Therapeutic advantage of combining calcium channel blockers and TRAIL in prostate cancer

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Abstract
Disruption of intracellular calcium initiates multiple cell-damaging processes, such as apoptosis. In normal cells, the levels of Ca\textsuperscript{2+} are low in the mitochondria, whereas in apoptotic cells, Ca\textsuperscript{2+} increases. Mitochondria uptake Ca\textsuperscript{2+} via an inner membrane channel called the uniporter and extrude it into the cytoplasm through a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Overload of Ca\textsuperscript{2+} in the mitochondria in CGP-treated cells leads to its damage, thus affecting cellular function and survival. The goal of these experiments was to determine the importance of mitochondrial calcium ([Ca\textsuperscript{2+}]\textsubscript{m}) in apoptosis of prostate cancer cells. Furthermore, we have examined the advantages of increasing the [Ca\textsuperscript{2+}]\textsubscript{m} and treating the cells with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a potent apoptotic agent. Our results show that, under these treatment conditions, inhibiting the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger using benzothiazepin CGP-37157 (CGP) did not induce apoptosis. However, combination of CGP and TRAIL increased the apoptotic response ~25-fold compared with control. Increase in apoptosis followed enhanced levels of [Ca\textsuperscript{2+}]\textsubscript{m} and was accompanied by pronounced mitochondrial changes characteristic of mitochondria-mediated apoptosis. Experiments with calcium ionophores showed that mere increase in cytosolic and/or mitochondrial Ca\textsuperscript{2+} was not sufficient to induce apoptosis. These results have therapeutic implications as inhibitors of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger are being used for treating some neurologic and cardiologic ailments, and TRAIL induces apoptosis preferentially in cancer cells. Furthermore, this system provides an excellent model to investigate the role of [Ca\textsuperscript{2+}]\textsubscript{m} in apoptosis.

Introduction
Calcium signaling mechanisms govern a multitude of vital cell functions that affect cell survival. As ionic calcium (Ca\textsuperscript{2+}) affects several signal transduction pathways, the cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{c}) is maintained at lower level (~100 nmol/L) in contrast to the extracellular concentration (~1 mmol/L). Cells regulate [Ca\textsuperscript{2+}]\textsubscript{c} mainly by controlling Ca\textsuperscript{2+} movement across the plasma membrane and in and out of key organelles, such as the endoplasmic reticulum and the mitochondria (1). Disruption of intracellular Ca\textsuperscript{2+} is a powerful activator of multiple cell-damaging processes, such as apoptosis or necrosis (2, 3). In normal cells, the endoplasmic reticulum is the largest reservoir of Ca\textsuperscript{2+}, with millimolar concentrations, ~10,000-fold higher compared with [Ca\textsuperscript{2+}]\textsubscript{c}. The levels of Ca\textsuperscript{2+} are low in the mitochondria, although mitochondria are known to accumulate Ca\textsuperscript{2+} during apoptosis especially when the [Ca\textsuperscript{2+}]\textsubscript{m} level is high (4–6). Mitochondria uptake Ca\textsuperscript{2+} via an inner membrane channel called the uniporter (7) and extrude it into cytoplasm through a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (8, 9). Overload of [Ca\textsuperscript{2+}]\textsubscript{m} leads to its damage, subsequently affecting cellular function and survival (1). It is well known that, in addition to serving as a buffer for Ca\textsuperscript{2+} (10), mitochondria are a powerful activator of multiple cell-damaging processes, such as apoptosis or necrosis (2, 3). In normal cells, the endoplasmic reticulum is the largest reservoir of Ca\textsuperscript{2+}, with millimolar concentrations, ~10,000-fold higher compared with [Ca\textsuperscript{2+}]\textsubscript{c}. The levels of Ca\textsuperscript{2+} are low in the mitochondria, although mitochondria are known to accumulate Ca\textsuperscript{2+} during apoptosis especially when the [Ca\textsuperscript{2+}]\textsubscript{m} level is high (4–6). Mitochondria uptake Ca\textsuperscript{2+} via an inner membrane channel called the uniporter (7) and extrude it into cytoplasm through a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (8, 9). Overload of [Ca\textsuperscript{2+}]\textsubscript{m} leads to its damage, subsequently affecting cellular function and survival (1). It is well known that, in addition to serving as a buffer for Ca\textsuperscript{2+} (10), mitochondria actively participate in apoptosis. Increase in [Ca\textsuperscript{2+}]\textsubscript{m} induces apoptosis through its effects on the mitochondrial membrane permeability leading to the formation of the permeability transition pore (PTP) at the contact sites between the inner and outer mitochondrial membranes. Activation of PTP accompanies the loss of mitochondrial membrane potential (MMP), expansion of the matrix, and the rupture of the outer mitochondrial membrane, through which cytochrome c is released into the cytoplasm (11). The key components of the PTP include adenine nucleotide translocase located on the inner mitochondrial membrane, voltage-dependent anion channel in the outer mitochondrial membrane, and cyclophilin D. An increase in the matrix Ca\textsuperscript{2+} opens the PTP by perturbing the interaction between cyclophilin D and the adenine nucleotide translocase (12). The mitochondrial cytochrome c is normally attached to the outer surface of the inner mitochondrial membrane, where it is involved in the respiratory electron transfer chain. Release of cytochrome c from the mitochondria to the cytosol is fast and is required for mitochondria-mediated apoptosis (13, 14). Once released into the cytoplasm, cytochrome c binds to cytosolic Apaf-1. The Apaf-1/cytochrome c complex undergoes multimerization (estimated to be an octamer) in an ATP-dependent manner (15, 16). After the formation of the multimeric complex, procaspase-9 is recruited into the complex, which is then activated, a necessary step for the activation of effector caspases, such as caspase-3 and caspase-7.

Since its discovery in 1995, the new member of the tumor necrosis factor–α family, tumor necrosis factor–related
apoptosis-inducing ligand (TRAIL), has attracted enormous interest for the treatment of cancer (17, 18). Significantly, TRAIL triggers apoptosis preferentially in tumor cells through the activation of specific death receptors DR4 (19) and DR5 (20–22). Activation of death receptors by TRAIL recruits the cytoplasmic adapter protein Fas-associated protein with a death domain, which then activates caspase-8 (FLICE, MACH, and Mch5) by induced-proximity protease activity (23). In some cells, caspase-8 propagates death signal directly through the activation of procaspase-3, whereas in others, the apoptotic signal is amplified via the mitochondria. In the latter, caspase-8 cleaves Bid, which translocates into the mitochondria and initiates changes in the mitochondria leading to the release of cytochrome c. Bid, a member of the BH3 domain only subgroup of Bcl2 family proteins, is a 22-kDa cytosolic protein that is cleaved by activated caspase-8 in cells treated with variety of apoptotic agents, such as TRAIL (24), histone deacetylase inhibitor (25), and UV radiation (26). The truncated 15-kDa Bid (tBid) is translocated into the mitochondria, where it participates in changes leading to the release of cytochrome c to the cytoplasm. TRAIL is being investigated as a potent apoptogenic drug due to several advantages, such as preferential effect on cancer cells, lack of adverse effects in preclinical animal studies, and effectiveness in combination with other cytotoxic drugs (25, 27–29).

The present experiments were designed to examine the advantages of combining TRAIL treatment with perturbations in the mitochondrial Ca\(^{2+}\). We have determined (a) whether alterations in [Ca\(^{2+}\)]\(_{m}\) affect prostate cancer cell survival and (b) whether changes in [Ca\(^{2+}\)]\(_{m}\) alter TRAIL-induced apoptosis. Mitochondrial Ca\(^{2+}\) was altered by affecting the function of pumps that are responsible for shunting Ca\(^{2+}\) in and out of mitochondria. The function of Na\(^{+}/Ca\(^{2+}\) exchanger was inhibited using benzothiazepin CGP-37157, a potent and specific inhibitor (8, 9), whereas the movement of Ca\(^{2+}\) into the mitochondria through the uniporter was inhibited using Ru360 or ruthenium red (7, 30, 31). We present data to show that inhibiting the extrusion of Ca\(^{2+}\) from the mitochondria sensitized mitochondria to TRAIL, resulting in highly synergistic apoptosis. These results have clinical implications, as blockers of Na\(^{+}/Ca\(^{2+}\) exchanger are presently being used for treating several cardiovascular and neurologic diseases (32, 33). Furthermore, these results provide a model to investigate the role of [Ca\(^{2+}\)]\(_{m}\) in TRAIL-induced, mitochondria-mediated apoptosis.

**Materials and Methods**

**Cell Culture and Experimental Design**

Prostate cancer cell lines DU145 and LNCaP (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 (Hyclone, Logan, UT) containing 10% fetal bovine serum, 0.5% penicillin-streptomycin (0.05 units/mL), and 0.1% fungizone (0.25 μg/mL). Experiments were conducted with the same medium but with fetal bovine serum concentration of 7.5%. Cells were seeded to reach a confluency of 80% after 24 hours. Fresh medium was added with or without benzothiazepin CGP-37157 (Calbiochem, San Diego, CA) for 30 minutes. In other groups, cells were treated with 200 ng/mL TRAIL (Biomol, Plymouth Meeting, PA) for 4 hours, thapsigargin (Calbiochem), or ionesomycin (Sigma-Aldrich, St. Louis, MO) for 24 hours. For dose curve experiments, cells were treated with increasing concentrations of CGP alone or in combination with TRAIL. Ru360 (EMD Biosciences, Inc., San Diego, CA), ruthenium red (EMD Biosciences), and cyclosporin A (Calbiochem) were added 15 minutes before TRAIL or combination treatments.

**Protein Extraction and Western Blotting**

Floating and attached cells were harvested and washed with PBS, and the cells were resuspended in lysis buffer (1× PBS, 1% Triton × 100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 0.5 μg/mL leupeptin, 1 μg/mL peptatin, 1 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin). Cells were incubated on ice for 30 minutes and centrifuged at 10,000 × g at 4°C for 10 minutes. The supernatant was collected and the protein concentration was estimated using Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA).

Cell extracts were separated on Tris-glycine gels (10-15%) and probed for caspase-3, caspase-8, caspase-9, or Bid. Positive signals were developed using Enhanced Chemiluminescence Plus (Amersham, Piscataway, NJ) and the signal was captured on a digital imager (Alpha Innotech 8900, San Leandro, CA) and/or exposed to Enhanced Chemiluminescence Hyperfilm, developed, and fixed. The blots were stripped and reprobed with β-actin that was used as loading control. Digitized signals were normalized against β-actin and expressed per unit protein.

**Measurement of Apoptosis**

Apoptosis was measured using the M30 Apoptosense kit (DiaPharma, West Chester, OH) or by flow cytometry. The M30 Apoptosense is an ELISA using a specific antibody against a neoepitope of cytokeratin 18 that is generated by the action of caspsases that are activated in response to apoptosis. This assay is highly sensitive and is specific to cell death due to apoptosis but does not measure necrotic cells. Apoptosense kit has been used successfully in measuring apoptosis in prostate cancer cells (25, 27, 34). On completion of the experiments, cells were harvested and total protein was extracted as described below. Protein extract was added to 96-well plates precoated with mouse monoclonal M30 antibody, and horseradish peroxidase tracer solution was added to the wells and incubated for 4 hours. Color was developed by adding tetramethylbenzidine solution and the absorbance was determined at 450 nm on a Spectra MAX 340 microplate reader (Molecular Devices Corp., Sunnyvale, CA). Standard solution supplied by the supplier was used for generating standard curves. To quantify apoptosis using flow cytometry, cells were stained for Annexin V-FITC (for early apoptosis) and 7-amino-actinomycin D (for late apoptosis). Cells were treated as indicated above and harvested by brief trypsinization and washed with PBS twice. Then, ~1 million...
cells/100 μL were suspended in binding buffer and subjected to Annexin V and 7-amino-actinomycin D staining, provided with the kit (Beckman Coulter, Miami, FL). Cells were incubated on ice and in dark for 15 minutes, and then binding buffer (400 μL) was added to each sample followed by reading within 30 minutes. The cells were analyzed on a Beckman Coulter Quanta flow cytometry system. The protocol was according to the manufacturer’s suggestions.

**Isolation of Mitochondrial Fraction**

Cells were washed and incubated in ice-cold buffer containing 20 mmol/L HEPES-KOH (pH 7.2), 10 mmol/L KCl, 1.6 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, and 250 mmol/L sucrose and protease inhibitor cocktail (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT). Cells were homogenized (100 strokes) on ice with a dounce homogenizer and centrifuged at 1,000 × g for 15 minutes to collect nuclear fraction. The supernatant was again centrifuged at 16,000 × g to obtain mitochondrial fraction that was washed and resuspended in mitochondrial extraction buffer [10 mmol/L Tris-HCl (pH 7.8), 0.1% Triton X-100]. The 1% bovine serum albumin and 5% dextran homogenizer was centrifuged at 30 minutes at 37°C.

**Measurement of Intracellular and Intramitochondrial Calcium**

Cells were detached with trypsin-EDTA, resuspended in fresh medium, washed and resuspended in PBS containing 1% bovine serum albumin and 5 μmol/L Fura 2-AM (Molecular Probes, Carlsbad, CA) or 4 μmol/L rhodamine 2 (Rhod 2)–AM (Molecular Probes), and incubated in dark at 37°C and 5% CO₂ for 40 minutes. Both Fura 2-AM and Rhod 2-AM were dissolved in DMSO containing 20% (w/v) Pluronic F-127 (Molecular Probes) to a stock solution of 5 mmol/L. Cells were washed with PBS and resuspended in HBSS (142 mmol/L NaCl, 5.6 mmol/L KCl, 1 mmol/L MgCl₂, 2 mmol/L KCl, 0.34 mmol/L Na₂HPO₄, 0.44 mmol/L KH₂PO₄, 10 mmol/L HEPES, 5.6 mmol/L glucose). For measurement of calcium, 2 to 3 million cells/mL buffer were transferred into a quartz cuvette placed in a temperature-controlled (37°C) sample compartment of a Hitachi F-2500 fluorescence spectrophotometer (Tokyo, Japan). Cells were treated by direct addition of the drugs into the cuvettes under continuous stirring. Fluorescence intensity was measured at 550 nm excitation and 580 nm emission wavelengths for Rhod 2 to measure mitochondrial calcium or using 340/380 nm excitation and 510 nm emission wavelengths for Fura 2 to measure cytosolic calcium.

**Detection of MMP (∆Ψₘ)**

Detection of ∆Ψₘ was evaluated using the potentiometric indicator, MitoCapture(Alexis Biochemicals, Lausen, Switzerland), which is a fluorescence-based method for distinguishing intact and disrupted mitochondria by detecting the changes in the MMP. Cells were grown in chamber slides to 80% confluency, treated as described above, and incubated with diluted MitoCapture for 30 minutes at 37°C. In healthy cells, MitoCapture accumulates and aggregates in the mitochondria, giving them a bright red fluorescence. In apoptotic cells, MitoCapture cannot aggregate in the mitochondria due to the altered MMP; thus, it remains in the cytoplasm in its monomeric form, fluorescing green. Signals were detected using a Carl Zeiss Axiopt fluorescent microscope and images were captured in a digital CCD camera.

**Measurement of Cellular ATP**

Cells were grown in 96-well plates to desired confluency and treated as described above. Cellular ATP was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega Corp., Madison, WI). The protocol was similar to the manufacturer’s suggestions, except that at the end of treatment, the cells were incubated with the supplied CellTiter-Glo reagent for 15 minutes. The treatment resulted in the lysis of the cell and generation of a luminescent signal proportional to the amount of ATP present. Luminescence signals were measured using Fluostar Optima (BMG Lab Technologies, Durham, NC).

**Results**

**Inhibiting the Na⁺/Ca²⁺ Exchanger and TRAIL Treatment–Induced Apoptosis in Prostate Cancer Cells, DU145**

To determine the effects of altered levels of [Ca²⁺]ₘ on the response to apoptotic agents, prostate cancer cells, DU145, were treated with TRAIL and increasing concentrations of CGP. CGP alone, up to a concentration of 100 μmol/L, did not induce noticeable apoptosis (Fig. 1A). As expected for DU145 cells, treatment with TRAIL alone induced ~7-fold apoptosis compared with untreated controls. Combination of low-dose CGP and TRAIL did not alter the apoptotic response compared with TRAIL alone but higher doses of CGP plus TRAIL induced highly significant apoptosis (~25-fold compared with control or 100 μmol/L CGP alone). The above results were obtained by using M30 Apoptosense ELISA, which measured the neoepitope of cytokeratin 18 that is generated by the activity of caspases. To use another assay for apoptosis, we used flow cytometric analysis using Annexin V as a marker. Results showed that combination of CGP and TRAIL increased Annexin V–positive cells (Fig. 1B). CGP alone did not induce apoptosis, whereas TRAIL treatment resulted in slight increase in apoptotic cells. These results agree with that generated using the M30 assay.

**Inhibition of the Uniporter Did Not Alter TRAIL-Induced Apoptosis**

As the mitochondrial milieu is affected not only by the alterations in Ca²⁺ efflux but also by Ca²⁺ influx into mitochondria, cells were treated with Ru360, a drug known to specifically block the uniporter. Inhibiting the uniporter by Ru360 alone did not induce apoptosis, even when the concentration was increased to 100 μmol/L (Fig. 1C). Furthermore, combining Ru360 with TRAIL did not alter TRAIL-induced apoptosis. To confirm lack of synergy when the uniporter was inhibited, the cells were treated with ruthenium red, a noncompetitive inhibitor of the mitochondrial uniporter that abolishes calcium influx into the mitochondria (30). Similar to Ru360, treatment with...
Ruthenium red alone did not result in apoptosis and its combination with TRAIL did not modify TRAIL-induced apoptosis (Fig. 1D). These results suggested that blocking the influx of Ca^{2+} did not affect mitochondrial response to the apoptotic effects of TRAIL.

Increasing Intracellular Ca^{2+} Is Not Sufficient to Increase CGP-Induced Apoptosis

It may be argued that the lack of response to inhibition of uniporter by Ru360 or ruthenium red is due to the fact that the uptake of Ca^{2+} into the mitochondria may be low in

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**Figure 1.** Effects of inhibiting the transport of Ca^{2+} into and out of the mitochondria and treatment with TRAIL on apoptotic response of prostate cancer cells. A, prostate cancer cells, DU145, were treated with increasing concentrations (1-100 μmol/L) of benzothiazepin CGP-37157 in the absence (CGP) or presence of 200 ng/mL TRAIL (CGP + TRAIL). Apoptosis was measured using M30 Apoptosense kit and is expressed as M30 antigen units/μg protein compared with control. B, flow cytometric analysis for Annexin V staining. DU145 cells were treated with vehicle (control), TRAIL, CGP-37157, or combination (Combo) as described earlier and stained for Annexin V. Bottom right quadrant, apoptotic cells. C, cells were treated with 1, 10, or 100 μmol/L Ru360 (RU or with both Ru360 and 200 ng/mL TRAIL (RU + TRAIL)). Apoptosis was measured and expressed as described earlier. D, DU145 cells were treated with 1, 10, 30, or 100 μmol/L ruthenium red (RRed) or with both ruthenium red and 200 ng/mL TRAIL (RRed + TRAIL). Apoptosis was measured and expressed as described earlier. E, DU145 cells were treated with 1 or 10 μmol/L calcium ionophore, ionomycin (Iono), or with both ionomycin and 80 μmol/L CGP-37157 (Iono + CGP) or with both thapsigargin and 80 μmol/L CGP-37157 (CGP + Thaps). Apoptosis was measured and expressed as described earlier. F, DU145 cells were treated with 1 or 10 μmol/L thapsigargin (Thaps) or with both thapsigargin and 80 μmol/L CGP-37157 (CGP + Thaps). Apoptosis was measured and expressed as described earlier. G, androgen-responsive prostate cancer cells, LNCaP, were treated with 1, 10, or 80 μmol/L CGP-37157 (CGP) or with both CGP and 200 ng/mL TRAIL. Apoptosis was measured and expressed as described earlier. *Inset*, expression of Bax and Bcl2 in DU145 and LNCaP cells. The blots were stripped and probed for β-actin that was used as loading control.
DU145. It has been shown that mitochondrial uptake of Ca\(^{2+}\) is significantly lower in some Bax-deficient cells (35, 36), such as DU145. Therefore, it was hypothesized that increasing the pool of [Ca\(^{2+}\)]\(_i\), may help increase the transport of Ca\(^{2+}\) into the mitochondria, leading to apoptosis. To test this hypothesis, the cells were treated with calcium ionophore, ionomycin (37), which is known to increase [Ca\(^{2+}\)]\(_i\). Interestingly, under the present experimental conditions, ionomycin did not induce apoptosis and was not sufficient in causing apoptosis in combination with CGP (Fig. 1E), suggesting that increased [Ca\(^{2+}\)]\(_i\), alone is not sufficient for inducing apoptosis. To test these further, cells were treated with thapsigargin that is also known to increase [Ca\(^{2+}\)]\(_i\), albeit via a different mechanism that include the endoplasmic reticulum mediation (38). At the low concentrations used in these experiments, thapsigargin alone induced little apoptosis (Fig. 1F). Treatment with CGP and the lowest tested concentration of thapsigargin (1 \(\mu\)mol/L) induced 6-fold apoptosis, a response that increased to 10-fold with higher concentration (10 \(\mu\)mol/L) of thapsigargin (Fig. 1E). It has been shown that perturbing the functions of the endoplasmic reticulum results in the induction of mitochondria-mediated apoptosis (39). Therefore, in these experiments, thapsigargin/CGP-induced apoptosis may be due to the endoplasmic reticulum-mitochondria-mediated apoptosis. Furthermore, it has been shown that treatment with thapsigargin increased transcription and translation of death receptor DR5, a key protein in TRAIL-induced apoptosis (40). Thus, thapsigargin-mediated apoptosis is not due to increased levels of [Ca\(^{2+}\)]\(_i\), alone, as increasing [Ca\(^{2+}\)]\(_i\), by treatment with ionomycin did not induce apoptosis.

**CGP- and TRAIL-Induced Apoptosis in Androgen-Responsive Prostate Cancer Cells, LNCaP.**

The first line of treatment for prostate cancer is androgen ablation therapy, which induces apoptosis in androgen-responsive cells. However, treatment options for androgen-independent cancer are limited; therefore, newer treatments are being investigated. In the present studies, androgen-independent DU145 prostate cancer cells were used to investigate the efficacy of combination therapy with TRAIL and CGP. To examine whether the combination therapy of TRAIL/CGP is also effective in androgen-responsive cells, LNCaP prostate cancer cells were treated with increasing concentrations of CGP and/or TRAIL (Fig. 1G). Similar to DU145 cells, even 80 \(\mu\)mol/L CGP by itself did not induce obvious apoptosis. TRAIL by itself did not induce apoptosis in LNCaP cells, as it is well documented by several laboratories, including ours, that LNCaP cells are resistant to TRAIL. However, it is impressive that even the TRAIL-resistant LNCaP cells were highly responsive to the combination of CGP and TRAIL, as the treatment resulted in >20-fold increase in apoptosis. These results confirmed that both androgen-responsive and androgen-independent prostate cancer cells respond to combination of CGP and TRAIL. Furthermore, DU145 cells are devoid of key members of the Bcl\(_2\) family, Bcl\(_2\) and Bax proteins, whereas LNCaP cells are wild-type for these proteins (Fig. 1G, inset). Thus, the combination of CGP and TRAIL induced apoptosis not only in the androgen-dependent and androgen-independent cells but also in cells that lack key Bcl\(_2\) proteins. As the responses of DU145 and LNCaP cells were similar, further experiments were conducted only in DU145 cells.

**Induction of CGP/TRAIL-Induced Apoptosis Follows Increased [Ca\(^{2+}\)]\(_m\).**

To confirm that the synergistic response of DU145 to TRAIL and CGP is indeed due to changes in mitochondrial Ca\(^{2+}\), the [Ca\(^{2+}\)]\(_m\) was measured using specific markers. As expected, the treatment with CGP significantly increased the levels of [Ca\(^{2+}\)]\(_m\) (Fig. 2A), although this treatment did not induce apoptosis (Fig. 1A). TRAIL by itself did not alter the [Ca\(^{2+}\)]\(_m\) (Fig. 2B), although it is well known that TRAIL-induced apoptosis involves the mitochondria. Similar measurements in cells treated with both TRAIL and CGP showed that the increased levels of [Ca\(^{2+}\)]\(_m\) were not different compared with CGP alone (Fig. 2C), confirming that the increase in [Ca\(^{2+}\)]\(_m\) is mainly due to CGP but not due to TRAIL treatment. Furthermore, this also indicated that synergistic apoptotic response to CGP and TRAIL (compared with CGP alone) was not due to increased accumulation of [Ca\(^{2+}\)]\(_m\), but may be due to increased sensitivity of the mitochondria to TRAIL in CGP-treated cells. This observation was supported by increased [Ca\(^{2+}\)]\(_m\).
in ionomycin-treated (Fig. 2D) or ionomycin/CGP-treated cells (Fig. 2E), although this treatment did not yield appreciable apoptosis (Fig. 1E). Thus, increased apoptotic response of DU145 cells to CGP and TRAIL does not seem to be due to increased \([\text{Ca}^{2+}]_{\text{m}}\) alone but is the result of increased sensitization of mitochondria to TRAIL caused by increased \([\text{Ca}^{2+}]_{\text{m}}\).

**Apoptosis Induced by the Inhibition of Na+/Ca2+ Exchanger and TRAIL Is Mediated through the Mitochondria**

Prior studies have suggested that \(\text{Ca}^{2+}\) overload in the mitochondria induced changes in the mitochondria, such as alterations in MMP and formation of PTP, key steps in the mitochondrial apoptotic response. We and others have established that the apoptotic response of prostate cancer cells to TRAIL involves similar mitochondrial changes. As the results described above indicate that combined treatment with TRAIL and CGP resulted in synergistic apoptosis, the involvement of the mitochondria in the above response was confirmed by determining the changes in the premitochondrial and postmitochondrial events. TRAIL treatment activated procaspase-8 (55 kDa) as seen by the presence of proteolytic bands of 40 and 23 kDa (Fig. 3A). Treatment with CGP alone did not activate caspase-8, which supports the apoptosis data (Fig. 1A). However, combination of both CGP and TRAIL resulted in significant increase in activated products, which correlated with higher apoptotic response in this group. Activation of procaspase-8 results in the truncation of Bid, a proapoptotic with higher apoptotic response in this group. Activation of caspase-9 is responsible for apoptosis significantly (Fig. 3A). Doses of 20 \(\mu\text{mol/L}\) or above reduced apoptotic response by \(\sim 50\%\), confirming that, in cells treated with both CGP and TRAIL, apoptosis was induced through the formation of the PTP.

Finally, dissipation of MMP has been shown to stop ATP synthesis, and the reduction in cellular ATP affects cellular homeostasis leading to cellular death by apoptosis, whereas further decrease in ATP causes necrotic death (32, 33). To determine whether the apoptotic changes due to TRAIL/CGP is mediated through alterations in ATP, cellular ATP was measured. Treatment of cells with TRAIL did not alter ATP levels compared with controls (Fig. 3F), which support our data that showed no increase in mitochondrial \(\text{Ca}^{2+}\) in these cells (Fig. 2B). However, treatment with CGP decreased ATP levels, which corroborates with significant increase in mitochondrial \(\text{Ca}^{2+}\) (Fig. 2A). Treatment with both TRAIL and CGP did not significantly alter the levels of ATP compared with CGP alone, suggesting that the drop in ATP is primarily due to CGP-induced increase in the mitochondrial \(\text{Ca}^{2+}\). Furthermore, this also supports our hypothesis that synergistic apoptosis in CGP plus TRAIL-treated cells is due to sensitization of mitochondria by CGP that facilitated TRAIL-induced, mitochondria-mediated apoptosis.

**Discussion**

Early research into apoptosis focused on the nucleus because of the discernible morphologic changes (41). However, data from the last few years have identified mitochondria as a key organelle involved in apoptosis. Recent research showed correlation between increased mitochondrial \(\text{Ca}^{2+}\) and mitochondria-mediated apoptosis. Furthermore, the demonstration that \(\text{Ca}^{2+}\) is required for several apoptotic events in the mitochondria solidified the idea that increased levels of mitochondrial \(\text{Ca}^{2+}\) is critical for apoptosis. For example, in some cells, inhibiting the transport of \(\text{Ca}^{2+}\) into the mitochondria through the uniporter reduced or blocked mitochondria-mediated apoptosis.
apoptosis, strengthening the suggestion that higher levels of \([\text{Ca}^{2+}]_{\text{m}}\) may be critical in this response (7, 30, 31). Earlier results from our and other laboratories have shown that TRAIL induced apoptosis in prostate cancer cells through the involvement of the mitochondria. Therefore, the goals of these experiments were 2-fold: (a) to examine whether TRAIL-induced apoptotic changes are mediated through alterations in \([\text{Ca}^{2+}]\) and (b) to determine the advantages of manipulating \([\text{Ca}^{2+}]_{\text{m}}\) on the response of the cells to TRAIL. We show that treatment of prostate cancer cells, DU145, with TRAIL induced apoptosis without altering the levels of \([\text{Ca}^{2+}]_{\text{m}}\) suggesting that TRAIL-induced apoptosis is due to other events, such as activation of procaspase-8 and truncation of tBid that affect the mitochondria (24, 42). TRAIL is an effective apoptotic agent by itself or in combination with other apoptotic drugs. We have shown synergistic increase in apoptotic response when TRAIL was combined with antiprogestin mifepristone (43) or by inhibiting the function of heat shock protein 90 (27, 34), inhibiting the proteasomal activity (34), or treatment with histone deacetylase inhibitor, SAHA (25). Our present results show that treatment with both TRAIL and CGP

Figure 3. Induction of apoptosis by the inhibition of \(\text{Na}^{+}/\text{Ca}^{2+}\) exchanger and TRAIL treatment is mediated through the mitochondria. DU145 cells were treated with vehicle (C), TRAIL (TR), CGP-37157 (CGP), or both as described in Materials and Methods. At the end of the experiments, cells were harvested and processed for extraction of total protein or for separating the cytosolic and mitochondrial fractions. A, total proteins were analyzed for the presence of procaspase-8 (55 kDa) and activated proteolytic products (40 and 23 kDa). The blots were stripped and reprobed for the presence of \(\beta\)-actin, which was used as loading control. Cytosolic and mitochondrial fractions were probed for the presence of total Bid (24 kDa) or tBid (15 kDa). \(\beta\)-Actin was used as loading control for cytosolic proteins, whereas the levels of Cox IV were used as loading control for mitochondrial fractions. Right, numbers, molecular mass in kDa. B, MMP was determined in control, TRAIL treated (TRAIL), CGP (CGP-37157), or both. Red fluorescence is indicative of intact mitochondria, whereas green fluorescence is indicative of loss of MMP. Yellow fluorescence is a combination of intact and apoptotic mitochondria. C, cytosolic and mitochondrial fractions were analyzed for the presence of cytochrome c. Expression of \(\beta\)-actin was used for loading controls of cytoplasmic fractions, whereas Cox IV was used as loading control for mitochondrial fractions. Right, numbers, molecular mass in kDa. D, total protein was analyzed for the activation of caspase-9, caspase-3, or poly(ADP-ribose) polymerase. \(\beta\)-Actin was used as loading control for cytosolic fractions, whereas Cox IV was the loading control for mitochondrial fractions. Right, numbers, molecular mass in kDa. E, to examine the participation of cyclophilin D in the formation of PTP, cells were treated with increasing concentrations of 20 to 50 \(\mu\)mol/L cyclosporin A (CA) alone or with 50 \(\mu\)mol/L CGP and 200 ng/mL TRAIL. Apoptosis was measured and expressed as described earlier. F, to measure the effect of treatment on cellular ATP, cells were treated as above and cellular ATP was measured using the CellTiter-Glo Luminescent Cell Viability Assay kit. The values were expressed as arbitrary units.
resulted in synergistic apoptosis, indicating the advantages of combining the two drugs to achieve increased apoptosis. Our observation that treatment with CGP altered the levels of mitochondrial Ca²⁺ suggested that altering mitochondrial milieu may be responsible for the apoptotic response to TRAIL. This was confirmed by our observations that blocking the uniporter and treatment with TRAIL did not affect apoptosis. Furthermore, to address the question whether global increase in cellular Ca²⁺ or specific increase in mitochondrial Ca²⁺ is responsible for facilitating apoptosis, cells were treated with ionomycin. As expected from published literature, ionomycin increased both [Ca²⁺]ₗ and [Ca²⁺]ₘ levels, but these changes did not translate into higher apoptosis, supporting the hypothesis that retention of Ca²⁺ in the mitochondria is responsible for the sensitization of the mitochondria to the effects of TRAIL, which resulted in significantly higher apoptosis. On the other hand, treatment with CGP and thapsigargin increased [Ca²⁺]ₗ, and concomitantly increased apoptosis, suggesting that thapsigargin-induced apoptosis is due to the disruption of the endoplasmic reticulum function and/or increased expression of DR5.

The involvement of the mitochondria in these events was confirmed by the observations that treatment with both CGP and TRAIL showed significantly higher levels of tBid and its transport into the mitochondria. Furthermore, we have shown the loss of MMP, release of the cytochrome c into the cytoplasm, activation of caspase-9 and caspase-3, all events indicating apoptotic response of the mitochondria. Although these changes have been recorded in cases of Ca²⁺ overload in the mitochondria, it is interesting that combination of TRAIL/CGP significantly increased these effects. Although our results show increase in [Ca²⁺]ₘ and classic apoptotic response of the mitochondria in CGP-treated cells, at this juncture, the exact mechanism(s) that affect apoptosis. Our observation that treatment with CGP altered the levels in [Ca²⁺]ₘ suggests that CGP was responsible for the increase in [Ca²⁺]ₘ, which led to decreased ATP and subsequent apoptosis. Thus, under these conditions, either apoptotic response does not require the involvement of Bcl2 family members or other proapoptotic members such as Bak may be sufficient for the response. Further experiments are being conducted to investigate these aspects.

Another reason for the increased apoptotic response may be the decrease in cellular ATP. Dissipation of MMP has been shown to stop ATP synthesis leading to the reduction in cellular ATP, which affects cellular homeostasis inducing apoptosis (44, 45). It is known that deprivation of ATP results in the loss of glycine followed by breakdown of the membranes leading to cell death. In the present study, measurement of ATP showed no differences between control and TRAIL-treated cells, whereas treatment with CGP in the absence or presence of TRAIL resulted in significant loss of ATP, which showed that one of the mechanisms of induction of apoptosis is by the loss of ATP. The observation that loss of ATP correlated with increased [Ca²⁺]ₘ suggests that CGP was responsible for the increase in [Ca²⁺]ₘ, which led to decreased ATP and subsequent apoptosis.

In conclusion, the above results show that retention of Ca²⁺ in the mitochondria facilitates mitochondria-mediated apoptosis. Our results provide an excellent model to investigate the role of Ca²⁺ in the mitochondria and to investigate the mitochondrial mechanisms leading to apoptosis. Furthermore, these results show therapeutic advantages of combining TRAIL and CGP, especially because TRAIL is a promising apoptogenic agent that preferentially affects cancer cells, whereas CGP belongs to a group of drugs that affect Na⁺/Ca²⁺ exchangers. Inhibitors of Na⁺/Ca²⁺ exchangers are presently being used for treating cardiovascular and neurologic diseases (32, 33). Thus, this article emphasizes the clinical advantages of combined therapy and, at the same time, offers an excellent experimental model for further investigation of the role of Ca²⁺ in the mitochondrial apoptosis.

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