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Antitumor Activity of Capsaicin on Human Colon Cancer Cells in Vitro and Colo 205 Tumor Xenografts in Vivo

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Capsaicin was reported to inhibit cancer cell growth. The aim of this study was to evaluate the antitumor potential of capsaicin by studying antitumor activity in vitro as well as in vivo. The in vitro studies are to examine the effects of capsaicin on human colon cancer colo 205 cells after exposure to capsaicin. The results showed that capsaicin induced cytotoxic effects in a time- and dose-dependent manner and increased reactive oxygen species (ROS) and Ca^{2+} but decreased the level of mitochondrial membrane potential ($\Delta\Psi_m$) in colo 205 cells. Data from Western blotting analysis indicated that the levels of Fas, cytochrome *c*, and caspases were increased, leading to cell apoptosis. Capsaicin decreased the levels of anti-apoptotic proteins such as Bcl-2 and increased the levels of pro-apoptotic proteins such as Bax. Capsaicin-induced apoptosis in colo 205 cells was also done through the activations of caspase-8, -9 and -3. In vivo studies in immunodeficient nu/nu mice bearing colo 205 tumor xenografts showed that capsaicin effectively inhibited tumor growth. The potent in vitro and in vivo antitumor activities of capsaicin suggest that capsaicin might be developed for the treatment of human colon cancer.

KEYWORDS: Capsaicin; human colon cancer colo 205 cells; apoptosis; Fas/FasL; caspases activation

INTRODUCTION

Cancer is the leading cause of death all over the world. One in every four deaths in the United States is from cancer. The incidence rate of colorectal cancer is 850,000/per year, and it causes 500,000 deaths in every part of the world (*1*). For males and females, the colon/rectum is the third leading site among all primary sites in Taiwan. The poor prognostic outcome of colorectal cancer is due to its resistance to current therapies, maintaining it as the leading cause of cancer-related death. Successful treatment with chemotherapeutic agents is largely dependent on their ability to trigger cell death in tumor cells. Therefore, novel inducers of apoptosis provide a new therapeutic approach for anticancer design. Several previous studies demonstrated that certain phytochemicals present in medicinal herbs exert antitumorigenic activity by inducing apoptosis in cancer cells.

It has long been noted that in Thailand, where much spicy food is consumed, there is a very low incidence of gastrointestinal cancers, including colorectal and stomach cancers, compared to the rest of Asia, including Japan and China. Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is widely used as a food additive and as an analgesic agent. In addition to its well-known role in nociception, it is mediated by vanilloid receptor 1 specifically expressed in dorsal root ganglion neurons. Capsaicin has also been considered as a potential anticancer agent as it inhibits cell proliferation and induces apoptosis in various types of cancer cells (*2*, *3*). Capsaicin is a major pungent ingredient of red pepper and is the active component of chili peppers, a plant belonging to the genus *Capsicum*. Natural capsaicin directly inhibits the growth of leukemic cells, and this was reported in several clinical studies conducted in Japan and China. Some studies described their early experimental findings as showing that daily consumption of hot peppers may actually prevent certain types of cancer, although these studies used pure capsaicin directly injected into isolated diseased cells in a laboratory setting (*4*).

Morre et al. reported that capsaicin acted against certain carcinogens and mutagens and, especially, was able to kill prostate cancer cells by causing them to undergo apoptosis (*5*). Morre et al. found that capsaicin preferentially repressed the growth of some transformed human and mouse cells (*5*, *6*). Hail

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and Lotan proved that capsaicin induced apoptosis in cultured cells derived from human cutaneous squamous cell carcinoma (SCC) through the increase in reactive oxygen species (ROS) generation and disruption of the mitochondrial transmembrane potential (*7*). Macho et al. demonstrated that capsaicinoid induced apoptosis in transformed cells through the involvement of ROS (*8*, *9*). The purpose of this research is to conduct a series of experiments that afford further insights into mechanisms underlying the apoptogenic action of capsaicin on human colon cancer cells inclusive of cytotoxicity, cell cycle arrest, and proteins involved in apoptosis. We also provide evidence suggesting that capsaicin causes apoptosis through extrinsic, intrinsic, and ER stress pathways.

MATERIALS AND METHODS

Chemicals and Reagents. Capsaicin, propidium iodide (PI), trypan blue, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium dihydrogen phosphate and dimethyl sulfoxide (DMSO) were obtained from Merck Co. (Darmstadt, Germany). RPMI-1640, penicillin−strptomycin, trypsin−EDTA, fetal bovine serum (FBS), and L-glutamine were obtained from Gibco BRL (Invitrogen, Grand Island, NY). Caspase-3, -8, and -9 activity assay kits were bought from OncoImmunin, Inc. (Gaithersburg, MD).

Human Colon Adenocarcinoma Colo 205 Cells. The human colon adenocarcinoma cell line (colo 205) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The colo 205 cells were placed into 75 cm^2 tissue culture flasks and grown at 37 °C under a humidified 5% CO_2 atmosphere in RPMI-1640 medium with 2 mM L-glutamine, 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Cell Viability of Colo 205 Cells Treated with Capsaicin Determined by Flow Cytometry. The colo 205 cells were seeded into 12-well plates at a density of 2×10^5 cells/well. After 24 h of incubation, cells were treated with various concentrations of capsaicin inclusive of 0, 25, 50, 100, 200, 300, 400, or 500 μM, whereas only DMSO (solvent) was added for the control regimen, and grown at 37 °C and 5% CO_2/95% air for the indicated time points. After treatment, the number of viable cells was determined by the flow cytometric assay as previously described (*10*, *11*).

Apoptotic Colo 205 Cell Determinations. Approximately 2×10^5 cells/well of colo 205 cells in 12-well plates with 0, 25, 50, 100, 200, 300, 400, or 500 μM capsaicin was incubated for 24 or 72 h. The cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol at −20 °C overnight, then resuspended in PBS containing 40 μg/mL PI, 0.1 mg/mL RNase, and 0.1% Triton X-100 in a dark room for 30 min at 37 °C, and analyzed with a flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an argon ion laser at 488 nm wavelength. Then the cell cycle and sub-G1 group (apoptosis) were determined as described previously (*11*, *12*).

Measurement of Intracellular ROS and Ca^{2+} Generations and Levels of Mitochondrial Membrane Potential ($\Delta\Psi_m$) after Treatment with Capsaicin by Flow Cytometry. Approximately 2×10^5 cells/well of colo 205 cells seeded into 12-well plates was treated with 0 μM (control) or 150 μM capsaicin and was incubated for 1, 3, 6, and 12 h to detect changes of ROS, Ca^{2+}, and $\Delta\Psi_m$. The cells were harvested and washed with PBS twice, resuspended in 500 μL of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) for ROS, 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester (Indo 1/AM) (3 μg/mL) for Ca^{2+}, and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (Calbiochem) (1 μmol/L) for $\Delta\Psi_m$. They then were incubated at 37 °C for 30 min and were analyzed by flow cytometry as described previously (*12*, *13*).

Caspase-3, -8, and -9 Activity Assays. Approximately 1×10^7 colo 205 cells was placed in 12-well plates, and cells were pretreated with or without specific inhibitors (15 μM) of caspase-3, -8, and -9 (Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK) individually, before being treated with 150 μM capsaicin and incubated for 24 h. Caspase-3, -8, and -9 activities were assessed by caspase colorimetric assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 mM digitonin, and 2 mM DTT). Cells were mixed with lysis buffer and put on ice and then centrifuged at 13000 rpm for 20 min. We then transferred the suspension into a new tube. After quantification, 50 μg of protein was incubated with caspase-3, -9, and -8 specific substrates (Ac-DEVD-pNA, Ac-LEHD-pNA, and Ac-IETD-pNA; R&D Systems) for 1 h at 37 °C. The caspase activity was determined by measuring OD_{405} of the released pNA (*14*), and the percentage of viable cells was also determined as described above (*15*).

Table 1. DNA Sequence Evaluation Using Primer Express Software[a]

primer name	primer sequence
homo caspase-3-F	CAGTGGAGGCCGACTTCTTG
homo caspase-3-R	TGGCACAAAGCGACTGGAT
homo caspase-8-F	GGATGGCCACTGTGAATAACTG
homo caspase-8-R	TCGAGGACATCGCTCTCTCA
homo caspase-9-F	TGTCCTACTCTACTTTCCCAGGTTTT
homo caspase-9-R	GTGAGCCCACTGCTCAAAGAT
homo AIF-F	GGGAGGACTACGGCAAAGGT
homo AIF-R	CTTCCTTGCTATTGGCATTCG
homo Endo G-F	GTACCAGGTCATCGGCAAGAA
homo Endo G-R	CGTAGGTGCGGAGCTCAATT
homo GAPDH-F	ACACCCACTCCTCCACCTTT
homo GAPDH-R	TAGCCAAATTCGTTGTCATACC

[a] Caspase-3, cysteine aspartic acid specific protease-3; caspase-8, cysteine aspartic acid specific protease-8; caspase-9, cysteine aspartic acid specific protease-9; AIF, apoptosis-inducing factor; Endo G, endonuclease G.

RNA Preparation and Real-Time Polymerase Chain Reaction (PCR). Approximately 2×10^5 cells/well of colo 205 cells in 6-well plates was treated with 150 μM capsaicin and was incubated for 0 (control) or 24 h. The total RNA was extracted from the colo 205 cells using a Qiagen RNeasy Mini Kit as described previously (*16*) after cotreatment with 150 μM capsaicin at 0 and 24 h of incubation time. A High-Vapacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for reverse transcription at 42 °C for 30 min, and then SYBR Green PCR Master Mix was added. An Applied Biosystems 7300 Real-Time PCR system was performed to analyze the products by C_T method. The DNA sequence of primers was evaluated by using Primer Express software as described in **Table 1** (*17*).

Protein Preparation and Western Blotting for Examination of the Levels of Proteins Associated with Cell Cycle and Apoptosis. Approximately 2×10^5 cells/well of colo 205 cells in 12-well plates was treated with 150 μM capsaicin and was incubated for 0, 6, 12, 18, or 24 h. Cells were harvested and then lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Triton X-100, and 5 mM EDTA containing both 1% protease inhibitor (Sigma Chemical Co.) and 1% phosphatase inhibitor mixture II (Sigma Chemical Co.). For total protein determination the cell lysates (40 μg of each land) were separated on a polyacrylamide gel followed by electrotransfer onto a sequi-blot polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA). Phosphorylation-specific rabbit polyclonal antibodies were purchased from New England Biolabs (Beverly, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA) was used as a secondary antibody for enhanced chemiluminescence (NEN Life Science Products, Inc., Boston, MA) as described previously (*11*). Western blotting for examining the effect of capsaicin on caspase-3, -8, and -9, Bcl-2, Bcl-x_L, xIAPs, cIAP1, Fas, FADD, Bax, Bak, Bad, cytochrome *c*, AIF, Apaf-1, p53, and p21 was performed, and quantitative evaluation of image from immunoblotting was done by using a computerized digital image system (NIH Image J).

In Vivo Antitumor Efficacy Study. Twenty-eight female BALB/c athymic nude mice (4−6 weeks old; 18−22 g) were purchased from the Laboratory Animal Center of National Applied Research Laboratories (Taipei, Taiwan). The animals were maintained under specific-pathogen-free conditions as previously described (*17*, *18*). The colo 205 cells were grown, harvested, washed with 1× PBS, and then resuspended in RPMI-1640 medium. The colo 205 cells (5×10^6 cells/mice) were subcutaneously (sc) injected into the flanks of mice for 10 days. After the tumor volume reached 150 mm^3, capsaicin treatment started. The 28 animals were randomly divided into 4 groups. Each group contained 7 animals. Group I is the control group that was treated with vehicle (1% DMSO) only.

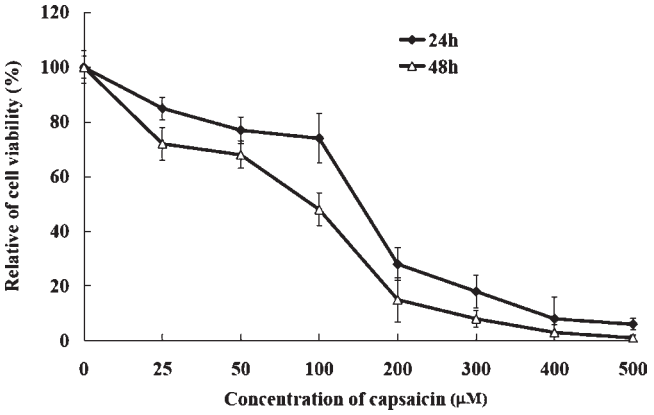

Figure 1. Capsaicin affects the total human colon cancer viable colo 205 cells. Cells were incubated with or without 0, 25, 50, 100, 200, 300, 400, or 500 μM capsaicin for 24 and 48 h and then were harvested for determination of the percentage of viable cells by flow cytometry as described under Materials and Methods.

Group II was treated with 5-FU (33 mg/kg). Group III was ip-treated with capsaicin at 1 mg/kg, and group IV was ip-treated with capsaicin at 3 mg/kg. At the end of the experiment (4 weeks after cell inoculation), the animals were anesthetized by CO_2 and sacrificed. Tumors from each animal were removed, measured, and weighed individually as described previously (*17*). Relative tumor growth (%) was calculated for the mean tumor volumes at day 30 as 100% (*19*).

Statistical Analysis. Differences between the capsaicin-treated and control groups were analyzed by the unpaired Student's *t* test and ANOVA analysis, a probability of $p < 0.05$ being considered to be significant.

RESULTS

Effects of Capsaicin on Colo 205 Cell Viability Detected by Flow Cytometry. Increasing the dose of capsaicin and/or time of incubation led to a decrease in the percentage of viable cells. Capsaicin at 500 μM significantly decreased the viable cells by almost 97%. Fifty percent of the viable cells were detected by treatment at 150 μM with 24 h of incubation (**Figure 1**).

Effects of Capsaicin on Colo 205 Cell Cycle Arrest and Apoptosis. The results from flow cytometric analysis shown in **Figure 2** indicated that after colo 205 cells were treated with various doses of capsaicin for 24 h of incubation, the increase in the percentage of cells in G0/G1 was dose-dependent (**Figure 2**). That the sub-G1 groups also appeared in the cell cycle meant apoptosis. **Figure 2** shows that when the dose of capsaicin was increased to 300 μM, about 60% apoptosis occurred, which is the highest apoptosis to occur in examined doses in colo 205 cells.

Effects of Capsaicin on the Levels of ROS, Ca^{2+}, and Mitochondrial Membrane Potential ($\Delta\Psi_m$) in Colo 205 Cells. With the loss of the outer mitochondrial membrane integrity and the release of cytochrome *c* from the mitochondria to the cytosol, the cells are committed to apoptosis (*20*, *21*). The production of ROS contributes to mitochondrial damage that may facilitate the further release of ROS into the cytoplasm (*21*). To address the possibility that capsaicin-induced apoptosis could be related to contributions from the mitochondrial pathway, colo 205 cells were treated with 150 μM capsaicin for the indicated periods of time. The changes in ROS production and $\Delta\Psi_m$ were examined, and the results are shown in **Table 2**. This is indicative that the capsaicin treatment induced a disruption of $\Delta\Psi_m$ (**Table 2**). In contrast, administration of capsaicin resulted in a right shift of the DCF and dihydroethidine fluorescence curves, indicating the increase in ROS (**Table 2**) and Ca^{2+} generations (**Table 2**).

Effects of Capsaicin on the Activities of Caspase-3, -8, and -9 and the Percentage of Viable Colo 205 Cells. To evaluate the effects of capsaicin on the activities of caspase-3, -8, and -9 and the percentage of viable colo 205 cells, we used the caspase activity assay and flow cytometric analysis. The results are shown in **Figure 3**. Capsaicin (150 μM) promoted caspase-3, -8, and -9 activities (**Figure 3A**) after a 24 h incubation; however, cells were pretreated with inhibitors of caspase-3, -8, and -9, respectively, which led to decreases in caspase-3, -8, and -9 activities and increases in the percentage of viable cells (**Figure 3B**). These result indicated that capsaicin-induced apoptosis is mediated by induction of caspase-8, -9, and -3 activities.

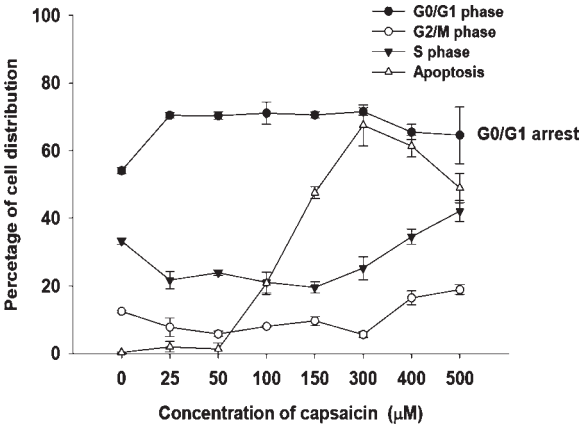

Figure 2. Capsaicin affects the distribution of cell cycle and apoptosis in human colon cancer colo 205 cells. Cells were incubated with or without 0, 25, 50, 100, 200, 300, 400, or 500 μM capsaicin for 24 h and then were harvested for determination of the distribution of cell cycle by flow cytometry as described under Materials and Methods.

Table 2. Flow Cytometric Analysis of Intracellular ROS, Ca^{2+}, and $\Delta\Psi_m$ Levels in Colo 205 Cells with Capsaicin Treatment[a]

	% of control		
time (h)	ROS	Ca^{2+}	$\Delta\Psi_m$
0	2.52 ± 2.55	1.34 ± 1.32	100.32 ± 5.69
1	38.26 ± 3.58***	92.32 ± 3.25***	75.63 ± 9.52***
3	35.36 ± 5.69 ***	55.36 ± 8.46***	52.36 ± 6.47***
6	36.95 ± 4.13***	72.46 ± 4.16***	33.65 ± 2.58 ***
12	41.23 ± 6.53***	38.25 ± 3.56***	21.65 ± 2.78 ***

[a] Colo 205 cells (2×10^5 cells/mL) were treated for 0, 1, 3, 6, and 12 h with capsaicin. 0 h was defined as control. The percentage of ROS, Ca^{2+}, and $\Delta\Psi_m$ stained by DCFH-DA, Indo-1/AM, and $DiOC_6$, respectively. The stained cells were determined by flow cytometry as described under Materials and Methods. Values are means ± SD ($n = 3$). Significantly different from the 0 h treatment at ***, $p < 0.001$.

Effects of Capsaicin on mRNA Expression Examined by Real Time-PCR. After 150 μM capsaicin treatment in colo 205 cells for 0, 24 h of incubation, gene expression of the mRNA levels of caspases-3, -8, and -9, AIF, and Endo G was observed. Compared with control, caspases-3, -8, and -9 and AIF were elevated except Endo G (not change) for 24 h of incubation (**Figure 4**).

Western Blotting for Examination of the Levels of Proteins Associated with Cell Cycle and Apoptosis. To confirm that the cytotoxic effect of capsaicin on colo 205 cells, as noted in flow cytometric assays, was due to apoptosis, colo 205 cells were cultured for 0, 6, 12, 18, and 24 h in the presence of the DMSO vehicle alone or 150 μM capsaicin. Cells were harvested from each treatment, and proteins were prepared for Western blot analysis of the extrinsic, intrinsic, and ER stress related protein expression. Western blots indicate in **Figure 5** that the levels of caspases-3, -8, and -9, Fas, FADD, Bax, Bad, cytochrome *c*, AIF, Apaf-1, p53, p21, smac/DIABLO, GADD153, and GRP78 were increased and

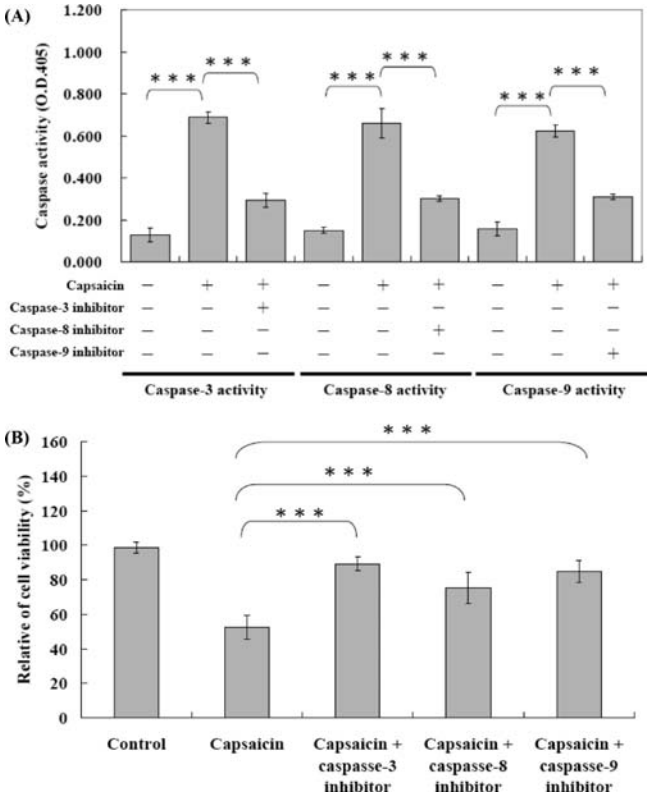

Figure 3. Capsaicin induced caspase-8, -9, and -3 activity in human colon cancer colo 205 cells. Cells were pretreated with caspase-3, -8, and -9 inhibitor and then were treated with 150 μM capsaicin for 24 h; cells were collected for determination of activities of caspase-3, -8, and -9 (**A**) and percentage of viable cells (**B**) as described under Materials and Methods. Data represent the mean ± SD of three experiments. ***, $p < 0.001$.

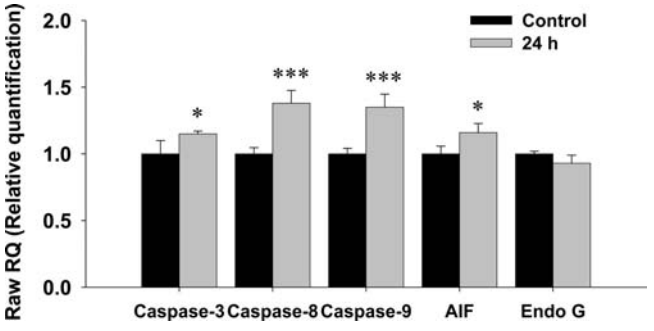

Figure 4. Effects of capsaicin on the gene expression (mRNA) of caspases-3, -8, and -9, AIF, and Endo G in human colon cancer colo 205 cells. The colo 205 cells were treated with or without 150 μM capsaicin for 0 and 24 h, and then cells were harvested for isolation of total RNA and then for real-time PCR to examine the gene expression of caspases-3, -8, and -9, AIF, and Endo G as described under Materials and Methods. *, $p < 0.05$ and ***, $p < 0.001$.

those of Bcl-2, Bcl-xL, and cIAP-1 were decreased and may have led to apoptosis.

Capsaicin Inhibited Tumor Growth of Tumor Xenograft in Nude Mice. We further examined the effects of capsaicin on in vivo tumor growth for 4 weeks in a colo 205 xenograft model. The 5-FU (33 mg/kg)-treated mice acted as a positive control. As summarized in **Figure 6** and **Table 3**, capsaicin inhibited colo 205 tumor growth in nude mice. Capsaicin was injected ip once every 3 days at two different doses (1 and 3 mg/kg). Tumor size and weight in the vehicle control group increased 6−8-fold over a period of 30 days (**Figure 6A−C**). Capsaicin inhibited tumor growth in xenograft nude mice. Capsaicin (1 and 3 mg/kg) used once every 3 days resulted in 26.6 and 19.8% T/C [**Table 3**, treatment group (T) over control (C) tumor volume ratio × 100%]. Relative to control animals, body weight changes did not show significant differences between control and capsaicin-treated groups (**Figure 6D**).

DISCUSSION

At least three apoptosis pathways had been addressed (*22*−*24*): First, the extrinsic pathway is initiated by ligation of transmembrane death receptors (Fas, TNF receptor, and TRAIL receptor) with their respective ligands (FasL, TNF, and TRAIL) to activate membrane-proximal caspases (caspases-8 and -10), which in turn cleave and activate effector caspases such as caspases-3 and -7. This pathway can be regulated by c-FLIP, which inhibits upstream initiator caspases, and apoptosis proteins (IAPs), which affect both initiator and effector caspases (*25*). Second, the intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins, such as cytochrome *c*, which is released from the mitochondrial intermembrane space to cytoplasm, to work together with the other two cytosolic protein factors, Apaf-1 (apoptotic protease activating factor-1) and procaspase-9, to promote the assembly of a caspase-activating complex termed the apoptosome, which in return induces activation of caspase-9 and thereby initiates the apoptotic caspase cascade (*23*, *26*, *27*). Third, the endoplasmic reticulum (ER) is the site of assembly of polypeptide chains destined for secretion or routing into various subcellular compartments. It also regulates cellular responses to stress and intracellular Ca^{2+} levels. A variety of toxic insults can result in ER stress that ultimately leads to apoptosis (*28*).

For the involvement of extrinsic apoptosis pathway, the related protein levels induced by capsaicin such as caspases-3, -8, and -9, Fas, and FADD increase. We may conclude that the extrinsic apoptosis pathway was affected by capsaicin. For the involvement of Bcl-2 family proteins in capsaicin-induced apoptosis, we investigated the Bcl-2 family molecules by regulating the release of cytochrome *c* from the mitochondria (*29*). The levels of Bcl-2 family members were detected by Western blot analysis. As shown in **Figure 5C**, exposure of colo 205 cells to 150 μM capsaicin caused a significant decrease in Bcl-xL, whereas there was an increase in Bax and Bad protein levels after treatments.

The caspase-independent pathway of intrinsic apoptosis was also triggered by the observation of the increase of AIF protein (**Figure 5B**) and gene expression (**Figure 4**). Due to Apaf-1 and smac/DIABLO elevation, the caspase-dependent pathway of intrinsic apoptosis may play an important role. Caspases are a family of cysteine proteases that play a central role during the executional phase of apoptosis (*30*). To explore whether capsaicin induces apoptosis by activation of caspases, caspase-3, -8, and -9 activity should be detected. The results shown in **Figure 5D** confirm that caspases-3, -8, and -9 were activated in colo 205 cells after 12 h of capsaicin treatment. The marked activations of caspases-3, -8, and -9 were observed after 18 h of capsaicin treatment. However, caspases-8 and -9 were slightly activated during late stages of capsaicin-induced apoptosis.

GADD153, also known as chop, is a highly stress-inducible gene that is robustly expressed following disruption of homeostasis in the ER (so-called ER stress). Although many studies have demonstrated that capsaicin induces cytotoxicity and apoptosis in human cancer cells, there is still no available information about the involvement of GADD153 in capsaicin-induced apoptosis. In this study, we concluded that GADD153 sensitizes cells

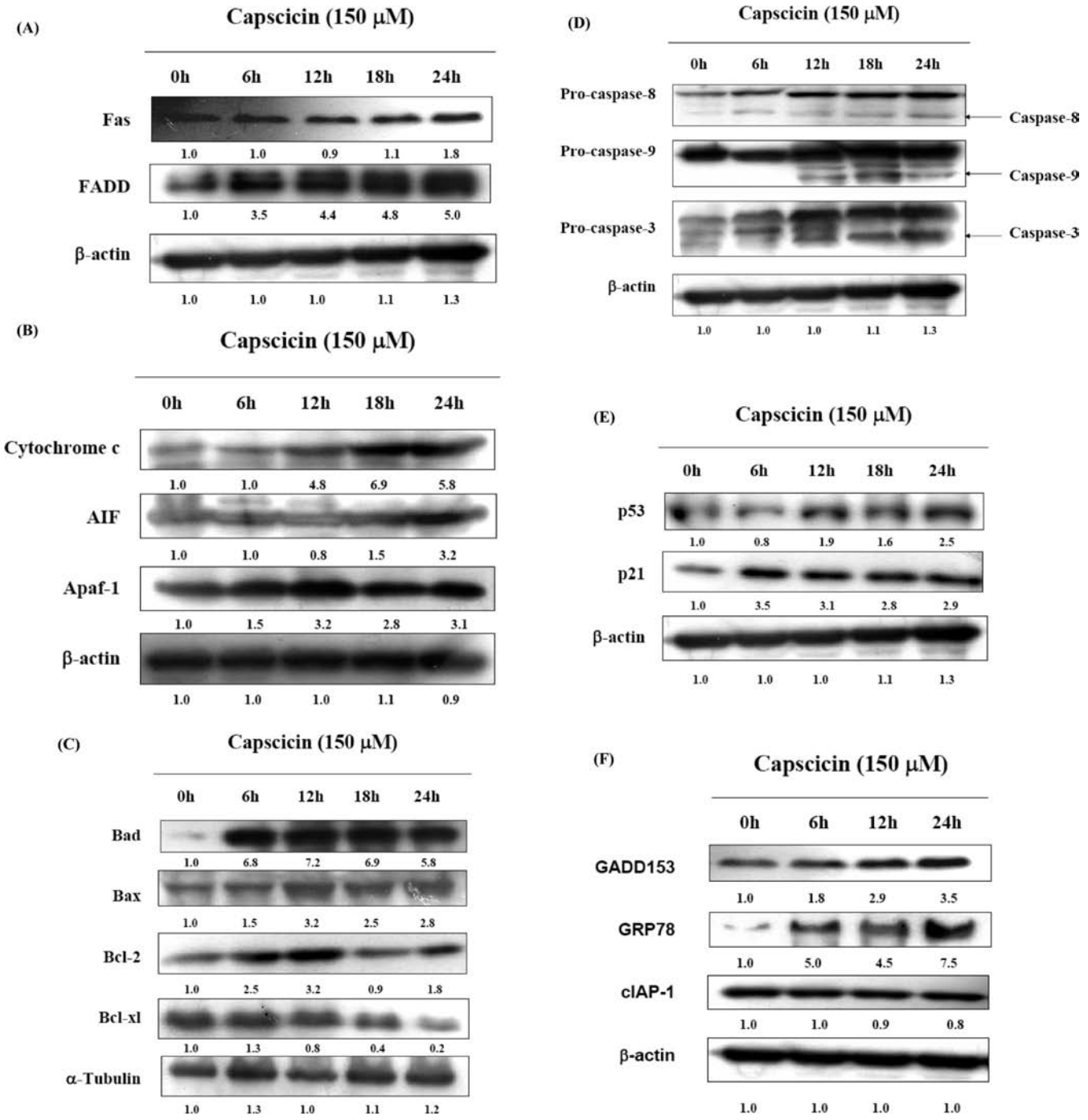

Figure 5. Effects of capsaicin on the levels of apoptotic associated proteins in human colon cancer colo 205 cells: (**A**) Fas and FADD; (**B**) cytochrome *c*, AIF, and Apaf-1; (**C**) Bax and Bad; (**D**) procaspase-3, -9, and -8; (**E**) p53, p21, and xIAPs; (**F**) GADD153, GRP78, smac/DIABLO, xIAPs, and cIAP1. Cells were incubated with or without 150 μM capsaicin for 0, 6, 12, 18, and 24 h incubations, and the cells were collected for Western blotting as described under Materials and Methods.

to ER stress through mechanisms that involve down-regulation of Bcl-2 and enhanced oxidant injury. GRP78 is also an ER chaperone protein. Our findings suggested that these events indicate that extrinsic, intrinsic, and ER stress apoptosis pathways were all induced by capsaicin. Accumulating evidence indicates that mitochondria play a pivotal role in the apoptotic process. Disruption of mitochondrial membrane potential is considered to be an indicator of mitochondrial damage and generally is defined as an early stage of apoptosis, preceding efflux of small molecules from the mitochondria (including cytochrome *c*, apoptosis-inducing factor, cIAPs, etc.) and followed by caspase-9/caspase-3 cascade activation.

It is becoming appreciated that the tumor suppressor protein p53 serves as a key player in the cellular response to a variety of extracellular and intracellular insults, such as DNA damage, oncogenic activation, and microtubule disruption (*31*, *32*), exerting its function mainly through transcriptional activation of target genes, such as the cyclin-dependent kinase (CDK) inhibitor p21, for arresting the cell cycle, and the proapoptotic protein Bax for inducing apoptosis (*33*, *34*). In this research, p53, p21, and Bax were increased and may have led to apoptosis. This paper describes the selective in vitro killing of colo 205 cells by capsaicin, the anticancer activity that is based on apoptotic cell death. The morphology changes of capsaicin-treated cells were typical of

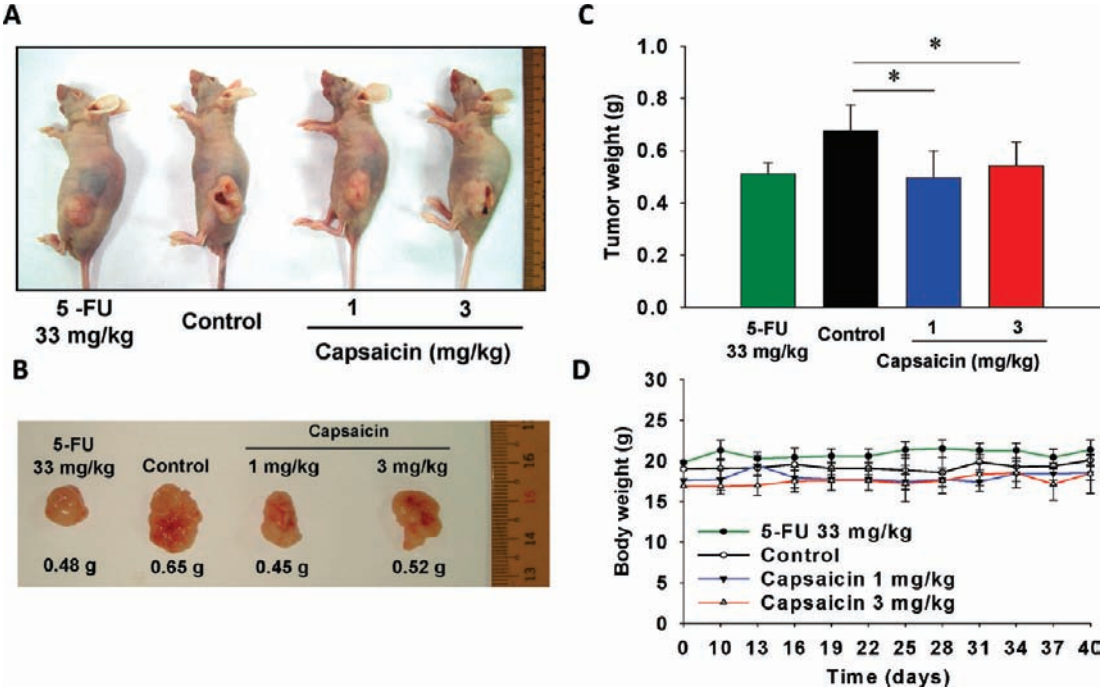

Figure 6. Capsaicin inhibited the tumor growth of tumor xenograft in nude mice. The colo 205 cells were injected subcutaneously into the flanks of male nu/nu mice. Representative tumors from animals were treated with or without capsaicin and 5-FU (**A**); (**B**) representative bearing xenograft solid tumors in nude mice, (**C**) effects of capsaicin and 5-FU on tumor weight; (**D**) effects of capsaicin and 5-FU on body weight of tumor xenograft mice. Data presented are the mean ± SD at 10−40 days post-tumor implantation; groups were compared and analyzed using the unpaired Student *t* test. ∗, $p < 0.05$.

Table 3. Effects of Tumor Weight and Inhibition Rate of Capsaicin on Leukemia Xenograft Tumor in the BALB/c$^{nu/nu}$ Mice Model[a]

dose or treatment	tumor weight (g)	inhibition rate (%)
control	0.656 ± 0.099	
5-FU 33 mg/kg	0.510 ± 0.043	24.54
capsaicin 1 mg/kg	0.496 ± 0.104***	26.62
capsaicin 3 mg/kg	0.552 ± 0.091***	19.83

[a] The nude mice were subcutaneously (sc) injected with colo cells (5×10^6 cells/mice), and the animals were randomly divided into four groups. At the end of treatment, each animal was measure for tumor weight as described under Materials and Methods. Results indicated tumor weight and inhibition rate (%), and data are expressed as the mean ± SD and analyzed by Student's *t* test. ***, $p < 0.001$.

apoptosis, nuclear condensation, DNA fragmentation, and caspase activation, all hallmarks of apoptosis. Similar observations have been reported in capsaicin-treated leukemic cells (*4*), human prostate cancer cells (*35*), human and mouse melanoma lines (*6*), and human cutaneous squamous cell carcinoma (*7*). The results from in vivo experiments show that the tumor grew faster in DMSO control group and slower in 5-FU- and capsaicin-treated groups. The tumor volumes and weights in the 5-FU- and capsaicin-treated groups were significantly smaller than those in the DMSO group after 40 days (**Figure 6**).

In addition, this research has demonstrated that capsaicin induces cytotoxicity and apoptosis in colo 205 cells. In conclusion, the capsaicin-induced apoptotic events can be summarized by the sequence presented in **Figure 7**. In this model, capsaicin-mediated ROS generation represents the central trigger for activation of the apoptotic cascade. Exposure of colo 205 cells to capsaicin resulted in generation of ROS and a decrease in $\Delta\Psi_m$, as well as in elevation of Bax and reduction of Bcl-xL levels. The movement of Bax to the mitochondria reduced the mitochondrial membrane potential, an event that results in the release of cytochrome *c* and subsequent activation of caspases-2, -3, and -9, and consequently cleaved specific substrates leading

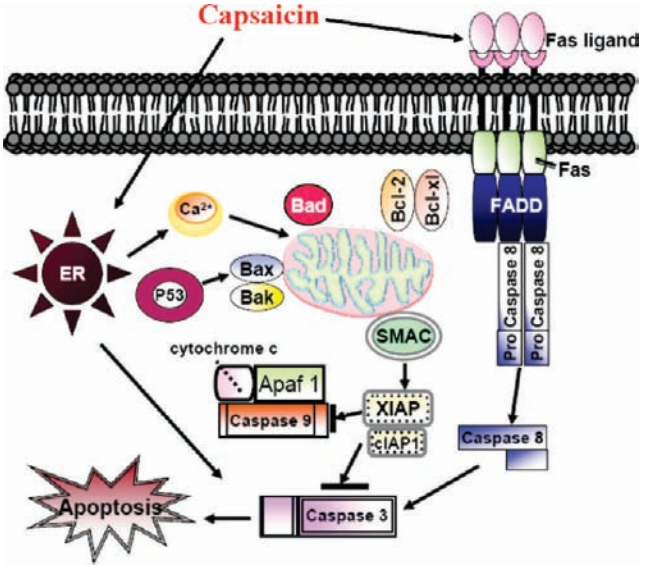

Figure 7. Possible signal pathways from capsaicin-induced apoptotic events.

to apoptotic changes. This observation is consistent with the conclusion of a recent study about the effect of capsaicin in pancreatic cancer cell lines (*36*). These findings may aid in the understanding of the mode of actions of capsaicin and provide a theoretical basis for the therapeutic use of this compound in the future.

ABBREVIATIONS USED

$\Delta\Psi_m$, mitochondrial membrane potential; Apaf-1, apoptosis protease-activating factor-1; DAPI, 4′,6,-diamidino-2-phenylindole; DiOC6, 3,3′-dihexyloxacarbocyanine iodide; ER, endoplas-

mic reticulum; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; ROS, reactive oxygen species; PI, propidium iodide.

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