Capsaicin-induced Apoptosis in Human Hepatoma HepG2 Cells

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Abstract. *Capsaicin, a pungent ingredient of red pepper, has been reported to possess antitumor activities. In this study, the effects of capsaicin on human HepG2 cells were investigated. Capsaicin reduced viability by PI incorporation in HepG2 cells in a dose and time dependent manner. Capsaicin promoted intracellular Ca^{2+} production and reactive oxygen species (ROS). The $\Delta\Psi_m$ significantly decreased after capsaicin treatment for 24 h. Co-treatment of HepG2 cells with capsaicin and BAPTA (an intracellular Ca^{2+} chelator) significantly reduced intracellular Ca^{2+} levels, prevented $\Delta\Psi_m$ disruption and inhibited apoptosis induction. The protein levels of Bcl-2 decreased and Bax increased in the mitochondrial fraction while the Bax protein decreased, and p53 and cytochrome c protein levels increased in the cytosolic fraction in HepG2 cells after capsaicin treatment for 24 h by Western blot. Immunostaining and confocal microscopic analysis also showed that capsaicin promoted cytoplasmic GADD153 expression and GRP78 nuclear translocation. The caspase-3 activity significantly increased after capsaicin treatment for 24 h. Our results indicated that the capsaicin-induced apoptosis in HepG2 cells may result from the elevation of intracellular Ca^{2+} production, ROS, disruption of $\Delta\Psi_m$, regulation of Bcl-2 family protein expression and caspase-3 activity.*

Capsaicin (*N*-vanillyl-8-methyl-1-nonenamide) is a pungent ingredient (0.1-1%) in varieties of red pepper of the genus Capsicum (1). Previous studies have shown the effect of capsaicin on genotoxicity and mutagenicity (2). Recently, many studies have focused on its chemopreventive effects, including regulation of cell proliferation and apoptosis in cancer cells (3). The expression of interleukin 1-beta converting enzyme family, Bcl-2 family and PI-3K/Akt signal transduction pathways are well known to induce mechanisms of apoptosis (4). Capsaicin suppressed the expression of inhibitor of caspase activated DNase to induce human melanoma cells apoptosis (3), and reduced the intracellular ratio of antiapoptotic Bcl-2 to pro-apoptotic Bax, and increased caspase-3 activity in hepatocarcinoma cells (5). In addition, capsaicin induced apoptosis through increased intracellular reactive oxygen species (ROS) and calcium levels in a carcinoma cell model (6).

In the induced pathway of apoptosis, mitochondria play a very important role, through the opening of their megachannel pores and the release of apoptogenic factors such as cytochrome *c* and apoptotic-inducing factor (7). Cytochrome *c* is an important regulating factor in the generation of mitochondrial membrane potential ($\Delta\Psi_m$). Outer membrane permeability resulting in cytochrome *c* release (8) is regulated by the Bcl-2 family proteins (9) which cause swelling of the mitochondrial matrix leading to the opening of the pores or channels in the outer mitochondrial membrane (10).

In this study, the involvement of intracellular Ca^{2+}, ROS, $\Delta\Psi_m$, Bcl-2 family, cytochrome *c* protein expression, and caspase-3 activity were investigated in hepatoma cells undergoing capsaicin-induced apoptosis.

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Materials and Methods

Chemicals and reagents. Capsaicin, propidium iodide (PI), Triton® X-100, Tris-HCl, trypan blue and ribonuclease-A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium phosphates, dimethyl sulfoxide (DMSO) and TE buffer were purchased from Merck Co. (Darmstadt, Germany). Ham's Dulbecco's modified Eagle's medium (HDMEM), L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Cell culture. HepG2 cell line (human hepatoma cells) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The HepG2 cells were placed into 75 cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 atmosphere in 90% HDMEM with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 10% fetal bovine serum, and 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) (Gibco BRL).

Cell viability determinations. HepG2 cells were plated in 12-well plates at a density of 2×10^5 cells/well and grown for 24 h. Different concentrations of capsaicin were then added to cells at a final concentration of 10, 50, 100, or 200 μM, while only adding DMSO (as solvent) for the control regimen and grown at 37˚C, 5% CO_2 and 95% air for different periods of time. Cells were harvested by centrifugation. To determine cell viability, the flow cytometric assay was used as described previously (11).

Apoptosis analysis. Apoptosis of HepG2 cells treated with different concentrations of capsaicin were examined by using flow cytometry analysis. Approximately 5×10^5 cells/well of HepG2 cells in 12-well plates with concentrations of 10, 50, 100, or 200 μM of capsaicin were incubated for different time periods. The zero concentration was defined as control. Cells were harvested by centrifugation and were fixed gently by addition of 70% ethanol at 4˚C overnight and then re-suspended in phosphate-buffered saline (PBS) containing 40 μg/ml PI and 0.1 mg/ml RNase and 0.1% Triton® X-100 in a dark room. After 30 minutes 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 wavelength. Apoptosis was determined and analyzed (11). Annexin V-FITC and PI double staining kit from PharMingen (San Diego, CA, USA) were used for apoptotic cell quantification (11, 12).

Reactive oxygen species (ROS) and intracellular Ca^{2+} assays. The level of ROS in HepG2 cells was examined by flow cytometry (Becton Dickinson FACS Calibur), using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma). Approximately 5×10^5 cells/well of HepG2 cells in 12-well plates with 0, 50, 100, 150 or 200 μM capsaicin were incubated for 1 h to detect ROS changes. The cells were harvested and washed twice, re-suspended in 500 μl of DCFH-DA (10 μM) incubated at 37˚C for 30 min and analyzed by flow cytometry (11, 13-14).

The level of Ca^{2+} in HepG2 cells was determined by flow cytometry (Becton Dickinson FACS Calibur) using Indo 1/AM (Calbiochem, La Jolla, CA, USA). Approximately 2×10^5 cells/well of HepG2 cells in 12-well plates with different concentrations (0, 50, 100, 150 and 200 μM) of capsaicin with or without 5 μM 1,2-bis (O-amino phenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) were incubated for 24 h to detect the changes of Ca^{2+} concentration (9, 15).

Mitochondrial membrane potential ($\Delta\Psi_m$) measurement. The level of $\Delta\Psi_m$ in HepG2 cells was determined by flow cytometry (Becton Dickinson FACS Calibur) using $DiOC_6$ (4 μmol/l). Approximately 5×10^5 cells/well in 12-well plates with different concentrations (0, 50, 100, 150 or 200 μM) of capsaicin with or without 5 μM BAPTA were incubated for 24 h to detect the changes of $\Delta\Psi_m$. The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 μmol/l) and incubated at 37˚C for 30 min then analyzed by flow cytometry (14).

Caspase-3 activity determination. Approximately 5×10^5 HepG2 cells/well in 12-well plates with concentrations of 0, 50, 100, 150 or 200 μM of capsaicin were incubated for different time periods. Cells were harvested by centrifugation and the media removed. A volume of 50 μl of 10 μM substrate solution (PhiPhilux is a unique class of substrates for caspase-3) was added to the cell pellet (1×10^5 cells per sample); the cells were not vortex mixed. Cells were incubated at 37˚C for 60 minutes, then washed once by adding 1 ml of ice-cold PBS and were re-suspended in 1 ml fresh PBS. Cells were analyzed with a flow cytometer (Becton-Dickinson) equipped with an argon ion laser at 488 nm wavelength. Caspase-3 activity was determined and analyzed (14).

Western blotting assay of apoptosis-regulating proteins. Approximately 5×10^5 HepG2 cells/well in 12-well plates with concentrations of 0, 50, 100, 150 and 200 μM of capsaicin were incubated for different time periods. After treatment cells were washed twice in PBS and suspended in homogenizing buffer (containing 20 mM Hepes-KOH at pH 7.5, 10 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulphonyl-fluoride (PMSF), 2 mg/ml aprotinin, 10 mg/ml leupeptin and 5 mg/ml pepstatin) and homogenized by a Dounce homogenizer (Bellco Biotechnology, Vineland, NJ, USA). After centrifugation at 23,100 ×g for 30 min at 4˚C, the supernatant was collected as the cytosolic fraction. The pellet containing mitochondria was re-suspended in lysis buffer (containing 150 mM NaCl, 0.5% Triton® X-100, 50 mM Tri-HCl at pH 7.4, 20 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate) as the mitochondrial fraction for further analysis or was frozen at –70˚C until use. Cytosolic p53, GADD153, GRP78, Smac/DIABLO, xIAP, cIAP1, Bax and cytochrome *c* and mitochondrial Bax and Bcl-2 expressions were determined by SDS-PAGE and immunoblot assay. Equal amounts of cytosol proteins were subjected to 10% polyacrylamide gel electrophoresis. Following electrophoresis, proteins separated on SDS-polyacrylamide gels were transferred to polyvinyldiene difluoride membranes. To block non-specific binding, membranes were incubated at 4˚C overnight with 5% skim milk, followed by a 30 min incubation at 37˚C with antiserum containing antibodies against p53, GADD153, GRP78, Smac/DIABLO, xIAP, cIAP1, Bax, Bcl-2 and cytochrome *c* from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The blots were developed using the enhanced chemiluminescence (ECL) kit (Amersham Life Science, USA). The intensity of each band was determined using an Image analyzer (AlphaImagerTM 2200; Alpha Innotech Co., USA) (11, 14).

Immunofluorescence microscopy. HepG2 cells (5×10^5 cells/well) plated on 4-well chamber slides were treated with or without 100 μM capsaicin for 24 hours then cells were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton®-X 100 in PBS for 1 hour with blocking of non-specific binding sites using 2% bovine serum albumin (BSA). Fixed cells were then incubated with antihuman GADD153 and GRP78 antibody (1:100 dilution) overnight and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution) from Santa Cruz Biotechnology followed by mitochondrial staining with MitiTracker. Cells were photographed under a Leica TCS SP2 Confocal Spectral Microscope (16).

Statistical analysis. Statistical analysis was performed using Student's *t*-test. Tukey's multiple comparison was used to determine significant differences among group means ($p<0.05$).

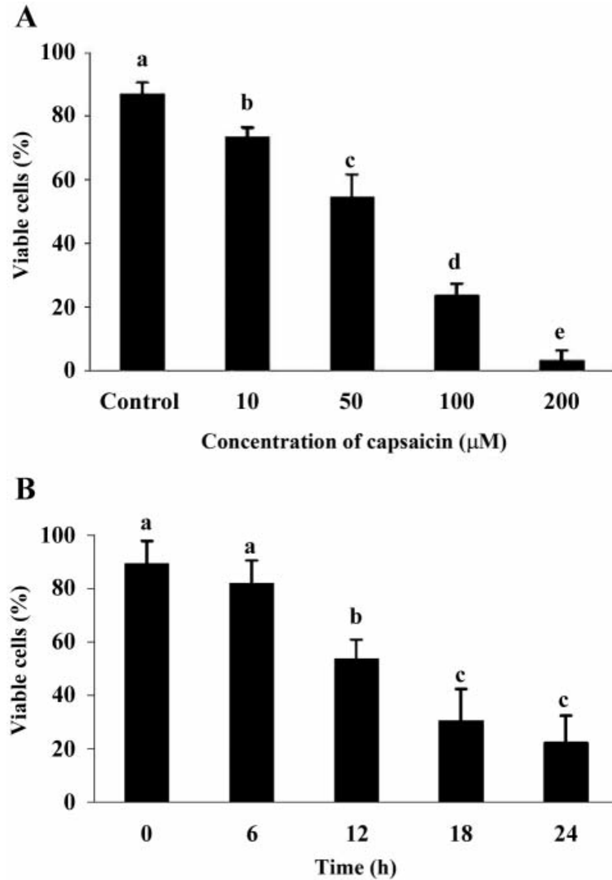

Figure 1. *Effects of capsaicin on the percentage of viable HepG2 cells. HepG2 cells (5×10^5 cells/ml) were treated with capsaicin (Panel A) for 24 h or 100 μM capsaicin for different time intervals (Panel B). The zero concentration was defined as control. Cells were collected and stained by PI dye. The stained and unstained cells were determined by flow cytometry as described in the Materials and Methods section. Values are means±SD (n=3). ^{abc}Groups not sharing the same letters are significantly different by Tukey's test (p<0.05).*

Table I. *Flow cytometric analysis of intracellular ROS and Ca^{2+} levels, $\Delta\Psi_m$ and caspase-3 activity in HepG2 cells with capsaicin treatment.*

Capsaicin (μM)	ROS	Ca^{2+}	$\Delta\Psi_m$	Caspase-3
	(fold of control)			
Control	1^c	1^b	1^a	1^c
10	$6.0±1.8^b$	$2.0±0.2^b$	$0.9±0.09^a$	$5.8±2.1^{b,c}$
50	$7.9±1.4^b$	$3.5±0.9^b$	$0.9±0.01^a$	$10.5±6.4^{b,c}$
100	$9.2±1.8^b$	$4.1±1.6^b$	$0.8±0.06^b$	$14.9±7.3^{a,b}$
200	$15.8±2.4^a$	$9.8±3.8^a$	$0.7±0.08^c$	$27.1±9.6^a$

HepG2 cells (5×10^5 cells/ml) were treated with various concentrations of capsaicin. The zero concentration was defined as control and all other variables expressed relative to it. ROS, Ca^{2+}, $\Delta\Psi_m$ and caspase-3 were determined by staining with DCFH-DA, Indo-1/AM, $DiOC_6$ and PhiPhilux respectively. The stained cells were determined by flow cytometry as described in the Materials and Methods section. Values are means±SD (n=3). Groups not sharing the same symbols are significantly different by Tukey's test ($p<0.05$).

Results

Effects of capsaicin on HepG2 cell viability. As shown in Figure 1A, when HepG2 cells were treated with 10, 50, 100 and 200 μM capsaicin for 24 h, the cell viability significantly decreased by 15, 37, 73 and 96% , respectively of that of the control group ($p<0.05$). After HepG2 cells were treated with 100 μM capsaicin for 12, 18 or 24 h, the cell viability significantly decreased ($p<0.05$) (Figure 1B). These results demonstrated that capsaicin reduced the percentage of viable cells in a dose- and time-dependent manner.

Effects of capsaicin on apoptosis. Figure 2A shows the induction of apoptosis by different concentrations of capsaicin in HepG2 cells. The cell cycle progress was significantly arrested in the sub-G1 phase (*i.e.* apoptosis occurred) when HepG2 cells were treated with 100 or 200 μM capsaicin for 24 h as compared with the control group ($p<0.05$). The percentages of induction of apoptosis were 41.5 and 73.4% of total cells, respectively (Figure 2B).

Effects of capsaicin on intracellular ROS, Ca^{2+} levels, $\Delta\Psi_m$, and caspase-3 activity. When HepG2 cells were treated with different concentrations of capsaicin, the changes of cells being stained for ROS and Ca^{2+}, $\Delta\Psi_m$ and caspase-3 activity were as shown in Table I. As compared with the control group, the proportion of cells stained for ROS significantly increased after HepG2 cells were treated with 10, 50, 100 or 200 μM capsaicin ($p<0.05$). When HepG2 cells were treated with 200 μM capsaicin, the proportion of cells stained for Ca^{2+} significantly increased as compared with the control group ($p<0.05$). The proportion of HepG2 cells stained for $\Delta\Psi_m$ however, significantly decreased after 100 or 200 μM capsaicin treatment, as compared with the control group ($p<0.05$). In addition, the activity of caspase-3 on HepG2 cells significantly increased after 100 and 200 μM capsaicin treatment as compared with the control group ($p<0.05$).

Effects of capsaicin on the levels of apoptosis-regulating proteins. As shown in Figure 3, the expression of apoptosis-regulation proteins in cytosolic and mitochondrial fractions of HepG2 cells were examined by Western blotting assay. Figure 3A indicated that in the mitochondrial fraction of HepG2 cells treat with 100 or 200 μM capsaicin significantly increased the level of Bax protein, as compared with the control group. The cytosolic Bax protein levels decreased after the cells were treated with 100 or 200 μM capsaicin, respectively, as compared with the control group (Figure

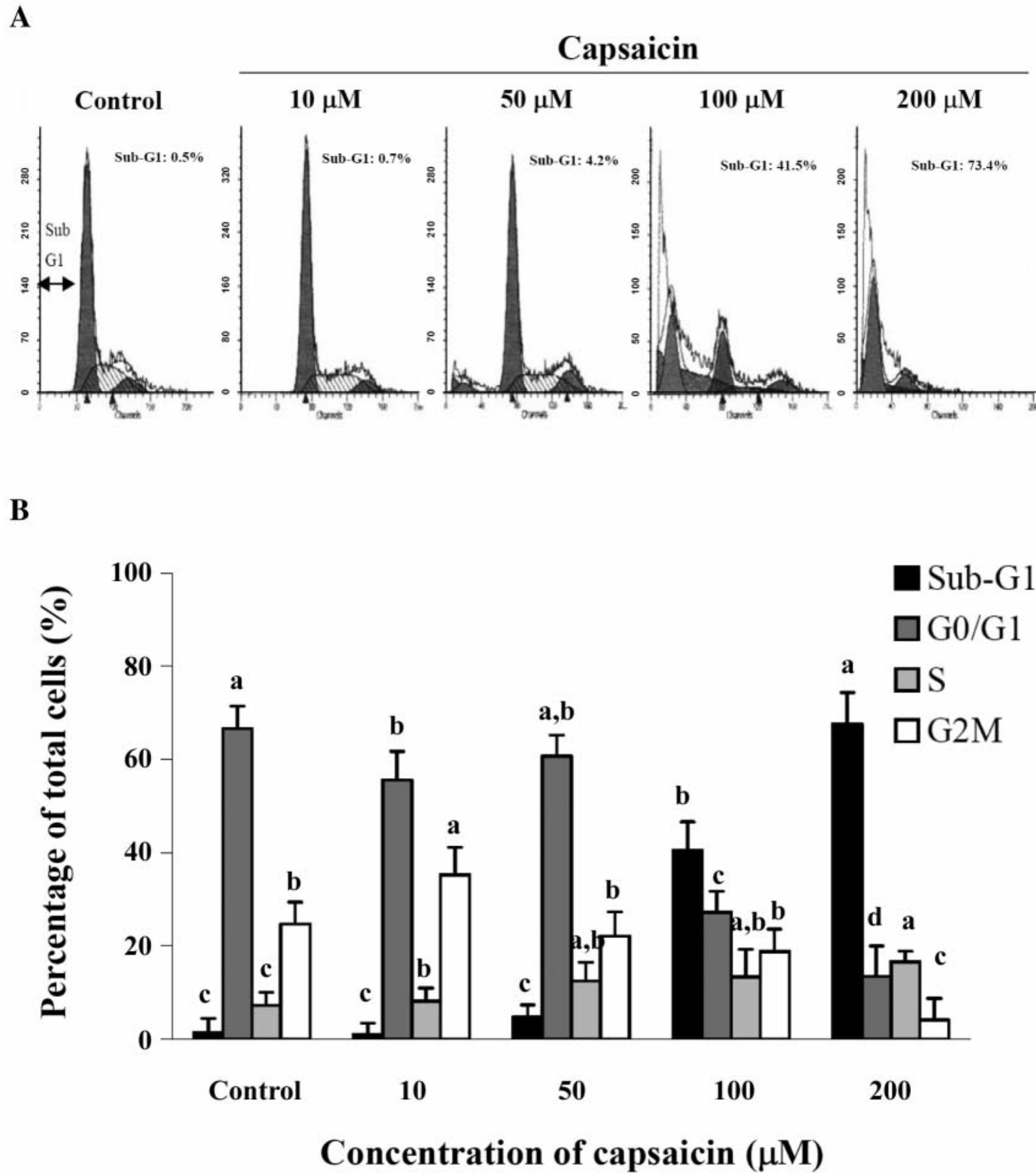

Figure 2. *Effects of capsaicin on cell cycle and apoptosis of HepG2 cells. HepG2 cells (5×10^5 cells/ml) were treated with capsaicin for 24 h. The zero concentration was defined as control. Viable cells were collected and were stained by Annexin V/ PI dye. The stained cells were determined for cell cycle and sub-G1 proportion (apoptosis) by flow cytometry as described in the Materials and Methods section. Panel A: representative profiles; panel B: percentage of phases of the cell cycle. Values are means±SD (n=3). [abc]Groups not sharing the same letters are significantly different by Tukey's test ($p<0.05$).*

3B). In addition, the p53 levels increased 1.3-, 1.6- and 2.1-fold, respectively, that of the control group in the cytosolic fraction of HepG2 cells after 50, 100 or 200 μM capsaicin treatment for 24 h (data not shown). The levels of cytosolic cytochrome *c* increased after treatment with different concentrations of capsaicin for 24 h (Figure 3B). The modulation of capsaicin on Bcl-2 family protein and cytochrome *c* expression occurred in a dose-dependent manner. In addition, as shown in Figure 3C, when cytosolic and mitochondrial fractions were isolated before and after capsaicin treatment, cytochrome *c* was shown to be released from mitochondria after capsaicin treatment.

Effects on endoplasmic reticulum stress protein in HepG2 cells after exposure to capsaicin. As shown in Figure 4, the levels of GADD153, GRP78 and Smac/DIABLO proteins

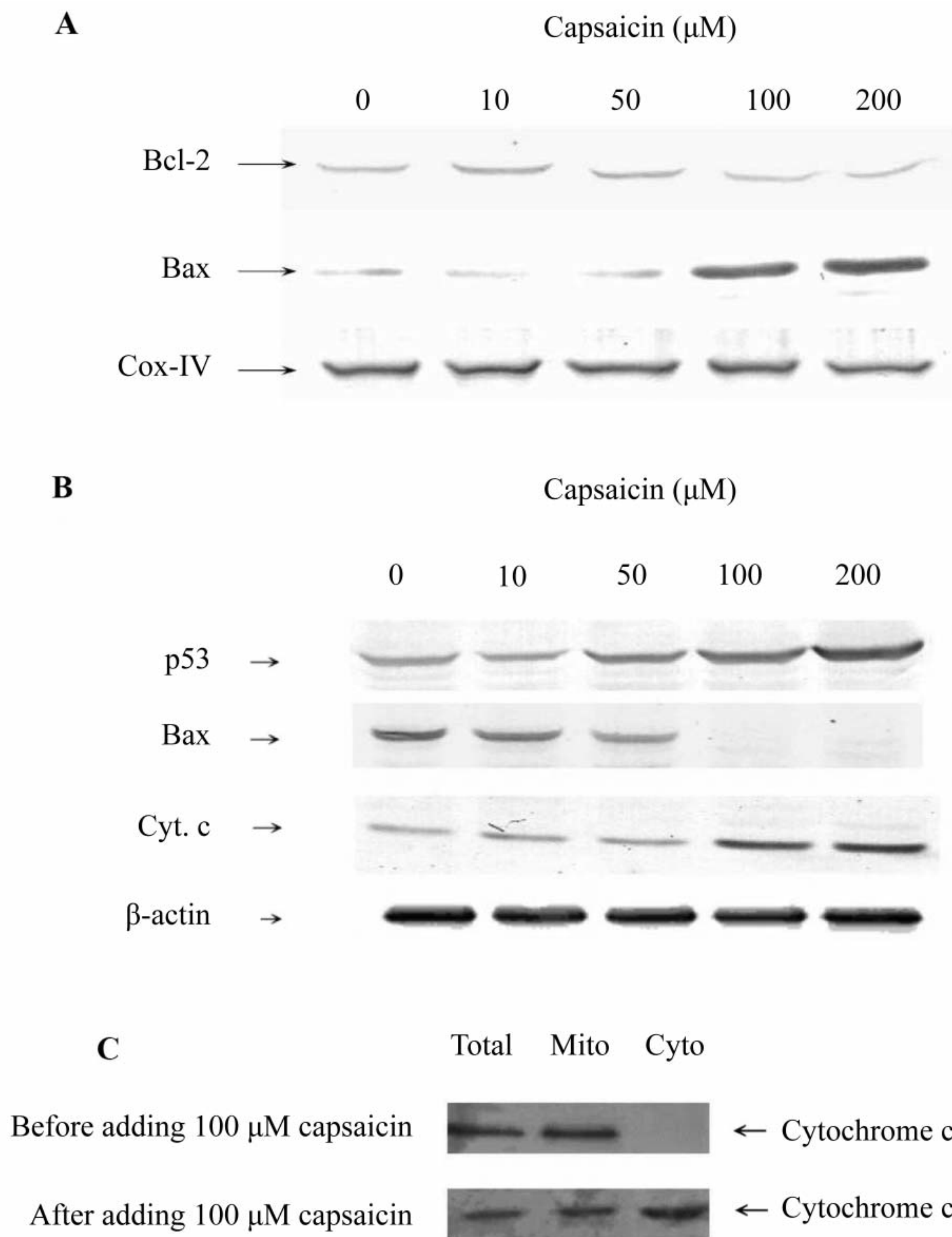

Figure 3. *Expression of p53, Bax, Bcl-2 and cytochrome c proteins after capsaicin treatment on HepG2 cells. HepG2 cells (5×10^5 cells/ml) were treated with capsaicin for 24 h. The zero concentration was defined as control. Cells were collected and lysed for protein determinations. Proteins were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes. The amount of total protein in each sample for the mitochondrial fraction (panel A) and the cytosolic fraction (panel B) by immunostaining was 20 and 15 μg, respectively. The cells were also harvested before and after treatment with capsaicin and then proteins were isolated from the mitochondrial and cytosolic fractions (panel C).*

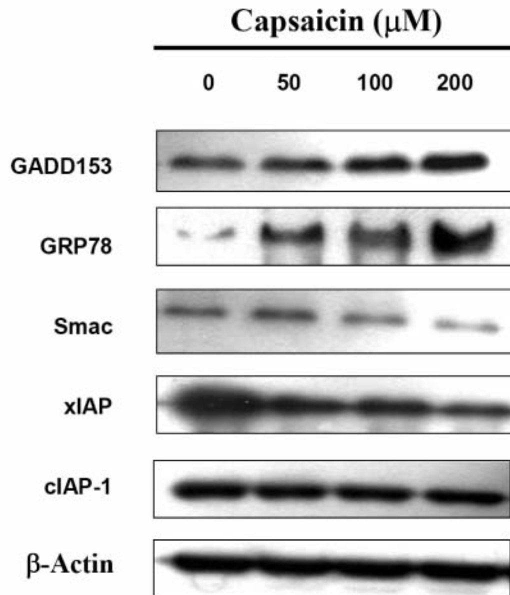

Figure 4. *Effects on ER stress proteins in HepG2 cells after exposure to capsaicin. The total proteins were also isolated from cells after treatment with 0, 50, 10 or 200 μM capsaicin to examine the GADD153, GRP78, Smac/DIABLO, xIAP and cIAP1. Proteins were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes. The amount of total protein in each sample for mitochondrial and cytosolic fractions was 20 and 15 μg, respectively.*

were increased in HepG2 cells after treatment with capsaicin but the levels of xIAP and cIAP1 proteins decreased. GADD153 and GRP78 are a hallmark of ER stress. xIAP and cIAP1 proteins are inhibitors of the caspase-3 pathway. Therefore, these results also confirmed that capsaicin induced apoptosis *via* caspase-3 activation.

Effects of capsaicin with or without BAPTA on intracellular Ca^{2+} levels, MMP, apoptosis and the protein levels of p53 and cytochrome c. To explore whether the Ca^{2+} was required for induction of apoptosis by capsaicin. HepG2 cells were co-treated with Ca^{2+} chelator (BAPTA) and capsaicin. Figure 5A shows that when HepG2 cells were co-treated with capsaicin and BAPTA, the intracellular Ca^{2+} concentration significantly decreased as compared with the capsaicin only group ($p<0.05$). At the same time, the $\Delta\Psi_m$ and apoptosis significantly decreased after HepG2 cells were co-treated with capsaicin and BAPTA as compared with the capsaicin only treated group ($p<0.05$) (Figures 5B, 5C, respectively). As shown in Figure 5D, when HepG2 cells were treated with capsaicin and BAPTA, the p53 protein levels were lower than that of the capsaicin only group. A similar response was obtained on the cytochrome *c* protein levels after co-treatment with capsaicin and BAPTA, and the cytochrome *c* protein level was lower than that of the capsaicin only group.

Effects of capsaicin on GRP78 nuclear translocation and cytoplasmic GADD153 expression in HepG2 cells. As in previous studies (17, 18), HepG2 cells (5×10^4 cells/well) treated or without 100 μM capsaicin for 24 h were plated on 4-well chamber slides. The results from immunostaining by confocal laser microscopic analysis indicated that the GRP78 protein was translocated into nuclei (Figure 6A) and cytoplasmic GADD153 expression was promoted (Figure 6B) in examined HepG2 cells. When compared to the control groups, both GRP78 and GADD153 were higher in the treated group.

Discussion

The actual signal pathway of apoptosis induced by capsaicin is still controversial. Irreversible damage due to oxidative stress on cellular macromolecules, *e.g.* protein, lipid, DNA and RNA, is due to multiple mechanisms (19).

Several studies showed that capsaicin induced apoptosis *via* increased Ca^{2+} and ROS levels, and reduced $\Delta\Psi_m$ in different carcinoma cells models (7, 15, 20). How capsaicin induced endoplasmic reticulum (ER) stress and the associated signal pathway of apoptosis is not clear. When capsaicin was added with BAPTA, an intracellular chelator of Ca^{2+}, the intracellular Ca^{2+} concentration and apoptosis induction significantly decreased ($p<0.05$) in this study. This means that the release of intracellular Ca^{2+} may be an important regulatory factor on early apoptosis induced by capsaicin. Mitochondrial Ca^{2+} sequestration is a prerequisite for ROS production and mitochondrial dysfunction (22). In this study, a dramatic ROS burst was observed in the early period of capsaicin treatment. It was reported that cancer chemopreventive agents induce apoptosis in part with generation of ROS and the disruption of redox homeostasis (23, 24). High levels of ROS not only induced cell death (25) but also caused DNA damage and genomic instability (26). HepG2 cells were pretreated with NAC then treated with capsaicin which led to reduced ROS production and also decreased the percentage of apoptosis (data not shown). These data suggest that apoptosis induced by capsaicin is dependent on ROS production. Our results also showed that capsaicin reduced $\Delta\Psi_m$ in HepG2 cells. Mitochondria were reported to play a key role in the regulation of apoptosis (27). Mitochondrial dysfunctions, including the loss of $\Delta\Psi_m$, permeability transition, and release of cytochrome *c* from the mitochondrion into the cytosol, are associated with apoptosis (28). Thus, a mitochondrial damage-dependent pathway might be involved in capsaicin-induced apoptosis in HepG2 cells. The results also showed capsaicin promoted caspase-3 activation (Table I).

Cytosolic p53 protein levels significantly decreased and cytochrome *c* release from mitochondria significantly decreased after HepG2 cells were co-treated with capsaicin and

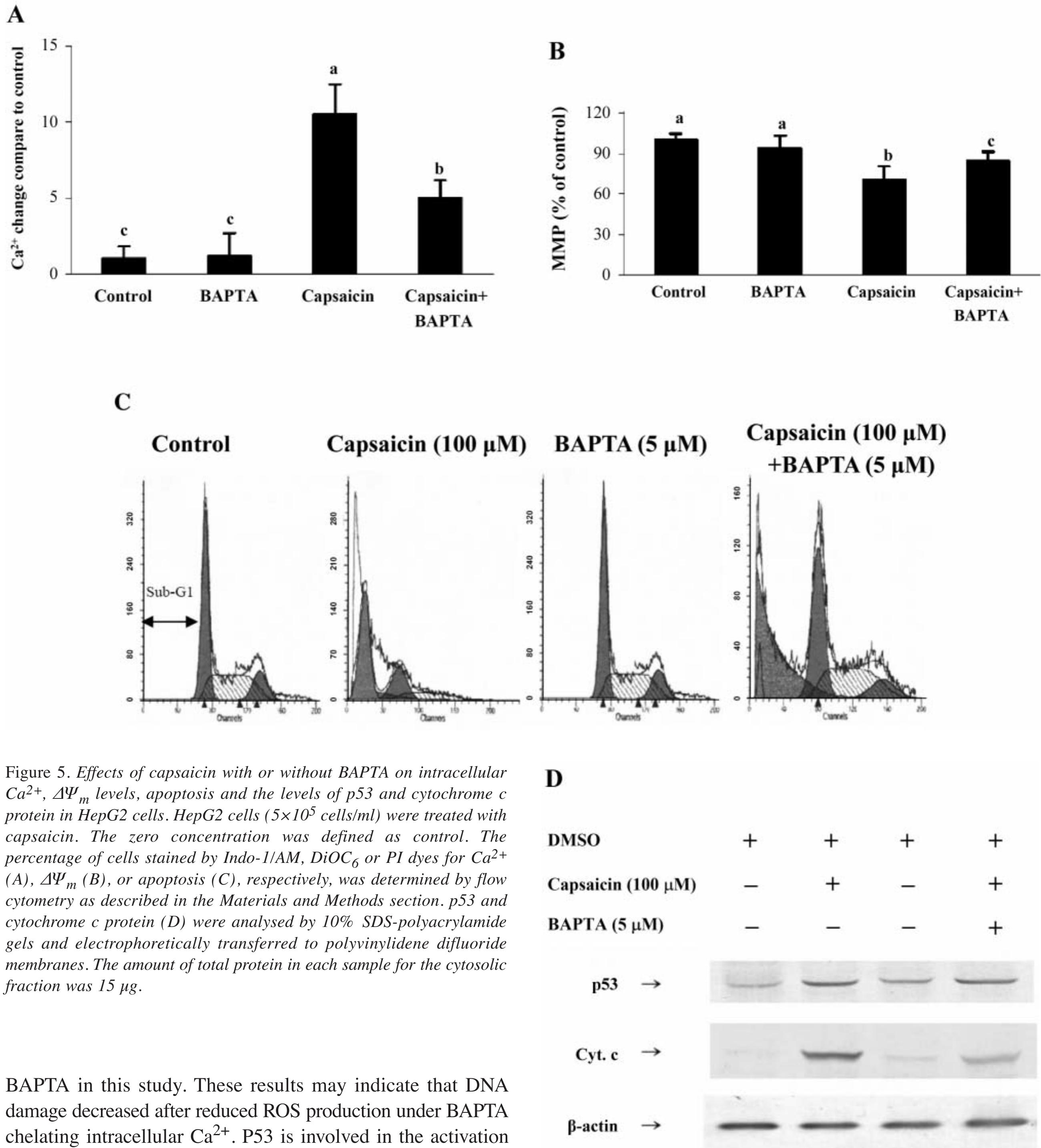

Figure 5. *Effects of capsaicin with or without BAPTA on intracellular Ca^{2+}, $\Delta\Psi_m$ levels, apoptosis and the levels of p53 and cytochrome c protein in HepG2 cells. HepG2 cells (5×10^5 cells/ml) were treated with capsaicin. The zero concentration was defined as control. The percentage of cells stained by Indo-1/AM, $DiOC_6$ or PI dyes for Ca^{2+} (A), $\Delta\Psi_m$ (B), or apoptosis (C), respectively, was determined by flow cytometry as described in the Materials and Methods section. p53 and cytochrome c protein (D) were analysed by 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes. The amount of total protein in each sample for the cytosolic fraction was 15 μg.*

BAPTA in this study. These results may indicate that DNA damage decreased after reduced ROS production under BAPTA chelating intracellular Ca^{2+}. P53 is involved in the activation of apoptosis after DNA damage induced by such agents as cisplatin. P53 could increase the transcription of many genes, such as *p21* or members of the Bcl-2 family, and the pattern of transcriptional regulation is critical in determining the cellular response to DNA damage (20, 29). In addition, intracellular Ca^{2+} levels may also regulate the expression of the Bcl-2 family, a protein family present in the mitochondrial outer membrane, nuclear envelope and endoplasmic reticulum (ER) (30). Our results also showed that capsaicin promoted the levels of cytoplasmic GADD153 and caused GRP78 nuclear translocation, which are indicators of ER stress, followed by Ca^{2+} released from the ER then finally causing apoptosis. Capsaicin also reduced the levels of xIAP and cIAP1; in the

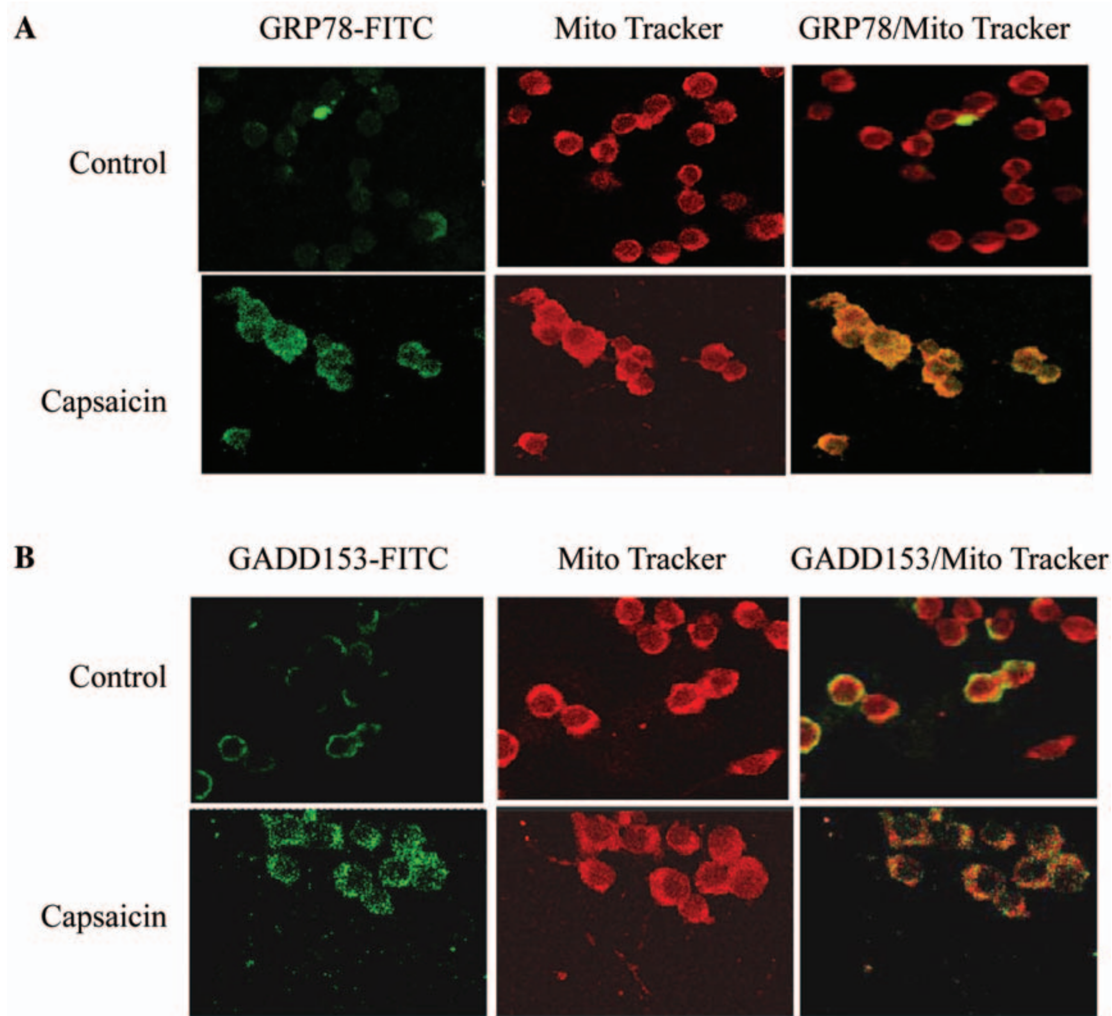

Figure 6. *Effects of capsaicin on GRP78 nuclear translocation and cytoplasmic GADD153 expression in HepG2 cells. HepG2 cells (5×10^5 cells/well; 12-well plates) cells were incubated in the absence or presence of capsaicin (100 μM) for 24 h. The cells were fixed and stained with primary antibodies to GRP78 (Panel A) and GADD153 (Panel B). FITC-labeled secondary antibodies were then used (green fluorescence) and the proteins were detected by a confocal laser microscopic system. The nuclei were stained by MitoTracker (red fluorescence). Areas of colocalization between GRP78 and GADD153 expressions and nuclei in the merged panels are yellow.*

HepG2 cells, both proteins have been shown to inhibit caspase-3 activation. Mitochondria play an important role in the induction of apoptosis *via* increasing the permeability of the outer mitochondrial membrane and decreasing the $\Delta\Psi_m$ (31, 32). There are many apoptosis regulatory factors, including Bcl-2 family members, cytochrome *c*, apoptosis protease-activating factor-1 (Apaf-1), which exist in mitochondria. Once the apoptosis signal is triggered, mitochondrial permeability transition is regulated and this leads to a decrease of mitochondrial membrane potential (33). In this study, capsaicin significantly affected the expression of Bcl-2 family members present in cytosolic and mitochondrial fractions. The levels of Bax, a pro-apoptotic Bcl-2 family member, increased in mitochondria after capsaicin treatment. In contrast, the levels of Bcl-2, an antiapoptotic Bcl-2 family member, decreased in mitochondria. In addition, cytochrome *c* release to the cytosol increased after capsaicin treatment. Previous studies have shown that cytochrome *c* release from mitochondria could be controlled by Bax. The translocation of Bax can alter the outer mitochondrial membrane permeability, leading to cytochrome *c* release from the mitochondria to the cytosol (31, 34-35). It was reported that insertion of Bax to the outer mitochondrial membrane and formation of Bax oilgomers may provide channels conducting large proteins, allowing transport of cytochrome *c* (36).

In conclusion, our results, as shown in Figure 7, clearly demonstrated that capsaicin induces apoptosis through elevating the levels of intracellular ROS and Ca^{2+}, promoting

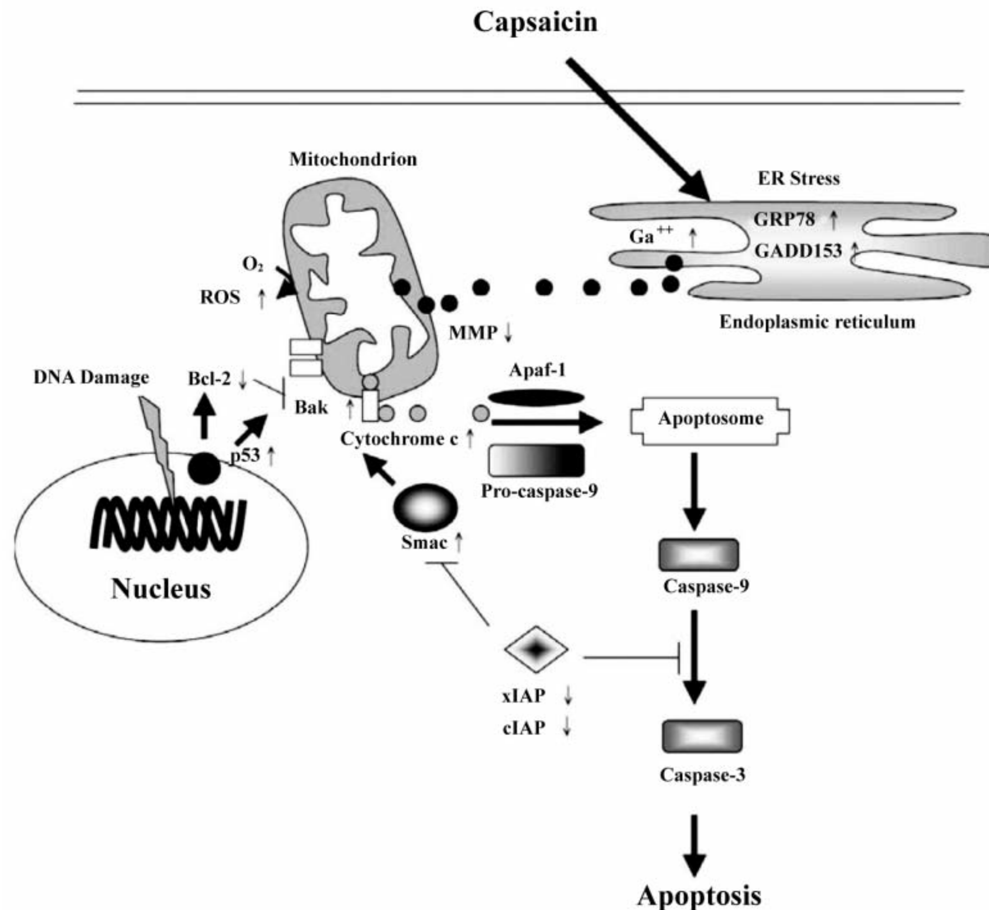

Figure 7. *Signals proposed in the model of apoptosis induced by capsaicin in HepG2 cells. Capsaicin induced an intracellular rise of Ca^{2+} and ROS production, and reduced $\Delta\Psi_m$. Ca^{2+} and Bcl-2 family expression could regulate the decrease of $\Delta\Psi_m$. Furthermore, reduced $\Delta\Psi_m$ could lead to cytochrome c release from the mitochondria to the cytosol. After caspase-9 and 3 are activated by cytochrome c, apoptosis occurs.*

the levels of Bax, GADD153 and GRP78, decreasing $\Delta\Psi_m$, Bcl-2, xIAP and cIAP1 and then increasing caspase-3 activation in HepG2 cells. ROS play an important role in the induction of apoptosis in HepG2 cells by capsaicin.

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References

1 Monsereenusorn Y, Kongsamut S and Pezalla PD: Capsaicin-a literature survey. Crit Rev Toxicol *10*: 321-339, 1982.

2 Richeux F, Cascante M, Ennamany R, Saboureau D and Creppy EE: Cytotoxicity and genotoxicity of capsaicin in human neuroblastoma cells SHSY-5Y. Arch Toxicol *73*: 403-409, 1999.

3 Gong XF, Wang MW and Ikejima T: Mechanisms of capsaicin-induced apoptosis of human melanoma A375-S2 cells. Zhonghua Zhong Liu Za Zhi *27*: 401-403, 2005.

4 McGill G and Fisher DE: Apoptosis in tumorigenesis and cancer therapy. Front Biosci *2*: d353-379, 1997.

5 Jung MY, Kang HJ and Moon A: Capsaicin-induced apoptosis in SK-Hep-1 hepatocarcinoma cells involves Bcl-2 down-regulation and caspase-3 activation. Cancer Lett *165*: 139-145, 2001.

6 Lee YS, Nam DH and Kim JA: Induction of apoptosis by capsaicin in A172 human glioblastoma cells. Cancer Lett *161*: 121-130, 2000.

7 Macho A, Calzado MA, Muñoz-Blanco J, Gómez-Díaz C, Gajate C, Mollinedo F , Navas P and Muñoz E: Selective induction of apoptosis by capsaicin in transformed cells: the role of reactive oxygen species and calcium. Cell Death Differ *6*: 155-165, 1999.

8 Waterhouse NJ, Goldstein JC, von Ahsen O, Schuler M, Newmeyer DD and Green DR: Cytochrome *c* maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. J Cell Biol *153*: 319-328, 2001.

9 Gross A, Jockel J, Wei MC and Korsmeyer SJ: Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J *17*: 3878-3885, 1998.

10 Brenner C and Kroemer G: Apoptosis, mitochondria – the death signal integrators. Science *289*: 1150-1151, 2000.
11 Wu CC, Chung JG, Tsai SJ, Yang JH and Sheen LY: Differential effects of allyl sulfides from garlic essential oil on cell cycle regulation in human liver tumor cells. Food Chem Toxicol *42*: 1937-1947, 2004.
12 Lin CC, Kao ST, Chen GW, Ho HC and Chung JG: Apoptosis of human leukemia HL-60 cells and murine leukemia WEHI-3 cells induced by berberine through the activation of caspase-3. Anticancer Res *26*: 227-242, 2006.
13 Shrestha SP, Tomita T, Weiss LM and Orlofsky A: Proliferation of *Toxoplasma gondii* in inflammatory macrophages *in vivo* is associated with diminished oxygen radical production in the host cell. Int J Parasitol *36*: 433-441, 2006.
14 Wu CC, Lin JP, Yang JS, Chou ST, Chen SC, Lin YT, Lin HL and Chung JG: Capsaicin induced cell cycle arrest and apoptosis in human esophagus epidermoid carcinoma CE 81T/VGH cells through the elevation of intracellular reactive oxygen species and Ca(2+) productions and caspase-3 activation. Mutat Res *601*: 71-82, 2006.
15 Kim JA, Kang YS and Lee YS: A phospholipase C-dependent intracellular Ca^{2+} release pathway mediates the capsaicin-induced apoptosis in HepG2 human hepatoma cells. Arch Pharm Res *28*: 73-80, 2005.
16 Hsu MH, Chen CJ, Kuo SC, Chung JG, Lai YY, Teng CM, Pan SL and Huang LJ: 2-(3-Fluorophenyl)-6-methoxyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (YJC-1) induces mitotic phase arrest in A549 cells. Eur J Pharmacol *559*: 14-20, 2007.
17 Chung JG, Yeh KT, Wu SL, Hsu NY, Chen GW, Yeh YW and Ho HC: Novel transmembrane GTPase of non-small cell lung cancer identified by mRNA differential display. Cancer Res *61*: 8873-8879, 2001.
18 Tang NY, Yang JS, Chang YH, Lu HF, Hsia TC, Lin WC and Chung JG: Effects of wogonin on the levels of cytokines and functions of leukocytes associated with NF-kappa B expression in Sprague-Dawley rats. In Vivo *20*: 527-532, 2006.
19 Czaja MJ: Induction and regulation of hepatocyte apoptosis by oxidative stress. Antioxid Redox Signal *4*: 759-767, 2002.
20 Perego P, Giarola M, Righetti SC, Supino R, Caserini C, Delia D, Pierotti MA, Miyashita T, Reed JC and Zunino F: Association between cisplatin resistance and mutation of *p53* gene and reduced bax expression in ovarian carcinoma cell systems. Cancer Res *56*: 556-562, 1996.
21 De Jong D, Prins FA, Mason DY, Reed JC, van Ommen GB and Kluin PM: Subcellular localization of the bcl-2 protein in malignant and normal lymphoid cells. Cancer Res *54*: 256-260, 1994.
22 Chinopoulos C and Adam-Vizi V: Calcium, mitochondria and oxidative stress in neuronal pathology. Novel aspects of an enduring theme. FEBS J *273*: 433-450, 2006.
23 Kluck RM, Martin SJ, Hoffman BM, Zhou JS, Green DR and New meyer DD: Cytochrome *c* activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system. EMBO J *16*: 4639-4649, 1997.
24 Xia Z, Lundgren B, Bergstrand A, DePierre JW and Nassberger L: Changes in the generation of reactive oxygen species and in mitochondrial membrane potential during apoptosis induced by the antidepressants imipramine, clomipramine, and citalopram and the effects on these changes by Bcl-2 and Bcl-xL – Triggering of ceramide generation through sphingomyelin hydrolysis. Biochem Pharmacol *57*: 1199-1208, 1999.
25 Droge W: Free radicals in the physiological control of cell function. Physiol Rev *82*: 47-95, 2002.
26 Wiseman H and Halliwell B: Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem J *313*: 17-29, 1996.
27 Gottlieb RA: Mitochondria: execution central. FEBS Lett *482*: 6-12, 2000.
28 Tang W, Liu JW, Zhao WM, Wei DZ and Zhong JJ: Ganoderic acid T from *Ganoderma lucidum* mycelia induces mitochondria mediated apoptosis in lung cancer cells. Life Sci *23*: 205-211, 2006.
29 Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC: Tumor suppressor p53 is a regulator of *bcl-2* and *bax* gene expression *in vitro* and *in vivo*. Oncogene *9*: 1799-1805, 1994.
30 Lao Y and Chang DC: Study of the functional role of Bcl-2 family proteins in regulating Ca(2+) signals in apoptotic cells. Biochem Soc Trans *35*: 1038-1039, 2007.
31 Green DR and Reed JC: Mitochondria and apoptosis. Science *281*: 1309-1312, 1998.
32 Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM and Kroemer G: Molecular characterization of mitochondrial apoptosis-inducing factor. Nature *397*: 441-446, 1999.
33 Regula KM, Ens K and Kirshenbaum LA: Mitochondria-assisted cell suicide: a license to kill. J Mol Cell Cardiol *35*: 559-567, 2003.
34 Goping I S, Gross A, Lavoie JN, Nguyen M, Jemmerson R, Roth K, Korsmeyer SJ and Shore GC: Regulated targeting of BAX to mitochondria. J Cell Biol *143*: 207-215, 1998.
35 Saito M, Korsmeyer SJ and Schlesinger PH: BAX-dependent transport of cytochrome *c* reconstituted in pure liposomes. Nat Cell Biol *2*: 553-555, 2000.
36 Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB and Korsmeyer SJ: tBID. A membrane-targeted death ligand, oligomerizes BAK to release cytochrome *c*. Genes Dev *14*: 2060-2071, 2000.
37 Gross A, McDonnell JM and Korsmeyer SJ: BCL-2 family members and the mitochondria in apoptosis. Genes Dev *13*: 1899-1911, 1999.

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