Multiple, disparate roles for calcium signaling in apoptosis of human prostate and cervical cancer cells exposed to diindolylmethane

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Abstract

Diindolylmethane (DIM), derived from indole-3-carbinol in cruciferous vegetables, causes growth arrest and apoptosis of cancer cells *in vitro*. DIM also induces endoplasmic reticulum (ER) stress, and thapsigargin, a specific inhibitor of the sarcoplasmic reticulum/ER calcium-dependent ATPase, enhances this effect. We asked whether elevated cytosolic free calcium $[Ca^{2+}]_i$ is required for cytotoxicity of DIM and thapsigargin in two cancer cells lines (C33A, from cervix, and DU145, from prostate). $[Ca^{2+}]_i$ was measured in real-time by FURA-2 fluorescence. We tested whether DIM, thapsigargin, and DIM + thapsigargin cause apoptosis, measured by nucleosome release, under conditions that prevented elevation of $[Ca^{2+}]_i$, using both cell-permeable and cell-impermeable forms of the specific calcium chelator BAPTA. DIM, like thapsigargin, rapidly mobilized ER calcium. C33A and DU145 responded differently to perturbations in Ca^{2+} homeostasis, suggesting that DIM induces apoptosis by different mechanisms in these two cell lines and/or that calcium mobilization also activates different survival pathways in C33A and DU145. Apoptosis in C33A was independent of increased $[Ca^{2+}]_i$, suggesting that depletion of ER Ca^{2+} stores may be sufficient for cell killing, whereas apoptosis in DU145 required elevated $[Ca^{2+}]_i$ for full response. Inhibitor studies using cyclosporin A and KN93 showed that Ca^{2+} signaling is important for cell survival but the characteristics of this response also differed in the two cell lines. Our results underscore the complex and variable nature of cellular responses to disrupted Ca^{2+} homeostasis and suggest that alteration Ca^{2+} homeostasis in the ER can induce cellular apoptosis by both calcium-dependent and calcium-independent mechanisms. [Mol Cancer Ther 2006;5(3):556–63]

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Introduction

3,3′-Diindolylmethane (DIM) is a major bioactive derivative of the dietary phytochemical indole-3-carbinol. Both compounds are effective in prevention and treatment of cancers in animal models and in human patients (1–5) as well as for treatment of recurrent laryngeal papillomatosis in humans [reviewed by Auborn (6)] and for mitigation of pathophysiologic effects of lupus in an animal model (7). *In vitro*, DIM is toxic to human cancer-derived cells at concentrations that approach those measured in tissues after pharmacologic administration (8); cell death is often accompanied by physiologic and cytologic end points typical of apoptosis (9–13).

Multiple changes in gene expression occur within hours of exposure to both DIM (14, 15) and indole-3-carbinol (16), followed soon after by cell cycle arrest (17–19), which may result at least in part from altered transcription factor interactions (14, 20). Early biochemical changes in cells exposed to DIM or indole-3-carbinol include activation of the aryl hydrocarbon receptor (21–24) and peroxisome proliferator-activated receptor γ (25), and circumstantial evidence exists for agonist/antagonist interactions with the receptors for estrogen (24, 26–29) and androgen (30). Among the individual regulatory proteins and signaling pathways affected by DIM and indole-3-carbinol are c-jun NH_2-terminal kinase (31, 32) and p38 mitogen-activated protein kinase (32), NAG-1 (15), AKT (33), nuclear factor κB (34, 35), bcl-2 (36), and other changes that activate the mitochondrial-mediated cell death pathway (37), induction of the family of growth arrest and DNA-damage (*GADD*) genes, and changes in mRNA levels of many other genes that could affect cell cycling and survival (14). It is unclear how DIM initiates these changes and which changes are crucial for the cytostatic and cytotoxic effects of DIM.

Many immediate biochemical responses to DIM are typical of cells undergoing endoplasmic reticulum (ER) stress (reviewed in ref. 38). However, cancer cells *in vitro* are also sensitized to DIM by other agents and conditions that cause nutritional stress (31), such as a combination of glucose limitation, starvation for amino acids, and induction of the hypoxia-inducible factor, or by the drug thapsigargin, which induces ER stress by disrupting

calcium homeostasis in the ER (39, 40). This latter observation suggests that Ca^{2+} dynamics play an important role in DIM cytotoxicity. In this communication, we examine the role of Ca^{2+} homeostasis and signaling in the response to DIM of two cell lines derived from cancers for which indole-3-carbinol (and/or DIM) is a potential therapeutic agent, based on clinical trials and preclinical studies (1, 2, 4). C33A is an estrogen-independent cervical cancer cell for which there are extensive *in vitro* data on DIM effects, and DU145 is an androgen-independent prostate cancer cell line that resembles C33A in its sensitivity to DIM.[3]

Materials and Methods

Cells and Cell Culture

C33A and DU145 cells were obtained from the American Type Culture Collection (Manassas, VA) and propagated in monolayer culture in DMEM containing 10% fetal bovine serum as described (14, 31).

Cell Viability

Cell viability was measured by a mitochondrial function assay involving reduction of tetrazolium dye (CellTiter96, Promega, Madison, WI) in 96-well plates according to the instructions of the manufacturer.

Apoptosis

Apoptosis was measured by an ELISA that detects release of nucleosomes into the cytoplasm (Cell Death Detection ELISA^{PLUS}, Roche, Indianapolis, IN). Results were normalized to cell number, determined by direct counting in a hemocytometer.

Calcium Clamping

Calcium clamping was accomplished by incubating monolayer cultures in DMEM without serum, containing 1.69 mmol/L BAPTA (glycine, *N,N′*-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-(carboxymethyl)]-, tetrasodium salt; cell impermeant; Molecular Probes, Eugene, OR) to prevent influx via store-operated Ca^{2+} channels (41) and 7 μmol/L esterified BAPTA-AM (*N,N′*-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-[2-[(acetyloxy) methoxy]-2-oxoethyl]-, bis[(acetyloxy)methyl] ester: cell permeant, Molecular Probes) added 1 hour before DIM, re-added at 12 hours, and present for the duration of the experiment (17–24 hours). BAPTA-AM is retained in cytosol after being hydrolyzed to BAPTA by esterases. The entire experiment was conducted in the absence of serum to minimize hydrolysis of the esterified BAPTA by serum esterases. Withholding serum also reduced the concentration of DIM required to obtain cytotoxic effects while not itself inducing ER stress.[3] These conditions together effectively buffer the $[Ca^{2+}]_i$ and have been called a ''calcium clamp'' (41). Clamping efficacy was determined by challenging clamped cells with 300 nmol/L thapsigargin and measuring calcium release as described below.

Intracellular (Cytosolic) Free Calcium $[Ca^{2+}]_i$ Measurement

The fluorescent calcium indicator FURA-2AM (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzoFURAn-5-oxy]-2-(2-amino-5-methylphenoxy)-ethane-*N,N,N′N′*-tetraacetic acid pentaacetoxymethyl ester, Molecular Probes) was used to measure changes in intracellular calcium. Cells were resuspended at a density of 10^6/mL for C33A and 5×10^5/mL for DU145 in HBSS or α-MEM containing Ca^{2+} and supplemented with 25 mmol/L HEPES, 0.1% bovine serum albumin, and 1.5 μmol/L FURA-2AM (Molecular Probes). Cell suspensions were incubated at 25°C with gentle stirring for 30 minutes and protected from light. This temperature was used to minimize redistribution of the FURA-2 to secondary intracellular compartments. After loading, cells were washed thrice in isotonic buffer without Ca^{2+} (132 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L Na_2HPO_4, 1.2 mmol/L NaH_2PO_4, 0.8 mmol/L $MgCl_2$) and resuspended in this buffer at a density of 5×10^5/mL. Changes in $[Ca^{2+}]_i$ were determined ratiometrically (340 nm/380 nm excitation, 512 nm emission) in 2-mL aliquots using a spectrofluorometer (Photon Technology International, Model 610 photomultiplier detection system). Calcium concentrations were calculated using the following equation (43): $[Ca^{2+}]_i = K_d(F_{380}\text{max}/F_{380}\text{min})\ (R - R_{\text{min}})\ /\ (R_{\text{max}} - R)$; a K_d value of 224 nmol/L was assumed for the binding of calcium to FURA-2. R_{max} and R_{min} were determined in each experimental group by the consecutive addition of 30 μmol/L digitonin (R_{max}) and 50 mmol/L EGTA (R_{min}).

Statistical Analysis of Data

SEs for replicate data were calculated and displayed as error bars in all figures. Differences between mean values for different treatments were calculated using two-tailed unpaired Student's t test and were considered to be significant at $P < 0.05$.

Inhibitors

Thapsigargin was obtained from Sigma Chemical Corp. (St. Louis, MO), dissolved in DMSO to make a 10 mmol/L stock, and stored frozen. U73122 and U73433 were obtained from Calbiochem (LaJolla, CA), dissolved in DMSO at 1 mmol/L, and used at a final concentration of 10 μmol/L. U73122 is an aminosteroid inhibitor of G-protein–coupled phospholipase C activation. U73433, the inactive analogue of U73122, was a negative control. U73122 activity was verified by its ability to cause a rapid increase in basal cytosolic calcium (data not shown) as previously described for this compound (43).

Results

Disruption of Calcium Homeostasis Sensitizes Cancer Cells to Growth Inhibition by DIM

Thapsigargin, a nonreversible inhibitor of the sarcoplasmic reticulum/ER–associated calcium-activated ATPase (SERCA; ref. 44), causes rapid depletion of ER Ca^{2+} stores and prolongs the transient elevation of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). The relative high potency of thapsigargin as a

[3] Our unpublished results.

sensitizing agent for DIM cytotoxicity led us to investigate the sensitizing effects of other agents that disrupt cellular calcium homeostasis. Figure 1 shows that ruthenium red, which inhibits mitochondrial uptake of Ca^{2+} ions from the cytosol (45), had a sensitizing effect similar to thapsigargin (90% loss of viability in the presence of the inhibitor together with DIM, compared with 20% reduction of viability in the presence of DIM alone). The calcium ionophore A23187 also enhanced DIM cytotoxicity (35% loss of viability). High doses of celecoxib inhibit the SERCA pump (46), and celecoxib, too, sensitized C33A cells to DIM (43% loss of viability). In contrast, tunicamycin, which induces ER stress by interfering with protein glycosylation in the ER lumen, did not sensitize cells to DIM under these conditions (Fig. 1).

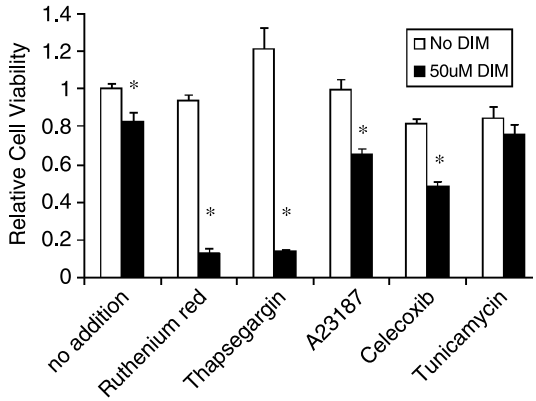

Figure 1. Disruption of calcium homeostasis increases the cytotoxic effect of DIM on C33A cells. Semiconfluent cells growing in monolayer were exposed to the indicated agents (ruthenium red, 25 μmol/L; thapsigargin, 300 nmol/L; A23187, 2.5 mmol/L; celecoxib, 20 μmol/L; tunicamycin, 5 μg/mL) with and without addition of 50 μmol/L DIM or 0.1% DMSO (solvent control, *no addition*) for 48 h, and cell viability was measured as described in Materials and Methods. Relative cell viability was calculated by normalizing the mean absorbance at 595 nm in eight replicate experimental samples to the mean of eight replicate cell cultures exposed only to DMSO. *Bars,* SD. The relative cytotoxic effect of DIM (% decrease in viable cell number relative to DMSO-treated cells) was significantly greater than control cultures in every case where a calcium disruptor was also used (*, $P < 0.05$) but not for tunicamycin-treated cells.

DIM Causes Rapid Mobilization of ER Ca^{2+} Stores

If disruption of Ca^{2+} homeostasis were either necessary or sufficient for DIM cytotoxicity, DIM alone might be expected to influence intracellular Ca^{2+} distribution. In DU145 cells, DIM caused a rapid elevation of $[Ca^{2+}]_i$, followed by partial clearance over a period of about 3 minutes (Fig. 2A). Subsequent addition of thapsigargin stimulated a small secondary increase in $[Ca^{2+}]_i$ that did not exceed the peak $[Ca^{2+}]_i$ attained after the initial DIM challenge. This kinetic profile was similar to that caused by thapsigargin (Fig. 2C), except that the peak $[Ca^{2+}]_i$ in DIM-treated cells was slightly lower and recovery (clearance of Ca^{2+} from the cytosol) was slower. C33A cells also responded to both DIM and thapsigargin challenges with a burst of cytosolic free Ca^{2+} but the kinetic profiles differed from DU145. Elevation of $[Ca^{2+}]_i$ developed much more slowly in DIM-treated C33A cells (Fig. 2B) and no recovery was evident for at least the 7-minute duration of the experiment. In contrast, thapsigargin released ER Ca^{2+} in C33A (Fig. 2D) with a time course very similar to that caused by DIM in DU145 (Fig. 2A). As in the case of DU145, subsequent addition of DIM to thapsigargin-treated C33A cells caused little further elevation of $[Ca^{2+}]_i$. These different patterns of Ca^{2+} mobilization were highly reproducible in three separate experiments. DIM and thapsigargin mobilization of Ca^{2+} stores in MCF-7 breast cancer cells had kinetics similar to DU145 (data not shown).

The results of Ca^{2+} release experiments are consistent with the hypothesis that both DIM and thapsigargin affect primarily the same intracellular pool of stored Ca^{2+}, most likely Ca^{2+} sequestered in the ER, because this pool is depleted by thapsigargin. A major physiologic pathway for ER Ca^{2+} release is activation of the ryanodine receptor by the second messenger inositol 1,4,5-trisphosphate, generated by the activation of phospholipase C. However, neither the phospholipase C inhibitor U73122 nor its inactive analogue U73433 altered the ability of DIM to trigger a burst of $[Ca^{2+}]_i$ (data not shown).

Elevated Cytosolic Calcium Contributes to DIM-Induced Apoptosis in DU145 but Is Not Required for DIM Cytotoxicity in C33A

Prolonged or inappropriate elevation of $[Ca^{2+}]_i$ has profound effects on cell physiology and survival (reviewed in ref. 47); however, an equally important consequence of chronic depletion of ER-sequestered Ca^{2+} is disruption of protein folding (40, 44, 47), leading to the condition of ER stress observed after treatment with many agents, including DIM (31). To distinguish between these two potential mechanisms of DIM-induced cytotoxicity, we buffered $[Ca^{2+}]_i$ by loading cells with the chelating agent BAPTA-AM and reducing external Ca^{2+} (calcium clamp) as described in Materials and Methods (44). Calcium clamp for 24 hours (renewed at 12 hours) prevented elevation of cytosolic calcium when cells were challenged with thapsigargin (Fig. 3A, *inset*), confirming that clamp conditions persisted throughout this period. Clamping conditions were rapidly established because addition of thapsigargin 1 hour after clamping did not result in detectable $[Ca^{2+}]_i$ elevation (data not shown). One hour after adding BAPTA-AM, cells were exposed to thapsigargin, DIM, or a combination of the two for a further 24 hours. Cytoplasmic extracts were then analyzed by ELISA for released nucleosomes as an indication of cellular commitment to apoptosis.

When DU145 cells were exposed to either thapsigargin or DIM for 24 hours, commitment to apoptosis was evident by a doubling in released nucleosomes compared with the basal level in untreated cells; Ca^{2+} clamping throughout the period of thapsigargin exposure nearly eliminated this increase (Fig. 3, *top left*). The same result was obtained when DIM was added together with thapsigargin, which caused a much more robust 15-fold increase in nucleosome release, illustrating the synergistic effects of the two agents (31). This increase was reduced

by calcium clamping to half the level measured in unclamped cells. Similar results were obtained when cell proliferation was used as an end point instead of apoptosis: DIM, thapsigargin, and the combination of the two all reduced the number of viable cells after 24 hours, and Ca^{2+} clamping protected cells against either agent alone although it failed to do so when the cells were treated with the more potent combination of the two agents (Fig. 3, *bottom left*), suggesting that the apoptotic response and growth inhibition may use different pathways. Clamping alone had no detectable effect on proliferation of DU145 cells. In contrast to DU145, the cervical cancer cell line C33A was not protected by Ca^{2+} clamping from growth inhibition and apoptosis caused by either agent alone or in combination (Fig. 3, *right*).

Calcium-Activated Processes Increase Cell Survival after Exposure to DIM

Two important pathways for Ca^{2+} signaling in the cytosol with profound and varied downstream effects involve calmodulin-mediated protein phosphorylation via calmodulin-dependent protein kinase II and calcineurin-catalyzed protein dephosporylation; each pathway has established roles in cell survival, proliferation, and apoptosis, depending on the experimental system (46). We asked whether either of these two pathways is involved either in the apoptotic effect of DIM and thapsigargin in DU145 cells or in the possible prosurvival effect of elevated calcium in DIM-treated C33A cells. Cells were treated with DIM and thapsigargin, singly and in combination, for 24 hours as above, but this time in the presence of either KN93, an inhibitor of calmodulin-dependent protein kinase II, or cyclosporin A, which inhibits calcineurin. Surprisingly, both inhibitors increased the apoptotic and growth-inhibitory effects of the potent combination of thapsigargin + DIM in both cell lines (Fig. 4). Nucleosome release in DU145 treated with both thapsigargin and DIM was stimulated between 3- and 5-fold in DU145 and nearly 3-fold in C33A. The effects of the inhibitors on cells treated with either DIM or thapsigargin alone were less dramatic and more varied. This may be ascribed, in part, to our deliberate use of thapsigargin and DIM at low concentrations chosen to show their synergistic effect when used in combination. However, even in these subacute conditions, cyclosporin A alone consistently increased apoptosis and decreased cell survival in C33A treated with either thapsigargin or DIM.

If calcineurin, the proximal target of cyclosporin A, were directly involved in an adaptive response of C33A cells to increased $[Ca^{2+}]_i$ treated with DIM, then cyclosporin A should make cells hypersensitive to DIM, resulting in cell killing at lower concentrations when compared with cells without cyclosporin A. Furthermore, this hypersensitivity should be enhanced when cells are also exposed to thapsigargin, which also releases ER Ca^{2+} stores. Results in Fig. 4 were consistent with this hypothesis. As a further test of this hypothesis, the dose response to DIM was

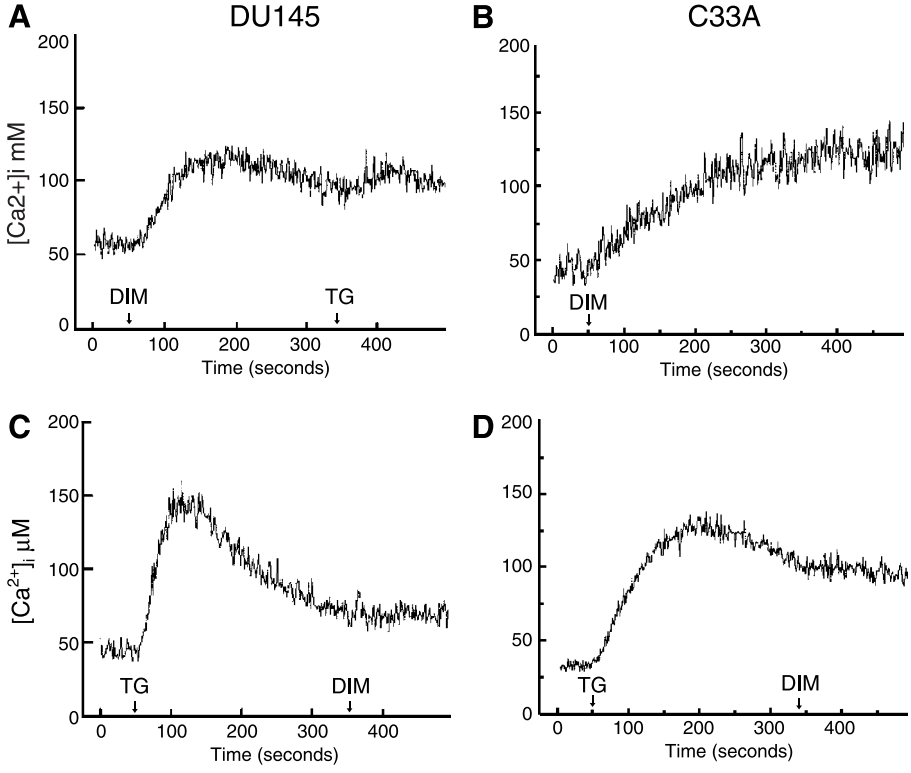

Figure 2. Cytosolic free calcium concentration in DU145 and C33A cells after acute exposure to DIM and thapsigargin. Cell suspensions were loaded with FURA-2AM, and $[Ca^{2+}]_i$ was measured continuously as described in Materials and Methods. Thapsigargin (300 nmol/L) and DIM (25 μmol/L) were added at indicated times (50 and 300 sec after the start of measurements). **A** and **C,** DU145. **B** and **D,** C33A. Representative of at least three separate experiments for each cell line, which all gave the same temporal patterns of calcium release and clearance.

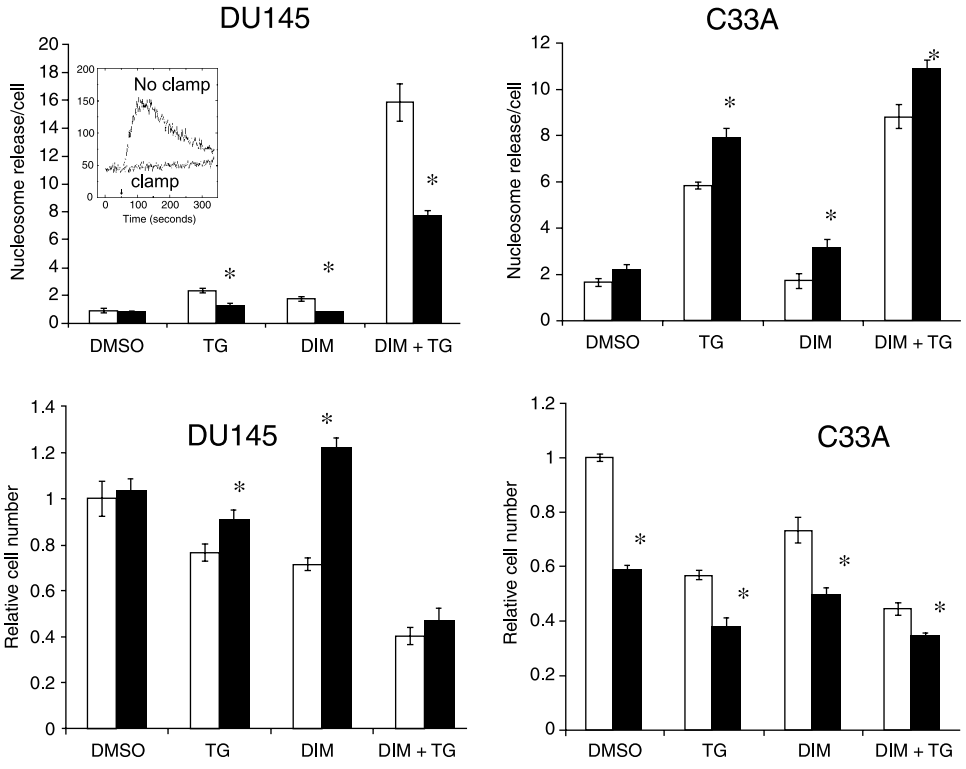

Figure 3. Effect of calcium clamping on cell growth and apoptosis after treatment with DIM and thapsigargin. Three separate sets of six replicate monolayer cultures of DU145 and C33A were exposed to clamp conditions or DMSO control as described in Materials and Methods; 1 h later, DIM (25 μmol/L), thapsigargin (300 nmol/L), or DIM + thapsigargin was added (or 0.1% DMSO as solvent control). Clamping was renewed after 12 h. Twenty-four hours after addition of DIM and thapsigargin, one culture from each set was trypsinized and cell number determined microscopically using a hemocytometer. A second culture was lysed and analyzed in triplicate for cytosolic chromatin by ELISA as described in Materials and Methods. This was repeated twice more for each condition. Relative cell number was determined by normalizing the cell counts in each condition to the average cell number in unclamped, DMSO-treated cultures. Nucleosome release/cell (apoptotic index) was calculated by dividing the average ELISA absorbance for each experimental condition by the relative cell number and setting the background level of nucleosome release in untreated C33A cells to a value of 1.0. *Columns,* mean apoptotic index for the three sets of cultures; *bars,* SE. *, $P < 0.05$, clamped versus unclamped culture. *Inset,* persistent effect of a 24-h calcium clamp on cytosolic free calcium concentration in DU145 cells after exposure to thapsigargin. Cells were grown for 24 h in the presence of both intracellular and extracellular calcium chelators (BAPTA-AM and BAPTA) or DMSO (solvent control) as described in Materials and Methods. $[Ca^{2+}]_i$ was measured continuously after thapsigargin challenge as in Fig. 2. Two traces are overlaid on the same figure. Thapsigargin data are from Fig. 2, which was done in parallel with the experiment reported in this figure.

determined in C33A in the presence or absence of thapsigargin and cyclosporin A (Fig. 5). Alone, each drug decreased the ID_{50} of DIM against C33A cells from ~60 to 45 μmol/L (in the presence of 10% serum) and the combination of cyclosporin A and thapsigargin further decreased the ID_{50} for DIM to 20 μmol/L.

Discussion

The multiple antiproliferative and proapoptotic effects of DIM on cancer-derived cells *in vitro* suggest that this diet-derived phytochemical might have clinical utility as a therapeutic or adjuvant therapeutic agent for some types of cancer. The range of cancer cell lines that have been reported to be sensitive to growth inhibition and apoptotic induction by DIM (and indole-3-carbinol) is impressively broad, including carcinomas of the cervix, prostate, breast, and liver; additionally, we have observed these effects in cell lines from ovarian and endometrial cancer, myelomas, and gliomas.[4] To date, the best clinical evidence for the efficacy of indole-3-carbinol in humans has been against cervical cancer (2). Because the parent compound, indole-3-carbinol, is converted quantitatively by acid catalysis in the stomach to DIM and a host of other less well-characterized compounds, it would seem reasonable to use DIM alone as a therapeutic agent, particularly because DIM exerts an array of cytotoxic effects on cancer cells *in vitro* nearly identical to those seen with indole-3-carbinol, but at lower doses and much more rapidly (11). It is important to understand why DIM is toxic to cancer cells *in vitro* whereas humans and animals can ingest either indole-3-carbinol or DIM without apparent ill effects. The sensitivity of stressed cells to DIM may provide a clue to its selective action, and disruption of intracellular Ca^{2+} homeostasis, which provokes and ER stress response, is a sensitizing condition for DIM cytotoxicity

[4] Unpublished data.

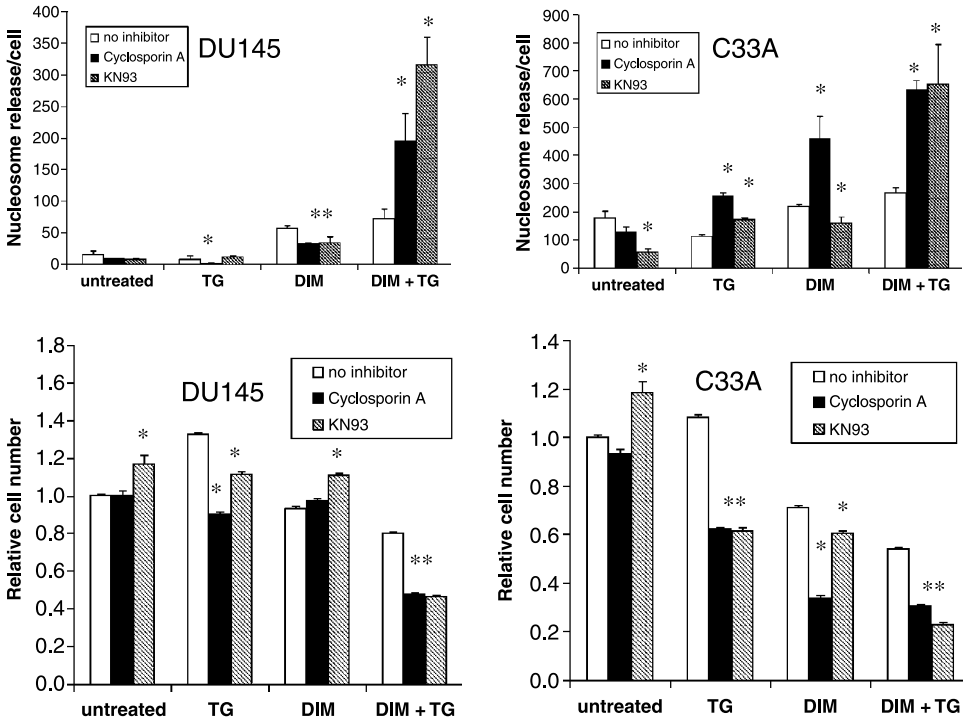

Figure 4. Effect of inhibitors on cell growth and apoptosis after treatment with DIM and thapsigargin. Three separate sets of six replicate monolayer cultures of DU145 and C33A as in Fig. 5 were treated with 5 μmol/L KN93 or 0.1 μmol/L cyclosporin A for 1 h in medium without serum and then supplemented with DIM, thapsigargin, or DIM + thapsigargin as in Fig. 5 for 24 h. Apoptotic index and relative cell number were determined as in Fig. 3.

(Fig. 1). In investigating the possible connection between Ca^{2+} dynamics and the effect of DIM on tumor cells, we obtained evidence for different cell-specific responses to DIM when calcium homeostasis is disrupted, either by DIM itself or by other pharmacologic agents. Using two end points, inhibition of cell proliferation and commitment to apoptosis, we found that elevation of $[Ca^{2+}]_i$ did not play a role in DIM cytotoxicity in the cervical cancer-derived cell line C33A because preventing this elevation did not prevent cell killing (Fig. 3). A similar result was reported for the effects of thapsigargin on the androgen-responsive LNCaP prostate cancer cell line (44). LNCaP cells are as sensitive to DIM-induced apoptosis as C33A,[3] further supporting the idea that Ca^{2+} signaling in the cytosol is not necessary for cell killing by agents that deplete ER Ca^{2+} stores. Lack of a role for cytosolic calcium signaling in apoptosis was also found in another experimental model, in which prostate cancer cells that overexpress BAX have chronically depleted ER Ca^{2+} stores (37). Thus, in several cases, depletion of ER Ca^{2+} seems to be more important for apoptotic signaling than elevation of cytosolic Ca^{2+}. However, our present study suggests that this is not true for all cancer cell lines. We found that preventing $[Ca^{2+}]_i$ elevation in an androgen-insensitive prostate cancer cell line, DU145, partially protected it from DIM cytotoxicity (Fig. 3), suggesting that at least a portion of the response of these cells to DIM requires either Ca^{2+} signaling in the cytosol or some other effect of altered calcium flux through cytosol, such as mitochondrial Ca^{2+} overload. Because protection of DU145 was not complete, however, it is likely that the loss of Ca^{2+} from the ER lumen may also play a role in the cytotoxic effect of DIM for in DU145 as it does for C33A (and LNCaP), presumably by inducing acute ER stress.

The role of Ca^{2+} in apoptosis of prostate cancer cell lines may be related to their state of progression. Some androgen-independent derivatives of LNCaP cells acquire sensitivity to a $[Ca^{2+}]_i$-dependent pathway to apoptosis when ER stores are depleted of Ca^{2+}, in addition to the ER stress–related pathway that predominates in the

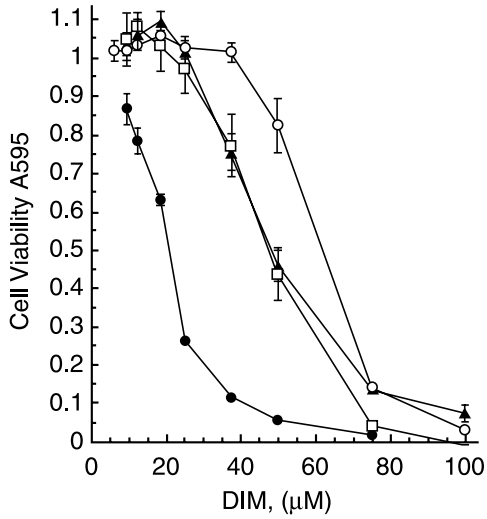

Figure 5. Effect of DIM on C33A cell viability in the presence of calcium-disrupting agents. Monolayer cultures growing in 96-well plates were treated with 0.1% DMSO (○), 1 μmol/L thapsigargin (▲), 0.1 μmol/L cyclosporin A (□), or thapsigargin + cyclosporin A (●). One hour later, varying concentrations of DIM were added to groups of eight cultures and cell viability was determined 48 h later as described in Materials and Methods.

androgen-sensitive parental LNCaP cells (48). Our results with DU145 are in accord with these observations in that DU145 is androgen independent and thus may have undergone a similar transition to a second mode of Ca^{2+} toxicity. From this perspective, C33A cervical cancer cells, which have a functioning estrogen receptor, behave much like the androgen-sensitive LNCaP prostate cancer cell line, in which $[Ca^{2+}]_i$ elevation seems not to play a role in cell death in response to agents like thapsigargin or DIM.

We do not yet know how DIM causes depletion of ER Ca^{2+}, but our results are consistent with some mechanisms and not with others. The inability of a phospholipase C inhibitor to prevent $[Ca^{2+}]_i$ elevation in response to DIM argues against the involvement of a receptor-activated mechanism involving inositol 1,4,5-trisphosphate signaling. In view of results reported for celecoxib, a plausible mechanism for DIM to induce Ca^{2+} release might be inhibition of the SERCA pump. This has not been tested directly. However, if DIM causes Ca^{2+} release by the same mechanism as thapsigargin, one might expect that ER Ca^{2+} stores would be completely depleted after acute treatment with a concentration of DIM sufficient to kill all the cells. This was not the case; addition of thapsigargin after DIM in DU145 cells released an additional pool of Ca^{2+} that was apparently refractory to mobilization by DIM (Fig. 2A). However, when ER Ca^{2+} stores were emptied after thapsigargin treatment, no further release could be detected on addition of DIM (Fig. 2C). If DIM does interfere with the SERCA pump, our results would be consistent with a weaker inhibition by DIM in comparison with thapsigargin, lowering steady-state Ca^{2+} levels in the ER lumen, which could be further emptied by thapsigargin treatment. We tested this hypothesis by treating MCF-7 cells, which behave similarly to DU145, with DIM for 17 hours and then measuring the effect of thapsigargin on $[Ca^{2+}]_i$. No further increase in $[Ca^{2+}]_i$ was observed after thapsigargin addition (data not shown). Thus, it is likely that DIM and thapsigargin mobilize Ca^{2+} from the same pool (the ER) but perhaps by different mechanisms. Other differences between thapsigargin and DIM were evident in the slower clearance of Ca^{2+} from the cytoplasm of DIM-treated cells (Fig. 3, A versus C) and in the much slower release of Ca^{2+} when C33A cells are exposed to DIM (Fig. 3, B versus D). These differences might reflect additional disruptive effects of DIM not seen with thapsigargin, some of which could be cell specific. For example, the slower clearance of $[Ca^{2+}]_i$ after DIM treatment could reflect interference with mitochondrial uptake or with one of the plasma membrane pumps that export intracellular Ca^{2+}. Whatever the explanation for the differences between DIM and thapsigargin reflected in their different kinetics of Ca^{2+} mobilization, our results support the conclusion that depletion of ER Ca^{2+} stores in both cell lines and the increase in $[Ca^{2+}]_i$ derived from ER stores in DU145 are proximal causes of downstream cytostatic and/or cytotoxic cellular responses to DIM. Circumstantial evidence that store-operated calcium channels do not contribute to DIM-induced cytotoxicity in either cell line is that during calcium clamping, external Ca^{2+} was maintained below the level required for these channels to open (44), yet DIM still exhibited both cytostatic and apoptotic activities in clamped cells.

The mechanism by which elevated $[Ca^{2+}]_i$ contributes to apoptosis in DU145 remains to be determined, as does the adaptive response pathway that operates in both cell lines under conditions of acute Ca^{2+} overload. Our inhibitor studies are consistent with cell-specific differences in both pathways. The potent cytotoxic effect of the combination of DIM + thapsigargin was enhanced by both cyclosporin A and KN93, suggesting that an adaptive survival response in both cell lines involves both calmodulin-dependent protein kinase II and calcinurin. On the other hand, calmodulin-dependent protein kinase II would seem to activate an apoptotic response in DU145 after mild perturbation of Ca^{2+} homeostasis (low doses of either DIM or thapsigargin). It also is possible that depletion of ER-sequestered Ca^{2+} triggers a survival pathway in C33A that is not activated in DU145 or that is less effective in this latter cell line. Further work is needed to clarify these issues. It is clear from the present work that Ca^{2+} dynamics play an important role in the response of cancer-derived cell lines to the dietary phytonutrient DIM and that the physiologic effects of Ca^{2+} mobilization are both varied and specific for an individual type of cancer cell.

A potential limitation on the use of DIM or indole-3-carbinol as adjuvant therapeutic agents is their apparent toxicity to nutritionally stressed cells. Thus, either agent would be counter-indicated in cases of chronic tissue injury (such as liver damage or ischemic conditions). Additionally, DIM is known to induce cytochrome *P*450s, which might reduce the available concentration of the primary chemotherapeutic agents, necessitating adjustment of their dosage. Further research is needed to evaluate these potential obstacles using animal models of ischemia and combination therapies employing a range of dosages to treat tumor models *in vivo*.

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