Reactive Oxygen Species Regulate Caspase Activation in Tumor Necrosis Factor–Related Apoptosis–Inducing Ligand–Resistant Human Colon Carcinoma Cell Lines


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Abstract

The effects of reactive oxygen species (ROS) on tumor necrosis factor–related apoptosis–inducing ligand (TRAIL)–induced apoptosis in solid cancers have yet to be clearly defined. In this study, we found that the classic uncoupler of oxidative phosphorylation, carbonyl cyanide m-chlorophenylhydrazone (CCCP), induced a reduction in \( \Delta \Psi_m \) and generation of ROS. This uncoupling effect enhanced TRAIL-induced apoptosis in TRAIL-resistant human colon carcinoma cell lines (RKO, HT29, and HCT8). Sensitization was inhibited by benzoxycarbonyl-valine-alanine-aspartate fluoromethylketone, indicating the requirement for caspase activation. CCCP per se did not induce apoptosis or release of proapoptotic factors from mitochondria. Generation of ROS by CCCP was responsible for TRAIL-induced Bax and caspase activation because scavenging ROS completely abrogated apical caspase-8 activation and further downstream events leading to cell death. Oxygen consumption by Bel-2 did not prevent the initial loss of \( \Delta \Psi_m \) and ROS generation following CCCP treatment, but did prevent cell death following TRAIL and CCCP exposure. Uncoupling of mitochondria also facilitated TRAIL-induced release of proapoptotic factors. X-linked inhibitor of apoptosis overexpression abrogated TRAIL-induced apoptosis in the presence of CCCP and decreased initiator procaspase-8 processing, indicating that additional processing of caspase-8 required initiation of a mitochondrial amplification loop via effector caspases. Of interest, depletion of caspase-9 in RKO cells did not protect cells from TRAIL/CCCP-induced apoptosis, indicating that apoptosis occurred via a caspase-9–independent pathway. Data suggest that in the presence of mitochondrial-derived ROS, TRAIL induced mitochondrial release of Smac/DIABLO and inactivation of X-linked inhibitor of apoptosis through caspase-9–independent activation of caspase 3.

Introduction

Tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) engages apoptosis via recruitment and rapid activation of caspase 8 (1). Activated caspase 8 subsequently initiates the activation of effector caspases including caspases 3, 6, and 7 (2). In type II cells, effector caspase activation requires amplification of death-inducing signaling complex signals by engagement of the mitochondrial intrinsic pathway. A critical step in the intrinsic pathway is the activation of Bax, leading to dissipation of the mitochondrial transmembrane potential (\( \Delta \Psi_m \)) and release of cytochrome c into the cytosol. Cytochrome c and apoptotic protease-activating factor-1 (Apaf-1), in the presence of ATP or dATP, are required for the cleavage of caspase 9 and subsequently the effector caspases (3). Once cleaved by caspase 8 during treatment with TRAIL, Bid translocates to the mitochondria and activates Bax, thus providing a link between the extrinsic and intrinsic apoptotic pathways (4, 5).

Reactive oxygen species (ROS) are known to induce a wide range of responses dependent on cell type and the levels of ROS within the cell (6, 7). High levels of ROS can lead to necrotic cell death, whereas low levels of ROS have been shown to induce apoptotic cell death (6, 7). Alteration in mitochondrial function can affect the response of tumor cells to apoptosis mediated by death receptors. Increase in mitochondrial respiration sensitized leukemic cells to tumor necrosis factor–induced apoptosis (8). Depletion of mitochondrial DNA can make tumor cells resistant to TRAIL-induced apoptosis (9). The uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) can enhance Fas-induced cell death, although CCCP alone does not have an apoptotic effect (10). Bcl-2 inhibitors also sensitize leukemic CEM cells to TRAIL-induced apoptosis by uncoupling of mitochondrial respiration (11). Recently, we have shown in human colon carcinoma cell lines that rottlerin, a mitochondrial uncoupler, induced a significant loss in \( \Delta \Psi_m \) and accelerated the onset of TRAIL-induced apoptosis in TRAIL-resistant human colon carcinoma cell lines (12). However, the precise mechanism by which the mitochondrial function contributes to death receptor–mediated apoptosis is still unclear.

In the present study, we have shown that CCCP sensitizes human colon carcinoma cell lines to TRAIL-induced apoptosis by enhancing caspase-8 and Bax activation, leading to the release of cytochrome c and Smac/DIABLO into the cytosol and degradation of X-linked inhibitor of apoptosis (XIAP). Data show that Bel-2, in addition to inhibiting these effects as well as cell death, also regulates apical processing of caspase 8. Similar data were observed in RKO cells overexpressing XIAP, indicating that in the presence of ROS, TRAIL-induced full processing of caspase 8 required a mitochondrial amplification loop involving downstream effector caspases. Our findings also provide direct evidence of TRAIL-induced caspase-3 activation in caspase-9–depleted RKO cells.

Materials and Methods

CCCP and 2,4-dinitrophenol were purchased from Sigma Chemical Co. (St. Louis, MO). N-Acetyl-cysteine, 2-dihydroethidium, and JC-1 dye were from Molecular Probes (Eugene, OR). For caspase inhibition or assay, benzoxycarbonyl-valine-alanine-aspartate fluoromethylketone (zVAD-fmk) from Enzyme Systems Products (Livermore, CA) was employed. The fluorogenic substrate N-acetyl-Leu-Glu-His-Asp-7-amino-4-methyl...
cumarin (Ac-LEHD-AMC; Alexis Biochemicals) was prepared as a 20 mmol/L stock in DMSO.

**Cell lines.** HT29 and HCT8 human colon carcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA). RKO was obtained from Dr. Michael Kastan of St. Jude Children’s Research Hospital (Memphis, TN). All cells were maintained in RPMI 1640 (Gibco, Carlsbad, CA) supplemented with 2 mmol/L glutamine and 10% FCS.

Production of recombinant human tumor necrosis factor–related apoptosis–inducing ligand. TRAIL was produced according to published procedures (13).

**Apoptosis assays.** Cells were plated at a density of 200,000 cells/well in 12-well plates, and after overnight attachment were treated with TRAIL (10-50 ng/mL) either in the absence or presence of CCCP (20 μmol/L) for up to 24 hours. Apoptotic cells were determined by Annexin V-propidium iodide staining. Cells were stained with 10 μL of Annexin V-antigen presenting cells (Becton Dickinson and Co., San Jose, CA) and 10 μL of propidium iodide (50 ng/mL) according to the instructions of the manufacturer, incubated for 15 minutes at room temperature in the dark, and immediately analyzed by flow cytometry. Alternatively apoptotic cells were detected as a sub-G1 (hypodiploid nuclei) fraction after propidium iodide staining and analysis using a Becton Dickinson FACScan (14). Cells were also pretreated with the caspase inhibitor zVAD-fmk (50 μmol/L) for 1 hour before TRAIL treatment.

**Caspase assays.** Before making the protein extract, floating cells were collected and combined with cells growing on the dish and washed twice with PBS. Cell lysates were prepared in caspase lysis buffer [25 mmol/L HEPES-NaOH (pH 7.4), 0.1% sucrose, 1% CHAPS, 2 mmol/L EDTA, 10 mmol/L DTT] and subsequently mixed with caspase assay buffer [25 mmol/L HEPES-NaOH (pH 7.4), 10 mmol/L DTT, and Ac-LEHD-AMC (50 μmol/L; caspase 9) or N-acetyl-Asp-Val-Asp-7-amino-4-methyl cumarin (Ac-DEVd-AMC; 50 μmol/L; caspase 3)]. After incubation at 37°C for 1 hour, the fluorometric detection of the cleaved AMC product was done on a CytoFluor Multifluid plate Reader series 2350 (Millipore) using a 400 nm excitation filter and a 530 nm emission filter.

**Western blot analysis.** Western blot analyses were carried out as described (15). Primary antibodies for the detection of caspase 8, caspase 9, Smac/DIABLO, and XIAP were from MBL (Woburn, MA), and for caspase 3, Bid, and poly(ADP-ribose) polymerase, from BD PharMingen (San Jose, CA). The cytochrome c monoclonal antibody was purchased from Clontech (San Jose, CA). Recognized proteins were detected using horseradish peroxidase–labeled secondary antibodies (Amersham Biosciences, Piscataway, NJ).

**Plasmid vectors and transfection.** The retroviral expression vector pMSCV-Bcl-2 (expressing human Bcl-2 and green fluorescent protein [GFP], separated by an internal ribosome entry site [IRES] sequence) was kindly provided by Dr. John Cleveland (St. Jude Children’s Research Hospital, Memphis, TN). A retroviral construct encoding human XIAP was prepared in Chaps buffer containing protease inhibitors, and total proteins of oxidative phosphorylation.

**Apoptosis–inducing ligand–induced apoptosis by uncouplers of oxidative phosphorylation.** To explore the effects of uncouplers of oxidative phosphorylation on tumor cell response to TRAIL, the effects of CCCP on TRAIL-induced cell death were analyzed in human colon carcinoma (HT29, RKO, and HCT8) cell lines. Tumor cells were pretreated with CCCP (20 μmol/L) for 1 hour before TRAIL, and apoptosis was determined 24 hours after treatment. As shown in Fig. 1, all cell lines examined were sensitized to TRAIL (30 ng/mL) in the presence of CCCP, with the percentage of cells undergoing apoptosis increasing to >80%. In contrast, culturing these cells with either TRAIL or CCCP alone had little effect. The broad-spectrum caspase inhibitor, zVAD-fmk, potently suppressed TRAIL-induced cell death in all three cell lines (Fig. 1A), suggesting that caspases remain critical for TRAIL-induced killing when mitochondrial respiration is uncoupled. The effect of a second inhibitor of oxidative phosphorylation, 2,4-dinitrophenol, on TRAIL-induced apoptosis was also examined. Data showed that 2,4-dinitrophenol enhanced TRAIL-induced cell death in RKO cells (Fig. 1B).

**Results**

Tumor cells are sensitized to tumor necrosis factor–related apoptosis–inducing ligand–induced apoptosis by uncouplers of oxidative phosphorylation.

**Evaluation of mitochondrial transmembrane potential.** Mitochondrial energization was determined by retention of JC-1 dye (17). Briefly, 2 × 10^6 cells were loaded with JC-1 dye (1 μg/mL) for 30 minutes at 37°C before the reaction was terminated. Cells were washed twice in PBS. Fluorescence was measured using a cytometer (λ excitation maximum 570 nm, λ emission maximum 595 nm; Becton Dickinson, Mountain View, CA).

**Cellular fractionation.** RKO cells were lysed in mitochondria lysis buffer (ApoAlert kit, Clontech) in a Dounce homogenizer and subjected to centrifugation at 700 × g to pellet nuclei. The post nuclear supernatant was centrifuged at 10,000 × g to pellet the mitochondria-enriched heavy membrane fraction, and the resulting supernatant was further centrifuged at 100,000 × g to obtain the cytosolic fraction. Total proteins (15 μg) were subjected to Western blot analysis.

**Immunoprecipitation of active Bax.** The detection of conformationally changed Bax has been described previously (18, 19). Briefly, cell lysates were prepared in Chaps buffer containing protease inhibitors, and total proteins (500 μg) were incubated with anti-Bax 6A7 antibody (2 μg; BD PharMingen) for 2 hours at 4°C, followed by addition of protein G agarose (20 μL) to precipitate the conformationally altered Bax protein. After extensive washing, the resulting immune complexes were subjected to Western blot analysis with anti-Bax (BD PharMingen) polyclonal antibody.

**Determination of reactive oxygen species.** Time course experiments were done to compare ROS production in RKO cells after the various different treatments. ROS production was detected using 2′,7′-dihydroethidium (10 μmol/L). Cells were incubated for 30 minutes at 37°C before the reaction was terminated. Cells were subsequently washed twice in PBS and analyzed by flow cytometry.

Results

Tumor cells are sensitized to tumor necrosis factor–related apoptosis–inducing ligand–induced apoptosis by uncouplers of oxidative phosphorylation. To explore the effects of uncouplers of oxidative phosphorylation on tumor cell response to TRAIL, the effects of CCCP on TRAIL-induced cell death were analyzed in human colon carcinoma (HT29, RKO, and HCT8) cell lines. Tumor cells were pretreated with CCCP (20 μmol/L) for 1 hour before TRAIL, and apoptosis was determined 24 hours after treatment. As shown in Fig. 1, all cell lines examined were sensitized to TRAIL (30 ng/mL) in the presence of CCCP, with the percentage of cells undergoing apoptosis increasing to >80%. In contrast, culturing these cells with either TRAIL or CCCP alone had little effect. The broad-spectrum caspase inhibitor, zVAD-fmk, potently suppressed TRAIL-induced cell death in all three cell lines (Fig. 1A), suggesting that caspases remain critical for TRAIL-induced killing when mitochondrial respiration is uncoupled. The effect of a second inhibitor of oxidative phosphorylation, 2,4-dinitrophenol, on TRAIL-induced apoptosis was also examined. Data showed that 2,4-dinitrophenol enhanced TRAIL-induced cell death in RKO cells (Fig. 1B).

Enhancement of tumor necrosis factor–related apoptosis–inducing ligand–induced caspase activation. To gain insight into the mechanism by which the apoptotic process is induced, activation of the receptor-proximal caspase 8 was determined as one of the earliest events after receptor aggregation. Treatment of cells with TRAIL alone induced processing of caspase 8, Bid, and caspase 9 at 30 minutes, but at 3 hours these proteins were no

longer cleaved, suggesting that TRAIL receptor signaling may be transient in RKO cells, thereby terminating the formation of active initiator caspases. Similar data were observed in TRAIL-resistant JR1 cells (20). Pretreatment with CCCP enhanced processing of caspase 8 by 1 hour after TRAIL stimulation, thereby preceding the onset of detectable apoptosis [poly(ADP-ribose) polymerase (PARP) cleavage; Fig. 1C]. Caspase-8 processing was sustained during the period of time examined and was followed by processing of the effector caspase 3 and PARP. Cleavage of Bid and caspase 9 in TRAIL/CCCP-treated cells suggests involvement of the mitochondrial loop in this process. Treatment of cells with CCCP alone had no effects on these proteins (Fig. 1C). Despite cleavage of caspase 8, Bid, and caspase 9, PARP, an apoptotic protease downstream of caspase 3, was not cleaved in cells treated with TRAIL alone. It has been shown that apoptotic stimuli initiate Bax conformational change and translocation to mitochondria where active Bax causes cytochrome c release and subsequent downstream caspase activation (21). To examine Bax activation after TRAIL treatment in the presence of CCCP, immunoprecipitation experiments were done with anti-Bax 6A7 antibody that recognizes only the conformationally changed Bax protein. As shown in Fig. 1C, TRAIL-induced Bax activation was weakly detected after 1 hour of treatment and increased after 2 to 3 hours, probably as a consequence of early caspase-8 activation. In cells treated with the combination of TRAIL and CCCP, dramatic activation of Bax was observed 2 to 3 hours after treatment (Fig. 1C). In RKO cells treated with CCCP alone, only slight activation of Bax was detected.

Carbonyl cyanide m-chlorophenylhydrazone–induced loss in ΔΨm and reactive oxygen species production. To elucidate whether CCCP may initiate a loss in ΔΨm in human colon carcinoma cell lines, and to further examine the mechanism by which CCCP sensitizes colon carcinoma cells to TRAIL-induced apoptosis, RKO cells were treated with CCCP (20 μmol/L) for 1 hour before treatment with TRAIL (50 ng/mL) for up to 24 hours. Apoptosis was determined as described in Materials and Methods. B, zVAD-fmk (50 μmol/L, coincubation) completely abolished TRAIL-induced apoptosis in CCCP-treated cells. Columns, mean of two determinations per point; bars, SD. C, CCCP enhances TRAIL-induced activation of caspases. RKO cells were treated with CCCP (20 μmol/L) 1 hour before and during TRAIL stimulation. After the time points indicated, lysates were prepared and analyzed by SDS-PAGE using specific antibodies as described in Materials and Methods. Bax immunoprecipitation was carried out as described in Materials and Methods.

**Figure 1.** CCCP sensitizes colon carcinoma cells to TRAIL-induced apoptosis. A, cells were pretreated with CCCP (20 μmol/L) for 1 hour before treatment with TRAIL (50 ng/mL) for up to 24 hours. Apoptosis was determined as described in Materials and Methods. B, zVAD-fmk (50 μmol/L, coincubation) completely abolished TRAIL-induced apoptosis in CCCP-treated cells. Columns, mean of two determinations per point; bars, SD. C, CCCP enhances TRAIL-induced activation of caspases. RKO cells were treated with CCCP (20 μmol/L) 1 hour before and during TRAIL stimulation. After the time points indicated, lysates were prepared and analyzed by SDS-PAGE using specific antibodies as described in Materials and Methods. Bax immunoprecipitation was carried out as described in Materials and Methods.

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![Figure 1](https://example.com/fig1.png)
caspase dependent. Generation of ROS was also examined by flow cytometry using 2-dihydroethidium. Treatment of RKO cells with CCCP initiated a caspase-independent ROS generation (Fig. 2B). TRAIL alone did not induce production of ROS, but in combination with CCCP, abrogated ROS production, which corresponded to the further collapse in mitochondrial function observed in TRAIL-treated RKO cells in the presence of CCCP (Fig. 2A). The presence of zVAD-fmk completely restored ROS production and abrogated cell death in RKO cells treated with the combination TRAIL and CCCP (Fig. 2B and C).

**Effect of Bcl-2 on tumor necrosis factor–related apoptosis–inducing ligand–induced apoptosis when mitochondrial respiration is uncoupled.**

The role of the cellular redox state during apoptosis was examined, particularly the role of ROS in regulating TRAIL-induced apoptosis in RKO cells. To elucidate the role of ROS in TRAIL-induced apoptosis in the presence of CCCP, we sensitized tumor cells to TRAIL by generating a mitochondrial redox state that could not block ROS generation following CCCP treatment, but protected mitochondria from further collapse, as judged by sustained ROS generation after TRAIL treatment in the presence of CCCP (Fig. 3C). These results rule out the possibility that Bcl-2 may express antioxidant activity following the generation of ROS, and also indicate that the basal redox state (2-dihydroethidium fluorescence) of these cells is comparable to control GFP cells and unaffected by the overexpression of Bcl-2.

To elucidate the role of ROS in TRAIL-induced apoptosis in the presence of CCCP, RKO cells were incubated with antioxidant N-acetyl-cysteine for 1 hour before CCCP treatment for 1 or 24 hours and subsequently treated with TRAIL for 24 hours. As seen in Fig. 3D, N-acetyl-cysteine completely blocked ROS generation following CCCP treatment and prevented TRAIL-induced apoptosis in the presence of CCCP. Collectively, these data suggest that ROS regulate TRAIL-induced apoptosis in RKO cells.
activation following treatment of cells with the combination of TRAIL and CCCP. Because the synergistic effect of CCCP on TRAIL-induced apoptosis was ROS dependent, we sought to determine the effects of ROS on proximal and effector caspase cleavage in TRAIL-treated RKO cells. Pretreatment with CCCP enhanced cleavage of caspase 8 at 5 hours after TRAIL treatment. Caspase-8 cleavage was followed by the cleavage of Bid, caspase 9, caspase 3, caspase 6, and PARP. Treatment of cells with TRAIL or CCCP alone had no effect on caspase cleavage. Pretreatment of cells with N-acetyl-cysteine completely abrogated the cleavage of

Figure 3. Bcl-2 prevents cell death in response to TRAIL in the presence of an uncoupler. RKO cells were stably transfected with a control vector (GFP) or Bcl-2 (GFP/Bcl-2), and transfected cells were sorted for GFP expression by FACS analysis. A, cells were pretreated with CCCP (20 μmol/L) for 1 hour and then incubated with TRAIL (50 ng/mL) for 24 hours. Apoptosis was determined as described in Materials and Methods following 24 hours of TRAIL treatment. B, effect of Bcl-2 overexpression on caspase-3 activity. Cells were pretreated with CCCP (20 μmol/L) for 1 hour and then incubated with TRAIL (50 ng/mL) for 3 hours, and lysates were subsequently assayed for caspase-3 activity, as described under Materials and Methods. C, ΔΨm collapse and ROS generation. Cells were pretreated with CCCP (20 μmol/L) for 1 hour and then incubated with TRAIL (50 ng/mL) for the indicated times. After treatment, cells were stained with dihydroethidium for ROS generation or with JC-1 for ΔΨm and analyzed by flow cytometry. In each case, ΔΨm and ROS were expressed as the mean fluorescence intensity. D, antioxidant N-acetyl-cysteine prevents CCCP-induced ROS generation and cell death following TRAIL treatment. Cells were pretreated with N-acetyl-cysteine (10 mmol/L) for 2 hours and subsequently incubated with CCCP (20 μmol/L), and ROS generation was monitored 1 and 24 hours following CCCP treatment. Cells were pretreated with N-acetyl-cysteine (10 mmol/L) for 2 hours then incubated with CCCP (20 μmol/L) for 1 hour. Apoptosis was determined 24 hours following TRAIL exposure as described in Materials and Methods. Columns, mean of two determinations per point; bars, SD. Representative of three independent experiments.
proximal caspase 8, as well as downstream effectors. The presence of N-acetyl-cysteine also abrogated TRAIL-induced cleavage of PARP in the presence of CCCP (Fig. 4A). These data suggest that ROS regulate a critical caspase activity in TRAIL-induced apoptosis when mitochondrial respiration is uncoupled. Immunoprecipitation experiments were subsequently done with anti-Bax 6A7 antibody that recognizes only the conformationally changed Bax protein. As shown in Fig. 4A, in cells treated with the combination of TRAIL and CCCP, Bax underwent a dramatic conformational change at 5 hours, which was completely inhibited by the presence of N-acetyl-cysteine, suggesting that the increased Bax activation in RKO cells treated with the combination of TRAIL and CCCP was ROS mediated (Fig. 4A).

Bcl-2 prevents caspase processing and Bax activation in tumor necrosis factor–related apoptosis–inducing ligand–stimulated cells. To probe the mechanism by which Bcl-2 inhibits cell death in RKO cells treated with TRAIL in the presence of CCCP, Bcl-2–overexpressing cells were treated with TRAIL for 5 hours. As indicated in Fig. 4A, Bcl-2 overexpression completely abrogated Bax activation in TRAIL + CCCP–treated cells. Despite cleavage of procaspase 3, cleavage of caspase 6 and PARP was completely abrogated, indicating that caspase-3 activity was reduced in Bcl-2–overexpressing cells compared with GFP cells (Figs. 3B and 4A). Because caspase 6 is known to process additional procaspase 8 (24), lack of caspase-3 activity in Bcl-2–expressing cells, and consequently lack of activation of caspase 6, probably explained the observed decrease in caspase-8 processing. Bid cleavage, and Bax activation compared with control cells (Fig. 4A).

Bcl-2 prevents release of Smac and cytochrome c from mitochondria. Due to the inability of caspase 3 to cleave caspase 6 and PARP in Bcl-2–overexpressing cells and because XIAP can bind and inhibit caspase-3 activation (25), these data suggested that Bcl-2 abrogated the release of proapoptotic factors from mitochondria that could interfere with XIAP inhibitory effects. Therefore, the release of mitochondrial proteins into the cytosol was examined during CCCP-induced sensitization of RKO cells to TRAIL. In RKO/GFP cells treated with TRAIL alone, release of Smac/DIABLO and cytochrome c could not be detected (Fig. 4B). In contrast to cells treated with TRAIL in the presence of CCCP, the release of cytochrome c and Smac/DIABLO from mitochondria was enhanced, whereas exposure to CCCP alone had no effect (Fig. 4B). Inhibition or degradation of XIAP was observed only in cells treated with the combination of TRAIL and CCCP. The presence of N-acetyl-cysteine completely abrogated Smac/DIABLO and cytochrome c release. In cells overexpressing Bcl-2, Smac/DIABLO and cytochrome c were not released and XIAP was not degraded. Data suggest that CCCP allows TRAIL-induced mitochondrial release of Smac/DIABLO necessary for the inactivation of XIAP and subsequent cell death, mediated by ROS, and abrogated by Bcl-2 expression.

Overexpression of X-linked inhibitor of apoptosis inhibits initiator procaspase-8 processing. To confirm that XIAP could inhibit TRAIL-induced apoptosis when mitochondria are uncoupled, RKO cells stably overexpressing XIAP were treated with TRAIL in the presence or absence of the uncoupler. The levels of TRAIL-induced apoptosis in XIAP-overexpressing cells were markedly reduced in comparison with those in cells transfected with vector alone (Fig. 5A). To verify that XIAP could regulate apical caspase-8 processing, the levels of cleavage of procaspases 8, 3, 6, and PARP were examined. XIAP overexpression significantly reduced the levels of procaspase-8 processing, activation of caspase 3, and cleavage of PARP (Fig. 5B and C). Processing of procaspase 6, a substrate of active caspase 3, to its active form, p18, was also reduced in XIAP-expressing cells compared with control GFP cells. Data suggest that XIAP regulates procaspase-8 processing probably by inhibiting caspase-3 and caspase-6 activation known to process additional procaspase 8 to amplify TRAIL signaling.

Impact of loss of caspase 9 on tumor necrosis factor–related apoptosis–inducing ligand–induced apoptosis when mitochondria are uncoupled. The synergistic effect of CCCP on TRAIL-induced apoptosis involves the release of cytochrome c from mitochondria (Fig. 4B), and a major target of Apaf-1/cytochrome c is procaspase 9, which is cleaved to generate caspase 9. To determine the role of caspase 9 in TRAIL-induced apoptosis in the presence of CCCP, two retroviral vectors containing shRNA targeting caspase 9, designated sh584 or sh479, were prepared, and their ability to influence caspase-9 levels was determined. Transfection of sh479 into RKO cells resulted in suppression of

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effects of Bcl-2 on TRAIL-induced activation of caspase, Bax, and release of proapoptotic factors from mitochondria. A, cells were pretreated with CCCP (20 μmol/L) for 1 hour and then incubated with TRAIL (50 ng/mL) for 5 hours. Cellular proteins were extracted from treated and untreated RKO/GFP and Bcl-2 cells. Immunoblotting and immunoprecipitation of the conformationally changed Bax were described in Materials and Methods. Bax expression in lysates serves as loading control. B, release of proapoptotic factors from mitochondria. RKO/GFP and Bcl-2 cells were pretreated with CCCP (20 μmol/L) for 1 hour and then incubated with TRAIL (50 ng/mL) for 5 hours. Cytoplasmic lysates were subsequently prepared as described in Materials and Methods and analyzed for their content of cytochrome c, Smac/DIABLO, and XIAP by Western blot analysis. Equal loading of cytoplasmic proteins was determined by reprobing of blots with β-actin–specific antibody.
caspase-9 expression, with no effect mediated by sh584 (Fig. 6A). No effect on the expression of β-actin or XIAP was detected. Subsequently, it was analyzed whether reduction in caspase-9 levels could affect caspase-9 activity and TRAIL-induced apoptosis in the presence of CCCP. Transfected cells were treated with CCCP for 1 hour and subsequently exposed to TRAIL. The activities of caspase 9 and caspase 3 were determined at 3 hours, and apoptosis at 24 hours, after TRAIL exposure. Figure 6A shows that transfection of RKO cells with sh479 completely abrogated caspase-9 activity and caspase-3 activity was assayed in the lysates as described under Materials and Methods.

These observations suggest that caspase-9 activation is not a required component in TRAIL-mediated apoptosis when mitochondria are uncoupled. Furthermore, treatment of cells for 24 hours with staurosporin and etoposide (VP-16), both known to induce the mitochondrial caspase activation pathway, induced apoptosis independently of caspase 9 (Fig. 6C).

**Discussion**

In this study, uncouplers of oxidative phosphorylation were shown to enhance TRAIL-induced apoptosis in human colon carcinoma cells. This enhancing effect required caspase activation and was totally dependent on ROS generation. Overexpression of Bcl-2 completely protected cells from TRAIL-induced apoptosis in the presence of the uncoupler, suggesting the involvement of the mitochondrial apoptotic pathway as described by Bax conformational change and release of apoptogenic factors from mitochondria. This is in agreement with our previous study demonstrating that rottlerin, also an uncoupler, enhanced TRAIL-induced apoptosis in colon carcinoma cells via a mitochondria-dependent pathway (12).

CCCP specifically acts to dissipate the proton gradient across the inner mitochondrial membrane. Although this effect will eventually lead to depletion of ATP within the cell, ATP levels did not drop significantly 24 hours following treatment with CCCP, suggesting that ATP disruption is not the relevant mechanism that enhances the TRAIL signal (data not shown). Furthermore, disruption of ATP generation may switch cell death from apoptosis to necrosis (26, 27). Because CCCP clearly enhanced the apoptosis-inducing capacity of TRAIL leading to caspase activation and PARP cleavage, it is unlikely that a block in ATP generation underlies the enhancing effect. TRAIL did not show the ability to generate ROS. However, TRAIL induced partial depolarization in a caspase-dependent manner. In CCCP-treated cells, a partial decrease in ΔΨm was observed and, in the absence of subsequent caspase activation, can be restored to normal levels (28). Addition of TRAIL to CCCP-treated cells caused a further decrease in ΔΨm, which was inhibited by zVAD-fmk. These observations confirm recent findings showing that after mitochondrial depolarization and caspase activation, active caspases cleave complexes I and II of the electron transport chain, resulting in a sustained loss of ΔΨm and induction of apoptosis (29).

The finding that production of ROS during CCCP treatment alone can be nontoxic suggests that they are not required for apoptosis per se as shown by others (30, 31). However, the presence of ROS during TRAIL treatment is likely to contribute to cell death. TRAIL-induced caspase-8 activation, Bax conformational change, and cleavage of downstream effectors were greatly enhanced following mitochondrial uncoupling. Antioxidant N-acetyl-cysteine prevented the activation of apical caspase 8 and subsequent cell death. It has been shown that degradation of Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein (FLIP) can sensitize tumor cells to TRAIL-induced apoptosis (32). However, the presence or absence of CCCP did not affect the levels of FLIP in TRAIL-treated RKO cells (data not shown). Mitochondrial uncoupling could overcome resistance to TRAIL at the level of the death-inducing signaling complex by releasing FLIP from TRAIL receptors, and increasing the recruitment of Fas-associated death domain and caspase 8 to the active death-inducing signaling complex. The fact that Bcl-2 or XIAP overexpression reduced procaspase-8 processing could overcome resistance to TRAIL at the level of the death-inducing signaling complex by releasing FLIP from TRAIL receptors, and increasing the recruitment of Fas-associated death domain and caspase 8 to the active death-inducing signaling complex. The fact that Bcl-2 or XIAP overexpression reduced procaspase-8 processing could overcome resistance to TRAIL at the level of the death-inducing signaling complex by releasing FLIP from TRAIL receptors, and increasing the recruitment of Fas-associated death domain and caspase 8 to the active death-inducing signaling complex.
enhances TRAIL-induced release of cytochrome c and Smac/DIABLO with concomitant inhibition of the function of XIAP. Therefore, activation of the mitochondrial apoptotic pathway is required for the execution of TRAIL-induced apoptosis in RKO cells.

Several studies have shown that Bcl-2 abrogates apoptosis by maintaining mitochondrial function (33, 34). In this report, we found that CCCP enhanced mitochondrial release of apoptogenic factors. These results are consistent with our recent report showing that TRAIL-induced release of proapoptotic factors from mitochondria was shown only in the presence of an uncoupler, and Bcl-2 overexpression inhibited this release and the induction of apoptosis (12). Overexpression of Bcl-2 in RKO protected cells from TRAIL-induced apoptosis in the presence of CCCP. Bcl-2 did not affect the partial decrease in ΔΨm and ROS generation induced by CCCP, but did prevent the second collapse in ΔΨm in cells treated with the combination of TRAIL and CCCP. This second collapse in ΔΨm is more likely caspase dependent because the pan-caspase inhibitor zVAD-fmk completely blocked further collapse in ΔΨm and cell death. These observations support the concept that Bcl-2 acts downstream of the point of ROS production during apoptosis and are inconsistent with the hypothesis that Bcl-2 itself may act as a ROS scavenger or induce ROS-scavenging activity. Caspase-8 processing and Bax activation are strongly reduced in Bcl-2–expressing cells, indicating that Bcl-2 regulates the levels of procaspase 8 processed after TRAIL stimulation by inhibiting the release of Smac/ DIABLO, cytochrome c, and the degradation of XIAP. Given that the activity of caspase 3 was abrogated, as well as the absence of cleavage of caspase 6 in Bcl-2–expressing cells treated with TRAIL and CCCP, it is more likely that effector caspases like caspase 3 and caspase 6 initiate an amplification loop required for additional processing of procaspase 8 (Fig. 7). Overexpression of XIAP completely abrogated TRAIL-induced apoptosis in the presence of CCCP. XIAP also inhibited the activation of caspase 3 and caspase 6 and additional procaspase 8 processing, indicating that inhibition of TRAIL signaling lies downstream of XIAP.

In addition to Smac/DIABLO, TRAIL-induced release of cytochrome c from mitochondria was also enhanced. Cytochrome c initiates apoptosis by inducing the formation of the caspase-9/Apaf-1 complex (35). However, although TRAIL + CCCP treatment induced cytochrome c release and subsequent caspase 9 cleavage, targeting caspase-9 expression and disruption of its activity using

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Effects of shRNA on caspase-9 expression and on TRAIL sensitivity in RKO cells. A, cells stably transfected with empty vector (psmc2) or caspase-9 shRNA (sh84 and sh479) were analyzed for caspase-9 expression levels. Cell extracts were examined for caspase-9 expression levels. *β*-Actin and XIAP were used as loading and specificity controls. Three hours after treatment with TRAIL in the presence or absence of CCCP, the activities of caspase 9 and caspase 3 were determined by a fluorimetric assay on cell extracts using the synthetic substrates Ac-LEHD-AMC (caspase 9) and Ac-DEVD-AMC (caspase 3). B, transfected cells were pretreated with CCCP (20 μmol/L) for 1 hour and then incubated with TRAIL (50 ng/mL) for 24 hours. Apoptosis and PARP cleavage were determined as described under Materials and Methods. C, transfected cells were treated with VP16 or STS for 24 hours and apoptosis was determined as described in Materials and Methods.
shRNA did not protect the cells from TRAIL + CCCP–induced apoptosis. Caspase 9 was also not involved in VP-16– and staurosporin-induced apoptosis. Other studies have shown that Apaf-1−/− MEF cells were not protected from staurosporin-induced cell death after 20 hours of treatment (36), and VP-16–induced apoptosis in caspase-9−/− thymocytes was only delayed. Also, caspase 9 is not required for Fas-induced apoptosis of caspase-9−/− thymocytes (37) or the toxicity of the agonistic anti-Fas antibody Jo2 in vivo in caspase-9−/− mice (38). These results suggest that TRAIL induced a caspase-9–independent activation of downstream caspases when mitochondrial respiration was uncoupled.

In conclusion, it has been shown that CCCP-induced ROS production can regulate caspase activation in TRAIL-resistant human colon carcinoma cells. In the presence of ROS, TRAIL-induced caspase activation was enhanced with concomitant release of proapoptotic factors from mitochondria (Fig. 7). It is also apparent that release of Smac/DIABLO and inactivation of XIAP may be considerably more important for TRAIL-induced apoptosis compared with the caspase-9/cytochrome c pathway. Thus, in the presence of ROS, TRAIL could be more efficient in the treatment of chemoresistant tumors, such as tumors lacking Apaf-1.

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**References**


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