

Research article

## CAPSAICIN INDUCES APOPTOSIS BY GENERATING REACTIVE OXYGEN SPECIES AND DISRUPTING MITOCHONDRIAL TRANSMEMBRANE POTENTIAL IN HUMAN COLON CANCER CELL LINES

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**Abstract:** Although genetic factors are a well-known cause of colorectal cancer, environmental factors contribute more to its development. Despite advances in the fields of surgery, radiotherapy and chemotherapy, the cure rates for colon cancer have not substantially improved over the past few decades. Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), the principal pungent ingredient of hot chili pepper, has exhibited an anti-tumor effect in many cell types. However, the mechanisms responsible for the anti-tumor effect of capsaicin are not yet completely understood. In this study, we investigated whether capsaicin induces apoptosis in colon cancer cell lines. Capsaicin decreased cell viability in a dose-dependent manner in Colo320DM and LoVo cells. In addition, capsaicin produced cell morphology changes and DNA fragmentation, decreased the DNA contents, and induced phosphatidylserine translocation, which is a hallmark of

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Abbreviations used:  $\Delta\Psi_m$  – mitochondrial transmembrane potential; MTT – 13-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; PI – propidium iodide; ROS – reactive oxygen species

apoptotic cell death. We showed that capsaicin-induced apoptosis is associated with an increase in ROS generation and a disruption of the mitochondrial transmembrane potential. A possible mechanism of capsaicin-induced apoptosis is the activation of caspase 3, a major apoptosis-executing enzyme. Treatment with capsaicin induced a dramatic increase in caspase 3 activity, as assessed by the cleavage of Ac-DEVD-AMC, a fluorogenic substrate. In conclusion, our results clearly showed that capsaicin induced apoptosis in colon cancer cells. Although the actual mechanisms of capsaicin-induced apoptosis remain uncertain, it may be a beneficial agent for colon cancer treatment and chemoprevention.

**Key words:** Capsaicin, Colon cancer cell line, Apoptosis, Mitochondrial transmembrane potential, Reactive oxygen species, Caspase 3

## INTRODUCTION

Colon cancer is one of the most common malignancies in Western society. Its incidence is increasing in East Asian countries, perhaps due to the adaptation of Western lifestyles, including diet. The dynamic balance in the colonic epithelium between cell production at the base and cell death at the surface of the colonic crypts is precisely regulated to maintain the cellular homeostasis of the tissue [1]. Since apoptosis is part of the final differentiation step for colonic enterocytes, a progressive inhibition of apoptosis may cause a cancerous transformation that further progresses to malignancy in the colon [2]. Apoptosis has been related to the survival rate in colorectal adenoma and carcinoma patients. Therefore, the factors or agents that control the balance between cell proliferation and death in the colonic epithelium are of considerable importance. Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the major pungent ingredient in red pepper, has long been used in food additives and drugs. It is used as a food additive worldwide, particularly in South East Asia and Latin America [3, 4]. Most capsaicin studies have focused on its neurophysiological effects [5-7]. These actions of capsaicin appear to be mediated by the stimulation of specific vanilloid receptors (VRs) that may have a therapeutic value, particularly in relieving pain [8]. There exists a controversy with regard to whether capsaicin exhibits carcinogenic or anti-carcinogenic effects, but recent studies have demonstrated that capsaicin induces apoptosis in certain types of normal and cancer cells, and that this action partially contributes to the observed biological and pharmacological effects of capsaicin. An *in vitro* study attributed the anti-proliferative effects of capsaicin to the inhibition of plasma membrane NADH oxidase activity in HeLa cells [9]. It was also shown that capsaicin induces apoptosis in many cancer cell types, including stomach, colon, liver, breast and prostate cancers and leukemia [10-15, 19] *in vitro*. According to the American Association for Cancer Research, capsaicin inhibited tumor formation in human prostate cancer cell cultures grown in mouse models *in vivo* [17]. However, the

effects of capsaicin on the growth of human colon cancer cells have not been extensively examined.

Programmed cell death (apoptosis) is essential for the appropriate development and function of multicellular organisms. Unnecessary, damaged, and potentially harmful cells must be eliminated to ensure structural and functional tissue homeostasis [16]. Dysregulated apoptosis that results in excessive, untimely or insufficient cell death is fundamental to the initiation and progression of many human diseases. Many studies have demonstrated that carcinogenic processes are significantly linked to the derangement of apoptosis-signaling pathways. Accumulating evidence suggests that inducing cancer cell apoptosis is useful in cancer treatment. Apoptosis is characterized by ultrastructural modification (cytoskeletal disruption, cell shrinkage and membrane blebbing), nuclear alteration (chromatin condensation and internucleosomal DNA cleavage), loss of plasma membrane phospholipid asymmetry, and biochemical change (activation of proteases) [18]. It was recently reported that the mitochondria play a key role in coordinating the activation of caspases (cysteinyll aspartate-specific proteases) through the release of cytochrome c in apoptosis triggered by various death stimuli [20]. Mitochondrial permeability transition is a critical event in cell death. It follows the inhibition of the mitochondrial electron transport chain, and is involved in the mechanism of mitochondrial dysfunction in apoptosis [21]. It was recently found that capsaicin caused a loss of nuclear DNA, preceded by intracellular superoxide production and mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) disruption [22]. In this study, we examined the effects of capsaicin on the growth of Colo320DM and LoVo human colon cancer cells to determine whether capsaicin induces apoptosis in them. We found that capsaicin inhibits the growth of Colo320DM and LoVo cells through the induction of apoptosis. We also found that capsaicin reduces  $\Delta\Psi_m$  breakdown and that capsaicin-induced apoptosis is mediated by oxidative damage of the mitochondria and the consequently increased caspase 3 activity.

## MATERIALS AND METHODS

### Reagents

Colo320DM and LoVo human colon cancer cell lines were purchased from the Korean cell line bank (KCLB). Capsaicin was purchased from Sigma. MTT (13-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide), propidium iodide (PI), DiOC<sub>6</sub>(3), dihydroethidine, hematoxylin solution and eosin were purchased from Sigma. The Annexin V-FITC Apoptosis Detection I kits and Caspase 3 Assay kit were purchased from Pharmingen.

### Cell culture

Colon cancer Colo320DM and LoVo cells were maintained in RPMI1640 medium supplemented with 10% FBS, 200 U/ml of penicillin and 200  $\mu$ g/ml of streptomycin at 37°C in a humidified incubator under 5% CO<sub>2</sub>/95% air.

**MTT assay**

The cells were incubated in 100  $\mu$ l of media with the indicated concentration of capsaicin for 24 h at an initial cell density of  $5 \times 10^5$  cells/ml in 96-well flat-bottomed plate in triplicate. An appropriate volume of the drug vehicle was added to the untreated cells. After incubation for 24 h, 10  $\mu$ l of MTT solution (5 mg/ml in PBS) was added to each well, and the incubation was carried out for 4 h. Then the supernatant was discarded and the crystal products were eluted with 100  $\mu$ l of DMSO. A colorimetric evaluation was performed using a spectrophotometer at 570 nm. The measurement of cell viability was shown as the percentage of cell growth inhibition induced by capsaicin in comparison with the control.

**Cell morphology**

Following incubation with capsaicin, cells were washed with cold phosphate-buffered saline (PBS), cytocentrifuged onto slide glass and fixed in 70% ethanol for 30 min at room temperature. The fixed cells were rinsed with cold PBS, stained with hematoxylin and eosin, and examined under a light microscope (400x). Apoptotic cells were identified by the typical morphological features (e.g. cell shrinkage, chromatin condensation, membrane blebbing and apoptotic body formation).

**Flow cytometer analysis for DNA contents**

To quantify the apoptosis via flow cytometry, cells treated with capsaicin for 24 h were trypsinized and washed with cold PBS, then resuspended in 1 ml of 70% cold ethanol and fixed at 4°C for 30 min. The cells were centrifuged to remove ethanol and washed again with cold PBS. The pellets were resuspended in 1 ml PI solution (0.1% Triton X-100, 0.1 mM EDTA, 50  $\mu$ g/ml PI in PBS) containing 50  $\mu$ g/ml RNase, and then incubated at 37°C for 10 min. The DNA content was analyzed with a FACScan. Apoptotic cells were found to be in the 'sub-G<sub>0</sub>/G<sub>1</sub>' peak.

**DNA fragmentation**

Cells in a 100-mm<sup>2</sup> culture dish were treated with capsaicin for 24 h, trypsinized, and collected with cold PBS. After centrifugation for 10 min at 4°C, the cells were washed with cold PBS and resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, 0.1 M EDTA, pH 8.0, 0.5% Triton X-100, 0.5 mg/ml proteinase K) and incubated overnight at 50°C. The lysates were centrifuged at 13,000 rpm for 15 min at 4°C to separate the soluble fragmented DNA from the intact chromatin pellet. Fragmented DNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1) and precipitated by adding 2 vol of absolute ethanol and 0.1 vol of 3 M sodium acetate. The purified DNA was treated with 10 mg/ml DNase-free RNase A for 1 h at 37°C. The pattern of DNA fragmentation was visualized by electrophoresis in a 2% agarose gel containing ethidium bromide, and photographed under UV light.

**Measurement translocation of phosphatidylserine**

Translocation of phosphatidylserine was determined using Annexin V-FITC Apoptosis Detection I kits. Cells were washed in cold PBS and exposed to Annexin V-FITC and PI according to the manufacturer's recommendation. Annexin V and PI staining was determined by a FACScan.

**Flow cytometer analysis of  $\Delta\Psi_m$  and ROS generation**

To evaluate the  $\Delta\Psi_m$  and ROS generation, cells were seeded at an initial density of  $5 \times 10^5$  cells/well in 24-well plates. The cells were trypsinized, washed with cold PBS and then incubated in cold PBS with DiOC<sub>6</sub>(3) (green fluorescent; 40 nM) and HE (red fluorescent after oxidation; 2  $\mu$ M) for 20 min at 37°C, followed by analysis on a FACScan.

**Caspase 3-like protease assay**

Caspase 3 activity was assessed with a caspase 3 assay kit. Briefly, cells were harvested and washed with cold PBS and then collected in a cell lysis buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM NaH<sub>2</sub>PO<sub>4</sub>/NaHPO<sub>4</sub>, 130 mM NaCl, Triton X-100, and a protein inhibitor (1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin). The suspension was kept on ice for 20 min, and the protein concentration was determined by the method of Bradford. For the enzyme assay, the lysates were diluted and incubated at room temperature with Ac-DEVD-AMC substrate in the presence or absence of the inhibitor Ac-DEVD-CHO. The AMC liberated from the Ac-DEVD-AMC was measured using a plate reader with an excitation wavelength of 380 nm and an emission wavelength range of 460 nm.

**Statistical analysis**

All the experiments were performed three times. The statistical software program Statistical Package for the Social Sciences (SPSS/PC+ 10.0) was used throughout. Significances were determined using Student's t test, and were accepted when *p* values were < 0.05.

**RESULTS****Capsaicin decreases cell viability in human colon cancer cell lines**

To examine whether capsaicin has a cytotoxic effect on the Colo320DM and LoVo human colon cancer cell lines, we performed the MTT assay. Cells were treated with various concentrations (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM) of capsaicin for 24 h (Fig. 1).

We observed that capsaicin decreased the cell viability in a dose-dependent manner. In order to determine whether capsaicin-induced cell death occurs via an apoptotic pathway, we investigated cell shrinkage and chromatin condensation, hallmarks of apoptotic cell death. Treatment with capsaicin was associated with a considerable reduction in cell viability and the induction of cell shrinkage and chromatin condensation in the Colo320DM and LoVo cells (Fig. 2).

This data indicates that capsaicin exerts a growth-inhibitory effect on these colon cancer cells.

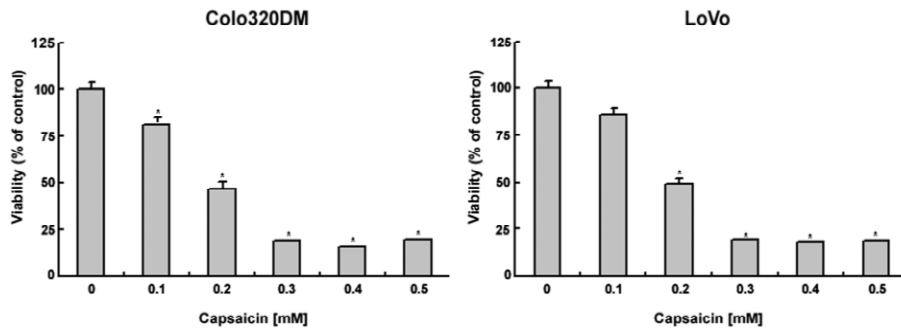


Fig. 1. The effects of capsaicin on the viability of human colon cancer cell lines. Colo320DM and LoVo cells were treated with various concentrations (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM) of capsaicin for 24 h. The cell viability was assessed via the MTT assay. This data is expressed as the means of triplicate determinations and is representative of three independent experiments (\* $p < 0.05$  compared to the control).

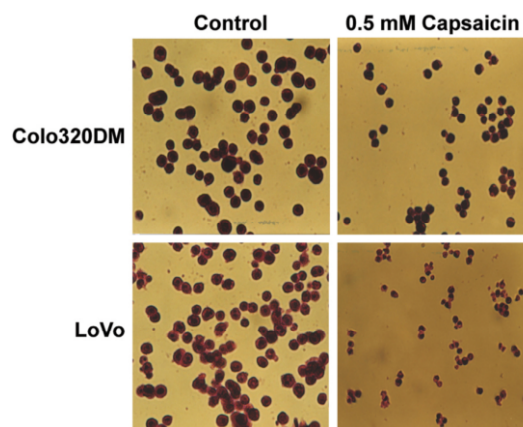


Fig. 2. The morphological characteristics of capsaicin-induced cell death. Colo320DM and LoVo cells were incubated with 0.5 mM capsaicin for 24 h, stained with hematoxylin and eosin, and then examined under a light microscope (400x). Untreated cells showed a normal distribution of chromatin, but the capsaicin-treated cells showed condensed chromatin. The cell volume of the capsaicin-treated cells was also lower than that of the normal cells.

### Capsaicin induces apoptosis in human colon cancer cell lines

To further characterize the mechanisms of cell death, we examined whether capsaicin induces apoptosis using three different assays (DNA fragmentation, PI staining, and Annexin V binding assay). As shown Fig. 3, treatment with the indicated concentrations of capsaicin for 24 h induced internucleosomal DNA

fragmentation in the Colo320DM and LoVo cells in a dose-dependent manner. As a next step, to detect the appearance of a specific sub-G<sub>0</sub>/G<sub>1</sub> apoptotic peak, we identified DNA content using PI staining.

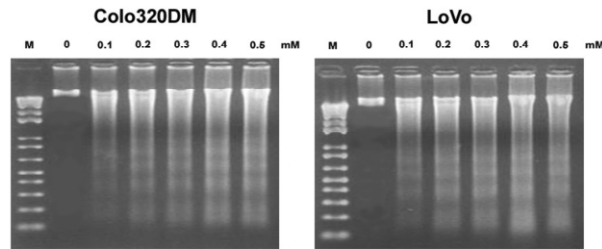


Fig. 3. Internucleosomal DNA fragmentation induced by capsaicin in Colo320DM and LoVo cells. Cells were cultured with 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM capsaicin for 24 h and washed in cold PBS. Genomic DNA was isolated from the capsaicin-treated cells as described in the Materials and Methods section, and analyzed via 2% agarose gel electrophoresis. The left lane of each photograph is a 100-bp DNA ladder.

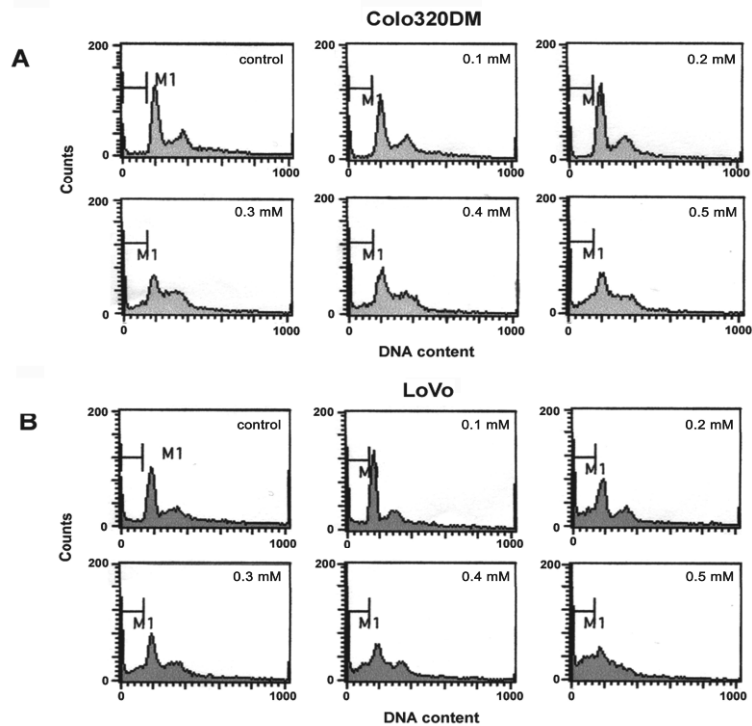


Fig. 4. The quantitative analysis of the DNA contents in capsaicin-induced cell death. Colo320DM (A) and LoVo (B) cells were treated with capsaicin at the indicated concentrations for 24 h. The cells were washed in cold PBS and fixed in 70% ethanol for 30 min at 4°C. Then the cells were stained with PI and immediately subjected to flow cytometry. The results are representative of three independent experiments.

The Colo320DM (Fig. 4A) and LoVo (Fig. 4B) cells were cultured for 24 h and treated with various concentrations of capsaicin. The endonuclease-driven loss of nuclear DNA that leads to hypoploidy was then measured using the DNA-intercalating dye PI after the permeabilization of cells with ethanol. Apoptosis-associated sub- $G_0/G_1$  peaks appeared on the flow cytometry histogram. The respective natural apoptotic rates for Colo320DM and LoVo cells are 4.13 and 9.84%. The various concentrations of capsaicin for 24 h resulted in an increase in the number of sub- $G_0/G_1$  peaks in the Colo320DM cells to 5.47, 6.84, 15.33, 16.01, and 21.97% and in LoVo cells to 14.64, 19.9, 27.40, 26.88, and 42.54%, respectively for the listed caspaicin concentrations. The quantitative analysis of flow cytometry revealed that the capsaicin-treated cells showed a loss of nuclear DNA, leading to sub- $G_0/G_1$  peaks in both cell lines in a dose-dependent manner. Translocation of phosphatidylserine to the outer surface of the cytoplasmic membrane, which is an early feature of apoptosis, was evaluated using the Annexin V binding assay (Fig. 5). Cells stained for Annexin V alone exhibit early apoptotic membrane changes with intact cell membranes and are in an early phase of apoptosis. Cells stained for both Annexin V and PI exhibit membrane disintegration, a finding consistent with necrosis or a late stage of apoptosis in cell cultures.

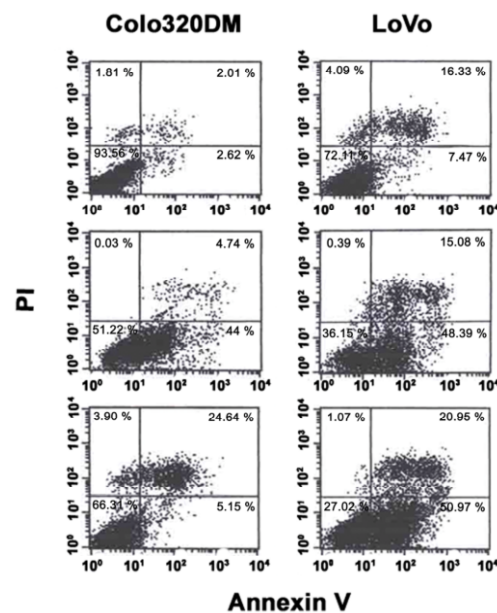


Fig. 5. The translocation of phosphatidylserine in Colo320DM and LoVo cells by capsaicin. Cells were incubated with 0, 0.3 and 0.5 mM capsaicin for 4 h and washed in cold PBS. The cells were excluded by double staining with PI and FITC-conjugated Annexin V for 15 min at 37°C and assessed with a flow cytometer. Capsaicin treatment resulted in early apoptotic membrane changes in a dose-dependent manner. The results are representative of three independent experiments.



The cells were incubated with 0, 0.3 or 0.5 mM capsaicin for 4 h. The percentage of early apoptotic cells among the Colo320DM cells (Fig. 5A) was 2.62% (0 mM), 44% (0.3 mM), and 5.15% (0.5 mM). Among the LoVo cells (Fig. 5B), it was 7.47% (0 mM), 40.39% (0.3 mM), and 50.97% (0.5 mM). Taken together, our data indicates that capsaicin induces apoptosis in these cells by producing rapid changes in the distribution of phosphatidylserine, followed by DNA fragmentation. In addition, the amount of fragmented DNA, the DNA content and the degree of Annexin V binding were higher in the relatively sensitive Colo320DM cells than in the LoVo cells when the same number of cells was analyzed.

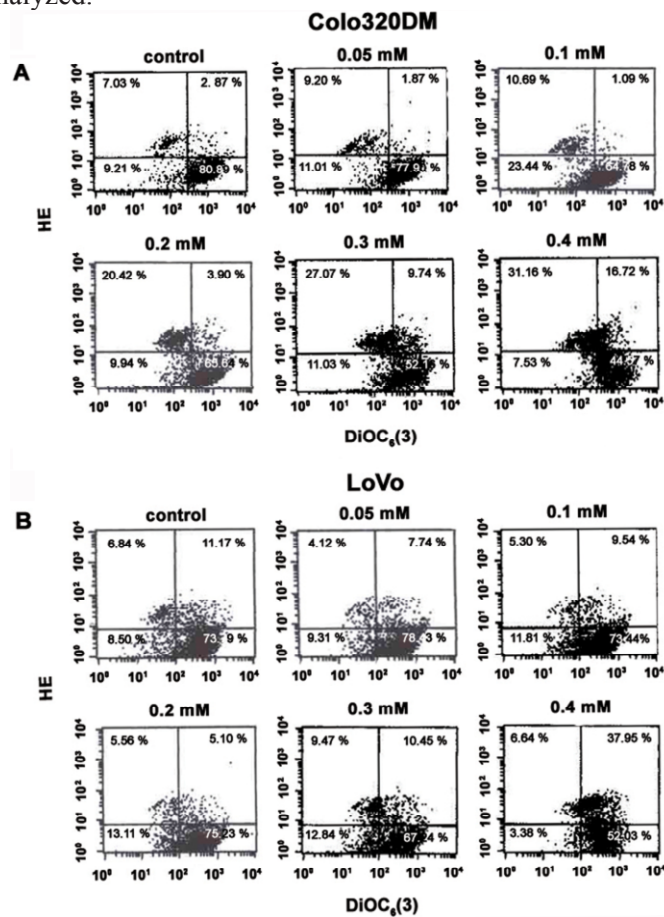


Fig. 6. A simultaneous assessment of  $\Delta\Psi_m$  disruption and ROS generation after treatment with capsaicin. The cells were treated with 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM capsaicin for 3 h, and then stained with the mitochondrial membrane potential sensitive dye DiOC<sub>6</sub>(3) and the ROS-oxidable probe HE. The mitochondrial membrane potential and ROS generation were analyzed with a flow cytometer. This result indicates that capsaicin decreased  $\Delta\Psi_m$  and increased ROS generation in Colo320DM and LoVo cells.

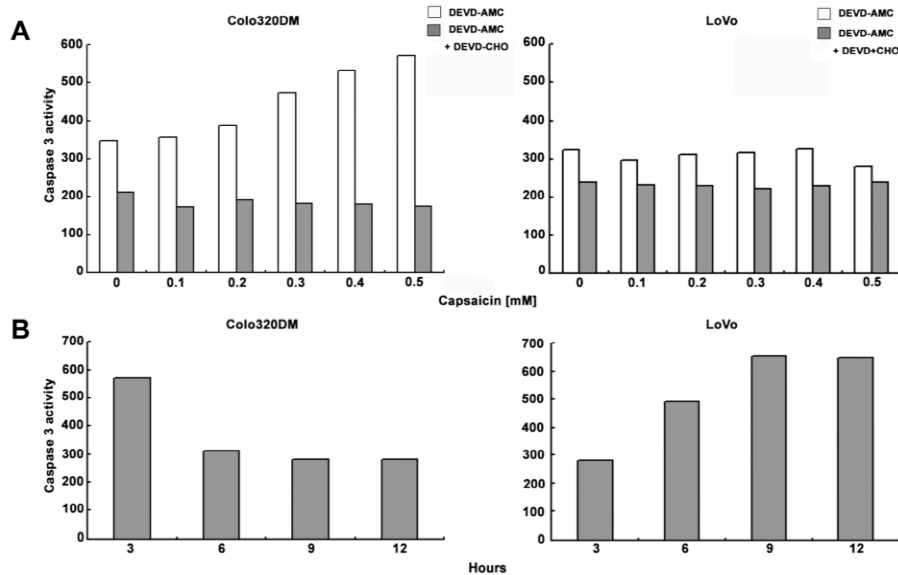


Fig. 7. The activation of caspase 3 in capsaicin-induced apoptosis. A – The cells were treated with the indicated concentrations of capsaicin for 3 h. Cell lysates were prepared and caspase 3 activity was determined by DEVD cleavage as described in the Materials and Methods section. B – The cells were treated with 0.5 mM of capsaicin for 3, 6, 12 and 16 h. Cell lysates were prepared and caspase 3 activity was determined by DEVD cleavage. These results indicate that the activation of caspase 3 was mediated in capsaicin-induced apoptosis. The results are representative of two independent experiments.

### The induction of apoptosis by capsaicin is preceded by $\Delta\Psi_m$ breakdown and reactive oxygen species (ROS) generation

There is accumulating evidence that ROS generation and  $\Delta\Psi_m$  disruption may serve as signaling events in apoptosis. We investigated both in double-staining experiments, using HE (non-fluorescent), which transforms to ethidium (Eth; red fluorescent) after its oxidation by ROS, and DiOC<sub>6</sub>(3) (green fluorescent), a cationic probe that accumulates in the mitochondria as a function of their potential. We detected the populations in the Colo320DM and LoVo cells treated with increasing concentrations of capsaicin. As shown in Fig. 6A, when the Colo320DM cells were cultured with various concentrations of capsaicin, the ROS generation increased and the  $\Delta\Psi_m$  decreased in a dose-dependent manner. Similarly, an increase in ROS content and a decrease in the  $\Delta\Psi_m$  were observed in the LoVo cells (Fig. 6B). These results strongly suggest a close relationship between the generation of ROS and the disruption of the  $\Delta\Psi_m$  in capsaicin-induced apoptosis.

### Capsaicin induces apoptosis through caspase 3 activation

Caspases are essential for the execution of cell death by various apoptotic stimuli (Cohen, 1997). To detect the enzymatic activity of caspase 3, the

executor of the caspase cascade, during capsaicin-induced apoptosis, we used the specific fluorogenic peptide substrate Ac-DEVD-AMC. The Colo320DM and LoVo cells were incubated with 0~0.5 mM capsaicin for 3 h. In the Colo320DM cells, caspase 3 activity increased in a dose-dependent manner, while pretreatment with a specific caspase 3 inhibitor (Ac-DEVD-CHO) prevented caspase 3 activity (Fig. 7A, left). However, in the LoVo cells, no change in the activation of caspase 3 was detected after capsaicin treatment for 3 h (Fig. 7A, right). As shown in Fig. 7B, we measured the activation of caspase 3 in a time-dependent manner. When the cells were incubated with 0.5 mM capsaicin for 3, 6, 12 and 16 h, the caspase 3 activity in the Colo320DM cells was high at approximately 3 h (Fig. 7B, left), whereas in the LoVo cells, the caspase 3 activity increased after 6 h (Fig. 7B, right). Our data indicates that the activation of caspase 3 is closely associated with capsaicin-induced apoptosis in the Colo320DM and LoVo cells.

## DISCUSSION

Apoptosis, a physiological mechanism of cell death, can be initiated by extracellular and intracellular mechanisms that trigger a complex machinery of proapoptotic proteases and mitochondrial changes, leading to the activation of specific endonucleases and DNA fragmentation [24]. In this study, we found that capsaicin inhibits the cellular growth of human colon cancer cells and induces apoptosis. The effects of capsaicin were investigated using two established human colon cancer cell lines, Colo320DM and LoVo, and it was found to inhibit the growth of those cell types by inducing apoptosis. To investigate the mechanism of capsaicin-induced apoptotic cell death, we examined apoptosis based on cell morphology, DNA fragmentation, loss of DNA content, and the translocation of phosphatidylserine via FACS analysis. Treatment with capsaicin decreased the cell viability; induced apoptotic morphological changes and translocation of phosphatidylserine from the inner to the outer membrane; produced nuclear DNA fragmentation; and reduced the DNA content in the colon cancer cells. In our experimental setting, the concentration of capsaicin needed to induce apoptosis is relatively high (about  $IC_{50}$  200  $\mu$ M). Various researchers have recently shown that 20~200  $\mu$ M of capsaicin was enough to induce apoptosis in solid tumors [13, 14, 19]. We think that the concentration of capsaicin required to induce apoptosis depends on the cell type.

Some previous papers reported that mitochondrial dysfunction is associated with apoptosis [20, 21]. The nuclear change is preceded by a reduction in the  $\Delta\Psi_m$ , the uncoupling of electron transport from ATP synthesis, and an increase in ROS generation [20, 24]. Recent studies have suggested that capsaicin induces apoptosis by increasing ROS generation in breast and hepatoma cancer cells [13, 14]. As disruption of the  $\Delta\Psi_m$  is a common metabolic alteration in all apoptotic processes, we evaluated the role of mitochondrial permeability transition. We determined that apoptosis induced by capsaicin is related to an

increase in ROS generation and the disruption of the  $\Delta\Psi_m$  in the mitochondrial membrane of the Colo320DM and LoVo cells. We suggest that capsaicin induces ROS generation leading to the oxidation of mitochondrial megachannel pores, which in turn allows disruption of the  $\Delta\Psi_m$  and apoptosis. Therefore, capsaicin-induced apoptosis may be mediated by  $\Delta\Psi_m$  breakdown and ROS generation.

Diverse groups of molecules are involved in the apoptosis pathway. One set of mediators implicated in apoptosis are the caspases. A member of this family, caspase 3 has been identified as being a key mediator of apoptosis in mammalian cells [23]. Recent evidence suggests that caspase 3 is required for DNA fragmentation and to produce morphological changes associated with apoptosis [25, 26]. However, many studies have provided compelling evidence that caspases play a pivotal role in the transduction of apoptotic signals. Therefore, we examined whether caspase 3 plays a critical role in capsaicin-induced apoptosis. Capsaicin induced the activation of caspase 3 rapidly in the Colo320DM cells in a dose-dependent manner, and this capsaicin-induced caspase 3 activity was attenuated by a well-known caspase 3-like protease inhibitor, Ac-DEVD-CHO. In the LoVo cells, although the caspase 3 activity did not change at 3 h in a dose-dependent manner, treatment with capsaicin activated caspase 3 in a time-dependent manner. A comparison of the capsaicin sensitivity between the Colo320DM and LoVo cells revealed that Colo320DM cells were more sensitive than LoVo cells to capsaicin-induced apoptosis. Further assessment of these pathways as the targets of capsaicin's effect is needed.

In conclusion, although the actual mechanisms of capsaicin-induced apoptosis remain uncertain, our results suggest that capsaicin induces apoptosis in colon cancer cells through the generation of ROS and the disruption of the  $\Delta\Psi_m$ , and via caspase 3-dependent mechanisms. We also think that a capsaicin analogue with anti-tumor activities but without the undesirable side effects of the natural vanilloids could be developed as a potential drug against human carcinoma.

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