Capsaicin Induces Apoptosis in SCC-4 Human Tongue Cancer Cells Through Mitochondria-Dependent and -Independent Pathways

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ABSTRACT: Although there have been advances in the fields of surgery, radiotherapy, and chemotherapy of tongue cancer, the cure rates are still not substantially satisfactory. Capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide) is the major pungent ingredient of hot chili pepper and has been reported to have an antitumor effect on many human cancer cell types. The molecular mechanisms of the antitumor effect of capsaicin are not yet completely understood. Herein, we investigated whether capsaicin induces apoptosis in human tongue cancer cells. Capsaicin decreased the percentage of viable cells in a dose-dependent manner in human tongue cancer SCC-4 cells. In addition, capsaicin produced DNA

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fragmentation, decreased the DNA contents (sub-G1 phase), and induced G0/G1 phase arrest in SCC-4 cells. We demonstrated that capsaicin-induced apoptosis is associated with an increase in reactive oxygen species and Ca^{2+} generations and a disruption of the mitochondrial transmenbrane potential ($\Delta\Psi_{m)}$. Treatment with capsaicin induced a dramatic increase in caspase-3 and -9 activities, as assessed by flow cytometric methods. A possible mechanism of capsaicin-induced apoptosis is involved in the activation of caspase-3 (one of the apoptosis-executing enzyme). Confocal laser microscope examination also showed that capsaicin induced the releases of AIF, ATF-4, and GADD153 from mitochondria of SCC-4 cells. Environ Toxicol 00: 000–000, 2010.

Keywords: capsaicin; human tongue SCC-4 cells; apoptosis; mitochondria; caspases-3

INTRODUCTION

Cancer is the major cause of death in the world. In the United States, one in every four deaths is from cancer. Oral and pharyngeal cancers number over 300,000 cases annually with male outnumbering female worldwide (Pintos et al., 2008). Based on the 2008 report from the Department of Health, R.O.C. (Taiwan), it was indicated that 9.6 individuals per 100,000 die annually from oral cancer in Taiwan. In the oral cavity, oral tongue squamous cell carcinoma (SCC) has been shown to be associated with the highest rate of metastasis when compared with other tumor sites (Sano and Myers, 2007). Although cures for oral cancer including surgery, radiotherapy, and current chemotherapeutic options are still inadequate, there is still focus on identifying new agents and novel targets for treating oral cancer (Ichimiya et al., 2005). The best strategy for treatment of cancer from chemotherapeutic agents is to induce apoptosis of cancer cells. It is well documented that some of the phytochemicals present in medicinal herbs exerts antitumorigenic activity via inducing apoptosis in cancer cells.

Capsaicin, a homovanillic acid derivate, is an active pharmaceutical ingredient of red pepper, genus *Capsicum*, and has been shown to induce apoptosis of many tumors cells (Hail, 2003; Ito et al., 2004; Amantini et al., 2007). Several reports have shown that reactive oxygen species (ROS) act as the principal signaling molecules in capsaicin-induced apoptosis (Lee et al., 2004; Zhang et al., 2008; Huang et al., 2009), but other reports also showed that capsaicin treatment reduced endogenous ROS levels in several cell lines (Oh et al., 2004; Baek et al., 2008). It was reported that p53 is important for capsaicin-induced growth arrest and apoptosis in human myeloid leukemia NB4 cells (Ito et al., 2004). Recently, it was reported that capsaicin induces apoptosis in prostate cancer cells including wild-type p53-expressing LNCaP cells, p53-null PC-3 cells, and p53-mutant DU-145 cells (Mori et al., 2006). It was also reported that capsaicin induced apoptosis in cultured cells derived from human cutaneous SCC via the increase in ROS generation and disruption of the mitochondrial transmembrane potential (Hail and Lotan, 2002). Therefore, the exact molecular mechanisms of capsaicin-induced apoptosis in human oral or tongue cancer cells still remain unclear. The purpose of this study is to investigate a series of experiments regarding the apoptogenic action of capsaicin on human tongue cancer cells inclusive of cytotoxicity, cell-cycle arrest, and proteins involved in apoptosis. We offer more information to show that capsaicin induced apoptosis via mitochondrial-dependent and ER stress pathway in human tongue cancer SCC-4 cells.

MATERIALS AND METHODS

Chemicals and Reagents

Capsaicin, dimethyl sulfoxide (DMSO), propidium iodide (PI), Triton X-100, and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI-1640, fetal bovine serum (FBS), penicillin–streptomycin, trypsin–EDTA, and glutamine were purchased from Gibco BRL (Invitrogen, Grand Island, NY). Caspase-3, -8, and -9 activity assay kits were obtained from OncoImmunin (Gaithersburg, MD).

Cell Line

The human SCC-4 cell line (human oral SCC) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in 75-cm^2 tissue-culture flasks at 37°C under a humidified 5% CO_2 and 95% air atmosphere as we have previously reported (Chen et al., 2009, 2010).

Determinations of Viability and Cell-Cycle Distribution of SCC-4 Cells

The SCC-4 cells were plated into 12-well plates at a density of 2×10^5 cells/well and maintained for 24 h. Cells were treated with 0, 200, 250, 300, and 400 μM capsaicin, while only adding DMSO (solvent) for the control regimen and grown at 37°C, 5% CO_2, and 95% air for 12, 24, 36, and 48 h. For viability determination, after treatment, cells were harvested, and the number of viable cells was determined by the flow cytometric assay cited previously (Tan et al., 2006; Ji et al., 2009). For cell cycle and sub-G1 phase determination, the harvested cells were washed with phosphate-buffered saline (PBS) and were fixed in 70% ethanol

at −20°C overnight then resuspended in PBS containing 40 μg/mL PI and 0.1 mg/mL RNase and 0.1% Triton X-100 in a dark room for 30 min at 37°C and analyzed by flow cytometry (Becton-Dickinson, San Jose, CA) equipped with an argon ion laser at 488-nm wavelength as described previously (Ji et al., 2009).

Determinations of DNA Damage and DNA Fragmentation

Approximately 2×10^5 cells/mL of SCC-4 cells in 12-well plates were treated with 200, 250, and 300 μM of capsaicin for 24 h. Cells in each treatment were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and then were examined and photographed using a fluorescence microscope as previously described (Ji et al., 2009). Approximately 1×10^6 cells/well of SCC-4 cells were treated with 0, 200, 250, 300, and 400 μM of capsaicin and incubated for 24 h. Cells from each treatment were harvested by centrifugation, and the DNA was isolated for DNA gel electrophoresis. It was then followed by EtBr staining and then examined and photographed under UV light as described previously (Chen et al., 2009; Yang et al., 2009).

Determinations of ROS, Ca^{2+} Production Levels, and Mitochondrial Membrane Potential ($\Delta\Psi_m$) in SCC-4 cells

Approximately 2×10^5 cells/mL of SCC-4 cells in 12-well plate were treated with 300 μM capsaicin for 0, 3, 6, 12, and 24 h. The cells from each treatment were harvested and washed twice by PBS then resuspended in 500 μL of 2,7-dichlorodihydrofluorescein diacetate (10 μM) (DCFH-DA contains fluorescence staining for ROS) and Indo 1/AM (3 μg/mL) (dye contains fluorescence for staining of Ca^{2+}) and $DiOC_6$ (1 μmol/l) (dye contains fluorescence for staining of MMP). All samples were then incubated at 37°C for 30 min to detect percentage of changes in ROS, Ca^{2+}, and $\Delta\Psi_m$ before flow cytometry as described previously (Lin et al., 2009; Lu et al., 2010).

Determinations of Protein Levels Associated with Cell-Cycle Arrest and Apoptosis in SCC-4 Cells After Exposure to Capsaicin

Approximately 1×10^6 cells/mL of SCC-4 cells were treated with 300 μM capsaicin for 0, 12, 24, 36, and 48 h, and then cells were harvested. The harvested cells were lysed in a radioimmunoprecipitation assay buffer containing PhosSTOP (Roche Applied Science). The Western blotting analysis was performed as previously described (Lin et al., 2009; Lu et al., 2010) using monoclonal anti-P21, -P16, -cyclin D, -Cyclin E, -CDK2, -CDK6, -AIF, -cytochrome c, -pro-caspase-9, -active-caspase-9, -pro-caspase-3, -PARP, -Bax, -Bid, -ATF6-α, -IRE1-α, -GRP78, -pro-caspase-12, -Fas ligand, and -act-caspase-8 (Santa Cruz Biotechnology). Then all samples were stained by secondary antibody. The signal was detected with an enhanced chemiluminescence Western blot analysis system (GE Healthcare) (Lin et al., 2009; Lu et al., 2010).

Confocal Laser Scanning Microscopy for Protein Translocation

Approximately 5×10^4 cells/well of SCC-4 cells were cultured on four-well chamber slides for treatment without or with 300 μM capsaicin for 24 h. Then cells on the slides were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton-X 100 in PBS for 1 h with blocking of nonspecific binding sites using 2% BSA as described previously (Chen et al., 2009; Kuo et al., 2009). Primary antibodies to AIF, ATF-4, and GADD153 (1:100 dilution) (green fluorescence) were used to stain the fixed cells overnight, washed twice with PBS before being stained with secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution), and followed by DNA staining with PI (red fluorescence). All samples were microphotographed using a Leica TCS SP2 Confocal Spectral Microscope (Chen et al., 2009).

Statistical Analysis

The results are showing in mean ± SD, and the difference between the capsaicin-treated and control groups were analyzed by Student's t test, a probability of $P < 0.05$ being considered significant.

RESULTS

Capsaicin Affected the Total Human Tongue Cancer Viability and Cell-Cycle Distribution of SCC-4 Cells

The results of flow cytometric analysis are shown in Figure 1(A,B), and they indicate that increasing the dose of capsaicin and/or time of incubation led to decrease the percentage of viable cells. The capsaicin at 350 μM significantly decreased the viable cells by almost 65% [Fig. 1(A)]. The cell-cycle distribution of SCC-4 cells after treatment with different doses of capsaicin for 0, 12, 24, 36, and 48 h is depicted in Figure 1(B). The cell cycle tended to be arrested at the $G_0/G1$ stage by dose-dependent [Fig. 1(B)] after exposure to capsaicin. The sub-G_1 peaks, indicating the proportions of apoptosis, increased in a dose-dependent manner when the concentration of capsaicin added increased [Fig. 1(B)].

Capsaicin Induced DNA Damage and Apoptosis in SCC-4 Cells

To investigate and confirm that capsaicin induced apoptosis via DNA damage, the SCC-4 cells were treated with

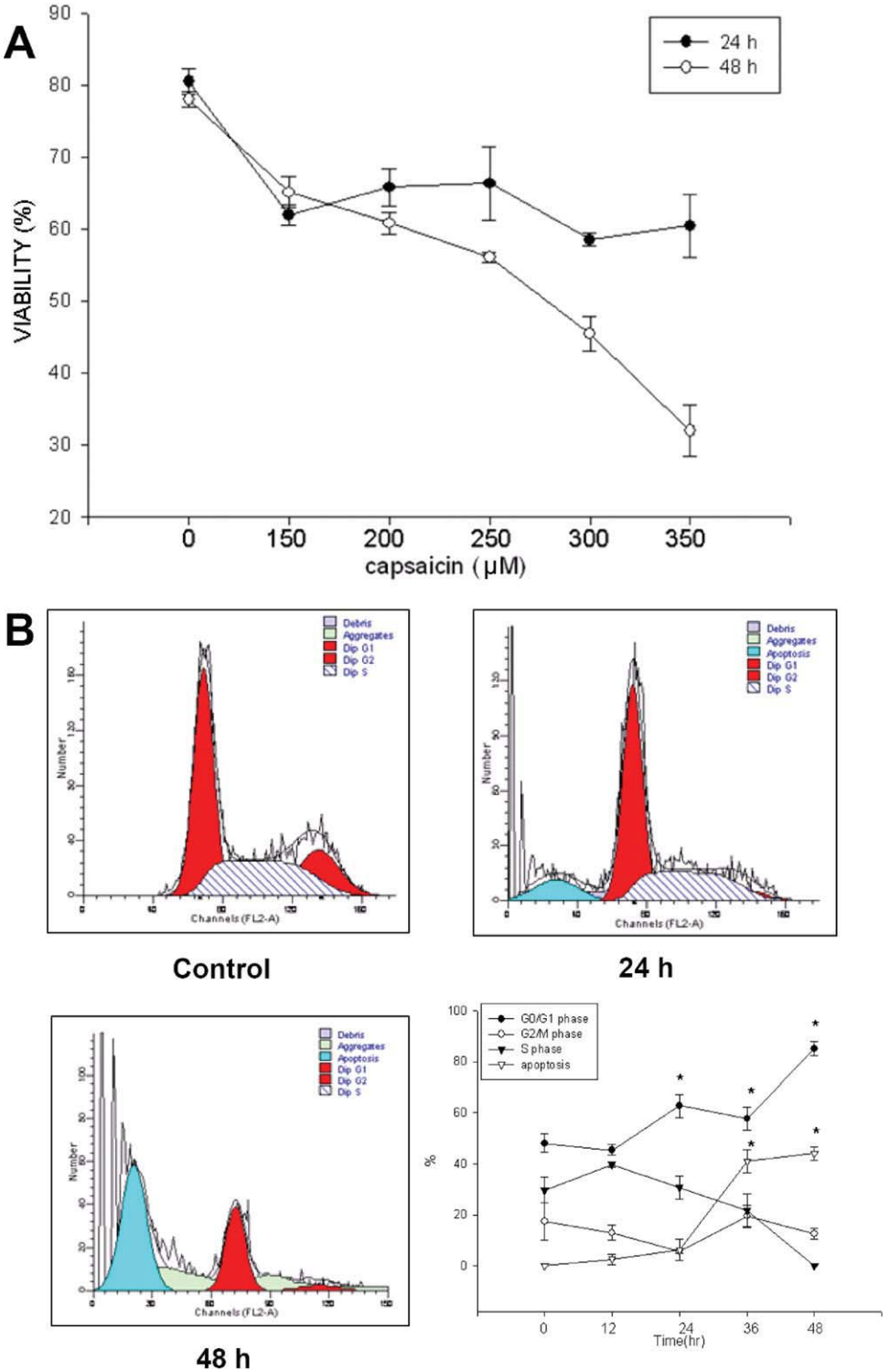

Fig. 1. Capsaicin affects the total human oral cancer viability and cell-cycle distribution of SCC-4 cells. Cells were incubated with or without 0, 150, 200, 250, 300, or 350 μM capsaicin for 24 h and 48 and then were harvested for determining the percentage of viable cells and the distribution of cell cycle by flow cytometry as described in Materials and Methods section. Data represent mean $\pm$ SD of three experiments. $^*P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

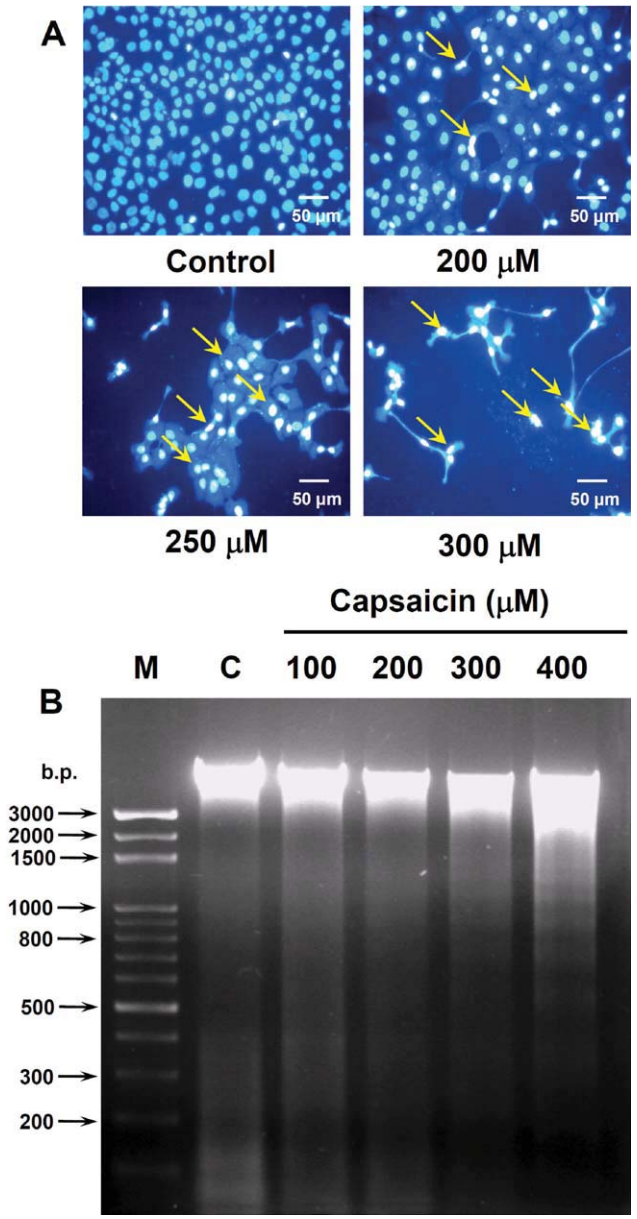

Fig. 2. Capsaicin induced DNA damage and apoptosis in SCC-4 cells. Cells were treated with different concentrations of capsaicin (0, 200, 250, 300, and 400 μM) for 24 and 48 before being harvested for DAPI staining (A) and for DNA gel electrophoresis (B) as described in Materials and Methods section. The arrow bar "↑" revealed the DNA condensation (an apoptotic characteristic). Scale bar, 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

capsaicin and isolated for DAPI staining and DNA fragmentation determination. The results are presented in Figure 2(A,B), which indicated that capsaicin induced DNA condensation [Fig. 2(A)] and DNA damage and fragmentation at 400-μM capsaicin treatment [Fig. 2(B)].

Capsaicin Affected the Productions of ROS and Ca^{2+} and the Levels of Mitochondria Membrane Potential ($\Delta\Psi_m$) in SCC-4 Cells

To investigate whether or not capsaicin induced apoptosis is involved in mitochondrial dependent, SCC-4 cells were treated with 300 μM capsaicin before cells were isolated for examining the levels of $\Delta\Psi_m$, and the results are shown in Figure 3(A), which indicated that capsaicin decreased the levels of $\Delta\Psi_m$ in SCC-4 cells. To address the possibility that the capsaicin-induced apoptosis could be related to contributions from the mitochondrial pathway, the changes in ROS and Ca^{2+} productions were further examined, and the results are shown in Figure 3(B,C). In contrast, administration of capsaicin resulted in a right shift of the DCF and dihydroethidine fluorescence curves, indicating the increase in ROS [Fig. 3(B)] and Ca^{2+} [Fig. 3(C)] generations. These effects are time-dependent manners.

The Effects of Capsaicin on the Associated Protein Levels of Apoptosis in Human Tongue Cancer SCC-4 Cells

To reconfirm that the cytotoxic effect of capsaicin on SCC-4 cells, as shown from flow cytometric assay, came from G0/G1 arrest and apoptosis, SCC-4 cells were cultured with 300 μM of capsaicin for 0, 12, 24, 36, and 48 h before being isolated for determination of protein levels and assayed by Western blotting. The results are shown in Figure 4(A–E). The figures indicated that capsaicin increased the levels of p21 and p16 [Fig. 4(A)] and decreased the levels of cyclin D, cyclin E, CDK2, and CDK6 [Fig. 4(A)] leading to the *G0/G1* phase arrest in SCC-4 cells. Capsaicin increased the levels of AIF, cytochrome c, active-caspase-9 [Fig. 4(B)], Bax [Fig. 4(C)], ATF6-a, IRE1-a, GRP78 [Fig. 4(D)], Fas ligand, and active-caspase-8 [Fig. 4(E)], but decreased the levels of pro-caspase-3 [Fig. 4(B)] and Bid [Fig. 4(C)] that led to apoptosis in SCC-4 cells.

The Effects of Capsaicin on the Location of AIF, ATF-4, and GADD153 in SCC-4 Cells

To confirm the translocation and levels of AIF, ATF-4, and GADD153 in SCC-4 cells after exposure to capsaicin, the SCC-4 cells were exposed to 300 μM of capsaicin for 24 and 48 h and stained and examined and photographed by confocal laser microscopy. The results showed in Figure 4 indicate that capsaicin promoted the levels of AIF, ATF-4, and GADD153 that reconfirm the results from Western blotting analysis.

DISCUSSION

Much evidence has shown that capsaicin induces apoptosis in many human cancer cells. However, the molecular

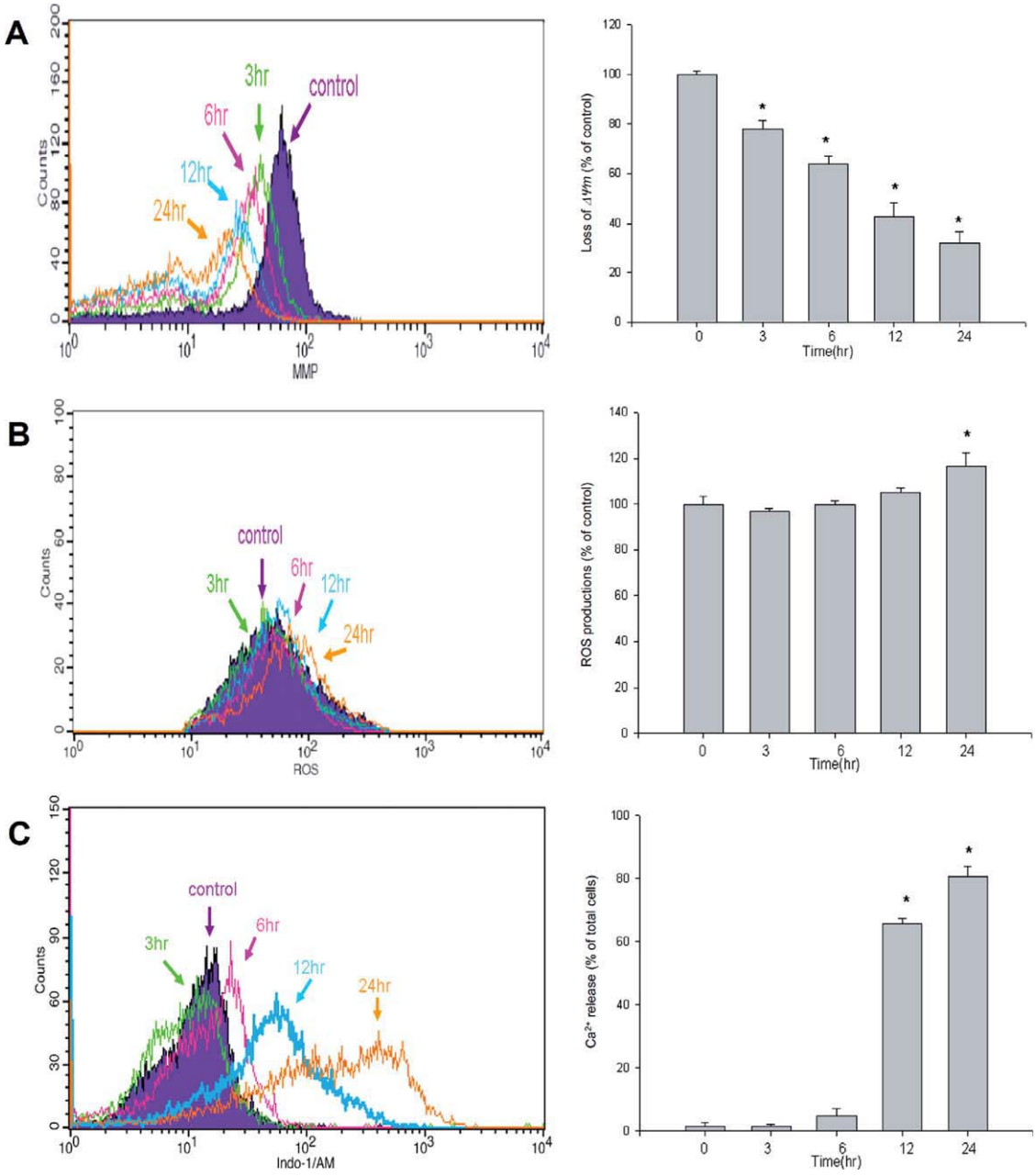

Fig. 3. Capsaicin affected the productions of reactive oxygen species (ROS) and Ca^{2+}, the levels of mitochondria membrane potential ($\Delta\Psi_{m)}$ in SCC-4 cells. Cells were treated with 300 μM of capsaicin for 0, 3, 6, 12, and 24 h before being collected and stained with $DiOC_6$ for the $\Delta\Psi_m$ levels determined (A), stained by 2,7-dichlorodihydrofluorescein diacetate for ROS levels determined (B) and stained by Indo 1/AM for Ca^{2+} levels determined (C) as described in Materials and Methods section. The % in *Y*-axis of the bar diagram refers to % cell count with fluorescence $^*P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mechanism that triggers capsaicin-induced apoptosis in human tongue cancer cells is unclear. Herein, we demonstrate that capsaicin-induced apoptosis is mediated in part through the mitochondria based on the observations: (a) we have found that capsaicin induced cytotoxic effects of SCC-4 cells in a dose- and time-dependent manner. Capsaicin-induced cell death is correlated with ROS. (b) Capsaicin treatment affected the levels of cell-cycle associated proteins, which led to G0/G1 arrest and induced apoptosis through mitochondrial-dependent pathways. (c) Capsaicin

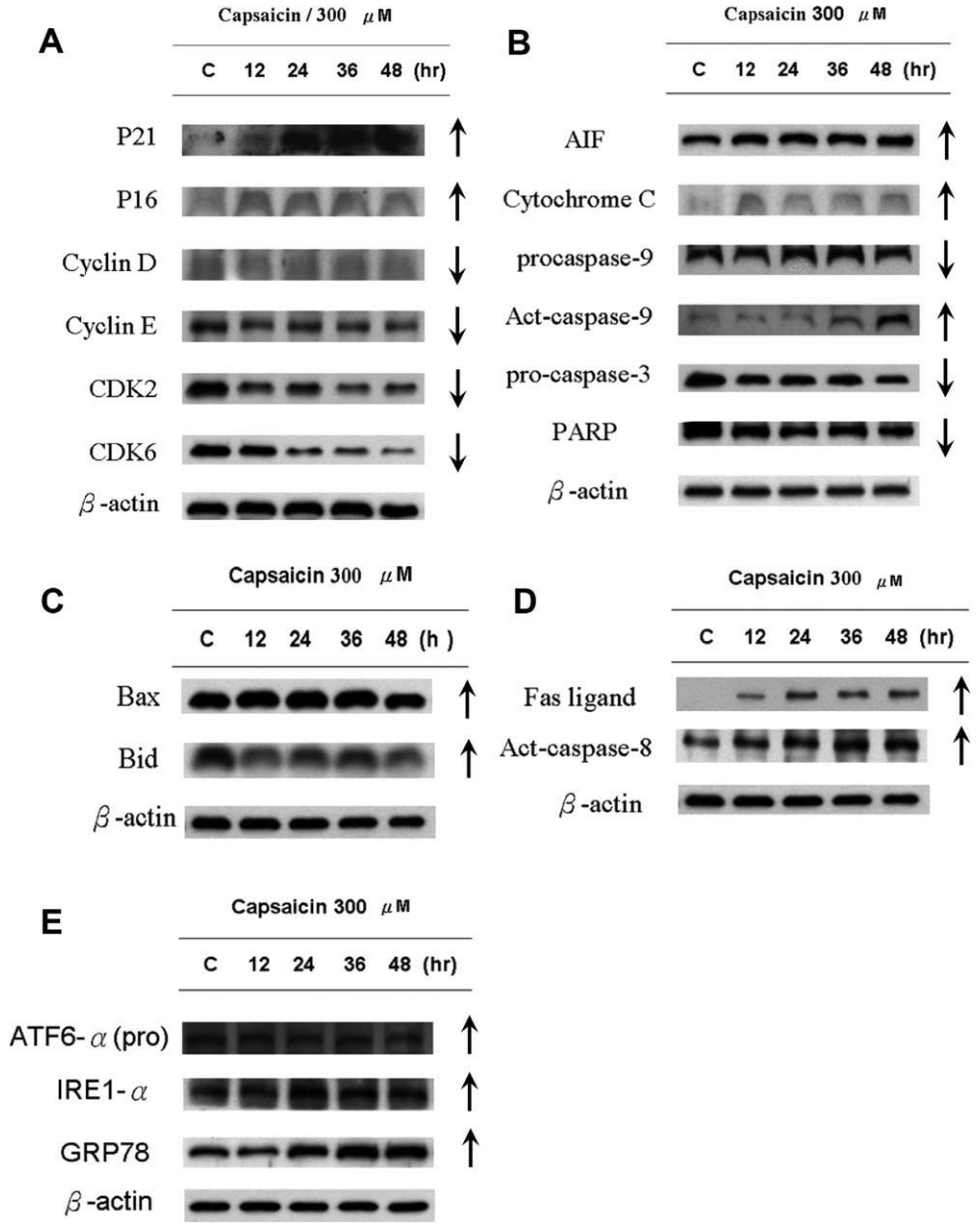

Fig. 4. The effects of capsaicin on the associated protein levels of apoptosis in human tongue cancer SCC-4 cells. Cells were incubated with or without 300 μM capsaicin for 0, 12, 24, 36, and 48-h incubations, and the cells were collected for Western blotting as described in Materials and Methods section. A: P21, P16, cyclin D, Cyclin E, CDK2 and CDK6; B: AIF, cytochrome c, pro-caspase-9, active-caspase-9, pro-caspase-3, PARP; C: Bax and Bid; D: ATF6-a; INEI-a, GRP78 and pro-caspase-12; E: Fas ligand and act-caspase-8 and Bid.

induced AIF release from mitochondria. (d) Capsaicin exposure also leads to dysfunction of mitochondria and induction of caspase-9 and caspase-3. All these findings clearly indicate that capsaicin-induced dysfunction of mitochondrial might be one of the critical events leading to apoptosis.

In this study, capsaicin does promote the ROS and Ca^{2+} levels; therefore, our results also showed that capsaicin treatment induces ER stress, which elicited a rise in intracellular Ca^{2+}, and subsequent mitochondrial membrane depolarization (decreased the levels of $\Delta\Psi_m$), followed by mitochondrial release of cytochrome c and AIF, then consequent activation of caspase-9 and -3. On the basis of these observations, we may suggest that Ca^{2+}-mediated signaling is involved in capsaicin-induced apoptosis, and this is also in agreement with other reports (Lee et al., 2004; Wu et al.,

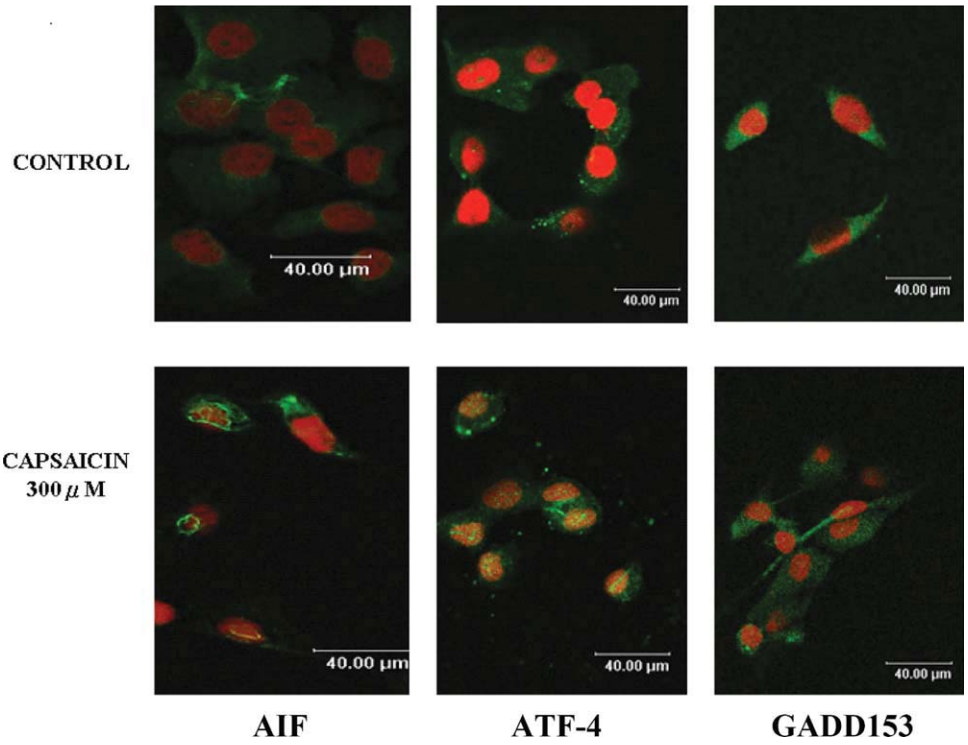

Fig. 5. Capsaicin promoted the release of AIF, ATF-4, and GADD153 in SCC-4 cells. The cells were exposed to 300 μM of capsaicin for 24 h and then were stained and examined and photographed by confocal microscopy as described in Materials and Methods section. Mitochondria and nuclei were counterstained with PI (red color), respectively. Control and capsaicin treatment: AIF, ATF-4, and GADD153. Scale bar, 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

2006; Huang et al., 2009). It was reported that AIF is a mitochondrial intermembrane flavoprotein (Vahsen et al., 2004). Other reports already demonstrated that apoptosis is induced when AIF is translocated from the mitochondria to the nucleus to induce caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation (Susin et al., 1999; Ye et al., 2002). Our results also showed that capsaicin increased the Bax level but decreased the Bcl-2 levels. It was reported that mitochondrial outer membrane permeabilization by proapoptotic Bcl-2 family members is involved in the release of AIF from the mitochondria (Otera et al., 2005). Increasing evidence have been found regarding the cancer chemotherapeutic agents modulating and interfering with mitochondrial function for promoting permeability of the mitochondrial membrane and cell death (Costantini et al., 2000). It means that capsaicin induced apoptosis partly through caspases-independent pathways. Our results also showed that capsaicin promoted the levels of GADD153 and Ca^{2+}. This is in agreement with other reports, which demonstrated the generation of oxidative stress in capsaicin-treated cells (Lee et al., 2004; Vahsen et al., 2004; Otera et al., 2005; Wu et al., 2006). In the SCC-4 cells, we have measured the production of ROS upon capsaicin exposure and antioxidant NAC partially recovered capsaicin-induced cell death (data not shown). The levels of 300 μM of capsaicin in the present study *in vitro* is higher than other phytochemicals, but it is lower

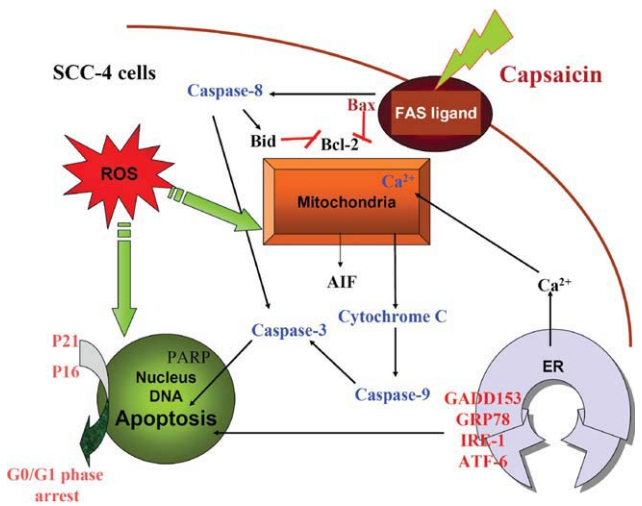

Fig. 6. The proposed possible signal pathways of capsaicin induced cell-cycle arrest and apoptosis in human tongue cancer SCC-4 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

than that of other reports, which showed that actual daily intake of capsaicin in some countries that use more chili peppers is much higher (estimated at 2.8 mg/kg body wt) (Lopez-Carrillo et al., 1994; Otera et al., 2005). Apparently, further investigations are needed in the future.

These observations from the present studies may suggest that capsaicin-induced apoptosis is likely mediated through the generation of oxidative stressed on the ROS and Ca^{2+} production. We found that capsaicin treatment induced the expression of ER stress-related proteins such as IRE1, GADD153/Chop, and GRP78/Bip (Figs. 4 and 5). It is well known that the oxidative stress could potentially damage mitochondria, which can again generate oxidative stress. Our results also showed that capsaicin increased the levels of Bax and p21, p16, and decreased the expression of Bcl-2 and the altered ratio of Bax/Bcl-2 proteins could further affect the integrity of mitochondria. It is well known that after cytochrome c is released from mitochondria into cytosol, it is followed by the sequential activation of caspase-9 and caspase-3 in various cancer cells (Shin et al., 2003; Ito et al., 2004; Jin et al., 2005; Wu et al., 2006; Zhang et al., 2008). Our earlier reports also showed that capsaicin induced apoptosis via increasing intracellular Ca^{2+} levels and leading to loss of the $\Delta\Psi_m$ (Wu et al., 2006). Consistent with this report, we found that capsaicin treatment increased the intracellular Ca^{2+} level and induced the loss of the $\Delta\Psi_m$ in SCC-4 cells.

In conclusion, our findings can be summarized as shown in Figure 6, which indicated that (1) capsaicin induced *G*0/*G*1 phase arrest via the decreasing of cyclin D and E, CDK2, and CDK6, but increased the levels of p21 and p16; (2) capsaicin induced apoptosis through the mitochondria-dependent and -independent pathways, and ROS plays an important role for ER stress before leading to apoptosis in SCC-4 cells.

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