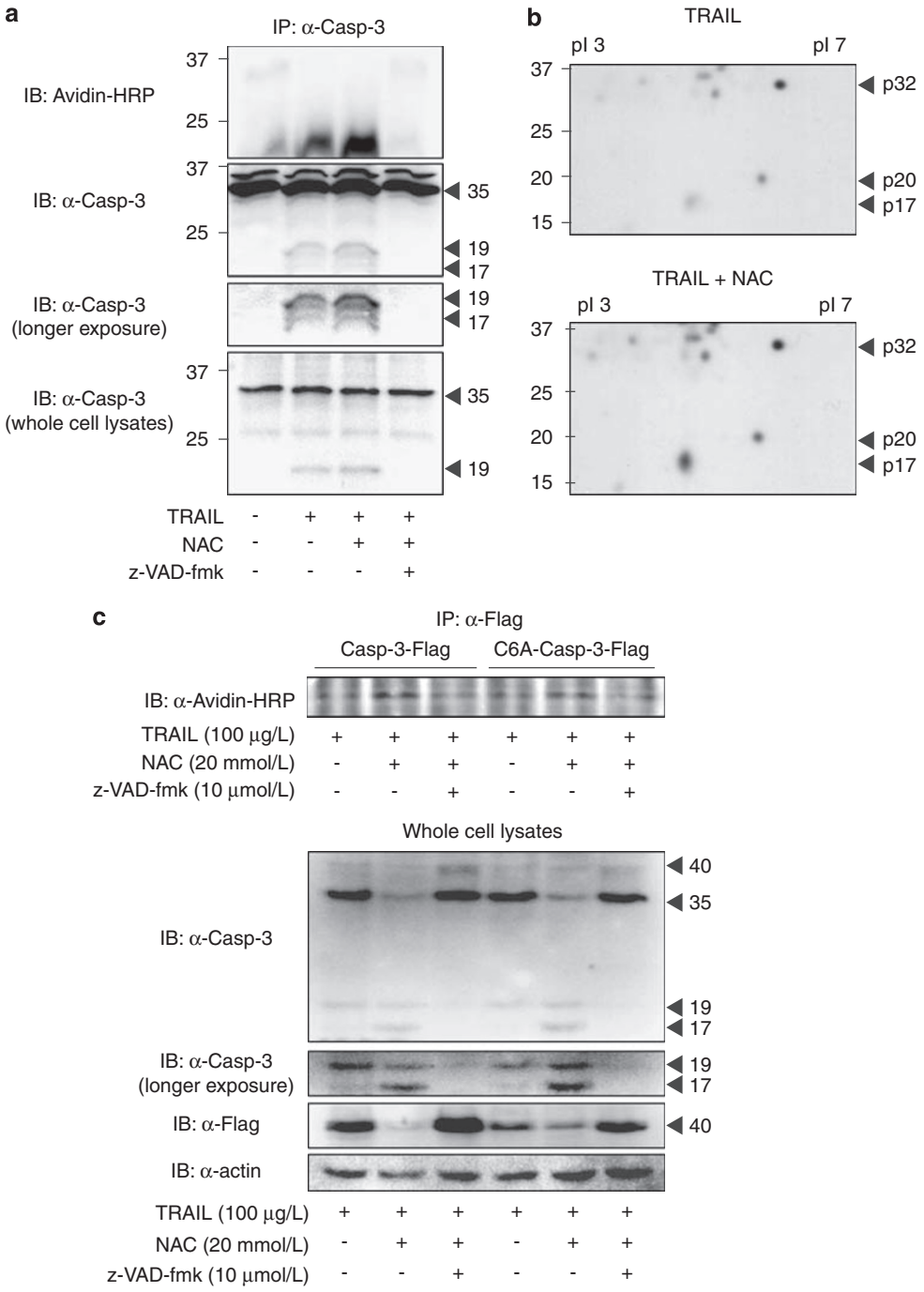

Figure 3 Cleaved fragments of caspase-3 are modified in an oxygen-dependent manner. (**a**) CRT-MG cells were incubated in the absence or presence of z-VAD-fmk or N-acetyl cysteine (NAC) for 1 h and treated with human recombinant tumor necrosis factor (TNF)-related apoptosis-inducing ligand (hrTRAIL) for 1 h. Soluble lysates were labeled with biotin-conjugated iodoacetamide (BIAM) in an acidic buffer (pH 6.5) and immunoprecipitated with an anti-caspase-3 antibody (upper panel). BIAM-labeled active caspase-3 was detected with HRP-conjugated streptavidin and ECL. The immunoprecipitate was also subjected to immunoblot analysis with an anti-caspase-3 antibody. Arrowheads indicate precursor and cleaved fragments of caspase-3. Whole-cell lysates were also examined by immunoblot analysis for caspase-3 (lower panel). (**b**) Cells were incubated in the absence or presence of NAC for 1 h before treatment with human recombinant (hr)TRAIL for 1 h. Soluble lysates were labeled with BIAM, and immunocomplexes precipitated with an anti-caspase-3 antibody were subjected to 2-D gel electrophoresis. Arrowheads indicate precursor and cleaved fragments of caspase-3. (**c**) Cells were transfected with either wild-type caspase-3 or the C6A-caspase-3 mutant. After 24 h, cells were incubated in the absence or presence of NAC or z-VAD-fmk for 1 h and treated with hrTRAIL for an additional 1 h. Soluble lysates were labeled with BIAM and immunoprecipitated with an anti-flag antibody (upper panel). Whole-cell lysates were also examined for caspase, flag-fused protein and β-actin by immunoblot analysis (lower panel)

cleavage at a DMQD/N site in the variable region (V3).[20] PKCδ has been shown to localize to the nucleus or the mitochondria in response to apoptotic stimuli and to act on specific targets for the induction of apoptosis.[35] Recent reports suggest that some PKC isoforms may exert pro-survival effects.[36] Previously, we showed that FasL, another TNF superfamily cytokine, can induce the caspase- and NOX4-dependent generation of intracellular ROS, which can

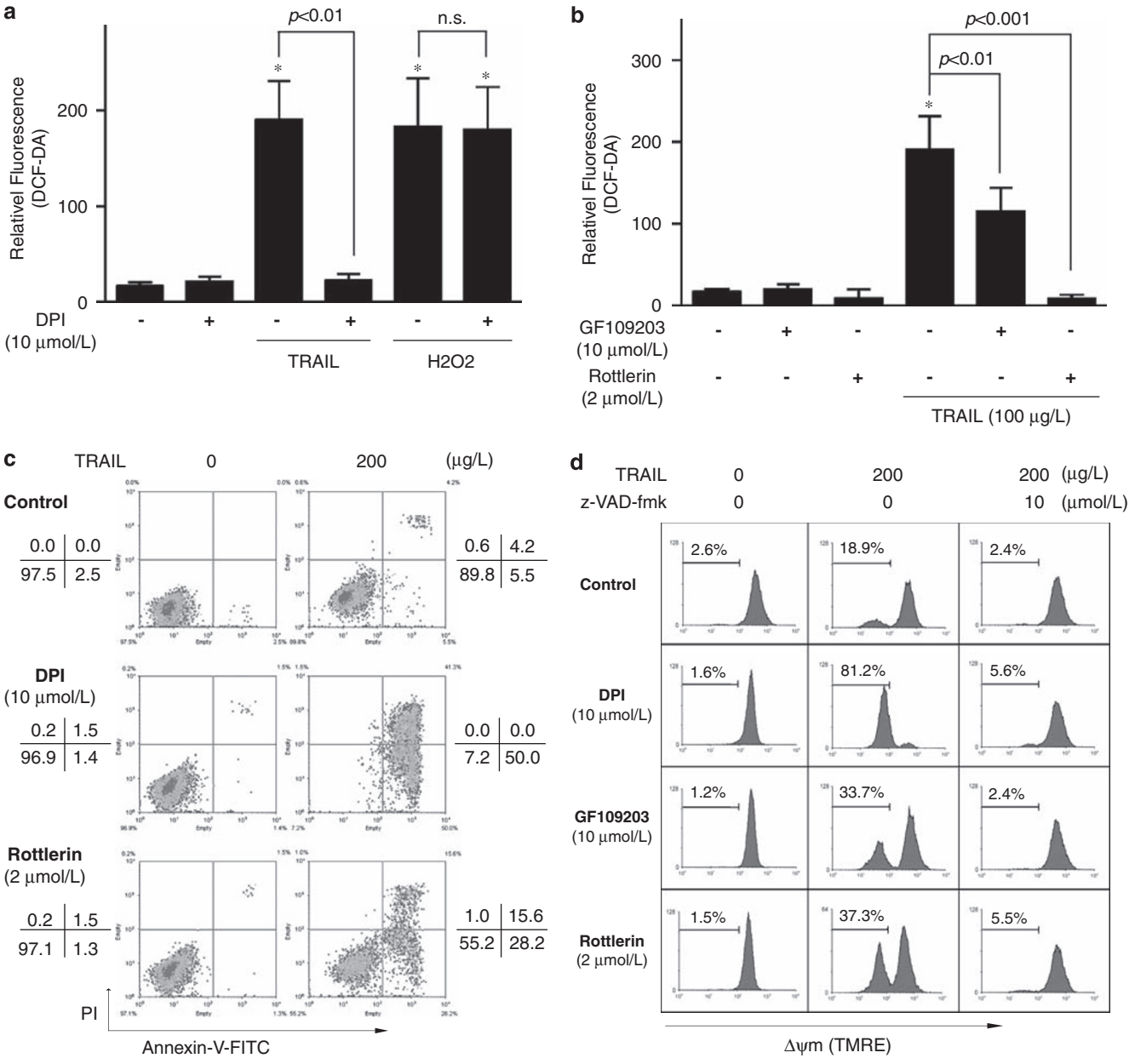

Figure 4 Protein kinase Cδ (PKCδ) and NADPH oxidase 4 (NOX4) mediate tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced reactive oxygen species (ROS) generation and subsequent cell death. (**a**) CRT-MG cells were incubated in the absence or presence of diphenyl iodonium (DPI) (10 μmol/l, 1 h) before treatment with human recombinant (hr)TRAIL (100 μg/l, 30 min) or H_2O_2 (1 mmol/l, 1 min), and intracellular reactive oxygen species (ROS) levels were examined. *$P \leqslant 0.001$ compared with unstimulated samples. (**b**) Cells were incubated in the absence or presence of GF109203 or rottlerin for 1 h before treatment with hrTRAIL for 30 min, and intracellular ROS levels were determined. *$P \leqslant 0.01$ compared with unstimulated samples. (**c**) Cells were incubated in the absence or presence of DPI or rottlerin for 1 h and treated with hrTRAIL for 6 h. Cell death was measured after staining with Annexin V and propidium iodide. (**d**) Cells were incubated in the absence or presence of DPI, GF109203, rottlerin, or z-VAD-fmk for 1 h and treated with hrTRAIL for 4 h. Cell death was determined after staining with TMRE. (**e**) Cells transiently transfected with scrambled small-interfering RNA (siRNA) or PKCδ- or NOX4-specific siRNA were treated with hrTRAIL (100 μg/l, 12 h), and the intracellular ROS levels were measured after staining with 2-7-dichlorofluorescin-diacetate (DCF-DA). The expression of PKCδ and NOX4 was assessed by immunoblot analysis (inset). (**f**) Cells transfected with scrambled siRNA or PKCδ- or NOX4-specific siRNA were examined for TRAIL-induced cell death. (**g**) Cells transfected with scrambled siRNA or PKCδ- or NOX4-specific siRNA were treated with hrTRAIL for 2 h. Cell lysates were examined for *in vitro* caspase-3-like activity. *$P \leqslant 0.01$ compared with scrambled siRNA samples. (**h**) Cells transfected with either PKCδ- or NOX4-specific siRNA were treated in the presence of hrTRAIL along with increasing doses (0–100 μmol/l) of H_2O_2 for 12 h. Cell death was determined by the MTT assay. *$P \leqslant 0.01$ compared with TRAIL-alone treated samples

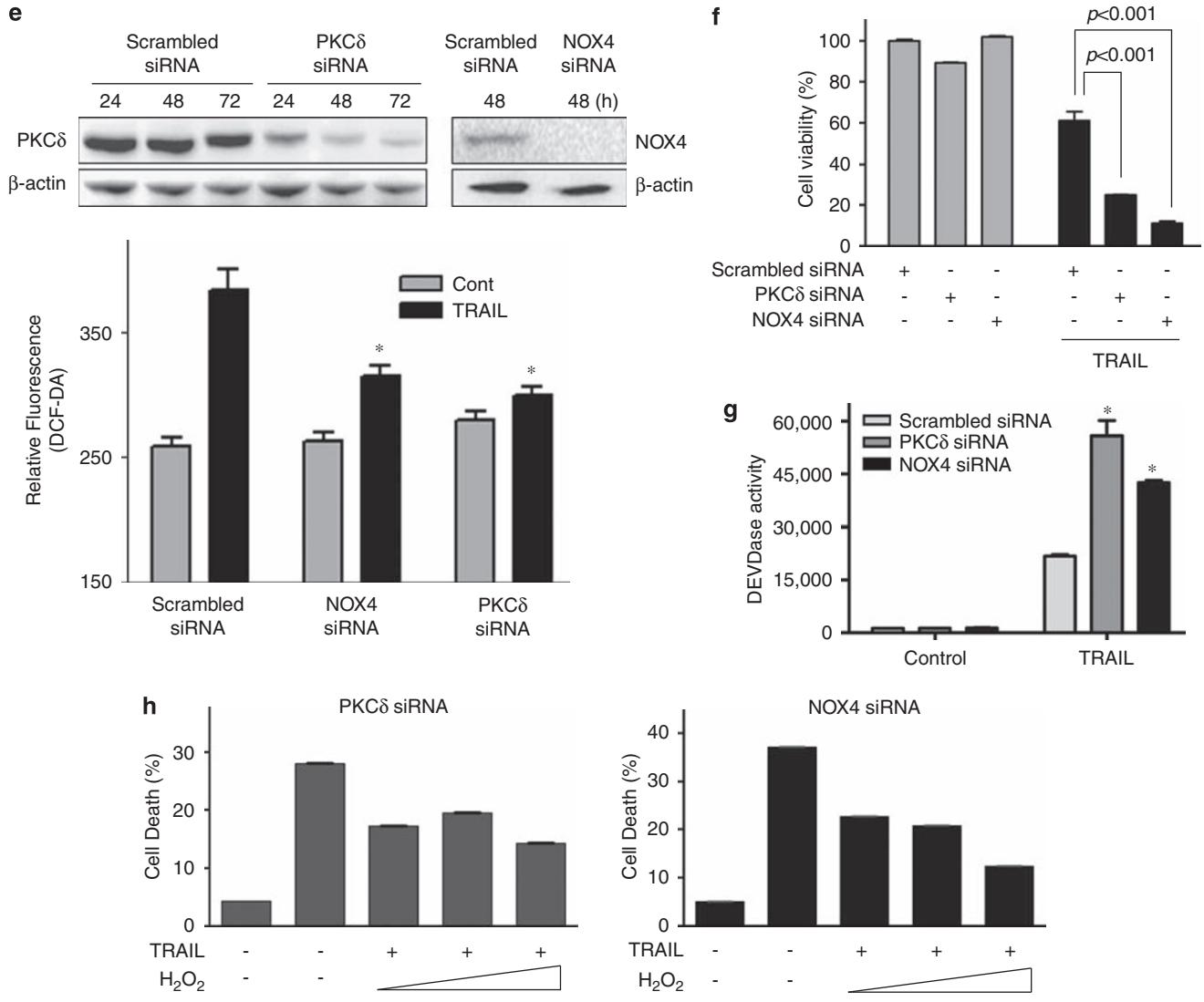

Figure 4 Continued

act as anti-apoptotic mediators.[13,37] We further confirmed that PKCδ is also involved in FasL-mediated signaling (Supplementary Figure 7).

Reactive oxygen species, particularly H_2O_2, have been recognized as second messengers in receptor-mediated signaling processes including proliferation, inflammation, and apoptosis. Although ROS generation has been shown to be critical for receptor-mediated activation of the NF-κB pathway, we did not observe any significant effect of NAC treatment on TRAIL-induced phosphorylation of IKK or on the phosphorylation and subsequent proteasome-dependent degradation of IκB (Supplementary Figure 6a). Furthermore, we showed that the TRAIL-induced expression of two NF-κB-responsive genes, monocytic chemoattractant protein-1 and interleukin-8, was not affected by pretreatment with NAC (Supplementary Figure 6b). Finally, we demonstrated that the expression of XIAP and c-FLIP$_L$, anti-apoptotic NF-κB-responsive proteins, was not affected by NAC pretreatment. These results clearly indicate that TRAIL-induced ROS generation is not involved in receptor-mediated NF-κB activation by human astrocytoma cells.

This study defines the novel molecular mechanism responsible for TRAIL-mediated ROS generation and its functional significance in TRAIL-mediated signal transduction as an anti-apoptotic signal. Receptor-mediated intracellular ROS function as negative regulators in caspase-dependent apoptosis, suggesting a potential therapeutic target for TRAIL-resistant malignant astrocytoma.

Materials and Methods

Cells and reagents. CRT-MG human astrocytoma cells were maintained in RPMI 1640 medium (JBI, Seoul, Korea) with 10 mmol/l HEPES (pH 7.2) (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 mg/l streptomycin, and 10% heat-inactivated fetal bovine serum (Invitrogen) as described previously.[5,38,39] NAC, melatonin, EGCG, DPI, H_2O_2, and PMA were obtained from Sigma (St Louis, MO, USA). Boc-D-fmk, z-VAD-fmk, z-DEVD-fmk, and z-IETD-fmk were purchased from Calbiochem (La Jolla, CA, USA). Human recombinant TRAIL (hrTRAIL) was generously provided by Dr K Kim (Yonsei University College of Medicine, Seoul, Korea), and hrTNF-α was obtained from R&D (Minneapolis, MN, USA). Polyclonal antibody specific for human NOX4 was

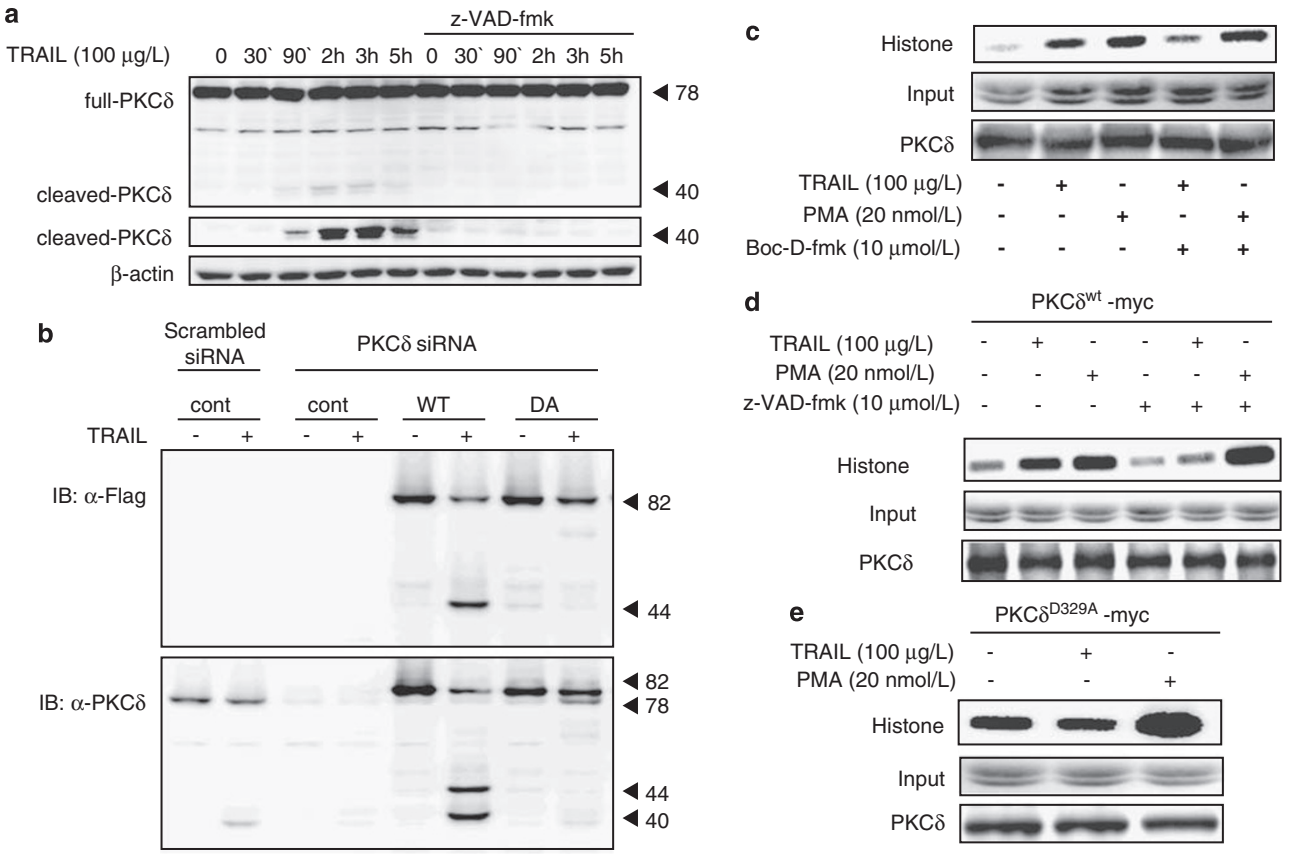

Figure 5 Caspase-dependent proteolytic activation of protein kinase Cδ (PKCδ) participates in tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated reactive oxygen species (ROS) generation and subsequent cell death. (**a**) CRT-MG cells were incubated in the absence or presence of z-VAD-fmk for 1 h and then treated with human recombinant (hr)TRAIL for varying lengths of time (0–5 h). Soluble lysates (50 μg total protein/aliquot) were subjected to immunoblot analysis with an anti-PKCδ antibody. Arrowheads indicate full-length (78 kDa) and cleaved (40 kDa) PKCδ fragments. Data shown are representative of three independent experiments. (**b**) Cells were transfected with small-interfering RNAs (siRNAs) and various siRNA-resistant PKCδ constructs for 48 h and then treated with hrTRAIL for various time periods. Cell lysates were subjected to immunoblot analysis for PKCδ and β-actin. (**c**) Cells were incubated in the absence or presence of Boc-D-fmk for 1 h and then treated with hrTRAIL (100 μg/l) or phorbol 12-myristate 13-acetate (PMA) (20 nmol/l) for 30 min. The *in vitro* kinase activity of PKCδ was assessed for its ability to phosphorylate a histone substrate after immunoprecipitation with an anti-PKCδ antibody. Substrate input is shown (middle panel). (**d**) Cells were transiently transfected with PKCδ^{wt}-myc. After 48 h, cells were incubated in the absence or presence of z-VAD-fmk for 1 h and treated with hrTRAIL or PMA for 30 min. *In vitro* kinase activity of PKCδ was determined after immunoprecipitation with an anti-myc antibody. (**e**) Cells transiently transfected with PKCδ^{D329A}-myc were treated with hrTRAIL or PMA for 30 min, and the *in vitro* phosphorylation of PKCδ was determined. Data shown are representative of three independent experiments

obtained from Abcam (Cambridge, MA, USA). Antibody against human PARP and flag-tagged proteins were obtained from Cell Signaling (Beverly, MA, USA).

Measurement of intracellular ROS levels. To measure intracellular ROS levels, the oxidation-sensitive fluorescent probe 2-7-dichlorofluorescin-diacetate (DCF-DA) (Molecular Probes, Eugene, OR, USA) was used. To study the time course of ROS production, cells were maintained in serum-free media for 16 h and then treated with hrTRAIL (100 μg/l) for varying lengths of time (0–60 min). To investigate the effect of various pharmacological inhibitors on TRAIL-induced ROS production, cells were maintained in serum-free media for 16 h, incubated in the absence or presence of an inhibitor for 1 h, and then treated with hrTRAIL for an additional 45 min. Cells were washed twice with Hank's buffered salt solution and incubated with DCF-DA (2.5 μmol/l) for 10 min. Then, cellular fluorescence was measured using an inverted epifluorescence microscope (Zeiss, Göttingen, Germany).

Plasmids and transfection. Human wild-type PKCδ was cloned into pCMV-3Tag-3A, and a mutation in the caspase cleavage site (pCMV-3Tag-PKC$\delta^{D\rightarrow A329}$) was generated from pCMV-3Tag-PKCδ by PCR site-directed mutagenesis using a Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The primers used were primer 1 (5′-GGAGCAAGCCAACAGTGGGAC-3′) and primer 2 (5′-GTCCCACTGTTGGCTTGCATGTCC-3′). The catalytic fragment of wild-type PKCδ was generated by PCR using full-length pCMV-3Tag-PKCδ as a template, starting at the caspase cleavage site (amino acid residue 378). Transient transfection was carried out using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The transfection efficiency was assessed to be higher than 50% by co-transfection of EGFP vector.

Mutation of six cysteine residues of caspase-3 into alanine residues was carried out by site-directed mutagenesis using a Quick Change site-directed mutagenesis kit (Stratagene). The cysteine-less C6A-caspase-3 mutation was verified by sequencing (Supplementary Figure 2a).

Double-stranded siRNA oligonucleotides were synthesized by Bioneer (Daejeon, Korea) as follows: NOX4, 5′-AAACCGGCAGAGTTTACCCAG-3′-dTdT; PKCδ, 5′-CCAUGAGUUUAUCGCCACC-3′-dTdT; scrambled (negative control), 5′-CCUACGCCAAUUUCGU-3′-dTdT). Transient transfection with siRNA was carried out using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was assessed by co-transfection of a FAM-labeled negative-control siRNA. The 'siRNA-resistant' PKCδ constructs (wild type and caspase-resistant DA mutant) were generated by introducing a silent mutation in the 3′ nucleotide of a codon in the middle of the siRNA binding site using a Quick change site-directed mutagenesis kit (Stratagene). The primers used were primer 1 (5′-CGTCGCTATGAACTCATGGTTCTTGATGTAG-3′) and primer 2 (5′-CATAGCGACGTTCTTTGGGCAACCCACCTTC-3′).

Immunoblotting and *in vitro* PKCδ kinase assay. Immunoblot analysis for caspase cleavage was carried out as previously described.[17] Cell lysates (50 μg)

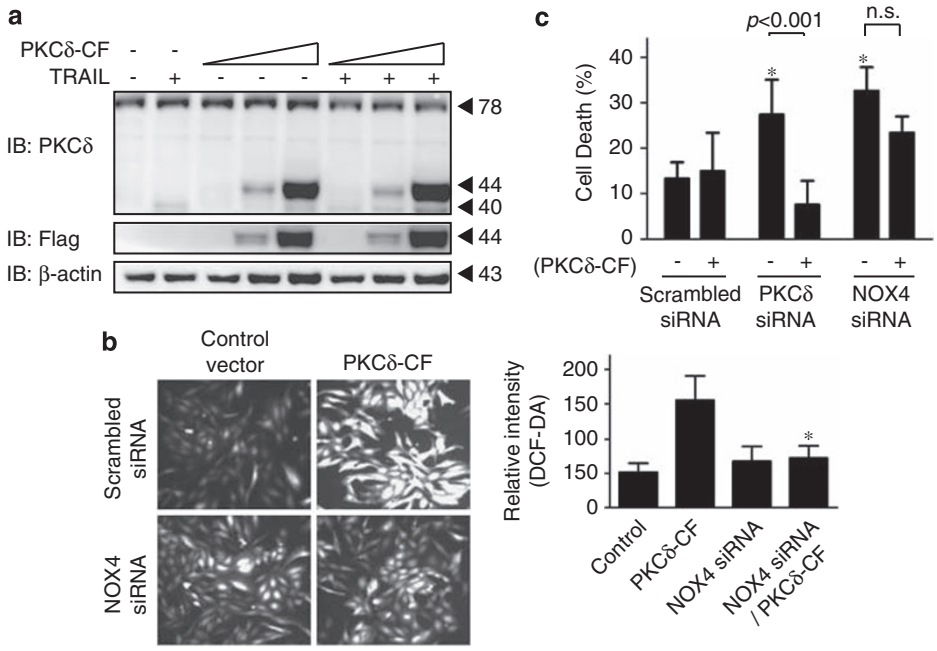

Figure 6 Caspase-cleaved catalytic fragment of protein kinase Cδ (PKCδ) is critical for tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated reactive oxygen species (ROS) generation and subsequent cell death in NADPH oxidase 4 (NOX4)-dependent manner. (**a**) Cells were transfected with various doses (up to 1 μg of DNA) of PKCδ-CF construct for 24 h and treated with human recombinant (hr)TRAIL for 2 h. Cell lysates were subjected to immunoblot analysis using antibodies against PKCδ, flag, and β-actin. (**b**) Cells were transfected with either scrambled or NOX4-specific small-interfering RNA (siRNA) for 24 h and then transfected with PKCδ-CF (0.5 μg of DNA) for an additional 24 h. *$P \leqslant 0.001$ compared with PKCδ-CF/scrambled siRNA-transfected samples. (**c**) Cells were transiently transfected with various siRNAs for 24 h, further transfected with PKCδ-CF for an additional 24 h, and treated with hrTRAIL (100 μg/l) for 12 h. Cell death was measured using the MTT assay. The data shown are representative of three independent experiments

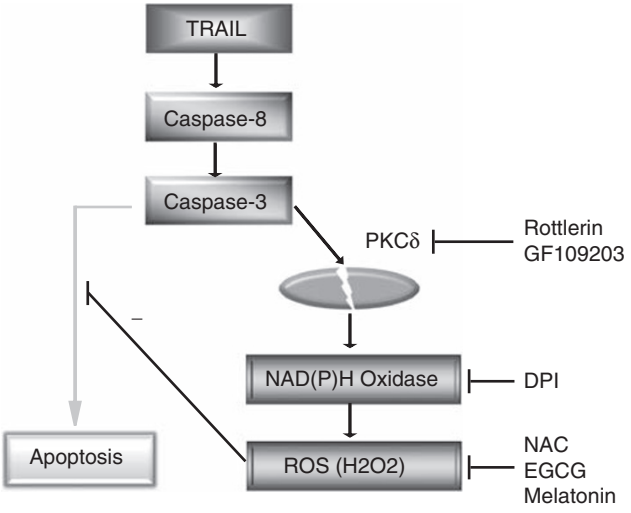

Figure 7 Proposed model for the role of the protein kinase Cδ (PKCδ)–NADPH oxidase 4 (NOX4)–reactive oxygen species (ROS) pathway on tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis

were separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies specific for human caspase-3 or caspase-8 (Cell Signaling). The blots were developed using chemiluminescence (AbFrontiers, Suwon, Korea).

Soluble lysates (200–500 μg) were incubated with 1 μg of anti-PKCδ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-myc antibody (AbFrontiers) for 12 h at 4 °C, followed by an additional 3-h incubation with Protein A/G beads (Pierce, Rockford, IL, USA). The immunocomplexes were incubated in 20 μl of kinase reaction buffer containing 2 μg of histone and 5.0 μCi of [γ-^{32}P]ATP for 60 min at 30 °C. Histone phosphorylation was stopped by boiling in Laemmli sample buffer, and samples were analyzed by 12% SDS-PAGE and autoradiography.

Measurement of cell death. Cell death was determined by staining with Annexin V (PharMingen, San Diego, CA, USA), a 35.8-kDa protein that has a strong affinity for phosphatidylserine. After treatment with hrTRAIL, cells were washed twice with phosphate-buffered saline, trypsinized, suspended in 200 μl of binding buffer, and stained with 0.5 ng of Annexin V-FITC and 2.5 ng of propidium iodide as previously described.[38] Within 30 min of staining, 1×10^4 cells were analyzed on a FACStar (Becton Dickinson, Mountain View, CA, USA). Cell death, including apoptosis and necrosis, was detected by staining with Annexin V and/or propidium iodide. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was also used to determine cell viability, as described previously.[13]

DEVDase activity measurement. Aliquots of the soluble lysate containing 20 μg total protein were incubated with 5 μg of the caspase-3/7 substrate Ac-DEVD-AMC (Calbiochem) in reaction buffer (100 mmol/l NaCl, 50 mmol/l HEPES, 10 mmol/l DTT, 1 mmol/l EDTA and 10 % glycerol) for 2 h at 37 °C. Sample fluorescence was determined using a plate reader (Labtech, Lewes, UK) with excitation and emission wavelengths of 360 nm and 460 nm, respectively.

Cells were incubated in the absence or presence of NAC for 1 h, and then treated with or without hrTRAIL for 2 h. Intracellular caspase-3/7-like activity was determined using a caspase-3 intracellular activity assay kit II (Calbiochem) according to the manufacturer's instructions. Intracellular fluorescence was measured using flow cytometry.

BIAM-labeling and 2-D gel electrophoresis. Cells were homogenized with lysis buffer containing 50 mmol/l of N-(biotinoyl)-N′-(iodoacetyl)ethylenediamine (BIAM) (Molecular Probes). Cell lysates were incubated for 1 h at room temperature with a nitrogen gas flush. The labeling reaction was terminated by the addition of β-mercaptoethanol to a final concentration of 50 mmol/l. The reaction mixture was centrifuged at 40 000 r.p.m. for 1 h at 4 °C and immunoprecipitated with an antibody specific for active caspase-3 (Santa Cruz Biotechnology). The BIAM-labeled immunocomplexes were subjected to two-dimensional (2-D) gel electrophoresis and detected with HRP-conjugated streptavidin using an enhanced chemiluminescence (ECL) system (AbFrontiers).

Statistical analysis. The data are presented as means ± standard deviation (S.D.). Levels of significance for comparisons between samples were determined by one-way analysis of variance using InStat software (GraphPad Software, San Diego, CA, USA).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)