Tumor Necrosis Factor Induces Apoptosis in Hepatoma Cells by Increasing Ca\(^{2+}\) Release from the Endoplasmic Reticulum and Suppressing Bcl-2 Expression*

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Tumor necrosis factor (TNF) plays an import role in the control of apoptosis. The most well known apoptotic pathway regulated by TNF involves the TNFR1-associated death domain protein, Fas-associated death domain protein, and caspase-8. This study examines the mechanism of TNF-induced apoptosis in FaO rat hepatoma cells. TNF treatment significantly increased the percentage of apoptotic cells. TNF did not activate caspase-8 but activated caspase-3, -10, and -12. The effect of TNF on the expression of different members of the Bcl-2 family in these cells was studied. We observed no detectable changes in the steady-state levels of Bcl-X\(_L\), Bax, and Bid, although TNF suppresses Bcl-2 expression. Dantrolene suppressed the inhibitory effect of TNF on Bcl-2 expression. TNF induced release of Ca\(^{2+}\) from the endoplasmic reticulum (ER) that was blocked by dantrolene. Importantly, the expression of Bcl-2 blocked TNF-induced apoptosis and decreased TNF-induced Ca\(^{2+}\) release. These results suggest that TNF induces apoptosis by a mechanism that involves increasing Ca\(^{2+}\) release from the ER and suppression of Bcl-2 expression.

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‡ The abbreviations used are: TNF, tumor necrosis factor; ER, endoplasmic reticulum; TGF-β, transforming growth factor-β1; TRADD, TNFR1-associated death domain protein; FADD, Fas-associated death domain; PI, propidium iodide; TUNEL, terminal dUTP nick end labeling; IETD-fmk, z-Ile-Glu(OMe)-Thr-Asp(OMe)-CH\(_2\)F. 

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TNF induces apoptosis via mobilization of intracellular Ca²⁺ and suppression of Bcl-2.

MATERIALS AND METHODS

Cell Culture—FaO rat hepatoma cells were maintained at 37 °C in DMEM (Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin (100 μg/ml)). Generation of FaO cells stably expressing Bcl-2 was previously described (28).

Reagents—Propidium iodide, SKF96365, TMB-8, and dantrolene, a blocker of intracellular calcium release from the sarcoplasmic reticulum that acts by binding to the ryanodine receptor, were purchased from Calbiochem. EGTA and thapsigargin were purchased from Sigma. Recombinant murine TNF was purchased from R & D Systems. All other chemicals were from standard sources and were molecular grade or higher. Caspase-8 inhibitor, IETD-fmk, partially blocked TGF-β1-mediated apoptosis but showed very little effect on TNF-mediated apoptosis. D, TUNEL-positive apoptotic cells at the respective incubation time were counted, and the percentage of apoptotic cells was graphed. Similar results were achieved in three separate experiments with comparable outcomes.

DNA Fragmentation Assay—FaO cells were plated at 5 × 10⁴ cells/eight-well chamber slide (Nalge Nunc International, Rochester, NY) and incubated for 24 h. The lysate was extracted with phenol, phenol/chloroform (1:1), and chloroform and precipitated with 2.5 volumes of ice-cold ethanol. The DNA was resuspended in Tris-EDTA buffer supplemented with 100 μg/ml RNase A. DNA samples were electrophoretically separated on 2% agarose gel for 2 h at 50 V.

Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) Assay—FaO cells were plated at 5 × 10⁴ cells/eight-well chamber slide (Nalge Nunc International, Rochester, NY) and incubated for 24 h. The cells were treated with TGF-β1 (5 ng/ml) or TNF (20 ng/ml) for 12 h and fixed with 4% paraformaldehyde (pH 7.4) for 10 min. Apoptotic cells were assessed by measuring DNA fragmentation in a standard TUNEL assay according to the instructions with the kit (In Situ Cell Death Detection Kit, POD; Roche Molecular Biochemicals).

Flow Cytometry Analysis—For the flow cytometry assay, FaO and FaO-Bcl-2 cells were grown in six-well plates and incubated for 24 h at 37 °C in the presence or absence of 20 ng/ml TNF-α. Cells were harvested and washed twice with phosphate-buffered saline (pH 7.4). After

FIG. 1. TNF-induced apoptosis in FaO rat hepatoma cells. A, cellular DNA fragmentation after TNF treatment (20 ng/ml). Genomic DNA was extracted from control, TGF-β1 (5 ng/ml) (12 h), and TNF-treated cells at 6, 12, and 24 h. Extracted DNA was resolved on 1.5% agarose gel. M, size markers. B, the TUNEL procedure was carried out, and pictures were taken under a light microscope (magnification, ×200). C, expression of Bcl-2 family proteins during TNF-induced apoptosis in FaO rat hepatoma cells. The effect of the TNF on Bcl-XL, Bcl-2, Bax, and Bid protein levels in FaO cells were determined. Cells were incubated with 20 ng/ml TNF. Cell lysates were made, and equivalent amounts of cellular proteins were separated by SDS-15% PAGE, blotted, and then probed with the appropriate antibody. Blots shown are typical of at least three individual experiments.

FIG. 2. Activation of caspases after TNF and TGF-β1 treatment. A and B, samples were taken at the indicated time points and assayed for caspase-3 and caspase-8 cleavage by Western blotting using caspase-3- and caspase-8-specific antibodies. The full-length caspase protein and cleaved products (sizes indicated in kDa) are indicated. C, examination of the effect of caspase-8 inhibitor on TNF-induced apoptosis in FaO cells by TUNEL apoptotic assay. Using the nucleus-specific staining 4',6-diamidino-2-phenylindole and TUNEL assay, an increase in the rate of nuclear fragmentation and apoptosis was observed in FaO cells following treatment with either TNF (20 ng/ml) or TGF-β1 (5 ng/ml). Pretreatment with caspase-8 inhibitor, IETD-fmk, partially blocked TGF-β1-mediated apoptosis but showed very little effect on TNF-mediated apoptosis. D, TUNEL-positive apoptotic cells at the respective incubation time were counted, and the percentage of apoptotic cells was graphed. Similar results were achieved in three separate experiments with comparable outcomes.
fixing in 80% ethanol for 30 min, cells were washed twice and resuspended in phosphate-buffered saline (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml propidium iodide (PI) and 50 μg/ml ribonuclease A for DNA staining. Cells were then analyzed by a FACScan cytometer (CELLQUEST program; Becton Dickinson). Red fluorescence due to PI staining of DNA was expressed on a logarithmic scale simultaneously with the forward scatter of the particles. Four thousand events were counted on the scatter gate. The number of apoptotic nuclei was expressed as a percentage of the total number of events.

Immunoblot Analysis—Whole-cell extracts were obtained in a 1% Triton X-100 lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM sodium chloride, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Western blotting was performed using anti-Bcl-2 (N-19; Santa Cruz Biotechnology, Inc.), anti-Bcl-Xs/L (S-18; Santa Cruz Biotechnology), anti-Bax (P-19; Santa Cruz Biotechnology), anti-Bid (M-20; Santa Cruz Biotechnology), anti-caspase-8 (p-20; Santa Cruz Biotechnology), anti-caspase-10 (Cell Signaling Technology Inc., Beverly, MA), and anti-rynodine receptor (N-1; Santa Cruz Biotechnology) antibodies. Monoclonal rat antibody against caspase-12 was kindly provided by Dr. Junying Yuan (Harvard Medical School, Boston, MA). The antibody recognizes the full-length unprocessed form as well as the processed fragments of their respective antigens. Protein samples were heated at 95 °C for 5 min and analyzed by SDS-10% PAGE. Immunoblot signals were developed by Super Signal Ultra chemiluminescent reagent (Pierce).
**RESULTS**

**TNF Induces Apoptosis in FaO Rat Hepatoma Cells**—Treatment of FaO rat hepatoma cells with TNF induced a 180–200-bp internucleosomal DNA cleavage as early as 12 h after TNF treatment (Fig. 1A). We have previously shown that TGF-β1 rapidly induces apoptosis in these cells (27, 28). Therefore, we used TGF-β1 treatment as a positive control for the apoptotic response. To show that the TNF-induced cell death is due to apoptosis, the TUNEL assay was also carried out (Fig. 1B). As indicated by the number of dark brown positive cells, there was a significant increase in the rate of apoptosis in a time-dependent manner following TNF treatment.

To characterize the mechanism of TNF-mediated apoptosis, the steady-state levels of several Bcl-2 family proteins were measured by Western blotting analysis. We detected no changes in expression of Bcl-X<sub>L</sub>, Bax, or Bid over the TNF treatment time course; nor were any cleaved products of these proteins detected (Fig. 1C). However, Bcl-2 expression decreased in a time-dependent manner (Fig. 1C).

**Caspases in TNF-mediated Apoptosis**—Since caspase-8 and caspase-3 have been implicated in TNF-induced apoptosis, we examined activation of caspase-8 and caspase-3 in TNF-induced apoptosis in FaO cells. TNF treatment of FaO cells induced a time-dependent processing of caspase-3 but not caspase-8 (Fig. 2A). We also used TGF-β1 as a control for the caspase activation. As expected, TGF-β1 treatment caused activation of both caspase-3 and caspase-8 (Fig. 2B). To confirm this observation, we examined the effect of inhibitor of caspase-8 on TNF-mediated apoptosis. The addition of an inhibitor of caspase-8 (IETD-fmk) reduced the percentage of TUNEL-positive nuclei induced by TGF-β1, but the presence of IETD-fmk showed very little effect on TNF-mediated apoptosis (Fig. 2, C and D). We next examined activation of caspase-10 in TNF-induced apoptosis in FaO cells. It is known that in some cases caspase-10 can functionally substitute for caspase-8 in death receptor signal transduction (32). The anti-caspase-10 antibody from Cell Signaling Technology does not detect the cleaved form of caspase-10. Treatment with TNF decreased the level of procaspase-10, whereas TGF-β1 treatment showed no effect on the level of procaspase-10, suggesting that TNF may induce apoptosis through the activation of caspase-10 (Fig. 3A).
Caspase-12, an ER-resident caspase, is specifically involved in apoptosis that results from ER stress (33–36). Caspase-12 participates in ER stress-induced apoptosis that is blocked by benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), a general caspase inhibitor (34). Alterations in Ca\(^2+\)/H\(^{+}\) homeostasis and accumulation of unfolded proteins in the ER cause ER stress. Using a polyclonal serum directed against caspase-12, a single band corresponding to the p53 proform of caspase-12 was detected. After treatment with TNF, smaller fragments between 17 and 40 kDa became visible representing active caspase-12 (Fig. 3, B and C). However, cleavage of pro-caspase-12 was not detected during TGF-\(\beta\)-induced apoptosis (Fig. 3C). These results suggest that the TNF-induced cell death is apoptosis and that the actual apoptotic pathway involves activation of caspase-3 and caspase-12.

Effects of Ca\(^2+\)/H\(^{+}\) on TNF-induced Apoptosis—Bcl-2 is known to regulate the flux of Ca\(^2+\) across the ER membrane, thereby abrogating Ca\(^2+\) signaling of apoptosis (20). To determine whether an increase in cytosolic Ca\(^2+\) was required for the induction of apoptosis by TNF, the action of extracellular calcium chelators, EGTA and SKF96365, was studied. In a Ca\(^2+\)-containing medium, TNF induced apoptosis in FaO cells. The low extracellular Ca\(^2+\) caused by either EGTA or SKF96365 did not alter TNF-induced apoptosis (Fig. 4, A and B). Dantrolene, a drug used to treat malignant hyperthermia, inhibits Ca\(^2+\) release from ER and has been shown to inhibit cell

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**Fig. 6.** Bcl-2 reduces the amount of releasable Ca\(^{2+}\) from intracellular stores. A Ca\(^{2+}\)-containing medium (A and C) or Ca\(^{2+}\)-free medium (B and D) containing 1 mM EGTA were used to assess intracellular Ca\(^{2+}\) changes in FaO cells (A and B) or FaO/Bcl-2 cells (C and D) (see the legend to Fig. 5 for details). In the bar graphs (E), the data represent the mean values of four replications, with bars indicating S.E. *, p < 0.05 compared with control.

**Fig. 7.** Dantrolene rescues Bcl-2 expression levels decreased by TNF in FaO rat hepatoma cells. A, ryanodine receptor expression in FaO cells was confirmed by Western blot analysis. B, FaO cells were treated with TNF (20 ng/ml) for 24 h with or without pretreatment with dantrolene for 1 h. Cell lysates were made, and equivalent amounts of cellular proteins were separated by SDS-15% PAGE, blotted, and then probed with the Bcl-2 antibodies. Blots shown are typical of at least three individual experiments. C, quantified results were obtained from three experiments using densitometry.

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Effects of TNF on Cellular Ca\(^{2+}\) Homeostasis—To support the view that TNF-induced apoptosis is triggered by the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores, we investigated whether TNF had a direct effect on intracellular Ca\(^{2+}\) homeostasis. FaO cells were loaded with the Ca\(^{2+}\) dyes, and changes in [Ca\(^{2+}\)]\(_i\) were measured. Treatment with TNF caused a relatively fast elevation in [Ca\(^{2+}\)]\(_i\), which peaked within 5 min and then decreased to a lower sustained level. In a Ca\(^{2+}\)-free medium, a smaller, transient increase was seen (Fig. 5, A, C, and H). However, TGF-β1 has no effect on intracellular Ca\(^{2+}\) homeostasis (Fig. 5G). This finding suggests that TNF induces both internal Ca\(^{2+}\) release and Ca\(^{2+}\) influx. The trace in Fig. 6B represents the internal Ca\(^{2+}\) release component. The increases in cytosolic Ca\(^{2+}\) elevation after TNF treatment in both Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free conditions, were significantly reduced in cells pretreated with dantrolene (Fig. 5, B, D, and H). This suggests that dantrolene reduces TNF-induced internal Ca\(^{2+}\) release. Dantrolene also inhibited internal Ca\(^{2+}\) release induced by a thapsigargin, a highly specific inhibitor of the ER-associated Ca\(^{2+}\) pump that is used to deplete internal Ca\(^{2+}\) stores (Fig. 5, E, F, and I).

We next determined the effect of Bcl-2 overexpression on TNF-induced calcium homeostasis. We previously generated FaO cell lines expressing Bcl-2 (28). These cell lines are individually isolated clones. In both Ca\(^{2+}\)-containing and Ca\(^{2+}\)-free medium, the increase of cytosolic Ca\(^{2+}\) after the addition of TNF was significantly reduced in FaO/Bcl-2 cells compared with FaO cells (Fig. 6).

Dantrolene Increases Bcl-2 Protein Level in FaO Cells—The skeletal muscle relaxant dantrolene is an inhibitor of Ca\(^{2+}\) release through ryanodine receptor. The expression of ryanodine receptor protein was confirmed in FaO cells by Western blot analysis using a specific antibody raised against the ryanodine receptor (Fig. 7A). We next examined the effect of dantrolene on Bcl-2 protein levels by Western blotting. As shown in Fig. 7, B and C, Bcl-2 protein levels increased significantly to 320% of the control after treatment with 25 μM dantrolene for 24 h. TNF treatment markedly decreased Bcl-2 protein level, but treatment with dantrolene blocked suppression of Bcl-2 expression by TNF, suggesting that TNF suppresses Bcl-2 expression by regulating the flux of Ca\(^{2+}\).

Dantrolene Blocks TNF-induced Apoptosis—To investigate whether dantrolene also blocks TNF-induced apoptosis in FaO cells, we measured nuclear incorporation of propidium iodide by fluorescence-activated cell sorting analysis. Cells were incubated in the absence or presence of TNF, TGF-β1 or thapsigargin was used as control. Dantrolene pretreatment robustly suppressed TNF-induced- or thapsigargin-induced population of apoptotic nuclei, whereas dantrolene did not block TGF-β1-induced apoptosis (Fig. 8). These results further suggest that TNF activates intracellular Ca\(^{2+}\) release from ER, which, in turn, induces apoptosis.

Overexpression of Bcl-2 has been shown to repress apoptosis by regulating ER-associated Ca\(^{2+}\) release. To investigate the protective effect of Bcl-2 on TNF-induced apoptosis, stable cell lines expressing Bcl-2 were generated (28) (Fig. 9A). As shown in Fig. 9A, Bcl-2 protein level was significantly increased. Most of the Bcl-2-overexpressing cell lines were completely resistant to TGF-β1-induced apoptosis. We next examined the propidium iodide incorporation in the FaO cells overexpressing Bcl-2. A
significant decrease in PI incorporation was seen after the addition of TNF in FaO/Bcl-2 cells compared with control cells (Fig. 9B).

Effects of Calcium Chelators and an Inhibitor of Intracellular Calcium Release on Activation of Caspase-3 and Caspase-12 Induced by TNF—Up to this point, our results suggest that ER-controlled calcium release is involved in TNF-induced apoptosis in FaO cells. The activation of caspase-12 is involved in a specific form of apoptosis in the ER unfolded protein response. TNF treatment increased cleaved active products of caspase-12. However, pretreatment with dantrolene or TMB-8 completely blocked activation of caspase-12 induced by TNF, whereas extracellular calcium chelators, EGTA or SKF96365, did not block the TNF-induced caspase-12 cleavage (Fig. 10).

DISCUSSION

Receptor-mediated apoptosis has been demonstrated for various growth factors, including TNF. The TNFs act via a large family of receptors expressed on the surface of the target cell, known as the TNF receptor superfamily (1–3). TNF is responsible for a diverse range of signaling events within cells, leading to either necrosis or apoptosis. TNF exerts many of its effects by binding to either a 55-kDa cell membrane receptor termed TNFR-1 or a 75-kDa cell membrane receptor termed TNFR-2. TNF induces apoptosis by more than one pathway. The most widely accepted pathway involves TRADD, FADD, and caspase-8 (39). Indeed, FADD- and caspase-8-deficient fibroblasts are resistant to TNFR1-induced apoptosis (40, 41). Ligation of TNFR1 by TNF recruits the TRADD to the receptor’s death domain, TRADD in turn recruits FADD, which recruits procaspase-8. TRADD also serves to recruit the serine/threonine kinase RIP and the TNF receptor-associated factor 2 (TRAF2), which are implicated in activation of the NF-kB and c-Jun N-terminal kinase/AP-1 pathways. This report shows that TNF also induces apoptosis through a caspase-8-independent mechanism in FaO rat hepatoma cells (Fig. 2). TNF treatment did not activate the cleavage of caspase-8; however, it induced activation of caspase-10. It has been suggested that in some cases caspase-10 can functionally substitute for caspase-8 in death receptor signal transduction (32). However, the role of caspase-10 is not clear in TNF-mediated apoptosis in FaO cells. TNF induces activation of caspase-12 in FaO cells. Caspase-12 is known to be essential for cell death induced by ER stress. Procaspase-12 is enriched in ER-containing microsomal fractions from the brain and is processed by the family of cytosolic calcium-dependent cysteine proteases, calpain (34). Procaspase-12 is cleaved, and the activated forms accumulate under ER stress conditions (35). Caspase-12-deficient mice are resistant to ER stress-induced apoptosis, but their cells are subject to apoptosis in response to other stimuli. Activation of caspase-12 can be directly stimulated by depletion of the ER calcium pool. Our results also support the concept that a rise in cytosolic free calcium concentration triggers caspase-12 activation and ER apoptotic signaling. Treatment with dantrolene, an inhibitor of calcium release from the ER, blocks the activation of caspase-12 and apoptotic signaling induced by TNF (Fig. 10).

The ER is a principal site for protein synthesis and folding and also serves as a cellular storage site for calcium (42, 43). Agents that block protein folding or export, inhibitors of protein glycosylation, and agents that affect calcium uptake and re-

FIG. 9. Antiapoptotic effect of Bcl-2 on TNF-induced apoptosis in FaO cells. A, expression of Bcl-2 protein in FaO cells which were stably transfected with the mammalian vector encoding bcl-2 was examined by Western blot analysis. Bcl-2FaO, B, FaO cells and Bcl-2-expressing FaO cells were incubated with 20 ng/ml TNF. A representative illustration is shown of PI incorporation measured in two cell lines by flow cytometry analysis. The number of apoptotic nuclei is expressed as a percentage of the total number of events.
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Fig. 10. Inhibition of TNF-induced caspase-3 and caspase-12 activation by intracellular Ca$^{2+}$ inhibitors. A, dantrolene (Dan) (50 μM), TMB-8 (20 μM), and EGTA (1 mM) were added 1 h before TNF treatment. Samples were taken at the indicated time points and assayed for caspase-3 cleavage by Western blotting using caspase-3-specific antibody. The full-length caspase protein and cleaved products (sizes indicated in kDa) are indicated. Con, control; B, cells were pretreated with either SK96365 or different doses of dantrolene and then treated with TNF (20 ng/ml) for 24 h. Samples were taken at the indicated time points and assayed by Western blotting analysis using caspase-12-specific antibody that recognizes full-length protein as well as the active form. Cell lysates were made, and equivalent amounts of cellular proteins were separated by SDS–15% PAGE, blotted, and then probed with the appropriate antibody. Blots were shown are typical of at least three individual experiments.

lease from the ER can all lead to ER stress and ultimately cell death (44–46). Several previous studies have implicated depletion of the ER calcium pool depletion in the initiation of apoptosis (19, 20, 47). TNF treatment also induces mobilization of Ca$^{2+}$ from intracellular stores in cultured sensory neurons (48). We confirmed that TNF treatment increases the release of Ca$^{2+}$ from the ER Ca$^{2+}$ pool and induces apoptosis in this study (Fig. 5).

Both the ER and mitochondria act as calcium stores controlling the capacitative calcium influx and cytoplasmic calcium homeostasis (49). It has been suggested that the ER Ca$^{2+}$ depletion caused by Bcl-2 overexpression is an integral part of the antiapoptotic program set by various agents (47). Ceramide induces a rise in cytoplasmic Ca$^{2+}$ concentration by releasing Ca$^{2+}$ from intracellular stores and activating the capacitative Ca$^{2+}$ entry pathway, resulting in prolonged mitochondrial Ca$^{2+}$ accumulation and alterations in organelle morphology (swelling and fragmentation) (50). Overexpression of Bcl-2 in WEHI7.2 cells blocks thapsigargin-induced cell death and thapsigargin-induced mobilization of ER Ca$^{2+}$. The effect of Bcl-2 on the release of Ca$^{2+}$ from the ER may be cell type-dependent. Other laboratories have reported different effects of Bcl-2 on the intracellular calcium pool (19, 20, 22, 23, 47). Three groups have shown that Bcl-2 lowers the steady-state level of Ca$^{2+}$ within the ER and inhibits apoptosis by reducing Ca$^{2+}$ influx across the ER membrane (19, 20, 23, 47). In contrast, another group (22) showed that the overexpression of Bcl-2 increased luminal Ca$^{2+}$ concentrations in the ER by up-regulating calcium pump (SERCA) expression in breast epithelial cells. Although we cannot explain these contrasting findings at present, they are probably related to the use of different cellular model systems. It is conceivable that cellular context dictates precisely how Bcl-2 will influence intracellular Ca$^{2+}$ pools. Importantly, the connection between ER Ca$^{2+}$ pool emptying and apoptosis is not disputed.

TNF treatment suppressed the protein level of Bcl-2 in FaO cells. This result is somewhat unexpected, given the evidence that Bcl-2 is capable of blocking Ca$^{2+}$ release from the ER, resulting in the protection of Ca$^{2+}$-induced toxicity. Although the mechanism of suppression of Bcl-2 by TNF in FaO cells remains to be investigated, this study suggests another apoptotic pathway triggered by TNF. It will be interesting to find whether the residual apoptotic response observed upon TNF treatment of FADD-deficient cells (41) is mediated through the mobilization of Ca$^{2+}$ from the ER.

Interference of Ca$^{2+}$ release from the ER increases Bcl-2 mRNA and protein levels. Treatment with dantrolene, an inhibitor of Ca$^{2+}$ release from the ER, prevented cell death, and this protection by dantrolene was associated with a marked increase in the protein levels of Bcl-2 in GT1 hypothalamic neurosecretory cells (38). As the ER Ca$^{2+}$ level has a prominent role in regulating protein synthesis (51), the effect of dantrolene on ER Ca$^{2+}$ could be the underlying mechanism for Bcl-2 induction. In another study, treatment with lithium, which interferes with Ca$^{2+}$ release from the ER (52), protected cell death induced by glutamate, and this protection was associated with increases in Bcl-2 mRNA and protein levels in cultured cerebellar neurons (53). Our study also supports the concept that inhibition of Ca$^{2+}$ release from the ER induces Bcl-2 expression. Treatment of FaO cells with dantrolene inhibited TNFα-induced apoptosis and increased the protein level of Bcl-2. Further investigation will be required to identify mechanisms contributing to the induction of the levels of Bcl-2 mRNA and protein by dantrolene. Regardless of the detailed mechanisms underlying the cytoprotective effects of dantrolene, the ability of dantrolene to induce Bcl-2 raises the possibility that this drug may be potentially useful in the treatment of some forms of neurodegenerative diseases.

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