Capsaicin and dihydrocapsaicin induce apoptosis in human glioma cells via ROS and Ca\(^{2+}\)-mediated mitochondrial pathway

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Abstract. Human glioma is the most common type of primary brain tumor and one of the most invasive and aggressive tumors, which, even with treatments including surgery, radiotherapy and chemotherapy, often relapses and exhibits resistance to conventional treatment methods. Developing novel strategies to control human glioma is, therefore, an important research focus. The present study investigated the mechanism of apoptosis induction in U251 human glioma cells by capsaicin (Cap) and dihydrocapsaicin (DHC), the major pungent ingredients of red chili pepper, using the Cell Counting Kit-8 assay, transmission electron microscopy analysis, flow cytometry analysis, laser scanning confocal microscope analysis and immunohistochemical staining. Treatment of U251 glioma cells with Cap and DHC resulted in a dose- and time-dependent inhibition of cell viability and induction of apoptosis, whereas few effects were observed on the viability of L929 normal murine fibroblast cells. The apoptosis-inducing effects of Cap and DHC in U251 cells were associated with the generation of reactive oxygen species, increased Ca\(^{2+}\) concentrations, mitochondrial depolarization, release of cytochrome c into the cytosol and activation of caspase-9 and -3. These effects were further confirmed by observations of the anti-tumor effects of Cap and DHC in vivo in a U251 cell murine tumor xenograft model. These results demonstrate that Cap and DHC are effective inhibitors of in vitro and in vivo survival of human glioma cells, and provide the rationale for further clinical investigation of Cap and DHC as treatments for human glioma.

Introduction

The incidence of primary malignant brain tumors has increased by 1.2% each year for the past 30 years, particularly in the elderly (1). Malignant glioma accounts for ~70% of primary brain tumor cases, with an annual incidence of ~5/100,000 and >14,000 new cases each year (2). Despite recent advances in therapy, significant progress in prognosis prediction and improved survival have not been achieved thus far (3), due to its resistance to radio- and chemotherapy, and a high rate of relapse. Therefore, novel strategies are required to treat human glioma.

Dietary agents are currently under consideration as effective and alternative means to control cancer. Previous studies have demonstrated that capsaicin (Cap), tea polyphenols, procyanidins and allicin are protective against various types of human tumor, including prostate cancer, breast cancer, liver cancer and gastric carcinoma (4-7). Cap and dihydrocapsaicin (DHC) are homovanillic acid derivatives with similar structures, and are the principle spicy components of hot chili peppers that are consumed worldwide, particularly in Southeast Asian and Latin American countries (8). Cap has been previously demonstrated to inhibit the proliferation of, or induce apoptosis in, numerous varieties of human cancer, including lung, prostate, pancreatic and breast cancer, in vitro and in vivo (9-12). Cap causes tumor cell cycle arrest in S or G\(_{1}/G\)_0 phase in NPC-TW 039 human nasopharyngeal carcinoma cells, MCF-7 human breast cells, BT-474 cells, SKBR-3 cells, MDA-MB231 cells and SCC-4 human tongue cells, in model systems in vitro and in vivo (13-15). Furthermore, Cap triggers...
apoptosis in >40 distinct tumor cell lines, primarily through the mitochondrial pathway or death receptor pathway (16). Cap induced apoptosis in AsPC-1 and BxPC-3 human pancreatic cancer cells through the mitochondrial death pathway, which was initiated by the generation of reactive oxygen species (ROS) and c-Jun N-terminal kinase (JNK) activation (11). In addition, intragastric administration of Cap significantly inhibits the growth of AsPC-1 pancreatic xenograft cells (11), and induces TRPV1-mediated apoptosis in RT4 urothelial cancer cells through the death receptor pathway by activating Fas cell surface death receptor (17). Gil and Kang (18) demonstrated that Cap inhibits the growth of A172 human glioblastoma cells and induces apoptosis by downregulation of B cell lymphoma 2 apoptosis regulator (Bcl-2) and activation of caspase-3. Maity et al (19) reported that Cap induces apoptosis in mouse neuro 2a cells via ubiquitin-proteasome system dysfunction. Notably, normal or noncancerous cells are less sensitive to the anti-proliferative or apoptotic effects of Cap compared with cancerous cells (16). DHC, an analog of Cap, inhibits the proliferation of HCT116, MCF-7 and Wi38 cells more potently than Cap, and induces autophagy in HCT 116 cells (20). Furthermore, DHC induces autophagy in A549 cells by downregulation of catalase, which leads to ROS accumulation and attenuation of microtubule-associated protein light chain 3 conversion (21).

However, the molecular mechanisms of Cap and DHC induction of apoptosis in U251 human glioma cells are not sufficiently understood. The present study aimed to investigate the effect of Cap and DHC on U251 human glioma cells and the mechanisms of this effect.

Materials and methods

**Chemicals and antibodies.** Cap, DHC (purity>99%) and trypsin were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Cell Counting Kit-8 (CCK-8), Fluo-3-AM, GENMED mitochondrial permeability transition pore (MPTP) living cell fluorescence detection kit and dimethyl sulfoxide (DMSO) were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). U251 cells were obtained from the National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China). L929 cells were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit and Cell Cycle Detection kit were purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). Caspase-3 activity assay kit, caspase-9 activity assay kit, Rhodamine 123 (Rh123), ROS assay kit and cytochrome C (cyto c) antibody (catalog no. AC909) were purchased from Beyotime Institute of Biotechnology (Haimen, China). UltraSensitive™ surface protein array (mouse/rabbit) immunohistochemistry (IHC) kit and 3,3'-diaminobenzidine (DAB) kit were obtained from Fuzhou Maixin Biotech Co., Ltd. (Fuzhou, China). Inverted fluorescence microscope and confocal laser scanning microscope were obtained from Nikon Corporation (Tokyo, Japan). Flow cytometry equipment was obtained from BD Biosciences (Franklin Lakes, NJ, USA).

**Cell culture.** U251 human glioma cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM L-glutamine and 1% penicillin-streptomycin solution. L929 murine fibroblast cells were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine and 1% penicillin-streptomycin solution. All cultures were maintained at 37°C in a humidified chamber of 95% air and 5% CO2. Cap and DHC were dissolved in 0.5% DMSO solution.

**Cell inhibition rate and cell survival.** Cell inhibition rate and cell survival were assessed by tetrazolium salt-based colorimetric detection in the CCK-8 assay. Cells were seeded in 96-well plates at an initial density of 5x10^3 cells/well. Following exposure to 50, 100, 150 and 200 µM Cap or DHC for 12 (U251 only), 24, 48 and 72 h, 10 µl CCK-8 solution was added to each well and the plate was incubated for 1 h. Cell viability was determined with a microplate reader by reading the absorbance at 450 nm (A450). The percentage of viable cells was calculated as follows: Cell viability (%)=[A450(treated)-A450(blank)]/[A450(control)-A450(blank)]x100%.

**Determination of apoptosis.** U251 cells (2x10^5) were plated in 6-well plates and allowed to attach for 24 h prior to exposure to 200 µM Cap and DHC for 12 h at 37°C. Apoptosis induced by Cap or DHC was evaluated using an Annexin V-FITC Apoptosis Detection kit according to the manufacturer's protocol. Briefly, U251 cells were detached, collected and suspended in 500 µl binding buffer, then 5 µl Annexin V-FITC and 5 µl propidium iodide (PI) was added and cells were incubated for 10 min at room temperature in the dark. Apoptotic cells were acquired using a FACS-Calibur flow cytometer (BD Biosciences) and the data was analyzed using CellQuest™ Pro software (BD Biosciences) and ModFit LT™ software (Verity Software House, Inc., Topsham, ME, USE).

**Transmission electron microscopy (TEM) analysis.** U251 cells (1x10^3/ml) were exposed to 200 µM Cap or DHC for 12 h at 37°C, detached and collected, postfixed in 1% buffered OsO4 for 2 h at 37°C, dehydrated in graded alcohol and embedded in Epon resin. Sections (60-nm thick) were cut and observed using TEM. A total of 5 slides were visualized, with 10 fields analyzed per slide.

**Cell cycle analysis.** U251 cells (2x10^5 cells/well) were plated in 6-well plates and allowed to attach for 24 h prior to exposure to 200 µM Cap or DHC for 12 h at 37°C. Control cells were treated with DMSO only. The cell cycle was analyzed using the Cell Cycle Detection kit according to the manufacturer's protocol. The cells were harvested, washed with phosphate-buffered saline (PBS), resuspended in Reagent A (100 µl), and incubated for 10 min at room temperature. Reagent B (100 µl) was then added, and plates incubated for a further 10 min at room temperature. Cells were stained with 150 µl PI for 15 min at room temperature in the dark, then fluorescence measured with a Becton-Dickinson FACS-Calibur flow cytometer and the data was analyzed using CellQuest Pro software and ModFit LT software.
**ROS generation.** Changes in intracellular ROS levels were detected by recording the oxidative conversion of cell permeable 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to fluorescein dichlorofluorescein (DCF) by flow cytometry. Following treatment with 200 µM Cap or DHC for 12 h, 2x10^5 cells were washed twice with PBS, then incubated with DCFH-DA at 37°C for 20 min. Cells were subsequently washed three times with serum-free DMEM, then collected and resuspended in PBS. The fluorescent signal intensity of DCF was detected using a FACSci-Calibur flow cytometer and the data was analyzed using CellQuest Pro software and ModFit LT software.

**Measurement of intracellular Ca²⁺ concentration ([Ca²⁺]i).** Following 12 h treatment with 200 µM Cap or DHC, 2x10^5 U251 cells were washed 3 times with HEPES-buffered salt solution (HBSS), then incubated with Fluo 3-AM (final concentration, 5 µM) at 37°C for 30 min in the dark. Cells were then washed 3 times with HBSS and incubated in fresh HBSS for a further 30 min to remove the remaining Fluo 3-AM. [Ca²⁺]i was then detected using a laser scanning confocal microscope, with 6 fields assessed per group.

**Measurement of mitochondrial membrane potential (MMP).** Rh123 was used to estimate MMP. Treated U251 cells (2x10^5 cells/well) were plated in 6-well plates and allowed to attach for 24 h, then exposed to 200 µM Cap or DHC for 12 h at 37°C. Following washing with Reagent A, the cells were incubated with 500 µl Reagent B and Reagent C at 37°C for 20 min in the dark. Subsequently, cells were washed twice with Reagent A, and the fluorescence detected with an inverted fluorescence microscope using 488 nm excitation (Ex) and 505 nm emission (Em) filter settings, with 6 fields assessed per group.

**Measurement of MPTP.** MPTP were detected using GENMED MPTP living cell fluorescence detection kits. U251 cells (2x10^5 cells/well) were plated in 6-well plates and allowed to attach for 24 h, then exposed to 200 µM Cap or DHC for 12 h at 37°C. Following washing with Reagent A, the cells were incubated with 500 µl Reagent B and Reagent C at 37°C for 20 min in the dark. Subsequently, cells were washed twice with Reagent A, and the fluorescence detected with an inverted fluorescence microscope using 488 nm excitation (Ex) and 505 nm emission (Em) filter settings, with 6 fields assessed per group.

**Cyto c expression in tumor xenografts.** For IHC analysis of cyto c, sections (5 µm thick) were cut, dewaxed, rehydrated and immersed in a 10 nM sodium citrate buffer (pH 6.0) and boiled for 30 min in a pressure cooker while maintaining the pressure of 181 kpa, then cooled and washed in PBS. Endogenous peroxidase activity was blocked with Immuno Staining Blocking buffer (Beyotime Institute of Biotechnology) for 1 h at room temperature to reduce nonspecific background staining. Subsequently, cyto C primary antibody diluted 1:1,000 in Immuno Staining Primary Antibody Dilution buffer (Beyotime Institute of Biotechnology) was added to the slides, incubated for 1 h at room temperature, then washed in PBS. Biotinylated anti-mouse immunoglobulin diluted 1:1,000 in Immunol Staining Primary Antibody Dilution buffer (Beyotime Institute of Biotechnology) and streptavidin conjugated to horseradish peroxidase, from the cyto C antibody kit, were subsequently applied for 30 min at room temperature. Finally, DAB was used for color development according to the manufacturer's protocol and sections were counterstained with hematoxylin. Negative control slides in the absence of primary antibody were included for each staining. Sections were observed under a TH4-200 light microscope (Olympus Corporation, Tokyo, Japan); 10 fields were examined per mouse.

**Statistical analysis.** Data are presented as the mean ± standard error for the indicated number of independently performed experiments. Data analyses were performed using SPSS v.20.0
Results

Effects of Cap and DHC on cell viability and induction of apoptosis in U251 human glioma cells and L929 normal murine fibroblast cells. The effects of Cap and DHC on the viability of U251 cells were examined by CCK-8 assay. Cap and DHC both significantly inhibited the proliferation of U251 cell lines in a dose- and time-dependent manner (Fig. 1A). Treatment of U251 cells for 72 h with 200 µM Cap and 200 µM DHC, resulted in 3.08±1.60 and 3.49±1.50% survival, respectively (P<0.001 and P<0.001, respectively; Fig. 1A), indicating that the majority of cells were killed by exposure to high concentrations of Cap and DHC. Cell viability of vehicle-treated cells (0.5% DMSO) remained >94.17%, indicating that 0.5% DMSO is not toxic to U251 cells (Fig. 1A). However, the viability of L929 normal mouse fibroblasts was not significantly affected by exposure to the equivalent concentrations of Cap and DHC that were highly cytotoxic to cancer cells (Fig. 1B).

Effects of Cap and DHC on cell cycle and apoptosis of human glioma U251 cells. Following exposure to Cap or DHC (200 µM) for 12 h, the proportion of U251 cells in the G0/G1 phase significantly increased from 71.47% in the control group to 75.78% with Cap (P=0.006; Fig. 2A) and 76.32% with DHC (P=0.003; Fig. 2A), while the proportion of cells in S phase also significantly increased from 12.02% in the control group to 18.43% with Cap (P=0.001; Fig. 2A) and 16.25% with DHC (P<0.001; Fig. 2A). The anti-tumor effect of Cap and DHC was, therefore, associated with G0/G1 and S phase cell-cycle arrest.

Annexin V-FITC/PI double staining assays were also performed to analyze cell apoptosis following 12 h treatment with 200 µM Cap or DHC. The proportion of apoptosed cells significantly increased from 14.16% in the DMSO-treated control group to 50.81% with Cap (P<0.001; Fig. 2B) and 42.79% with DHC (P<0.001; Fig. 2B). Necrosis was also significantly increased from 3.45% in the control group to 7.17% with Cap (P=0.001; Fig. 2B) and 11.15% with DHC (P<0.001; Fig. 2B).

TEM also revealed that treatment with either 200 µM Cap or 200 µM DHC for 12 h resulted in condensed cytoplasm, nuclei displaying peripheral chromatin condensation, which had broken up into rounded bodies, and cells developing into apoptotic bodies, compared with the integrated cellular morphology of the untreated cells (Fig. 2C).

Cap and DHC treatment increases ROS generation and [Ca2+]i in U251 cells. ROS can initiate the loss of MMP, mitochondrial translocation of pro-apoptotic proteins, including Bcl-2 associated protein X apoptosis regulator (Bax) and Bcl-2 associated agonist of cell death (Bad), and release of cyto c (23). Intracellular ROS levels in U251 cells were, therefore,
evaluated by DCFH-DA assays and flow cytometry detection. DCFH-DA is intracellularly hydrolyzed to DCFH, which is oxidized by ROS to generate fluorescent DCF. Following 12 h exposure to Cap or DHC, mean DCF fluorescence intensity in U251 cells increased from 3.45 in the control group to 5.84 with Cap and 6.41 with DHC (Fig. 3A).

High levels of Ca\(^{2+}\) can open mitochondrial permeability transition pores, depolarize mitochondrial membrane potential, activate caspase-9 and caspase-3, initiate the mitochondrial apoptosis pathway, to induce cell apoptosis (24). Fluo 3-AM was, therefore, used to observe the intracellular concentration of Ca\(^{2+}\). The mean fluorescence intensity in U251 cells increased from 333.79 in the control group to 1,116.46 (P<0.001) with Cap and 1,240.66 (P<0.001) with DHC, respectively (Fig. 3B), indicating an increase in the concentration of intracellular Ca\(^{2+}\).
Cap and DHC treatment increases MPTP formation and reduces MMP. MPTP formation is one of the principle regulators of the mitochondrion during cell apoptosis. The opening of MPTPs can lead to matrix swelling, rupture of the outer membrane of mitochondrion and a release of intermembrane space proteins into the cytoplasm, ultimately resulting in a loss of MMP. The presence of MPTPs was, therefore, detected using a GENMED MPTP living cell fluorescence detection kit. Following exposure to Cap or DHC for 12 h, the fluorescence intensity in U251 cells was decreased compared with the control group (Fig. 3C), indicating the formation of MPTPs.

Depolarization of the MMP induces release of cyto c and apoptosis-inducing factor (AIF) from the intermembrane space into the cytoplasm, resulting in fragmentation of nucleus and cell apoptosis (25). The mean fluorescence intensity in U251 cells decreased from 980.90 in the control group to 216.13 following 12 h exposure to 200 µM Cap (P<0.001) and 222.48 with 200 µM DHC (P<0.001), respectively (Fig. 3D), indicating the loss of MMP.

Cap and DHC treatment activates caspase-9 and -3. Apoptotic cell death is inevitable following caspase-3 activation, and is a vital factor in mitochondrial pathway-induced apoptosis. Caspase-9 (Fig. 4A and B) and -3 (Fig. 4C and D) activity were dose-dependently increased in U251 cells exposed to Cap or DHC for 12 h, compared with control group, indicating that Cap- and DHC-induced apoptosis is associated with the activation of caspase-9 and -3.

Cap and DHC treatment inhibits the growth of U251 human glioma tumor xenografts. In vitro experiments are valuable for quick, convenient, and large-scale screening of latent anti-tumor agents, however the effects observed in cell-based assays require further investigation in suitable pre-clinical in vivo models prior to clinical trials. Tumor xenografts in nude mice is an accepted animal model for pre-clinical evaluation of potential anti-tumor agents. The effect of Cap and DHC on the development of U251 glioma tumor xenografts was, therefore, evaluated in nude mice. The rate of U251...
glioma tumor xenograft development was attenuated in mice administered with Cap or DHC in both the low dose group (5 mg/kg, every 3 days; Fig. 5A). The mean tumor volume at 23 days in control mice (2126.48±211.28 mm$^3$) was ~2.8-fold larger than tumors from the Cap (10 mg/kg)-treated group (821.15±215.63 mm$^3$; P<0.001) and ~2.95-fold larger than tumors from the DHC (10 mg/kg)-treated group (785.83±218.31 mm$^3$; P<0.001; Fig. 5A). Similar results were obtained with mice treated with 5 mg/kg Cap (P<0.001) and DHC (P<0.001) treated mice compared with control mice (Fig. 5A). The mean wet weight of tumors from control mice was 2.30±0.44 g, compared with 0.92±0.33 g in Cap (10 mg/kg)-treated mice (P<0.001; Fig. 5B) and 0.71±0.34 g in DHC (10 mg/kg)-treated mice (P<0.001; Fig. 5B). A similarly significant anti-tumor effect was demonstrated in mice treated with 5 mg/kg Cap or DHC compared with control mice (P<0.001 and P<0.001, respectively; Fig. 5B). However, the differences between high-dose and low-dose treatments with the same drug were not statistically significant (Fig. 5B). As previous reported, alcohol may certain antitumor effects (26). In addition, no difference in average body weight was observed between the control group, Cap- and DHC-treated mice throughout the experiment (data not shown). The liver index (liver weight/body weight×100) and spleen index (spleen weight/body weight×100) in Cap- and DHC-treated mice were also not significantly different compared with the control group (data not shown), indicating that Cap and DHC are not toxic to mice. Histological analysis of the tumors by H&E staining revealed liquefaction and necrosis in tumors from Cap- and DHC-treated mice, compared with vigorous growth in control and vehicle-treated mice (Fig. 5C). No ulcers or erosion, were observed in the stomachs of any mice, nor were any apparent side effects observed during the histological analysis of the hearts, spleens, lungs, livers and kidneys of the mice (data not shown). IHC analysis of cyto c expression revealed numerous cyto c-positive cells in the tumors of Cap- and DHC-treated mice, but few in the control or vehicle-treated groups (Fig. 5D), indicating that cyto c was released from mitochondria to the cytoplasmic matrix, inducing U251 cell apoptosis. Cap and DHC have, therefore, been demonstrated to significantly reduce U251 human glioma tumor development with no apparent negative side effects on the organs.

Discussion

Human glioma is the most common type of primary brain tumor, and one of the most invasive and aggressive tumors, which, even with treatments, including surgery, radiotherapy and chemotherapy, often relapses and exhibits resistance to conventional treatment methods. Apoptosis and angiogenesis are two potential targets for novel strategies to treat this disease (27). Resistance to radiotherapy and chemotherapy are common consequences of the dysregulation of apoptosis, therefore, inducing apoptosis is an important feature of various chemotherapy drugs, including temozolomide and
Cisplatin (28,29). Cap, the most abundant pungent chili pepper component, has been widely examined as an anti-cancer agent, and induces cell apoptosis in numerous cancer cell lines, in vitro and when explanted into rodents (11,30,31). The present study demonstrated that Cap and DHC, the second most abundant capsaicinoids in chili pepper, are anti-cancer agents against U251 human glioma cells and are not toxic to L929 normal murine fibroblast cells. The apoptotic effects of Cap and DHC in U251 cells were associated with the generation of ROS, increased intracellular Ca\(^{2+}\), disruption of MMP and the release of cyto c to the cytosol, which ultimately resulted in the activation of caspase-9 and caspase-3 cascade (Fig. 6).

Cell cycle arrest is a prospective therapeutic target in cancer as it is a downstream focal point for oncogenic signaling pathways (32). Previous reports have demonstrated that Cap inhibits cell proliferation by cell-cycle arrest: MCF-7 human breast cancer cells and BT-20 cells were arrested in the S phase following 72 h treatment with 200 µM Cap (12). Thoennissen et al (14) demonstrated that Cap arrests MCF-7, T47D, BT-474, SKBR-3 and MDA-MB231 (ER-positive and -negative) human breast cancer cells in the G\(_0\)/G\(_1\) phase at concentrations of 50-200 µM. Similarly, SCC4 human tongue cancer cells, NPC-TW 039 nasopharyngeal carcinoma cells, RT4 urothelial cancer cells, CE 81 T/VGH esophage epithelial carcinoma cells, HL-60 leukemia and NB4 myeloid leukemia cells were also arrested in the G\(_0\)/G\(_1\) phase following treatment with Cap (13,15,17,33-35). In the current study, the anti-proliferation effect of Cap and DHC was associated with G\(_0\)/G\(_1\) and S phase cell cycle arrest following 12 h treatment at a concentration of 200 µM (Fig. 2A).

The most common apoptotic pathway induced by Cap is reported to be the mitochondrial pathway, which is particularly susceptible to ROS and Ca\(^{2+}\). A sustained increase in intracellular Ca\(^{2+}\) increases the production of ROS and eventually triggers cell apoptosis. Excess H\(_2\)O\(_2\) inhibits the activity of Na\(^+\)/H\(^+\) antiporters, which leads to intracellular acidification and creates a microenvironment for apoptosis (36). Furthermore, ROS directly damage the mitochondrial electron transport chain, activate caspases and induce cell apoptosis (29). Cap boosts the generation of ROS in human pancreatic cancer cells by inhibiting mitochondrial complex I and III and destroying mitochondrial functions (37). Initiation of the mitochondrial pathway by excess ROS and sustained an increase in intracellular Ca\(^{2+}\) results in the formation of MPTPs, which leads to the dissipation of the MMP. The opening of MPTPs elevates the permeability of inner mitochondrial membranes, allowing

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**Figure 5.** Cap and DHC inhibit the growth of U251 human glioma tumor xenografts in a BALB/c nude mouse xenograft model. (A) Average tumor volume, measured using vernier calipers. Data are presented as the mean ± standard error (n=6-7 mice per group). (B) Average wet weight of tumors following 3 weeks of treatment. Data are presented as the mean ± standard error (n=6-7 mice per group). *P<0.05, **P<0.01 vs. control. (C) Morphological changes in tumors, visualized by hematoxylin and eosin staining. (D) Immunohistochemical analysis of cytochrome c immunoreactivity in dissected tumors. Cap, capsaicin; DHC, dihydrocapsaicin.
molecules < 1.5 kDa to enter the mitochondria, resulting in swelling of the mitochondrial matrix (24,38). The outer mitochondrial membranes subsequently rupture, releasing numerous pro-apoptotic proteins from the intermembrane space into the cytosol (24,38). These pro-apoptotic proteins are divided into two groups: One group includes AIF and endonuclease G, which act as through a caspase-independent pathway to induce cell apoptosis; the other group, containing cyto c and second mitochondrial-derived activator of caspases (Smac/Diablo), activate caspases. Cyto c induces the oligomerization of apoptotic protease activating factor 1 (Apaf-1), which subsequently activates caspase-9. The activated cyto c, Apaf-1 and caspase-9 then form the apoptosome and, ultimately, activate caspase-3, resulting in nuclear fragmentation (39). Once caspase-3 is activated, cell apoptosis is irreversible (29,40).

The present study indicated that Cap and DHC induced apoptosis in human glioma cancer cells via a reactive oxygen species and Ca\(^{2+}\)-mediated mitochondrial pathway, which was associated with opening the MPTP, dissipating the MMP, releasing cyto c to the cytosol and activating caspase-9 and caspase-3. Cap, capsaicin; DHC, dihydrocapsaicin; ROS, reactive oxygen species; MPTP, mitochondrial permeability transition pore; MMP, mitochondrial membrane potential; cyto C, cytochrome C.

Figure 6. Apoptosis signaling activated by Cap and DHC in U251 cells. Cap and DHC induce apoptosis in human glioma cancer cells through a reactive oxygen species and Ca\(^{2+}\)-mediated mitochondrial pathway, which is associated with opening the MPTP, dissipating the MMP, releasing cyto c to the cytosol and activating caspase-9 and caspase-3. Cap, capsaicin; DHC, dihydrocapsaicin; ROS, reactive oxygen species; MPTP, mitochondrial permeability transition pore; MMP, mitochondrial membrane potential; cyto C, cytochrome C.

In conclusion, the present study demonstrated that Cap and DHC induce apoptosis in human glioma cancer cells through a reactive oxygen species and Ca\(^{2+}\)-mediated mitochondrial pathway, which caused MPTP formation, dissipation of
the MMP, and release of cyto c to the cytosol, resulting in activation of caspase-9 and caspase-3 (Fig. 6). In addition, intragastric administration of Cap and DHC in vivo was demonstrated to suppress the development of U251 human glioma xenograft tumors, with no apparent side effects. Consequently, these findings provide a basis for developing Cap and DHC as potential therapeutic approaches against glioma.

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