Examining the Role of Mitochondrial Respiration in Vanilloid-Induced Apoptosis

Numsen Hail, Jr., Reuben Lotan

Background: The vanilloids capsaicin and resiniferatoxin are natural products that contain a vanillyl moiety (4-hydroxy-3-methoxybenzyl). Both vanilloids can induce apoptosis in certain cell types by a mechanism that has not been fully elucidated but may involve plasma membrane or mitochondrial targets. We investigated the role of mitochondrial respiration in vanilloid-induced apoptosis. Methods: Cytofluorometric analysis was used to evaluate the effects of vanilloids on apoptosis, Ca$^{2+}$ mobilization, hydroperoxide generation, and DNA content in cells from two human cutaneous squamous cell carcinoma (SCC) cell lines (parental cells) and in their respiration-deficient clones. Oxygen consumption by the cells was determined polarographically. Results: The majority of the parental SCC cells underwent apoptosis after a 12-hour exposure to 100 µM capsaicin or 10 µM resiniferatoxin. The induction of apoptosis was associated with the mitochondrial permeability transition (i.e., an increase in the permeability of the inner mitochondrial membrane associated with the opening of a nonspecific pore). Exposure of parental cells to either vanilloid was not associated with an increase in intracellular free Ca$^{2+}$ levels but was associated with a rapid increase in hydroperoxide generation and a decrease in oxygen consumption. After vanilloid treatment, the respiration-deficient clones generated less hydroperoxide and were resistant to the mitochondrial permeability transition and the induction of apoptosis. Moreover, vanilloid treatment inhibited cell proliferation in the respiration-deficient clones by promoting G1 arrest. Conclusions: Vanilloid-induced apoptosis in the parental SCC cells appears to involve the inhibition of mitochondrial respiration. The apoptotic effects promoted by vanilloid treatment in parental SCC cells, as well as the antiproliferative effects observed in their respiration-deficient clones, suggest that vanilloids may be useful for preventing or treating skin cancers or other hyperproliferative skin disorders. [J Natl Cancer Inst 2002;94:1281–92]

Studies of natural products provide opportunities to reveal interesting biology and generate leads pertaining to specific cellular targets, activities, and therapeutic manipulations (1). Both capsaicin, the pungent constituent of hot peppers of the genus Capsicum, and resiniferatoxin, a complex diterpenoid isolated from the latex of certain succulent African Euphorbias, contain a vanillyl moiety (Fig. 1) and are considered archetypal vanilloids (2). Vanilloids can promote numerous acute cellular effects. Many of these effects are associated with interactions between vanilloids and vanilloid receptors, specific membrane recognition sites that act as voltage-independent and relatively nonselective cation channels. Vanilloid receptors are expressed almost exclusively by primary sensory neurons that are associated with nociception and neurogenic inflammation. It is generally accepted that vanilloid receptors mediate the effects of capsaicin and resiniferatoxin in these cells. These effects are manifested by neuronal excitation followed by desensitization due to elevations in intracellular free Ca$^{2+}$ ([Ca$^{2+}$]i), reviewed in (2).

In addition to the vanilloid receptor-mediated effects, capsaicin can also exert receptor-independent effects by acting as a
coenzyme Q analogue. Thus, capsaicin has been shown to inhibit the plasma membrane NADH oxidase, which is a component of the plasma membrane electron transport system (3,4), and complex I of the mitochondrial electron transport chain (5–9), to alter membrane structure and function (10–12), to promote the generation of reactive oxygen species (13–15), to depolarize mitochondria of intact cells (16), and to induce apoptosis in transformed cells and in activated T cells (4,13,17,18). Resiniferatoxin has also been reported to inhibit the NADH oxidase located in plasma membranes (4), to promote the generation of reactive oxygen species (13,15,18), and to induce apoptosis in transformed cells (4,13,18).

As a medicinal agent, capsaicin is used topically to treat the pain and inflammation associated with a variety of diseases (1), and both capsaicin and resiniferatoxin are being evaluated in clinical trials (2,19) for their efficacy in treating various pathologic conditions. Capsaicin reportedly protects against experimentally induced mutagenesis and tumorigenesis in various model systems, including benzo[a]pyrene-induced pulmonary adenomas and carcinomas in mice and vinyl carbamate-induced skin tumors in mice, suggesting that it may be an important dietary chemopreventive agent (20). Furthermore, topical capsaicin can suppress phorbol ester-induced activation of nuclear factor-kappa B (NF-κB)/Rel and activator protein 1 (AP-1) transcription factors in mouse epidermis. The suppression of these transcription factors by capsaicin may account for its chemopreventive effects on both mouse skin tumorigenesis and inflammation (21–23).

Most antineoplastic agents inhibit tumor cell proliferation by inducing apoptosis (24–27). Many of these agents target the mitochondria, thereby triggering the downstream effectors of apoptosis directly, irrespective of the upstream control mechanisms (e.g., p53) or the status of caspases (proteolytic enzymes associated with apoptosis induction) and/or endogenous caspase inhibitors (24). Although observations at various levels (3,4,13–18) have raised the possibility that vanilloids exert their antitumor effects via their interactions with targets in the plasma membrane and/or mitochondrial electron transport, direct evidence that these putative targets are involved in the pro-apoptotic activity of vanilloids is still unavailable. We used human cutaneous squamous cell carcinoma (SCC) cells and their respiration-deficient derivatives to examine whether capsaicin- and/or resiniferatoxin-induced apoptosis are mechanistically associated with the modulation of mitochondrial respiration.

**METHODS**

**Cell Culture and Reagents**

The COLO 16 cell line was derived from a metastatic lesion in a female patient who succumbed to metastatic SCC (28). The SRB-12 cell line was derived from cells taken from an epidermal lesion on a patient undergoing skin cancer treatment at The University of Texas M. D. Anderson Cancer Center. Both cell lines were routinely cultured in keratinocyte basal medium supplemented with 100 ng/mL recombinant epidermal growth factor and 0.4% bovine pituitary extract (BioWhittaker/Clonetech, San Diego, CA) unless otherwise specified. Respira-
deficient cells depleted of mitochondrial DNA (p0 clones) generated from COLO 16 and SRB-12 cells (29,30) were cultured in enriched media consisting of Dulbecco’s modified Eagle medium containing 4.5 mg/mL glucose (Sigma Chemical Co., St. Louis, MO) supplemented with 110 µg/mL pyruvate, 50 µg/mL uridine, 100 ng/mL ethidium bromide (all from Sigma Chemical Co.), and 2% dialyzed (3500 molecular weight cutoff) fetal bovine serum (Life Technologies, Grand Island, NY). All cell cultures were incubated at 37 °C in humidified air containing 5% CO2. Treatments with vanilloids or other agents were performed on subconfluent cultures. In some experiments, p0 clones and parental COLO 16 cells were treated with the indicated agents after the cells were cultured for 24 hours in enriched media lacking ethidium bromide.

Capsaicin, carbonyl cyanide m-chlorophenylhydrazone (CCCP), dimethyl sulfoxide (DMSO),-ionomycin, potassium cyanide (KCN), and resiniferatoxin were purchased from Sigma Chemical Co. Dihydroethidium, 2′,7′-dichlorofluorescein diacetate, 3,3′-diethoxyloxycarbocyanine iodide (DiOC6(3)), and Fluo-3 acetoxyethyl ester were purchased from Molecular Probes, Inc. (Eugene, OR).

**Assays for Apoptosis**

Control (DMSO-treated) cells and cells treated with the specified concentrations of vanilloids or CCCP for the indicated times were harvested by trypsinization (5 minutes in 1 mL of 0.25% trypsin [Sigma Chemical Co.] diluted in Ca2+-free phosphate-buffered saline [PBS]) and then combined with their respective culture media, which had been removed prior to trypsinization. The cells were pelleted by centrifugation (4 minutes, 300g) and then fixed and stained by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique to detect intranucleosomal DNA fragmentation by using the protocol provided in the apoptosis detection kit (Phoenix Flow Systems, Inc., San Diego, CA) with slight modifications (32). The TUNEL technique labels the 3′-hydroxyl termini of DNA that are generated during apoptosis with fluorescein isothiocyanate (FITC)-conjugated dUTP (31). Cells were also stained with propidium iodide (PI) (Phoenix Flow Systems, Inc.) to indicate
relative DNA content. Cell samples were analyzed for FITC-labeled dUTP incorporation and PI uptake by flow cytometry. Experiments involving the TUNEL assay were typically conducted by using triplicate samples for each treatment, and each experiment was repeated at least two times.

Protocols to evaluate mitochondrial inner transmembrane potential (ΔΨ\text{m}) dissipation, superoxide production, and caspase activity were adapted from previously published methods (33) with modifications (30). For concurrent determinations of ΔΨ\text{m} dissipation and superoxide production, cells in 10-cm plastic tissue culture plates were treated with the designated agent for the indicated time. Twenty minutes before the cells were harvested, DiOC\text{6}(3) and dihydroethidium were added directly to the culture medium to final concentrations of 30 nM and 5 μM, respectively. The cells were harvested by trypsinization, pelleted, resuspended in 1 mL Ca\text{2+}-free PBS at 37°C, and analyzed immediately for DiOC\text{6}(3) and ethidium fluorescence intensity by flow cytometry. Caspase activity was detected with PhiPhiLux-G1D2 (Oncoimmunin Inc., Gaithersburg, MD), a cell-permeant fluorogenic caspase substrate (aspartate-glutamate-valine-aspartate [DEVD]-rhodamine) that is cleaved in a DEVD-dependent manner to produce fluorescent molecules of rhodamine (33). Cells were treated with the indicated agent in 10-cm plastic tissue culture plates as described above for the TUNEL assay. Following treatment, the cells were harvested by trypsinization, incubated in 50 μL of 10 μM PhiPhiLux-G1D2 reagent, and washed according to the manufacturer’s recommendations. The resulting cell suspension was analyzed immediately for rhodamine fluorescence intensity by flow cytometry. Experiments assessing the dissipation of ΔΨ\text{m} and superoxide production or caspase activity were typically conducted by using duplicate samples for each treatment, and each experiment was repeated at least three times.

Assay for Ca\text{2+} Mobilization

The procedure for assessing increases in [Ca\text{2+}]\text{c} using Fluo-3 acetoxymethyl ester was adapted from a previously described method (14). Briefly, cells in 10-cm plastic tissue culture plates were incubated for 30 minutes at 37°C with 4 μM Fluo-3 acetoxymethyl ester diluted in Krebs–Ringer buffer (10 mM Na\text{H}2\text{PO}4, 120 mM NaCl, 4.5 mM KCl, 0.7 mM Na\text{H}2\text{PO}4, 1.5 mM Na\text{H}2\text{PO}4, and 0.5 mM Mg\text{Cl}2 [pH 7.4 at 37°C]; Sigma Chemical Co.). Fluo-3 acetoxymethyl ester is cell permeant and is converted to Fluo-3 by the activity of cellular esterases. The resulting cell suspension was analyzed immediately for Fluo-3 fluorescence intensity by flow cytometry. Experiments assessing increases in [Ca\text{2+}]\text{c} were conducted by using duplicate samples for each treatment, and each experiment was repeated at least three times.

Assay for Hydroperoxide Generation

We measured hydroperoxide production in DMSO- and vanilloid-treated cells using 2′,7′-dichlorofluorescin diacetate, as previously described (30). Briefly, cells in 10-cm plastic tissue culture plates were incubated for 20 minutes with 20 μM 2′,7′-dichlorofluorescin diacetate diluted in Krebs–Ringer buffer. 2′,7′-Dichlorofluorescin diacetate is cell permeant and is cleaved by esterase activity to form 2′,7′-dichlorofluorescin, which is retained inside the cell. 2′,7′-Dichlorofluorescin can then be converted to the fluorescent product 2′,7′-dichlorofluorescin (DCF) in the presence of hydroperoxide (30). The cells were washed once with 5 mL of Krebs–Ringer buffer to remove the residual dye and then treated for 30 minutes with capsaicin, resinafetoxin, or DMSO diluted to the appropriate concentrations in Krebs–Ringer buffer. After treatment, the cells were harvested by trypsinization, washed in 5 mL of Ca\text{2+}-free PBS at 37°C, pelleted by centrifugation, resuspended in 1 mL of Ca\text{2+}-free PBS at 37°C, and analyzed immediately for DCF fluorescence intensity by flow cytometry. Experiments assessing hydroperoxide generation were typically conducted by using duplicate samples for each treatment, and each experiment was repeated at least three times.

Assessment of Cellular DNA Content

The DNA content of vanilloid- and DMSO-treated cells was examined by using a hypotonic solution of PI, as described previously (34). Following treatment, cells were harvested, as described above, for the TUNEL assay. The resulting cell pellet was resuspended in 1 mL of hypotonic PI solution (50 μg/mL PI, 0.1% sodium citrate, and 0.1% Triton X-100 [all from Sigma Chemical Co.] dissolved in Ca\text{2+}-free PBS) at 4°C and stored overnight at 4°C. The cell suspensions were analyzed the next day for PI fluorescence intensity by flow cytometry. Experiments assessing cellular DNA content were typically conducted by using duplicate samples for each treatment, and each experiment was repeated at least three times.

Flow Cytometry

All flow cytometric procedures were performed with a Coulter XL flow cytometer, and data analysis was accomplished by using System II XL software (Coulter Corp., Miami, FL). Approximately 10,000 events (cells) were evaluated for each sample. In all cytofluorometric determinations, cell debris and clumps were excluded from the analysis of the cell suspensions by gating.

Measurement of Oxygen Consumption in Cultured Cells

Oxygen consumption was measured polarographically by using a Clark-type oxygen electrode and a Biological Oxygen Monitor (Model 5300; Yellow Springs Instrument Co., Yellow Springs, OH), as described previously (30,35). Briefly, capsaicin, resinafetoxin, KCN, or DMSO was added directly to the medium of cells cultured in 10-cm plastic tissue culture plates. Approximately 30 minutes later, the cells were harvested by trypsinization, pelleted by centrifugation, and resuspended at a density of approximately 2 × 10^6 cells/mL in fresh medium at 37°C, and 3 mL of the cell suspension was placed in a 3-mL respiration chamber in a circulating water bath at 37°C. Oxygen consumption was measured over a 10-minute period after equilibration of the electrode in the respiration chamber. Respiration rates (nmol O_2/minute) were normalized for 10^6 cells, assuming an oxygen concentration of 220 μM in air-saturated medium at 37°C (36). Cell viability was routinely checked via trypan blue exclusion after oxygen consumption was measured.
Statistical Analysis

The 95% confidence intervals of mean values obtained from triplicate samples were calculated using Microsoft Excel software (version 5.0; Microsoft Corporation, Seattle, WA).

RESULTS

Effect of Vanilloids on Apoptosis in SCC Cells

Exposure to capsaicin or resiniferatoxin rapidly induces apoptosis in certain transformed cells and in activated T cells (4,13,17,18). We found that a 12-hour exposure to increasing concentrations of capsaicin was sufficient to promote increasing levels of apoptosis in COLO 16 cells (Fig. 2, A). The 100- and 200-μM concentrations of capsaicin appeared to be equally effective in promoting TUNEL-positive cells in the majority of the treatment population. On a molar basis, resiniferatoxin was more potent than capsaicin in inducing apoptosis in these cells. A 12-hour exposure to 10 μM resiniferatoxin was sufficient to promote apoptosis in approximately 60% of COLO 16 cells. This concentration of resiniferatoxin was roughly 10-fold lower than the concentration of capsaicin needed to promote a similar level of apoptosis. These results are in agreement with those from previous reports (4,13,18) of the apoptosis-inducing effects of these agents in other cell types. Exposure of either COLO 16 or SRB-12 cells to 100 μM capsaicin or 10 μM resiniferatoxin for 6 or 12 hours resulted in a similar time-dependent increase in the percentage of TUNEL-positive cells (Fig. 2, B).

Effects of Vanilloid Treatment on Mitochondrial Permeability Transition in SCC Cells

Several events typically occur in the mitochondria and cytoplasm of apoptotic cells before DNA fragmentation. Three such events—the dissipation of ΔΨm, enhanced superoxide production, and caspase activation—are associated with the mitochondrial permeability transition (i.e., an increase in the permeability of the inner mitochondrial membrane associated with the opening of a nonspecific pore) (37,38). We have reported previously (32,35) that the mitochondrial permeability transition is required for DNA fragmentation in SCC cells exposed to synthetic retinoids. We therefore evaluated the dissipation of ΔΨm, superoxide production, and caspase activation in vanilloid-treated SCC cells to determine whether the mitochondrial permeability transition was associated with cell death. The dissipation of ΔΨm, which is detected by the decreased retention of the cationic probe DiOC6(3), and enhanced superoxide production, which is detected by the oxidation of dihydroethidium to ethidium, can be measured concurrently to monitor the mitochondrial permeability transition in intact cells (33). Control (DMSO-treated) COLO 16 cells were gated because we assumed that they would exhibit high DiOC6(3) fluorescence and low ethidium fluorescence. Therefore, most of the cell population is situated in the lower right quadrant of the upper left panel of Fig. 3, A. The percentages in each of the four quadrants of the panels in Fig. 3, A and B, indicate the proportion of cells in the sample population exhibiting specific patterns of DiOC6(3) fluorescence intensity and ethidium fluorescence intensity [i.e., cells with high DiOC6(3) fluorescence intensity and low ethidium fluorescence intensity appear in the lower right quadrant, cells with high DiOC6(3) fluorescence intensity and high ethidium fluorescence intensity appear in the upper right quadrant, cells with low DiOC6(3) fluorescence intensity and low ethidium fluorescence intensity appear in the lower left quadrant, and cells with low DiOC6(3) fluorescence intensity and high ethidium fluorescence intensity appear in the upper left quadrant]. Each contour outline represents the relative frequency of cells in the sample population exhibiting varying degrees of fluorescence intensity of the indicated dye, which increases as the contour outlines approach the innermost area of the contour plot.

A 2-hour exposure to capsaicin shifted approximately 20% of the cell population to low DiOC6(3) fluorescence intensity but did not markedly increase the ethidium fluorescence intensity. After a 4-hour exposure to capsaicin, approximately 63% of the cell population had shifted to low DiOC6(3) fluorescence, and 39% of these cells exhibited high ethidium fluorescence. After an 8-hour exposure to capsaicin, approximately 95% of the cell population had shifted to low DiOC6(3) fluorescence, and approximately 56% of these cells exhibited high ethidium fluorescence. Resiniferatoxin treatment promoted similar time-dependent changes in DiOC6(3) and ethidium fluorescence intensity in COLO 16 cells (Fig. 3, B). These results suggest that treatment of SCC cells with vanilloids is associated with the progressive dissipation of ΔΨm and enhanced superoxide production, which exemplify the disintegration of mitochondria and electron transport that is characteristic of the mitochondrial permeability transition (38).

Fig. 2. Evaluation of vanilloid-induced apoptosis in cutaneous squamous cell carcinoma cells. A) COLO 16 cells were treated with the indicated concentrations of capsaicin (CAP) or resiniferatoxin (RTX) for 12 hours and then examined for apoptosis by using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique. B) COLO 16 (left) and SRB-12 cells (right) were treated for 6 or 12 hours with dimethyl sulfoxide (Control), 100 μM CAP, or 10 μM RTX and then examined for apoptosis by using the TUNEL technique. Data in A and B are expressed as the mean of triplicate samples. Error bars represent 95% confidence intervals.

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The mitochondrial permeability transition also results in the release of cytochrome $c$ and other soluble mitochondrial factors that are capable of inducing caspase activity (33,39). We found that COLO 16 cells exposed to capsaicin or resiniferatoxin for 6 hours displayed increased cleavage of a fluorogenic caspase substrate compared with control (DMSO-treated) cells (Fig. 3, C). Caspase activity was determined after a 6-hour exposure of COLO 16 cells to 100 $\mu$M CAP, 10 $\mu$M RTX, or an equal volume of the vehicle dimethyl sulfoxide (control). After treatment, COLO 16 cells were incubated with PhiPhiLux-G1D2 (a cell-permeant fluorogenic caspase substrate (aspartate–glutamate–valine–aspartate [DEVD]–rhodamine) that is cleaved in a DEVD-dependent manner to produce fluorescent molecules of rhodamine. The fluorescence intensity of rhodamine is indicated by the $x$-axis. The relative frequency of cells (count) exhibiting varying degrees of rhodamine fluorescence intensity in vanilloid- or dimethyl sulfoxide-treated samples is indicated by the $y$-axis. Data depicted in A–C are representative of those obtained in three independent experiments.

**Effect of Vanilloid Treatment on Ca$^{2+}$ Mobilization in SCC Cells**

Among the various stimuli that can trigger mitochondrial permeability transition, Ca$^{2+}$ has been investigated extensively and, consequently, is considered to be a prototypical inducing agent (24,40,41). In certain cell systems, vanilloids function by increasing the [Ca$^{2+}$]$_i$ level via their interactions with vanilloid receptors (2). Therefore, we examined the effects of capsaicin and resiniferatoxin treatment on [Ca$^{2+}$]$_i$ levels in SCC cells. We used the Ca$^{2+}$-sensitive dye Fluo-3 (14) to monitor fluctuations in [Ca$^{2+}$]$_i$. As a positive control for increases in [Ca$^{2+}$], COLO
16 cells were exposed to the Ca^{2+} ionophore ionomycin (42) for 30 minutes. Compared with DMSO-treated control cells, cells treated with ionomycin displayed an increase in Fluo-3 fluorescence intensity, indicating an increase in [Ca^{2+}], (Fig. 4). By comparison, 30-minute exposures to capsaicin or resiniferatoxin had no effect on the fluorescence intensity of Fluo-3 compared with DMSO-treated control cells. This was also the case for cells exposed to capsaicin or resiniferatoxin for 60 minutes (data not shown). Similar results were obtained in SRB-12 cells following 30- and 60-minute treatments with either vanilloid (data not shown). These results imply that Ca^{2+} mobilization is not directly associated with vanilloid-induced mitochondrial permeability transitions and apoptosis in SCC cells. Capsaicin reportedly promotes the formation of ion pseudochannels in planar lipid bilayers (11) and alters membrane dynamics (10,12). However, the data in Fig. 4 suggest that a 30- or 60-minute exposure to capsaicin was not sufficient to disrupt the plasma membranes of SCC cells.

**Effect of Vanilloids on Hydroperoxide Production and Respiration in SCC Cells**

Exposure to capsaicin or resiniferatoxin promotes the generation of reactive oxygen species (i.e., hydroperoxide or superoxide) in transformed cells (13–15,18), and we have reported previously (30) that capsaicin promotes rapid hydroperoxide production in SCC cells. As we have already shown (Fig. 3, A and B), both capsaicin and resiniferatoxin enhanced superoxide generation in SCC cells. However, the increase in superoxide generation, as detected by an assay that measured the oxidation of dihydroethidium to ethidium, was not evident until after the cells had been exposed to vanilloids for approximately 4 hours. We therefore evaluated hydroperoxide production in vanilloid-treated cells with the use of 2',7'-dichlorofluorescin. 2',7'-Dichlorofluorescin can serve as a peroxidase substrate during the dismutation of hydrogen peroxide, and its rate of oxidation to DCF is greatly enhanced by peroxidase activity (43). We found that a 30-minute exposure of COLO 16 cells to capsaicin (Fig. 5, A) or resiniferatoxin (Fig. 5, B) was sufficient to promote an approximately threefold increase in the mean DCF fluorescence intensity compared with that produced by DMSO treatment in control cells. Similar results were obtained with SRB-12 cells (data not shown). The ability of vanilloids to promote rapid production and inhibition of respiration in cutaneous squamous cell carcinoma cells. COLO 16 cell cultures were incubated for 20 minutes with 20 μM 2',7'-dichlorofluorescin diacetate diluted in Krebs–Ringer buffer. The cells were washed with Krebs–Ringer buffer and then treated for 30 minutes with 100 μM capsaicin (CAP) (A) or 10 μM resiniferatoxin (RTX) (B) diluted in Krebs–Ringer buffer. Control cells were treated for 30 minutes with an equal volume of the vehicle dimethyl sulfoxide diluted in Krebs–Ringer buffer. After treatment, the cells were harvested and analyzed immediately by flow cytometry for DCF fluorescence intensity (y-axis). The relative frequency of cells (count) exhibiting varying degrees of Fluorescence intensity in vanilloid-, ionomycin-, or dimethyl sulfoxide-treated samples is indicated by the y-axis. The vertical dotted lines approximate the mean channel number for Fluor-3 fluorescence intensity in dimethyl sulfoxide-treated cells. The data are representative of those obtained in three independent experiments.

![Image](88x583 to 291x761)

**Fig. 4.** Assessment of intracellular free calcium modulation by vanilloids in cutaneous squamous cell carcinoma cells. COLO 16 cell cultures were incubated for 30 minutes at 37 °C with 4 μM Fluo-3 acetoxymethyl ester diluted in Krebs–Ringer buffer. Fluo-3 acetoxymethyl ester is cell permeant and is converted to Fluor-3 by the activity of cellular esterases. The fluorescence intensity of Fluor-3 is enhanced after it binds to intracellular free calcium. The cell cultures were washed once with Krebs–Ringer buffer to remove the residual dye and then treated for an additional 30 minutes with 1 μM ionomycin, 100 μM capsaicin (CAP), 10 μM resiniferatoxin (RTX), or an equal volume of the vehicle dimethyl sulfoxide (DMSO). The cells were harvested, washed with Ca^{2+}-free phosphate-buffered saline, and analyzed immediately for Fluor-3 fluorescence intensity (y-axis) by flow cytometry. The relative frequency of cells (count) exhibiting varying degrees of Fluor-3 fluorescence intensity in vanilloid-, ionomycin-, or dimethyl sulfoxide-treated samples is indicated by the y-axis. The vertical dotted lines approximate the mean channel number for Fluor-3 fluorescence intensity in dimethyl sulfoxide-treated cells. The data are representative of those obtained in three independent experiments.

![Image](351x266 to 567x542)

**Fig. 5.** Vanilloids promote hydroperoxide production and inhibit respiration in cutaneous squamous cell carcinoma cells. COLO 16 cell cultures were incubated for 20 minutes with 20 μM 2',7'-dichlorofluorescin diacetate diluted in Krebs–Ringer buffer. Control cells were treated for 30 minutes with an equal volume of the vehicle dimethyl sulfoxide diluted in Krebs–Ringer buffer. After treatment, the cells were harvested and analyzed immediately by flow cytometry for DCF fluorescence intensity (y-axis). The relative frequency of cells (count) exhibiting varying degrees of Fluor-3 fluorescence intensity in vanilloid- or dimethyl sulfoxide-treated samples is indicated by the y-axis. The data in A and B are representative of those obtained in three independent experiments. C) Rates of oxygen consumption were determined polarographically in COLO 16 and SRB-12 cells exposed for 30 minutes to 100 μM CAP, 10 μM RTX, or 2.5 mM potassium cyanide (KCN). Control cells were treated for equivalent times with an equal volume of the vehicle dimethyl sulfoxide. Results are expressed as the mean of triplicate samples. Error bars represent 95% confidence intervals.
hydroperoxide production (Fig. 5, A and B) prior to enhanced superoxide generation (Fig. 3, A and B) may be evidence of a condition of oxidative stress in SCC cells that appears to be initially modulated, to some degree, by cellular antioxidant enzymes such as superoxide dismutases and peroxidases.

We have previously reported that capsaicin, while able to promote hydroperoxide production in SCC cells, can considerably decrease the hydroperoxide generation triggered in these cells after exposing them to the synthetic retinoid N-(4-hydroxyphenyl)retinamide (30). Because the pro-oxidant effect of N-(4-hydroxyphenyl)retinamide in SCC cells appears to depend on an enzymatic process in the mitochondrial electron transport chain (30), it would appear that, in this situation, capsaicin was inhibiting mitochondrial respiration. Indeed, a 30-minute exposure to capsaicin or resiniferatoxin was sufficient to inhibit oxygen consumption by approximately 60% in COLO 16 cells and 50% in SRB-12 cells, implying a reduction in mitochondrial respiration (Fig. 5, C). The complex IV inhibitor cyanide (44) was used as a positive control to illustrate that cellular oxygen consumption was predominantly of mitochondrial origin in these SCC cells.

Vanilloid-Induced Hydroperoxide Generation in $\rho^0$ Cells

Capsaicin reportedly inhibits electron transfer at complex I in isolated mitochondria (6) and submitochondrial particles (5,7–9) by functioning as a coenzyme Q antagonist. If capsaicin were to inhibit complex I in the mitochondria of intact cells, we would expect, as illustrated above (Fig. 5, C), that oxygen consumption would also be inhibited because the turnover of complex I is typically rate-limiting in respiration under physiologic conditions (45,46). In this scenario resiniferatoxin, by virtue of its vanillyl moiety, would also be predicted to function as a coenzyme Q antagonist and inhibit mitochondrial respiration at complex I. However, to date, no activity has been documented for resiniferatoxin. Inhibition of mitochondrial electron transport can promote the production of nonenzymatic reactive oxygen species due to redox cycling of reduced electron carriers upstream of the site of inhibition (44,47). This activity could explain the enhanced hydroperoxide generation promoted by capsaicin and resiniferatoxin (Fig. 5, A and B) before induction of the mitochondrial permeability transition. Capsaicin and resiniferatoxin can also inhibit the plasma membrane NADH oxidase by functioning as a coenzyme Q antagonist (3,4). The inhibition of the plasma membrane NADH oxidase has been reported to be associated with the pro-oxidant and pro-apoptotic properties of capsaicin and resiniferatoxin in certain transformed cells and activated T cells (13,14,18). If the initial pro-oxidant effects of capsaicin and resiniferatoxin (Fig. 5, A and B) were solely associated with inhibition of the plasma membrane NADH oxidase, this oxidative stress could ultimately inhibit mitochondrial respiration, because hydrogen peroxide and other reactive oxygen species can interfere with electron transport and disrupt mitochondrial function (44,48).

To directly address the question regarding the probable cause of vanilloid-induced hydroperoxide production, we examined the effects of vanilloid exposure on hydroperoxide generation in $\rho^0$ clones derived from COLO 16 and SRB-12 cells. The $\rho^0$ clones are deficient in mitochondrial respiration because they have been depleted of mitochondrial DNA (30,35) that encodes specific subunits of the mitochondrial electron transport chain enzymes (including complex I) (49,50). $\rho^0$ clones derived from human B-type lymphoblastoid cells have been reported to increase the activity of the plasma membrane electron transport system by approximately fourfold to maintain redox homeostasis following the loss of mitochondrial electron transport (51). If this were also the case in the $\rho^0$ clones derived from human SCC cells, and if the inhibition of the plasma membrane NADH oxidase were the sole cause of hydroperoxide production, we would predict that capsaicin or resiniferatoxin would stimulate more hydroperoxide generation in the $\rho^0$ clones than they would in the parental cells. We found that this was not the case. COLO 16 $\rho^0$ cells that were exposed to capsaicin or resiniferatoxin after incubation with 2',7'-dichlorofluorescin diacetate displayed a slight, yet discernable and reproducible, increase (approximately 25%) in DCF fluorescence intensity compared with DMSO-treated control COLO 16 $\rho^0$ cells (Fig. 6, A), which was considerably less than the approximately threefold increase observed in parental COLO 16 cells (Fig. 5, A and B). Similar results were observed for $\rho^0$ clones derived from SRB-12 cells (Fig. 6, B). Under similar assay conditions, $\rho^0$ cells were able to generate levels of DCF fluorescence intensity comparable to that of parental cells exposed to 500 $\mu$M hydrogen peroxide for 30 minutes, which served as a positive control for the assay (data not shown). This result suggests that the decreased production of hydroperoxide observed in the vanilloid-treated $\rho^0$ clones was not due to reduced uptake of the probe 2',7'-dichlorofluorescin diacetate, reduced conversion of the probe by esterase activity to
generate 2',7'-dichlorofluorescin, and/or the activation of peroxidase activity to produce the fluorescent product DCF. Together, these results indicate that most of the initial hydroperoxide produced in parental SCC cells exposed to capsaicin or resiniferatoxin was of mitochondrial origin and that its production was probably associated with the inhibition of mitochondrial respiration. Yet even in the absence of mitochondrial respiration in the \( \rho^0 \) clones, vanilloids could still promote some hydroperoxide generation, suggesting the possible involvement of the plasma membrane NADH oxidase in this process.

**Effects of Vanilloids on Mitochondrial Permeability Transition and Apoptosis in \( \rho^0 \) Clones**

Although there appeared to be a link between the inhibition of mitochondrial respiration and vanillloid-induced cell death in parental COLO 16 and SRB-12 cells, it was also possible that the hydroperoxide generation, decreased respiration, mitochondrial permeability transition, and apoptosis we observed were triggered by some other cytotoxic event able to induce apoptosis. To address this possibility, we examined whether exposure of \( \rho^0 \) cells to vanilloids could trigger events associated with the mitochondrial permeability transition. Control (DMSO-treated) \( \rho^0 \) cells were gated (as described for Fig. 3, A) during flow cytometry, because we assumed that they would exhibit high DiOC\(_6(3)\) fluorescence and low ethidium fluorescence (Fig. 7, A). The protonophore CCCP (47) was used as a positive control to determine if the \( \Delta_P \psi_m \) could be modulated in COLO 16 \( \rho^0 \) cells by decreasing the retention of DiOC\(_6(3)\). We predicted that CCCP would dissipate the \( \Delta_P \psi_m \) of COLO 16 \( \rho^0 \) cells, thereby decreasing the retention of DiOC\(_6(3)\) by these cells, because it has been reported that cells depleted of mitochondrial DNA shift the maintenance of \( \Delta_P \psi_m \) from the electron transport chain to the F1-ATPase, which hydrolyzes ATP to pump protons out of the mitochondrial matrix (52). Indeed, a 2-hour exposure of COLO 16 \( \rho^0 \) cells to CCCP shifted approximately 60% of the cell population to low DiOC\(_6(3)\) fluorescence intensity (Fig. 7, A), indicating that \( \Delta_P \psi_m \) was present in \( \rho^0 \) cells. Four- and 8-hour exposures to capsaicin or resiniferatoxin that were capable of inducing signs of the mitochondrial permeability transition in parental COLO 16 and SRB-12 cells were examined in COLO 16 \( \rho^0 \) cells. However, these short-term exposures to either vanillloid had no profound effects on the fluorescence intensities of both probe compared with DMSO-treated \( \rho^0 \) cells (data not shown). Even after a 24-hour exposure of the COLO 16 \( \rho^0 \) cells to either vanillloid, there were no marked differences between these cells and DMSO-treated cells (Fig. 7, A). Similar results were obtained for SRB-12 \( \rho^0 \) cells (data not shown).

Furthermore, microscopic examination of the \( \rho^0 \) clones at various times during a 48-hour exposure to capsaicin or resiniferatoxin revealed no signs of a subpopulation of apoptotic cells (i.e., shrunken and detached cells), whereas such a subpopulation was commonly observed in parental cells exposed to these vanillloids for 12 hours (data not shown). This finding suggested that the \( \rho^0 \) clones were not undergoing apoptosis, at least in a mitochondrial permeability transition-dependent manner.

It was still possible that the \( \rho^0 \) cells were undergoing apoptosis in a mitochondrial permeability transition-independent fashion. To address this possibility, we used the TUNEL assay to determine whether DNA fragmentation had occurred in \( \rho^0 \) cells treated with DMSO, CCCP, capsaicin, or resiniferatoxin. Exposure of COLO 16 \( \rho^0 \) cells to CCCP for 24 hours promoted DNA fragmentation in approximately 56% of the cell population (Fig. 7, B). As already noted (Fig. 7, A), a 2-hour exposure to CCCP promoted the dissipation of \( \Delta_P \psi_m \) in COLO 16 \( \rho^0 \) cells. Together, these data suggest that the mitochondrial permeability transition was probably associated with cell death caused by CCCP treatment. By contrast, a similar duration of exposure of COLO 16 \( \rho^0 \) cells to capsaicin or resiniferatoxin failed to increase the levels of TUNEL-positive cells (data not shown), which was also the condition observed after a 48-hour exposure.

In fact, the numbers of TUNEL-positive cells in the vanillloid-treated cultures were lower than those in DMSO-treated control cultures (Fig. 7, B). For example, approximately 0.5% of the cells in the capsaicin- or resiniferatoxin-treated populations were TUNEL-positive, whereas 6.5% of the cells in DMSO-treated control cultures were TUNEL-positive. SRB-12 \( \rho^0 \) cells were also examined for DNA fragmentation after 24- and 48-hour exposures to capsaicin or resiniferatoxin, and these results are summarized along with those obtained for COLO 16 \( \rho^0 \) cells (Fig. 7, C). We observed fewer TUNEL-positive cells in COLO 16 \( \rho^0 \) and SRB-12 \( \rho^0 \) cells treated for 24 or 48 hours with either vanillloid than in the respective control (DMSO-treated) cells.

Similar results were obtained in the \( \rho^0 \) clones even when the concentrations of capsaicin and resiniferatoxin were doubled, to 200 \( \mu M \) and 20 \( \mu M \), respectively (data not shown).

To determine whether the culture conditions were in some way associated with the survival of vanillloid-treated \( \rho^0 \) cells, parental COLO 16 cells were cultured in the same medium as the \( \rho^0 \) clones. Under these conditions, a 24-hour exposure of the parental cells to capsaicin resulted in an increase in the number of TUNEL-positive cells to a level that was approximately 10-fold greater than that in the DMSO-treated control cells (Fig. 7, D). This finding indicates that the culture conditions did not specifically favor cell survival in the vanillloid-treated \( \rho^0 \) cells. Together, these results strongly suggest that mitochondrial respiration is associated with the apoptogenic effects of capsaicin and resiniferatoxin in parental SCC cells.

**Effect of Vanilloids on Proliferation of \( \rho^0 \) Cells**

As illustrated in Fig. 7, B, a 48-hour vanillloid treatment resulted in the accumulation of COLO 16 \( \rho^0 \) cells in which would be predicted to be the G\(_1\) subpopulation relative to the distribution of cells in the DMSO-treated control population. The cellular DNA content is indicated by the intensity of PI staining, which is displayed along the x-axis for each of the respective populations. Furthermore, microscopic examination of \( \rho^0 \) cell cultures exposed to capsaicin or resiniferatoxin for 24 or 48 hours revealed that they had lower cell densities than the respective DMSO-treated cells (data not shown). Cell counts of DMSO- and vanillloid-treated cultures were routinely determined before the TUNEL analysis (Fig. 7, C). For each of the \( \rho^0 \) clones, the cell number obtained after 24- and 48-hour exposures to capsaicin or resiniferatoxin was less than that in cells treated with DMSO for the same periods, indicating that vanillloid exposure inhibited the proliferation of these cells (Fig. 8, A). We also compared the DNA profile of a population of COLO 16 \( \rho^0 \) cells treated with capsaicin for 24 hours with that of a population of COLO 16 \( \rho^0 \) cells treated with DMSO for 24 hours (Fig. 8, B). The capsaicin-treated cells exhibited an increase in the G\(_1\) subpopulation (the peak occurring around 400 fluorescence units of PI on the x-axis of the histograms) and a decrease in the S and
G2/M subpopulations (counts occurring above 500 fluorescence units of PI on the x-axis of the histograms) compared with that of the DMSO-treated control cells. Similar results were obtained in COLO 16 ρ0 cells exposed to resiniferatoxin for 24 hours (data not shown). These results imply that vanilloid exposure inhibited proliferation of the ρ0 clones by promoting G1 arrest. By comparison, parental COLO 16 cells exposed to capsaicin for 12 hours exhibited a substantial hypoploid sub-population (the peak occurring below 300 fluorescence units of PI on the x-axis of the histograms), which is indicative of a subpopulation of apoptotic cells, compared with that of DMSO-treated control cells (Fig. 8, C). This observation, along with the results presented in Fig. 2 and 3, implies that apoptosis induction was associated with vanilloid-induced growth inhibition in the parental SCC cells.

**DISCUSSION**

Several studies have established that mitochondria and mitochondrial-derived factors are involved in the process of apoptosis (33,53–55). Mitochondria are attractive targets for cancer prevention and chemotherapy because there is growing evidence...
A variety of structurally diverse hydrophobic compounds can disrupt bioenergetics by inhibiting mitochondrial electron transport (5, 58). In addition, most of the classical inhibitors of complex I and complex III are naturally occurring compounds (58–60). A structure–activity relationship exists among complex I inhibitors because these agents act at or close to the coenzyme Q-binding site of the enzyme (58–60). The vanillyl moieties of capsaicin and resiniferatoxin (circled in Fig. 1) are structurally similar to the cyclic portion of coenzyme Q, which could account for the fact that vanilloids act as coenzyme Q antagonists. This assumption is reasonable if one considers that the capsaicin-binding site in complex I overlaps with that of rotenone (5), which also inhibits electron transfer to coenzyme Q (58). The hydrophobic domains attached to the vanillyl moieties of capsaicin and resiniferatoxin correspond to the isoprenoid chain of coenzyme Q. The relative hydrophobicity of these domains may also dictate the activities of these compounds, and different hydrophobicities could explain why resiniferatoxin was as effective as capsaicin in inhibiting respiration in SCC cells, even when it was used at a 10-fold lower molar concentration than capsaicin. For example, on a molar basis, capsaicin is far less potent than rotenone as an inhibitor of complex I in submitochondrial particles (58), and respiration in SCC cells can be inhibited to approximately the same degree by using either 1 μM rotenone or 100 μM capsaicin (Hail N, Lotan R: unpublished observation). This observation implies that rotenone can partition more effectively than capsaicin into the hydrophobic core of complex I to gain entry to the coenzyme Q-binding site. This quality could also apply to resiniferatoxin.

The apoptotic effects (i.e., mitochondrial permeability transition, caspase activity, and DNA fragmentation) of capsaicin and resiniferatoxin appeared to be triggered via the inhibition of mitochondrial respiration in the parental SCC cells. We speculate that, given the results of this and previous studies, the antiproliferative effect of capsaicin and resiniferatoxin observed in the parental COLO 16 cells could be associated with hydroperoxide production and/or inhibition of an enzymatic process associated with the plasma membrane transport system. The capacity of capsaicin and resiniferatoxin to promote apoptosis in parental SCC cells and to inhibit the proliferation of their derivatives are important biologic effects with respect to carcinogenesis. Further examination of these agents in the prevention or treatment of skin cancers or other hyperproliferative skin diseases is therefore essential. Capsaicin is currently used topically to treat the pain and inflammation associated with a variety of diseases (1). In addition, topical capsaicin has been shown to be of clinical benefit in suppressing the skin lesions of psoriasis (61, 62), and Zostrix HP® (75 mg/mL, ~2.45 mM capsaicin) and Zostrix® (25 mg/mL, ~0.818 mM capsaicin) are used to treat herpes zoster (63). It is conceivable that intervention in the process of skin carcinogenesis could be attained by treating cutaneous preneoplastic lesions topically with a much lower concentration of capsaicin than those contained in the above-mentioned formulations (because the concentration of capsaicin required for induction of cell death in parental SCC cells, and for growth arrest in ρ0 cells, was approximately 8–25 times lower). Given that resiniferatoxin appeared to be more potent than capsaicin in triggering cell death and growth arrest, we would expect that this agent might also have value in prevention or treatment of hyperproliferative skin diseases. In light of this and previous reports, one might envision the use of capsaicin, or the more potent agent resiniferatoxin, formulated in an emollient or a patch, as a treatment for skin cancers, dysplastic lesions, and psoriatic plaques. In conclusion, agents such as capsaicin and resiniferatoxin, which appear to target mitochondrial respiration in the SCC cells examined in this study, may be useful in the prevention or treatment of skin cancers and/or other hyperproliferative skin disorders by promoting endogenous apoptosis-inducing mechanisms.


NOTES

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