Involvement of Bax, Bcl-2, Ca^{2+} and Caspase-3 in Capsaicin-induced Apoptosis of Human Leukemia HL-60 Cells

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Abstract. *The role of Ca^{2+} on the effects of capsaicin on human leukemia HL-60 cells in vitro and the molecular mechanisms of capsaicin-induced apoptosis were investigated. The flow cytometric analysis indicated that capsaicin decreased the percentage of viable HL-60 cells, via the induction of G_0/G_1-phase cell cycle arrest and apoptosis. Capsaicin-induced G_0/G_1-phase arrest involved the suppression of CDK2 and the cyclin E complex, which are check-point enzymes for cells moving from G_0/G_1- to S-phase. Capsaicin-induced apoptosis was associated with the elevation of intracellular reactive oxygen species and Ca^{2+} production, decreased the levels of mitochondrial membrane potential, promoted cytochrome c release and increased the activation of caspase-3. An intracellular Ca^{2+} chelator (BAPTA) significantly inhibited capsaicin-induced apoptosis. Capsaicin-induced apoptosis was time-and dose-dependent. These results suggest that the capsaicin-induced apoptosis of HL-60 cells may result from the activation of caspase-3 and the intracellular Ca^{2+} release pathway.*

Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is a major, pungent ingredient of the red pepper, with possible tumorigenicity and genotoxicity (1). It was also reported to be active against certain carcinogens and mutagens (2, 3). Capsaicin was found to inhibit the growth of various immortalized and malignant cells (4, 5) and to induce apoptosis in transformed (6, 7) and HL-60 cells (8). Capsaicin inhibited tumor growth and induced apoptosis *in vivo* in NOD/SCID mice with no toxic effects, therefore indicating that it may have a potential as a novel therapeutic agent for the treatment of leukemia (8).

The cell cycle represents a series of tightly integrated events, involving the cyclins and cyclin-dependent kinases (CDKs) and some of the inhibitors of these molecules (9-11). When activated, the CDKs provide a means for the cell to move from one phase of the cell cycle to the next (G_1 to S or G_2 to M). If cyclin and/or CDKs are affected, cell cycle arrest occurs. DNA damage within an intact cell results in the triggering of the apoptotic machinery.

Many bioactive substances, such as taxol, have been shown to exert their anticancer activity by blocking cell cycle progression and triggering tumor cell apoptosis, which have become major indicators of an anticancer effect (12, 13).

In the present study, capsaicin was shown to inhibit human HL-60 cells, through the induction of G_0-G_1 cell cycle arrest *via* the inhibition of cyclin E and the induction of apoptosis through the production of Ca^{2+} and activation of caspase-3.

Materials and Methods

Chemicals and reagents. Capsaicin, Trypan blue, ribonuclease-A (RNase), propidium iodide (PI), triton X-100 and Tris-HCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium phosphates, dimethyl sulfoxide (DMSO) and the TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI 1640 medium, glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Human myelocytic leukemia HL-60 cell line. The human leukemia HL-60 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The HL-60 cells

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were placed into 75-cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 atmosphere in 90% RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% FBS and 2% penicillin-streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin).

Flow cytometric assays for cell viability of HL-60 cells treated with or without capsaicin. The HL-60 cells were plated in 12-well plates at a density of $2x10^5$ cells/well and were grown for 24 h. Capsaicin was then added to the cells at final concentrations of 0, 25, 50, 75 and 100 μM. Only DMSO (solvent) was added for the control regimen and the cells were grown at 37˚C, in 5% CO_2 and 95% air for 6, 12, 24, 48 and 48 h. The viability of the HL-60 cells (%) was assayed by flow cytometry, as described previously (14).

Flow cytometric assays of cell cycle and apoptosis in HL-60 cells treated with capsaicin. Approximately $5x10^5$ cells/well of HL-60 cells in 12-well plates with capsaicin at 0, 25, 50, 75 and 100 μM were incubated in an incubator for various time-periods. The cells were harvested by centrifugation, fixed gently by adding 70% ethanol in phosphate-buffered saline (PBS) at 4˚C overnight and were then re-suspended in PBS containing 40 μg/mL PI, 0.1 mg/mL RNase and 0/1% triton X-100, in a dark room. After 30 min at 37˚C, the cells were analyzed with a flow cytometer (Becton-Dickinson, San Jose, CA, USA), equipped with an argon ion laser at 488 nm wave-length. The cell cycle was determined and analyzed, as previously described (13). Apoptotic cells were quantified by Annexin V-FITC and PI double staining using a staining kit from PharMingen (San Diego, CA, USA) (14).

Flow cytometric assays for caspase-3 activity in HL-60 cells treated with or without capsaicin. Approximately $5x10^5$ cells/well of HL-60 cells in 12-well plates with capsaicin concentrations of 0, 25, 50, 75 and 100 μM were incubated for various time-periods. The cells were harvested by centrifugation and the medium was removed. Fifty μL of a 10 μM PhiPhilux solution (PhiPhilux is a unique substrate for caspase-3) were added to the cell pellet ($1x10^5$ cells per sample). The cells were not vortexed, but incubated at 37˚C for 60 min, followed by washing once with the addition of 1 mL of ice-cold PBS and re-suspension in 1 mL fresh PBS. The cells were analyzed with a flow cytometer (Becton-Dickinson) equipped with an argon ion laser at 488 nm wave-length. The caspase-3 activity was determined and analyzed, as previously described (15).

Flow cytometric assays for reactive oxygen species (ROS) in HL-60 cells after treatment with capsaicin. The level of ROS in the HL-60 cells was examined by flow cytometry (Becton Dickinson FACS Calibur), using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). Approximately $5x10^5$ cells/well of HL-60 cells in 12-well plates were incubated with various capsaicin concentrations (0, 25, 50, 75 and 100 μM) for 24 h. The cells were harvested, washed twice, re-suspended in 500 μL of DCFH-DA (10 μM), incubated at 37˚C for 30 min and analyzed by flow cytometry (16).

Flow cytometric assays for mitochondrial membrane potential ($\Delta\Psi_m$) in HL-60 cells after treatment with capsaicin. The $\Delta\Psi_m$ of the HL-60 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using $DiOC_6$ (4 mol/L). Approximately $5x10^5$ HL-60 cells/well in 12-well plates were incubated with various capsaicin concentrations (0, 25, 50, 75 and 100 μM) for 24 h to detect the changes in $\Delta\Psi_m$. The cells were harvested, washed twice, re-suspended in 500 μl of $DiOC_6$ (4 mol/L), incubated at 37˚C for 30 min and subsequently analyzed by flow cytometry (16).

Flow cytometric assays for Ca^{2+} concentrations in HL-60 cells after treatment with capsaicin. The level of Ca^{2+} of the HL-60 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using Indo 1/AM (Calbiochem, La Jolla, CA, USA). Approximately $2x10^5$ HL-60 cells/well in 12-well plates were incubated with various capsaicin concentrations (0, 25, 50, 75 and 100 μM) for 24 h. The cells were harvested, washed twice, re-suspended in Indo 1/AM (3 μg/mL), incubated at 37˚C for 30 min and analyzed by flow cytometry (16, 17).

Detection of Ca^{2+} concentrations, $\Delta\Psi_m$ values and apoptosis in HL-60 cells after pre-treatment with BAPTA followed by treatment with capsaicin. The Ca^{2+} level and $\Delta\Psi_m$ of the HL-60 cells were determined by flow cytometry (Becton Dickinson FACS Calibur), using Indo 1/AM (Calbiochem). Approximately $5x10^5$ HL-60 cells/well in 12-well plates were pre-treated with BAPTA (an intracellular Ca^{2+} chelator) before the addition of 100 μM of capsaicin and incubation for 24 h to detect the changes in Ca^{2+} concentration and the $\Delta\Psi_m$ values. The cells were harvested and washed twice, once for apoptosis analysis and the other for re-suspension in Indo 1/AM (3 μg/mL), incubated at 37˚C for 30 min and analyzed by flow cytometry (16, 17).

Western blotting to examine the effect of capsaicin on cyclin E, CDK1, CDK2, Bax, Bcl-2 and cytochrome c expressions in HL-60 cells. The total protein was collected from HL-60 cells treated with or without various concentrations of capsaicin for 48 h before the cyclin E, CDK1, CDK2, Bax, Bcl-2 and cytochrome c levels were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, as described previously (16).

Statistical analysis. The Student's *t*-test was used to analyze the differences between the capsaicin-treated and control groups.

Results

Capsaicin decreased the percentage of viable HL-60 cells. After the HL-60 cells had been treated with or without various concentrations of capsaicin for various time-periods, the percentage of viable cells was determined by flow cytometry. The data demonstrated that capsaicin decreased the percentage of viable cells in a dose- (Figure 1A) and time-dependent (Figure 1B) manner.

Capsaicin induced G_0/G_1 arrest and apoptosis in HL-60 cells. After the HL-60 cells had been treated with or without various concentrations of capsaicin for various time-periods, the cell cycle and apoptosis (sub-G_1 group) were analyzed by flow cytometry. The data demonstrated that capsaicin induced both G_0/G_1-phase arrest (Figure 2A) and apoptosis (Figure 2B). Both effects were dose- and time-dependent.

Capsaicin induced caspase-3 activity in HL-60 cells. After the HL-60 cells had been treated with or without various

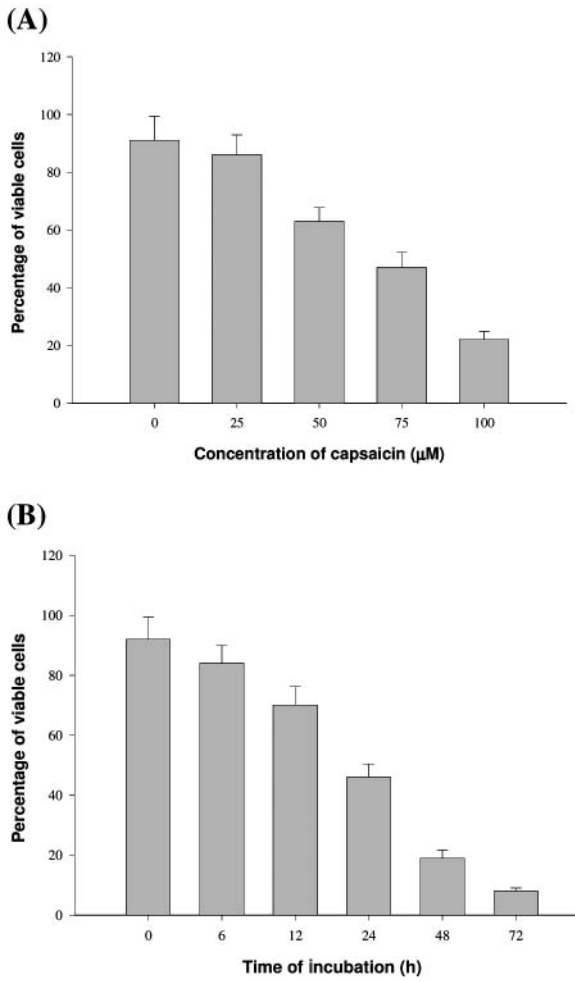

Figure 1. *The percentage of viable HL-60 cells after capsaicin treatment. The HL-60 cells (2x10^5 cells/well; 12-well plates) were plated in medium with different concentrations of capsaicin (A) for 24 h or with 70 μM capsaicin for 6, 12, 24, 48 and 72 h (B). The cells were collected by centrifugation and the viable cells were determined by flow cytometry, as described in Materials and Methods. Each point is the mean±S.D. of three experiments. *p<0.05.*

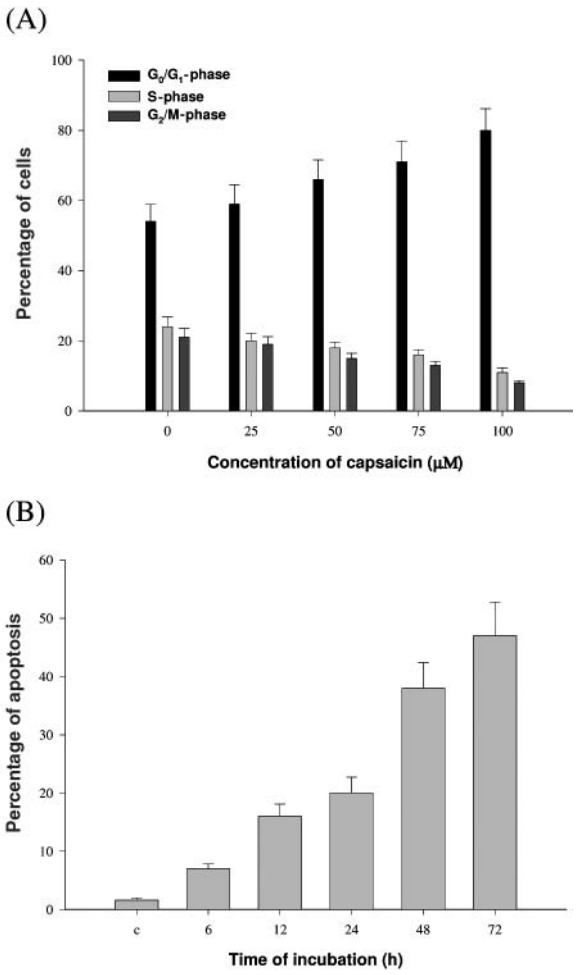

Figure 2. *Flow cytometric analysis of the effects of capsaicin on the HL-60 cell cycle and sub-G_1 group. The HL-60 cells were exposed to various concentrations of capsaicin for 48 h or to 70 μM capsaicin for 6, 12, 24, 48 and 72 h, and the cells were harvested and analyzed for cell cycle (panel A: the percentage of cells in phase) and sub-G_1 group (panel B: the percentage of cells in apoptosis) were analyzed by flow cytometry, as described in Materials and Methods. The data represents mean±S.D. of three experiments. *p<0.05.*

concentrations of capsaicin for 24 h, the cells were harvested and caspase-3 activity was analyzed by flow cytometry. The data indicated that capsaicin induced caspase-3 activity (Figure 3A) in a dose-dependent manner. The inhibitor of caspase-3 (z-VAD-fmk) decreased the caspase-3 activity and the percentage of apoptotic cells (Figure 3A).

Capsaicin induced the production of reactive oxygen species (ROS) in HL-60 cells. After HL-60 cells had been treated with or without various concentrations of capsaicin for 2 h, ROS production was analyzed and quantitated by flow cytometry. Capsaicin was found to induce ROS production in a dose-dependent manner (Table I).

Capsaicin induced Ca^{2+} production in HL-60 cells. After the cells had been treated with or without various concentrations of capsaicin for 6 h and harvested, the Ca^{2+} production was analyzed and quantified by flow cytometry. The data demonstrated that capsaicin induced Ca^{2+} production in a dose-dependent manner (Table II). The representative profiles given in Figure 4 indicate that the Ca^{2+} production increased over time.

Capsaicin decreased the levels of mitochondrial membrane potential ($\Delta\Psi_m$) in HL-60 cells. After the cells had been treated with or without various concentrations of capsaicin for 12 h, the levels of $\Delta\Psi_m$ were analyzed and quantitated by

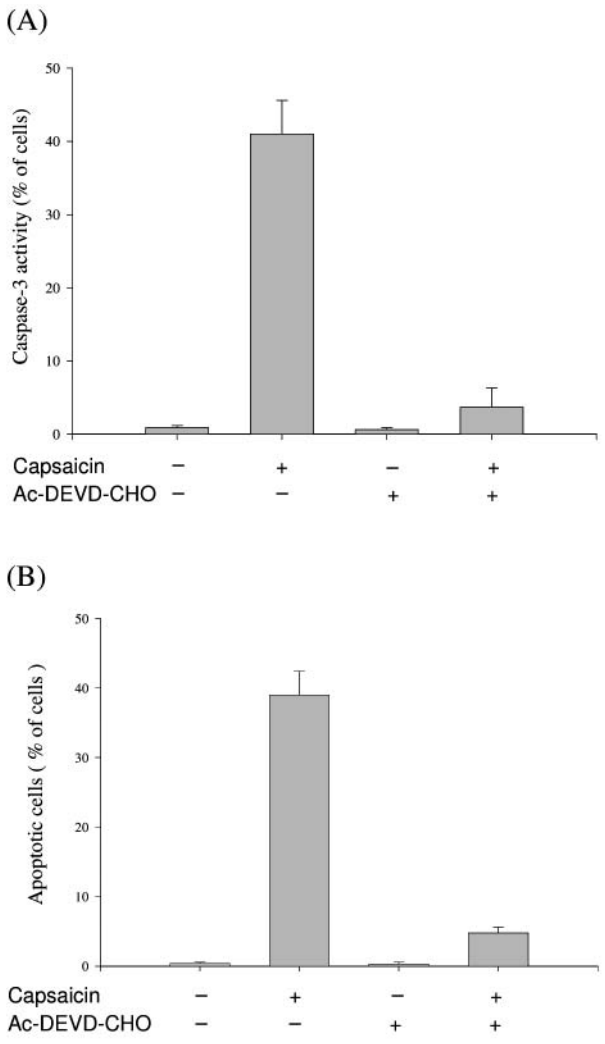

Figure 3. *Flow cytometric analysis of the effects of capsaicin on caspase-3 activity and apoptosis in HL-60 cells. The cells were incubated with 70 μM capsaicin with or without Ac-DEVD-CHO treatment for caspase-3 activity (A) and apoptosis determination (B), as described in Materials and Methods. The data represents mean±S.D. of three experiments. *p<0.05.*

Table I. *Flow cytometric analysis of reactive oxygen species in human leukemia HL-60 cells with or without 2-h capsaicin treatment.*

Capsaicin (μM)	Percentage of cells stained by DCFH-DA (% control)
0	0.5±0.2
25	8.8±1.2*
50	19.5±2.1*
75	28.6±2.4*
100	47.8±3.9*

Values are mean±S.D. n=3. The zero concentration was defined as the control. The percentage of cells stained with DCFH-DA was determined by flow cytometry, as described in Materials and Methods. *differs between capsaicin and control. $p<0.05$.

Table II. *Flow cytometric analysis of Ca^{2+} concentrations in human leukemia HL-60 cells with or without capsaicin treatment.*

Capsaicin (μM)	Percentage of cells stained by Indo-1/AM (% control)
0	2.4±0.6
25	11.2±1.2
50	24.2±2.2*
75	32.8±3.9*
100	44.1±3.6*

Values are mean±S.D. n=3. The HL-60 cells ($5x10^5$ cells/ml) were treated with various concentrations of capsaicin. The zero concentration was defined as the control. The percentage of cells stained by Indo-1/AM was determined by flow cytometry, as described in Materials and Methods. *differs between capsaicin and control. $p<0.05$.

Table III. *Flow cytometric analysis of mitochondrial membrane potential in human leukemia HL-60 cells with or without capsaicin treatment.*

Capsaicin (μM)	Percentage of cells stained by $DiOC_6$
0 (control)	92.1±8.7
25	81.0±7.1
50	62.2±5.7*
75	46.4±4.2*
100	28.3±3.2*

Values are mean±S.D. n=3. The HL-60 cells ($5x10^5$ cells/ml) were treated with various concentrations of capsaicin. The zero concentration was defined as the control. The percentage of cells stained by $DiOC_6$ was determined by flow cytometry, as described in Materials and Methods. *differs between capsaicin and control. $p<0.05$.

flow cytometry. The data indicated that capsaicin lowered the mitochondrial $\Delta\Psi_m$ in a dose-dependent manner (Table III).

Effects of the calcium antagonist BAPTA (Ca^{2+} chelator) on the influence of capsaicin on the levels of Ca^{2+}, MMP and apoptosis in HL-60 cells studied by flow cytometric analysis. The results showed decreased Ca^{2+} release leading to increased levels of MMP and a decreased percentage of apoptosis (Figure 4 A, B and C).

Capsaicin affected the expressions of cyclin D3, cyclin E, CDK1, CDK2, Bax, Bcl-2 and cytochrome c in HL-60 cells. In order to characterize the molecular mechanism of capsaicin-induced G_0/G_1 arrest followed by apoptosis in HL-60 cells, the expressions of cell cycle- and apoptosis-associated proteins were examined by Western blotting. The results indicated that the decreased levels of cyclin D3, CDK1 and cyclin E (Figure 5) may have led to G_0/G_1 arrest.

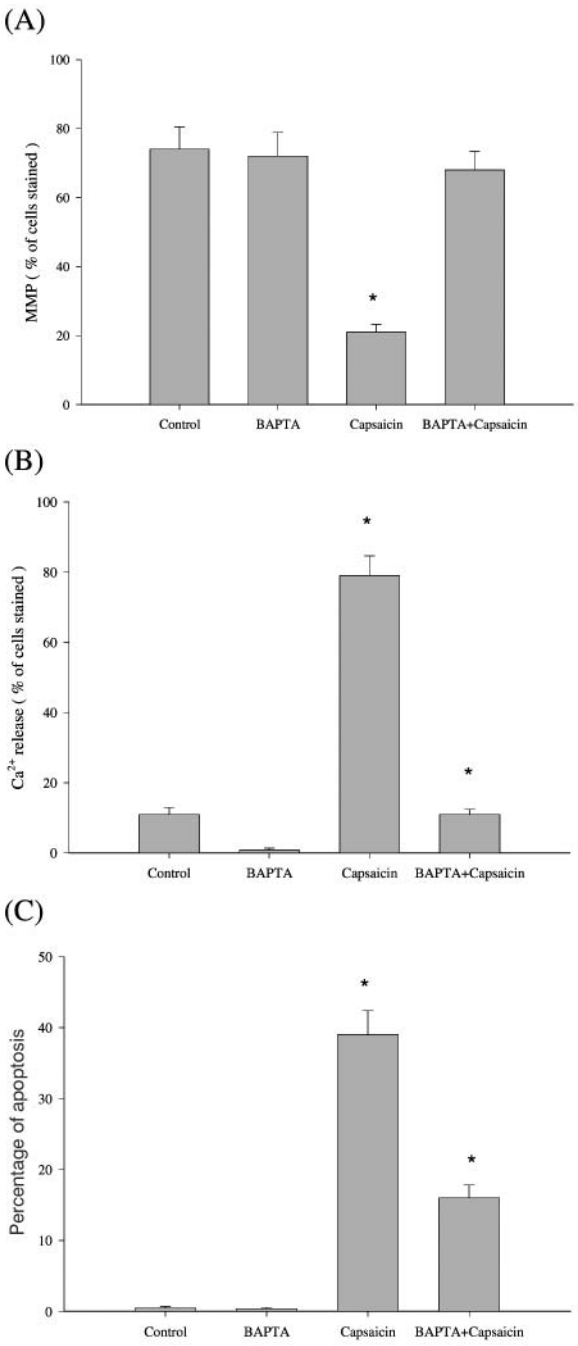

Figure 4. *Effects of the calcium antagonist BAPTA (Ca^{2+} chelator) on the influence of capsaicin on the levels of Ca^{2+}, MMP and apoptosis in HL-60 cells by flow cytometric analysis. The cells were pre-treated with BAPTA for 3 h then treated with capsaicin and harvested for Ca^{2+}, MMP and apoptosis determination, as described in Materials and Methods. The data represent mean±S.D. of three experiments. *p<0.05.*

Capsaicin increased the expressions of Bax and cytochrome c but decreased the expression of Bcl-2, which may have led to apoptosis.

Discussion

The present study focused on the role of Ca^{2+} in capsaicin-induced HL-60 leukemia cell apoptosis as well as on cell cycle arrest. Capsaicin caused an increase in the production of ROS and Ca^{2+} accompanying a down-regulation of mitochondrial membrane potential ($\Delta\Psi_m$), thereby casusing apoptosis. These findings suggest that Ca^{2+} plays a potential role in capsaicin-induced apoptosis in HL-60 cells. To date, the anticancer activity of capsaicin is still controversial, but our results clearly demonstrated that capsaicin induced G_0/G_1-phase arrest and apoptosis in the examined cells. This finding is in agreement with other reports which demonstrated capsaicin-induced G_0/G_1-phase arrest and apoptosis in human leukemia cells (8). Futher, our data showed the role of Ca^{2+} in the capsaicin-induced apoptosis of HL-60 cells. The Western blotting data also indicated that capsaicin decreased the levels of cyclin D3, cyclin E and CDK1, but not that of cyclin A, to lead to G_0/G_1 arrest. Currently, attention has been focused on the inhibition of CDK activity, which may lead to cell cycle arrest and represents a productive strategy for the discovery and design of novel anticancer agents specifically targeting the cell cycle. The CDK inhibitors (lavopiridol, UCN-01, olomoucine, rocosvitine, butyrolactone I, indirubin-5-sulfonate and indirubin-3'-monoxime) inhibit the growth of cancer cells by causing cell cycle arrest and apoptosis (18).

Although capsaicin had been shown to bind to the cytoplasmic domains of the TRPV1 receptor in neurons and promote neurotoxicity, resulting in cell death (19), it was reported that capsaicin-induced apoptosis was not inhibited by capsazepine or ruthenium red (the TRPV1 receptor antagonists) (20). Therefore, capsaicin-mediated apoptosis cannot be induced through a TRPV1-independent pathway (21, 22). Our results demonstrated that capsaicin induced apoptosis *via* the mitochondria-dependent pathway and promoted the expression of Bax and the release of cytochrome c as well as the activation of caspase-3, but decreased the levels of Bcl-2 in HL-60 cells. These results are in agreement with other reports that capsaicin can modulate the process of apoptosis in tumor cells through caspase activation (23, 24), a decrease of Bcl-2 expression and an increase in Bax expression (25). Our data also revealed that capsaicin induced ROS production in HL-60 cells, in agreement with a previous report demonstrating that capsaicin can function as a coenzyme Q antagonist to promote ROS production by interfering with electron transport (26).

It is well known that mitochondria play an important role in the regulation of apoptosis (27, 28) and mitochondrial dysfunctions, including the loss of mitochondrial membrane potential ($\Delta\Psi_m$), permeability transition and release of cytochrome c from the mitochondria into the cytosol, are associated with apoptosis (29). Our results showed that

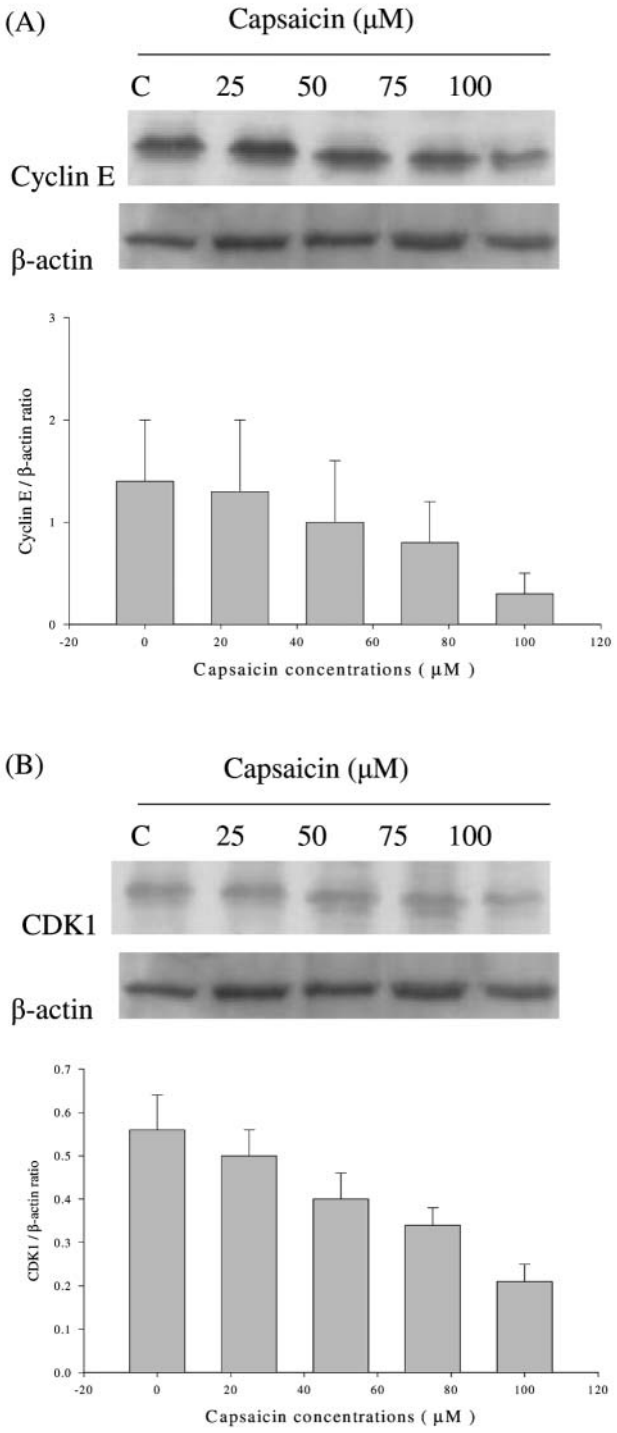

Figure 5. *Representative Western blot showing changes in the levels of cyclin E, CDK1 in HL-60 cells after treatment with capsaicin. The cells ($5x10^6$/ml) were treated with 0, 25, 50, 75 and 100 μM capsaicin for 24 h and the cytosolic fraction and total protein were prepared and determined, as described in Materials and Methods. The evaluation of the levels of the associated protein levels, cyclin E (A); CDK1 (B) were estimated by Western blot, as described in Materials and Methods.*

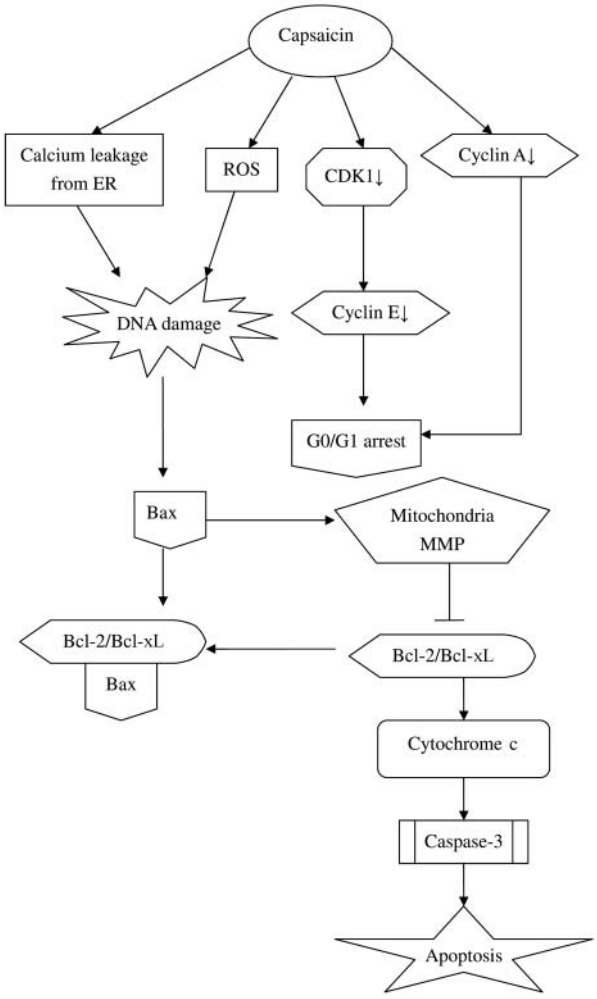

Figure 6. *Proposed model of the capsaicin mechanism of action for G_0/G_1 arrest and apoptosis in HL-60 cells. Capsaicin induced decreases in CDK1, cyclin E and cyclin A, leading to G_0/G_1 arrest. Capsaicin increased the production of ROS and Ca^{2+} and decreased the MMP levels and cytochrome c release, leading to caspase-3 activity that induced apoptosis in the HL-60 cells.*

capsaicin induced the rapid loss of $\Delta\Psi_m$ and release of cytochrome c, followed by the activation of caspase-3. Thus, a mitochondrial damage-dependent pathway is involved in capsaicin-induced apoptosis. Similar results were obtained in our earlier studies on capsaicin-induced apoptosis in esophageal cancer CE 81T/VGH cells (30).

It is well known that caspases play an important role in the initiation of apoptosis. The caspases can be grouped into apoptotic initiators (such as caspase-8) and apoptotic effectors (such as caspase-3), based on their substrate specificities and target proteins (31). In this study, capsaicin-induced apoptosis was observed at 12 h, and then at 72 h. Capsaicin promoted the activity of caspase-3, as analyzed by flow cytometry. The caspase-3 inhibitor (Ac-DEVD-CHO) inhibited capsaicin-

induced caspase-3 activity and led to a decrease in the percentage of apoptosis. These results demonstrated that capsaicin-induced apoptosis might involve a caspase-3-mediated mechanism. Figure 6 presents a proposed model of the capsaicin mechanism of action leading to apoptosis.

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