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Effects of Capsaicin on Induction of Apoptosis and Inhibition of Adipogenesis in 3T3-L1 Cells

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Currently, at the beginning of the 21st century, obesity has become the leading metabolic disease in the world. It is a serious health problem in industrialized countries. Previous research has suggested that decreased preadipocyte differentiation and proliferation and decreased lipogenesis are mechanisms to reduce obesity. In the present study, the effects of capsaicin on the induction of apoptosis and inhibition of lipid accumulation in 3T3-L1 preadipocytes and adipocytes were investigated. The results demonstrated that capsaicin decreased cell population growth of 3T3-L1 preadipocytes, assessed with the MTT assay. Flow cytometric analysis of 3T3-L1 preadipocytes exposed to capsaicin showed that apoptotic cells increased in a time- and dose-dependent manner. Treatment with capsaicin decreased the number of normal cells and increased the number of early apoptotic and late apoptotic cells in a dose-dependent manner. The treatment of cells with capsaicin caused the loss of mitochondria membrane potential ($\Delta\Psi$m). The induction of apoptosis in 3T3-L1 preadipocytes by capsaicin was mediated through the activation of caspase-3, Bax, and Bak, and then through the cleavage of PARP and the down-regulation of Bcl-2. Moreover, capsaicin significantly decreased the amount of intracellular triglycerides and glycerol-3-phosphate dehydrogenase (GPDH) activity in 3T3-L1 adipocytes. Capsaicin also inhibited the expression of PPARγ, C/EBPα, and leptin, but induced up-regulation of adiponectin at the protein level. These results demonstrate that capsaicin efficiently induces apoptosis and inhibits adipogenesis in 3T3-L1 preadipocytes and adipocytes.

KEYWORDS: Capsaicin; adipogenesis; 3T3-L1 cells; apoptosis; protein expression

INTRODUCTION

Obesity is an important topic in the realm of public health and preventive medicine, because it is considered to be a risk factor associated with the genesis or development of various diseases, including coronary heart disease, hypertension, type 2 diabetes mellitus, cancer, respiratory complications, and osteoarthritis (*1*). Currently, at the beginning of the 21st century, obesity has become the leading metabolic disease in the world (*2*). Recent reports have proposed mechanisms to reduce obesity, including decreased energy/food intake and increased energy expenditure, decreased preadipocyte differentiation and proliferation, decreased lipogenesis, and increased lipolysis and fat oxidation (*3*). The preadipocytes play a key role by differentiating into mature adipocytes and increasing fat mass. Obesity is characterized at the cell biological level by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes in adipose tissue (*4*). Hausman et al. (*5*) indicated that adipogenesis is a process wherein the preadipocytes differentiate into adipocytes. MacDougald and Mandrup (*6*) also indicated the major differentiation programmed is coordinated by several positive and negative adipogenic molecules, including a variety of growth factors, cytokines, and hormones.

Capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide) is a major pungent ingredient in red pepper that is widely used as a spice (*7*). The pepper is a potent analgesic, anti-inflammatory (*8*), and causes desensitization to different chemical irritants upon long-term treatment. Capsaicin has been reported to decrease energy intake (*9*), decrease the adipose tissue weight, and decrease the serum triacylglycerol content by enhancing energy metabolism (*10*). Capsaicin inhibits the growth of various immortalized and malignant cells (*11*) and induces apoptosis in transformed cells (*12*). The chemical structure of capsaicin is shown in **Figure 1**. Cell apoptosis is important for destruction of undesired cells during development and homeostasis of multicellular organisms and is characterized by distinct morphological changes such as plasma membrane blebbing, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation (*13*). There are two main pathways leading to apoptosis. The first of these depends on the participation of mitochondria, and the second is involved in the interaction of a death receptor with its ligand. Many proteins are known to be involved in the process of programmed cell death. Caspase are a family of cysteine proteases that are activated during the execution phase of the cell apoptotic process

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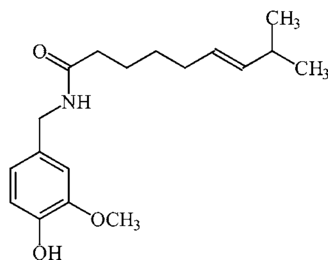

Figure 1. Chemical structure of capsaicin.

(*14*). Pro- and antiapoptotic members of the Bcl-2 family regulate the mitochondria pathway (*15*). Our previous study revealed that some phenolic acids (including gallic acid, *o*-coumaric acid, *m*-coumaric acid, and chlorogenic acid) caused an improved inhibition of cell population growth and induction of apoptosis in 3T3-L1 preadipocytes (*16*). However, the literature regarding the effect of capsaicin on induction of apoptosis and inhibition of adipogenesis in 3T3-L1 preadipocytes and adipocytes is unclear. In the present study, we investigated the effects of capsaicin on the induction of preadipocytic apoptosis and inhibition of adipocytic triglyceride content. The murine 3T3-L1 cell line was used in this study due to its widespread use as a cell model for adipose cell biology research over the course of several decades (*17*).

MATERIALS AND METHODS

Materials. Capsaicin, MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], propidium iodide (PI), ribonuclease (RNase), oil red O, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), insulin (INS), and anti-rabbit or anti-mouse secondary horseradish peroxidase antibodies were purchased from the Sigma Chemical Co. (St. Louis, MO). Anti-chicken secondary horseradish peroxidase antibody was purchased from the Bethyl Laboratories, Inc. (Montgomery, TX). Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum, and antibiotic mixture (penicillin−streptomycin) were purchased from the Gibco BRL Co. (Grand Island, NY). Anti-capsase-3, anti-PARP [poly(ADP-ribose) polymerase], and anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-Bcl-2, anti-Bax, and anti-Bak antibodies were obtained from Pharmingen (San Diego, CA). Anti-PPARγ (peroxisome proliferator−activated receptor−gamma) antibody was obtained from Upstate (Lake Placid, NY). Anti-C/EBPα (CCAAT enhancer binding protein alpha) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-adiponectin antibody was obtained from BioVision (Mountain View, CA). Anti-leptin antibody was obtained from Chemicon (Temecula, CA). Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). Polyvinyldifluoride (PVDF) membranes for Western blotting were obtained from Millipore (Bedford, MA). All other chemicals were reagent grade.

Cell Culture. The mouse embryo 3T3-L1 cells (BCRC 60159) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). 3T3-L1 preadipocytes were incubated in culture medium included DMEM, 10% calf serum, 1.5 g/L sodium bicarbonate, and 100 U/mL penicillin−streptomycin. Adipocyte differentiation was induced by the adipogenic agents (0.5 mM IBMX, 1 μM DEX, and 1 μM INS) that were added to culture medium. Afterward, the medium was changed to normal culture medium and was freshly replaced every 48 h. The cells were harvested 8 days after the initiation of differentiation. The cell culture condition was 37 °C in a humidified 5% CO_2 incubator.

MTT Assay. The MTT assay was performed according to the method of Mosmann (*18*). 3T3-L1 preadipocytes were plated into 96-well microtiter plates at a density of 1 × 10^4 cells/well. After 24 h, the culture medium was replaced by 200 μL serial dilutions (0−250 μM) of capsaicin and the cells were incubated for 24, 48, and 72 h. The final concentration of the solvent was less than 0.1% in the cell culture medium. Culture solutions were then removed and replaced by 90 μL of culture medium. Ten microliters of a sterile, filtered MTT solution (5 mg/mL) in phosphate-buffered saline (PBS, pH = 7.4) was added to each well to reach a final concentration of 0.5 mg MTT/mL. After 5 h, the unreacted dye was removed, and then the insoluble formazan crystals were dissolved in DMSO (200 μL/well) and measured spectrophotometrically in a FLUOstar galaxy spectrophotometer (BMG Labtechnologies Ltd., Germany) at 570 nm. The cell population growth percentage (%) was expressed as the percentage of cell growth compared to the control, and it was calculated by A_{570nm}[capsaicin]/A_{570nm}[control] × 100. The IC_{50} was calculated as the capsaicin concentration under which 50% inhibition of cell population growth occurred compared to that of untreated controls.

LDH Leakage Assay. The lactate dehydrogenase (LDH) leakage activity assay was performed using a commercial kit (Sigma Chemical Co., St. Louis, MO). 3T3-L1 preadipocytes were incubated with 0−250 μM capsaicin for 24−72 h and then analyzed for LDH leakage into the culture media. The total LDH activity was determined after the cells were thoroughly disrupted by sonication. The percentage of LDH leakage was then calculated to determine the membrane integrity. The amount of LDH leakage was expressed as a percentage of total activity (activity in the medium)/(activity in the medium + activity of the cells) × 100.

Cell Apoptosis Analysis by PI Staining. Cell apoptosis was assayed by the PI (propidium iodide) staining method (*19*). The 3T3-L1 preadipocytes were stimulated with 0−250 μM capsaicin for 24, 48, and 72 h, respectively. Briefly, cells were harvested by trypsin−EDTA (TE) solution (0.05% trypsin and 0.02% EDTA in PBS), washed with PBS twice, and fixed in 80% ethanol at 4 °C for 30 min, followed by incubation with 100 μg/mL RNase for 30 min at 37 °C. The cells were then stained with 40 mg/mL PI for 15 min at room temperature and subjected to flow cytometric analysis of DNA content using a FACScan flow cytometer (Becton-Dickinson Immunocytometery Systems USA, San Jose, CA). Approximately 1 × 10^4 counts were made for each sample. The percentage of distribution of cell apoptosis was calculated by CELL Quest software.

Annexin V-FITC/PI Double-Staining Analysis by Flow Cytometry. Annexin V-FITC/PI double staining of the cells was determined using the Annexin V-FITC kit (ANNEX100F, SEROTEC, U.K.). To detect early apoptosis, late apoptosis, and necrosis induced by capsaicin, 3T3-L1 preadipocytes (1 × 10^6 cells/dish) were added to a 6 cm dish and treated for 72 h at 37 °C in 3 mL of culture medium containing testing agents at final concentrations of 0, 50, 100, and 250 μM. 3T3-L1 preadipocytes (1 × 10^5) were then stained for 10 min at room temperature with FITC-conjugated Annexin V-FITC and PI in a Ca^{2+}-enriched binding buffer (Annexin V-FITC kit) and analyzed by a FACScan flow cytometer. Annexin V-FITC and PI emissions were detected in the FL 1 and FL 2 channels of a FACScan flow cytometer, using emission filters of 525 and 575 nm, respectively. The Annexin V-FITC−/PI− population was regarded as normal healthy cells, while the Annexin V-FITC+/PI− cells were taken as a measure of early apoptosis, Annexin V-FITC+/PI+ as late apoptosis, and Annexin V-FITC−/PI+ as necrosis. Approximately 1 × 10^4 counts were made for each sample. The percentage of distribution of normal, early apoptotic, late apoptotic, and necrotic cells was calculated using CELL Quest software.

Mitochondria Membrane Potential (ΔΨm) Assay. Mitochondria membrane potential was determined by using the MitoPTTM 100 test kit (Immunochemistry Technologies, Bloomington, MN). 3T3-L1 preadipocytes were seeded in 12-well plates. After 24 h, the cells were treated with 0−250 μM capsaicin for 6, 12, and 24 h, respectively. Routine passage consisted of rinsing cells in 12-well plates once with PBS, followed by harvesting with 0.1 mL of TE solution, addition of 1 mL of culture medium, and thorough dispersion. Aliquots of the resulting cell suspensions were placed in eppendorf tubes containing 1 mL of the culture medium at a concentration of 1 × 10^6 cells per eppendorf. After being centrifuged (1000 rpm, 5 min), cells were incubated with 10 μg/mL JC-1 at 37 °C for 15 min in a humidified 5% CO_2 incubator. Cells were collected and washed with 1× assay buffer

(MitoPTTM 100 test kit). The cells were resuspended in the same solution and analyzed by a FLUOstar Galaxy fluorescence plate reader with an excitation wavelength of 485 nm and an emission wavelength of 590 nm for red fluorescence. Apoptotic cells generate a lower level of red fluorescence, and changes in the mitochondria membrane potential (ΔΨm) can be most accurately assessed by comparing the red fluorescence of untreated cells to the red fluorescence of those treated with capsaicin.

Measurement of Caspase-3 Activity. After treatment with capsaicin, 3T3-L1 preadipocytes were collected, washed with PBS, and lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris−HCl, pH 8, 2 mM dithiothione, 10 μg/mL pepstatin A, 2 mM phenylmethanesulfonyl fluoride, and 10 μg/mL leupeptin) for 20 min at 4 °C followed by centrifugation (10 000*g*) for 30 min. Caspase-3 activity was assayed in 50 μL reaction mixtures with a fluorogenic reporter substrate peptide specific for caspase-3. The substrate peptide (200 μM) was incubated at 37 °C with cytosolic extracts (50 μg of total protein) in reaction buffer (100 mM HEPES, 10% sucrose, 10 mM dithiothreitol, 0.1% 3-[(3-chloamidopropyldimethylammonio)-1-propanesulfonate]. Fluorescence was measured after 2 h (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a FLUOstar Galaxy fluorescence plate reader (BMG LabTechologies, GmbH, Offenburg, Germany).

Measurement of Triglyceride Content. The 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were incubated with 0−250 μM capsaicin for 72 h at 37 °C in a humidified 5% CO_2 incubator. Cells were collected and lysed in lysis buffer (1% Triton X-100 in PBS). The total triglyceride content in the cells was determined using a commercial triglyceride assay kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). The protein concentration was determined by using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Oil Red O Staining. The oil red O working solution was prepared as described by the method of Ramirez-Zacarias et al. (*20*). 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were incubated with 0−250 μM capsaicin for 72 h at 37 °C in a humidified 5% CO_2 incubator. Cells were washed twice with PBS and then fixed with 10% neutral formalin for at least 20 min at room temperature. After the 10% neutral formalin was removed, 100% propylene glycol was added to each well for 3 min. Cells were decolorized with 60% propylene glycol before staining for 1 h with the oil red O working solution and then washed exhaustively with water. The staining dye of cells was extracted with isopropyl alcohol (1 mL/well) and measured spectrophotometrically in a FLUOstar Galaxy spectrophotometer (BMG Labtechnologies Ltd., Germany) at 510 nm. The oil red O-stained material (OROSM) was expressed on a per cell basis using the cell number determined from similar plates. The relative oil red O-stained material (OROSM, %) relative to control wells containing cell culture medium without capsaicin was calculated by A_{510nm}[capsaicin]/A_{510nm}[control] × 100.

Glycerol-3-Phosphate Dehydrogenase Activity. The 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were incubated with 0−250 μM capsaicin for 72 h at 37 °C in a humidified 5% CO_2 incubator. Cells were carefully washed twice with ice-cold PBS on 3T3-L1 adipocytes and lysed in 25 mM Tris/1 mM EDTA, pH 7.5 for measurement of glycerol-3-phosphate dehydrogenase (GPDH) specific activity. GPDH activity was determined according to the procedure of Wise and Green (*21*). Protein concentration was determined by Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Enzyme activity was expressed as units of activity/mg protein.

Western Blot Analysis. The 3T3-L1 preadipocytes were incubated with 0−250 μM capsaicin for 12 and 24 h at 37 °C in a humidified 5% CO_2 incubator. 3T3-L1 adipocytes were incubated with 0−100 μM capsaicin for 12 and 24 h at 37 °C in a humidified 5% CO_2 incubator. Cells were collected and lysed in ice-cold lysis buffer (20 mM Tris−HCl (pH 7.4), 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin, and 1 mM PMSF). The protein concentration of the extracts was estimated with a Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. The caspase-3, PARP, Bak, Bax,

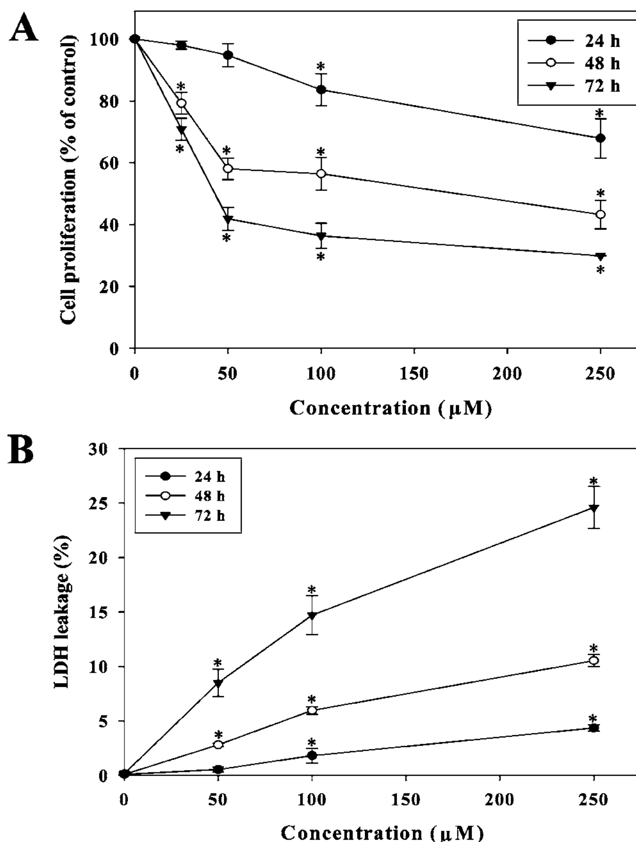

Figure 2. Effect of capsaicin on the inhibition of cell population growth (**A**) and cytotoxicity (**B**) in 3T3-L1 preadipocytes. Cells were treated with 0–250 μM capsaicin for 24, 48, and 72 h. Reported values are the mean ± SD ($n = 3$); $*p < 0.05$ compared with the control.

Bcl-2, and β-actin proteins were assessed in preadipocytes. The PPARγ, C/EBPα, adiponectin, leptin, and β-actin proteins were assessed in adipocytes. The total proteins (50−60 μg) were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using a 12% polyacrylamide gel. The proteins in the gel were then transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in PBST (0.05% v/v Tween-20 in PBS, pH 7.2) for 1 h. Membranes were incubated with primary antibody (1:5000) at 4 °C overnight and then with secondary antibody (1:5000) for 1 h. Membranes were washed three times in PBST for 10 min between each step. The signal was detected by using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). The relative expression of proteins was quantified densitometrically using the software LabWorks 4.5 and calculated according to the reference bands of β-actin.

Statistical Analysis. Each experiment was performed in triplicate. The results were expressed as the mean ± SD. Statistical analysis was performed using SAS software. Analysis of variance was performed using ANOVA procedures. Significant differences ($p < 0.05$) between the means were determined by Duncan's multiple range tests.

RESULTS

Inhibition of Population Growth in 3T3-L1 Preadipocytes. To assess whether capsaicin inhibited the population growth of 3T3-L1 preadipocytes, cells were treated with 0−250 μM capsaicin and the cell population growth was determined by using an MTT assay. As shown in **Figure 2A**, capsaicin decreased the cell population growth in a time- and dose-dependent manner with an IC_{50} value of 45 μM. LDH activity in the medium and within cells was also measured in order to evaluate the influence of cell injuries on 3T3-L1 preadipocytes. The cytotoxic effect of capsaicin on 3T3-L1 preadipocytes is

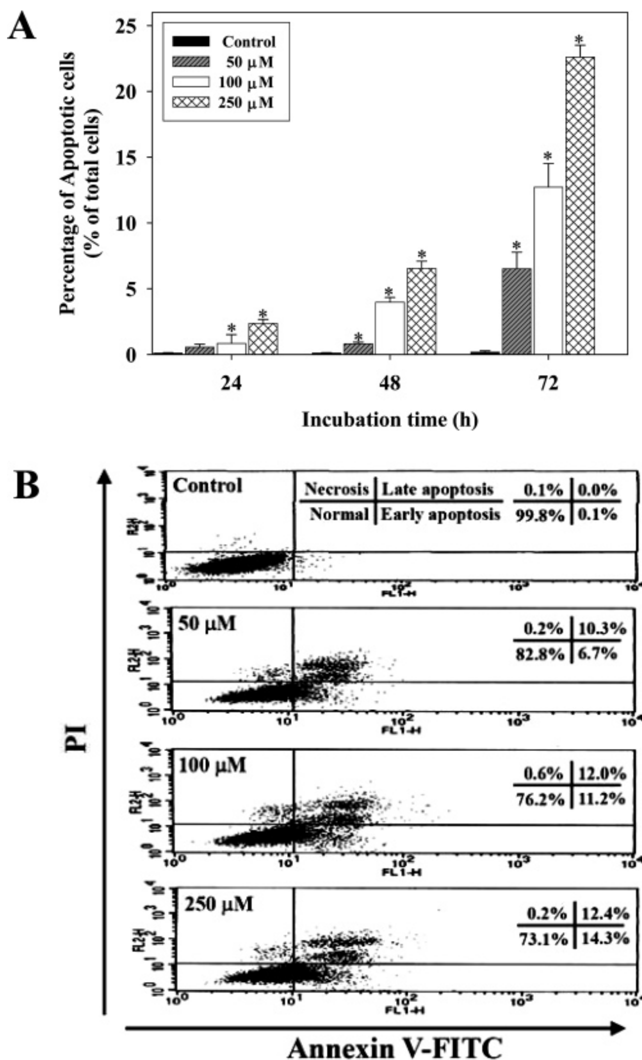

Figure 3. Flow cytometric analysis of capsaicin-mediated cell apoptosis in 3T3-L1 preadipocytes. (**A**) PI stained. Cells were treated with 0–250 μM capsaicin for 24, 48, and 72 h. (**B**) Annexin V-FITC/PI double stained. Cells were treated with 0–250 μM capsaicin for 72 h. The percentage of apoptotic/necrotic cells was calculated by CELL Quest software (mean ± SD, $n = 3$); $*p < 0.05$ compared with the control.

shown in **Figure 2B**. Capsaicin caused significant LDH leakage ($p < 0.05$) as compared with the control.

Capsaicin-Induced Apoptosis in 3T3-L1 Preadipocytes. To quantify the degree of apoptosis, the amount of sub-G_1 DNA was analyzed by flow cytometry. As shown in **Figure 3A**, flow cytometric analysis of capsaicin-mediated cell apoptosis of 3T3-L1 preadipocytes indicated that the increase of apoptotic cells occurred in a time- and dose-dependent manner. To quantify the modes of cell death (apoptosis or necrosis) induced by capsaicin, 3T3-L1 preadipocytes were treated with capsaicin for 72 h, stained with Annexin V-FITC and PI, and analyzed by flow cytometry. As shown in **Figure 3B**, flow cytometric analysis demonstrated that treatment of cells with capsaicin decreased the number of normal cells (Annexin V-FITC−/PI−) in a dose-dependent manner. The apoptotic cells including early apoptotic (Annexin V-FITC+/PI−) and late apoptotic cells (Annexin V-FITC+/PI+) were increased in a dose-dependent manner. When the treatment concentrations were increased, the percentage of normal cells decreased from 99.8% (control) to 73.1% (250 μM). The percentage of apoptotic cells (including early apoptotic and late apoptotic) increased from 0.1% (control) to 26.7% (250 μM).

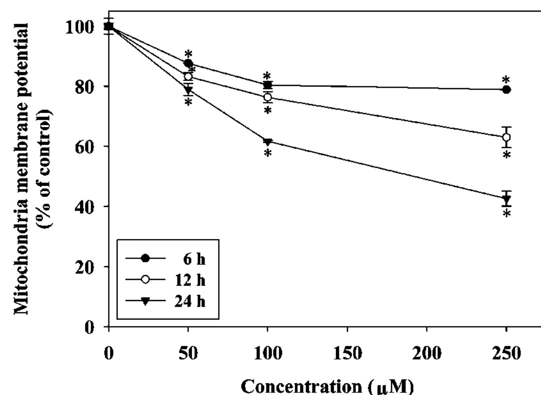

Figure 4. Effect of capsaicin on mitochondria membrane potential ($\Delta\Psi$m) in 3T3-L1 preadipocytes. Cells were treated with 0–250 μM capsaicin for 6, 12, and 24 h. Reported values are the mean ± SD ($n = 3$); $*p < 0.05$ compared with the control.

Collapse of Mitochondria Membrane Potential ($\Delta\Psi$m). **Figure 4** shows the effect of capsaicin on mitochondria membrane potential ($\Delta\Psi$m) in 3T3-L1 preadipocytes. A significant decrease in fluorescence intensity was observed when 3T3-L1 preadipocytes were treated with capsaicin for 6−24 h. The results demonstrated that the early damage was due to the change in mitochondria membrane potential, which may further activate the intrinsic pathway of apoptosis.

Modulation of Apoptosis-Related Activity and Proteins by Capsaicin. Caspases are essential for the execution of cell death by various apoptotic stimuli (*22*). To monitor the enzymatic activity of caspase-3 during apoptosis induced by capsaicin, we used the specific fluorogenic peptide substrate (Ac−DEVD−MCA) for the detection of caspase-3 activity. As shown in **Figure 5A**, activation of caspase-3 was significantly ($p < 0.05$) increased in capsaicin-treated cells at 12 and 24 h. There was an approximately 3.3-fold increase of caspase-3 activity resulting from treatment with 250 μM capsaicin for 24 h.

The effects of capsaicin on the protein expression of caspase-3, PARP, Bak, Bax, and Bcl-2 in 3T3-L1 preadipocytes are shown in **Figure 5B**. Caspase-3 is a member of the caspase family that has been shown to play an essential role in apoptosis induced by a variety of stimuli (*23*). Capsaicin (250 μM) significantly ($p < 0.05$) stimulated caspase-3 expression in a time- and dose-dependent manner and with a maximal increase of 2.82-fold after 24 h. However, activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. This cleavage leads to its inactivation, thus preventing the futile DNA repair cycle. Treating cells with 250 μM capsaicin significantly ($p < 0.05$) induced PARP cleavage with a maximal cleavage of 0.37-fold occurring after 24 h. The imbalance of expression of anti- and proapoptotic protein after the stimulus is one of the major mechanisms underlying the ultimate fate of cells in the apoptotic process. It is well-known that the proteins of the Bcl-2 family play a pivotal role in cells undergoing apoptosis by interfering with the caspases (*24*). Capsaicin resulted in a significant ($p < 0.05$) increase in Bak and Bax expression from 1.00 (control) to 2.20 and 3.50 (100 μM, 24 h), respectively. Treating cells with capsaicin significantly ($p < 0.05$) decreased Bcl-2 expression from 1.00 (control) to 0.25 (100 μM, 24 h).

Inhibition of Intracellular Triglyceride and GPDH activity in 3T3-L1 Adipocytes. **Figure 6** shows the effect of capsaicin

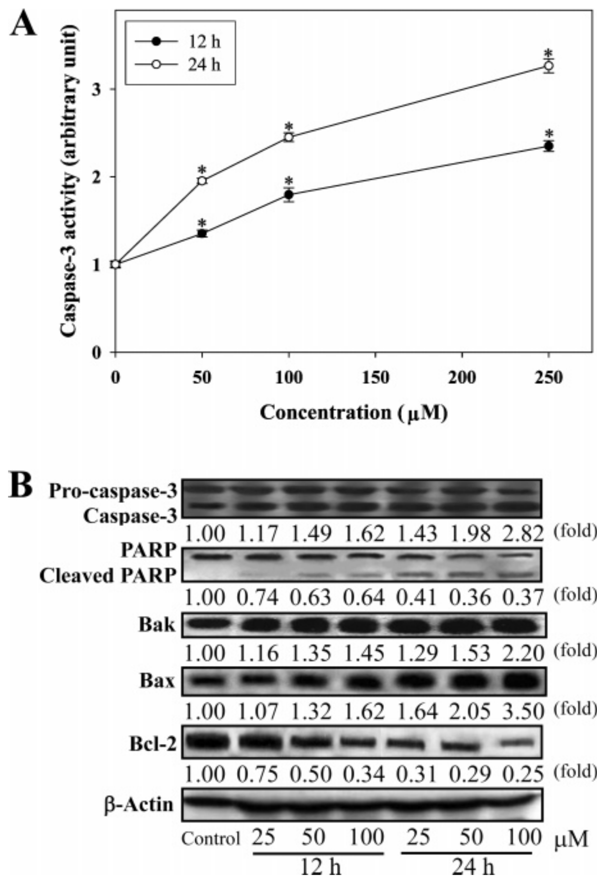

Figure 5. Effect of capsaicin on caspase-3 activity and apoptotic protein levels in 3T3-L1 preadipocytes. Cells were treated with 0–250 μM capsaicin for 12 and 24 h. Results are the mean ± SD ($n = 3$). Protein levels were analyzed by Western blot analysis. The relative expression of caspase-3, PARP, Bak, Bax, and Bcl-2 in 3T3-L1 preadipocytes was quantified densitometrically using the software LabWorks 4.5 and calculated according to the reference bands of β-actin (mean, $n = 3$); $*p < 0.05$ compared with the control.

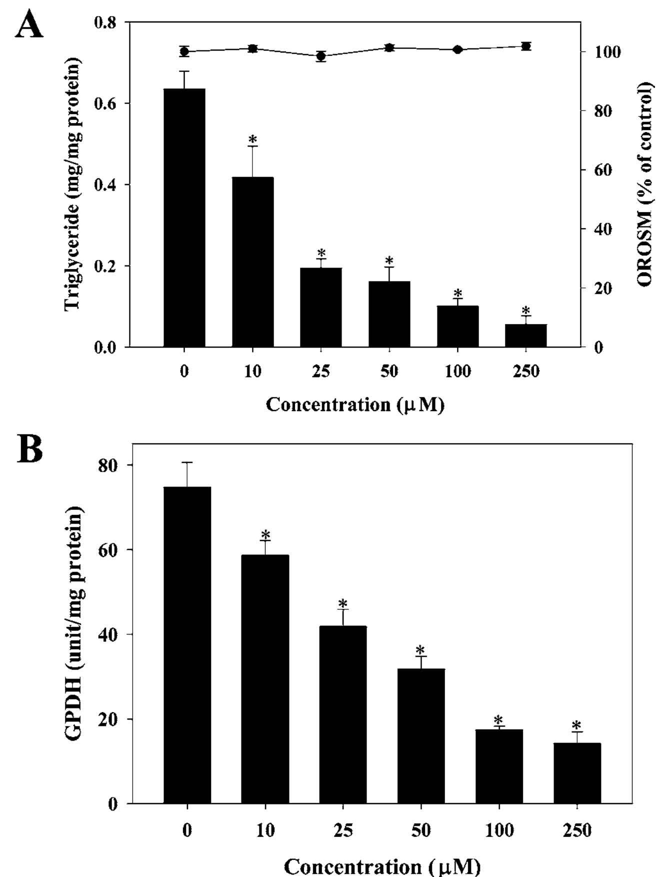

Figure 6. Effect of capsaicin on the inhibition of intracellular triglycerides (**A**) and GPDH activity (**B**) in 3T3-L1 adipocytes. Cells were treated with 0–250 μM capsaicin for 72 h. Results are the mean ± SD ($n = 3$); $*p < 0.05$ compared with the control.

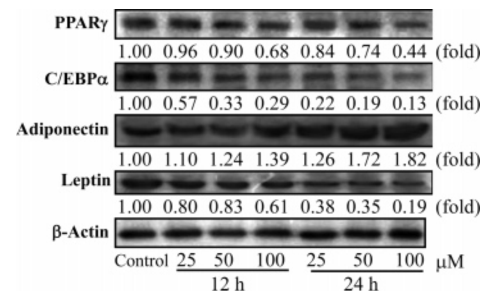

Figure 7. Effect of capsaicin on protein expression of PPARγ, C/EBPα, adiponectin, and leptin in 3T3-L1 adipocytes. Cells were treated with 0–100 μM capsaicin for 12 and 24 h. Protein levels were analyzed by Western blot analysis. The relative expression of PPARγ, C/EBPα, adiponectin, and leptin in 3T3-L1 adipocytes was quantified densitometrically using the software LabWorks 4.5 and calculated according to the reference bands of β-actin.

on the inhibition of intracellular triglyceride content and GPDH activity in 3T3-L1 adipocytes. The results demonstrated that the inhibition of intracellular triglyceride in 3T3-L1 adipocytes occurred in a dose-dependent manner when cells were exposed to capsaicin at the concentrations of 0−250 μM (**Figure 6A**). The OROSM showed that cell number in 3T3-L1 adipocytes was not influenced by the treatment with capsaicin. However, the addition of capsaicin to 3T3-L1 adipocytes resulted in a marked decrease of GPDH activity in a dose-dependent manner (**Figure 6B**).

Modulation of Adipocyte Differentiation-Related Protein by Capsaicin. PPARγ is known as a key station protein that is expressed early in the adipocyte differentiation of 3T3-L1 cells and prior to C/EBPα (*25*). The effects of capsaicin on the protein expression of PPARγ, C/EBPα, adiponectin, and leptin in 3T3-L1 adipocytes are shown in **Figure 7**. The protein expression of PPARγ, C/EBPα, and leptin was decreased in a time- and dose-dependent manner in 3T3-L1 adipocytes treated with capsaicin. Treatment of 3T3-L1 adipocytes with capsaicin induced a significant ($p < 0.05$) up-regulation of adiponectin expression in a time- and dose-dependent manners. It was maximally up-regulated to 1.82-fold after cells were treated with 100 μM capsaicin for 24 h.

DISCUSSION

Obesity is a chronic, stigmatized, and costly disease that is rarely curable and is increasing in prevalence throughout most of the world (*26*). The 3T3-L1 preadipocyte line has been well characterized in its ability to undergo complete differentiation into mature adipocytes (*27*). Adipose tissue consists of adipocytes, which store triacylglycerol as a fuel for the body. Excess adipose tissue leads to insulin resistance, thereby increasing the risk of type 2 diabetes mellitus and cardiovascular disease (*28*). Wang and Jones (*29*) proposed that decreased preadipocyte proliferation and adipocyte lipogenesis are mechanisms of

antiobesity. The 3T3-L1 preadipocytes are not cancer cells, but the preadipocytes have been differentiated into mature adipocytes. So, we focused on the effects of capsaicin on induction of preadipocytic apoptosis and inhibition of adipocytic lipid accumulation.

The goal of this research was to study the inhibition of adipogenesis and adipocyte differentiation with a natural chemical. The results of the MTT and LDH assays clearly indicated that capsaicin caused the inhibition of cell population growth of 3T3-L1 preadipocytes (**Figure 2**, parts **A** and **B**). In the cell apoptosis analysis by PI staining, treatment of 3T3-L1 preadipocytes with capsaicin increased the induction of cell apoptosis in a time- and dose-dependent manner (**Figure 3A**). Annexin V-FITC binds to phosphatidylserine and can be used to detect the early stages of apoptosis (*30*). Our data showed that the treatment of 3T3-L1 preadipocytes with capsaicin increased the apoptotic cell population in a dose-dependent manner (**Figure 3B**). Detection of the mitochondria membrane potential event provided an early indication of the initiation of cellular apoptosis. In general, changes in the membrane phosphatidyl serine externalization are generally observed at a stage later than the loss of mitochondria membrane potential (*31*). Mitochondria play an essential role in cell death signal transduction such that permeability transition pore opening and collapse of the $\Delta\Psi$m results in the rapid release of caspase activators such as cytochrome *c* into the cytoplasm (*32*). These results demonstrated that the treatment of 3T3-L1 preadipocytes with capsaicin increased the loss of mitochondria membrane potential in a time- and dose-dependent manner (**Figure 4**).

In an attempt to reveal the molecular mechanisms underlying capsaicin-induced apoptosis of 3T3-L1 preadipocytes, caspase-3 activity (**Figure 5A**) and the protein levels of various key apoptosis-linked gene products including caspase-3, PARP, Bak, Bax, and Bcl-2 (**Figure 5B**) were determined. The data also showed that capsaicin-induced apoptosis is controlled through caspase activation. Caspase-3 is one of the candidates for cell death-inducing proteases that cleave PARP (*33*). Subsequent Western blot analysis disclosed progressive proteolytic cleavage of PARP in 3T3-L1 preadipocytes after treatment with capsaicin. Moreover, capsaicin can modulate the process of apoptosis in tumor cells through caspase activation, a decrease of Bcl-2 expression, and an increase in Bax expression (*34*). In the present study, the data showed that the decreased level of antiapoptotic Bcl-2 protein and increased level of proapoptotic Bax and Bak proteins may play a key role in capsaicin-induced apoptosis of 3T3-L1 preadipocytes. Yang et al. (*35*) indicated that esculetin-mediated adipocyte apoptosis involves the mitochondrial pathway.

Our data indicated that the exposure of 3T3-L1 adipocytes to capsaicin caused a significant decrease ($p < 0.05$) in the content of intracellular triglycerides and GPDH (**Figure 6**). However, it did not affect the cell number of 3T3-L1 adipocytes. One report has indicated that CLA treatment of rats does not decrease adipocyte number (*36*). Mochizuki and Hasegawa (*37*) demonstrated that when adipocytes were exposed to green tea catechins, EGCG inhibited lipogenesis, while (+)-catechins increased lipogenesis. Mori and Hasegawa (*38*) indicated that powdered green tea inhibited insulin-induced lipogenesis and increased the level of superoxide dismutase (SOD) activity in 3T3-L1 adipocytes. In the present study, the results indicated that treatment with capsaicin markedly decreased PPARγ, C/EBPα, and leptin protein expression in 3T3-L1 adipocytes (**Figure 7**). The promoters of several adipogenic genes are regulated by PPARγ and C/EBPα transcription factors (*39*). Moreover, PPARγ target genes in adipose tissue are directly implicated in lipogenic pathways (*40*). Adiponectin is an adipocytokine that has been shown to have antiatherogenic, anti-inflammatory, and antidiabetic roles (*41*). Leptin is a protein hormone secreted mainly by adipose tissue, and it inhibits food intake and stimulates thermogenesis (*42*). Jeon et al. (*43*) indicated that red yeast rice extracts induced the down-regulation of adipogenic transcription factors and gene expression in 3T3-L1 adipocytes. Our data suggested that capsaicin decreased the levels of PPARγ, C/EBPα, and leptin and then increased the level of adiponectin in 3T3-L1 adipocytes.

Bioavailability is the extent to which a nutrient in a food constituent can be absorbed and used by the body after ingestion. The reported indicated that the intake of capsaicin in a typical Indian or Thai diet was about 128 μg/kg human body weight (*44*). The concentrations of capsaicin in the gastric fluid (1−3 L) would be equivalent to 8−25 μM for an adult human with a body weight of 60 kg. However, data presented here indicated that capsaicin efficiently induces apoptosis and inhibits adipogenesis in 3T3-L1 cells at concentrations below 50 μM. The range of concentrations used in the present study was consistent with those in another study on the effect of capsaicin in 3T3-L1 cells (*45*).

In conclusion, the results of this study clearly showed that capsaicin could inhibit the population growth and the induction of apoptosis in 3T3-L1 preadipocytes. Capsaicin induced apoptosis through the collapse of mitochondria membrane potential, activation of caspase-3, Bax, and Bak, and then cleavage of PARP and down-regulation of Bcl-2. Capsaicin also inhibited lipid accumulation and the protein expression of PPARγ, C/EBPα, and leptin, but induced up-regulation of adiponectin in 3T3-L1 adipocytes. These results demonstrate that capsaicin efficiently suppresses adipogenesis in 3T3-L1 preadipocytes and adipocytes.

ABBREVIATIONS

Annexin V-FITC, annexin V-fluorescein isothiocyanate; C/EBPα, CCAAT enhancer binding protein alpha; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GPDH, glycerol-3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; IC_{50}, 50% growth inhibitory concentrations; INS, insulin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; $\Delta\Psi$m, mitochondria membrane potential; OROSM, oil red O-stained material; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; PPARγ, peroxisome proliferator−activated receptor−gamma; PVDF, polyvinyldifluoride; RNase, ribonuclease; TE, trypsin−EDTA.

LITERATURE CITED

(1) Kopelman, P. G. Obesity as a medical problem. *Nature* **2000**, *404*, 635−643.

(2) Formiguera, X.; Canton, A. Obesity: epidemiology and clinical aspects. *Best Pract. Res. Clin. Gastroenterol.* **2004**, *18*, 1125−1146.

(3) Wang, Y. W.; Jones, P. J. Conjugated linolic acid and obesity control: efficacy and mechanisms. *Int. J. Obes. Relat. Metab. Disord.* **2004**, *28*, 941−955.

(4) Furuyashiki, T.; Nagayasu, H.; Aoki, Y.; Bessho, H.; Hashimoto, T.; Kanazawa, K.; Ashida, H. Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARγ2 and C/EBPα in 3T3-L1 cells. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 2353−2359.

(5) Hausman, D. B.; DiGirolamo, M.; Bartness, T. J.; Hausman, G. J.; Martin, R. J. The biology of white adipocyte proliferation. *Obes. Rev.* **2001**, *2*, 239−254.

(6) MacDougald, O. A.; Mandrup, S. Adipogenesis: forces that tip the scales. *Trends Endocrinol. Metab.* **2002**, *13*, 5−11.
(7) Cordell, G. A.; Araujo, O. E. Capsaicin: identification, nomenclature, and pharmacotherapy. *Ann. Pharmacother.* **1993**, *27*, 330−336.
(8) Surh, Y. J. Anti-tumor promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: a short review. *Food Chem. Toxicol.* **2002**, *40*, 1091−1097.
(9) Yoshioka, M.; St-Pierre, S.; Drapeau, V.; Dionne, I.; Doucet, E.; Suzuki, M.; Tremblay, A. Effects of red pepper on appetite and energy intake. *Br. J. Nutr.* **1999**, *82*, 115−123.
(10) Kawada, T.; Hagihara, K. I.; Iwai, K. Effects of capsaicin on lipid metabolism in rats fed a high fat diet. *J. Nutr.* **1986a**, *116*, 1272−1278.
(11) Morre, D. J.; Chueh, P. J.; Morre, D. M. Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1831−1835.
(12) Macho, A.; Sancho, R.; Minassi, A.; Appendino, G.; Lawen, A.; Munoz, E. Involvement of reactive oxygen species in capsaicinoid-induced apoptosis in transformed cells. *Free Radical Res.* **2003**, *37*, 611−619.
(13) Vermeulen, K.; Van Bockstaele, D. R.; Berneman, Z. N. Apoptosis: mechanisms and relevance in cancer. *Ann. Hematol.* **2005**, *84*, 627−639.
(14) Woo, K. J.; Jeong, Y. J.; Park, J. W.; Kwon, T. K. Chrysin-induced apoptosis is mediated through caspase activation and Akt inactivation in U937 leukemia cells. *Biochem. Biophys. Res. Commun.* **2004**, *325*, 1215−1222.
(15) Fan, T. J.; Han, L. H.; Cong, R. S.; Liang, J. Caspase family proteases and apoptosis. *Acta Biochim. Biophys. Sin.* **2005**, *37*, 719−727.
(16) Hsu, C. L.; Huang, S. L.; Yen, G. C. Inhibitory effect of phenolic acids on proliferation of 3T3-L1 preadipocytes in relation to their antioxidant activity. *J. Agric. Food Chem.* **2006**, *54*, 4191−4197.
(17) Green, H.; Kehinde, O. Sublines of mouse 3T3 cells that accumulate lipid. *Cell* **1974**, *1*, 113−116.
(18) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(19) Takada, E.; Toyota, H.; Suzuki, J.; Mizuguchi, J. Prevention of anti-IgM-induced apoptosis accompanying G1 arrest in B lymphoma cells overexpressing dominant-negative mutant form of c-Jun N-terminal kinase 1. *J. Immunol.* **2001**, *166*, 1641−1649.
(20) Ramirez-Zacarias, J. L.; Castro-Munozledo, F.; Kuri-Harcuch, W. Quantitation of adipose conversion and triglycerides by staining intercytoplasmic lipids with Oil Red O. *Histochemistry* **1992**, *97*, 493−497.
(21) Wise, L. S.; Green, H. Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells. *J. Biol. Chem.* **1979**, *254*, 273−275.
(22) Cohen, G. M. Caspases: the executioners of apoptosis. *Biochem. J.* **1997**, *326*, 1−16.
(23) Proter, A. G.; Janicke, R. U. Emerging roles of caspase-3 on apoptosis. *Cell Death Differ.* **1999**, *6*, 99−104.
(24) Antonsson, B.; Martinou, J. C. The bcl-2 protein family. *Exp. Cell Res.* **2000**, *256*, 50−57.
(25) Tontonoz, P.; Hu, E.; Graves, R. A.; Budavari, A. I.; Spiegelman, B. M. mPPARγ2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev.* **1994**, *8*, 1224−1234.
(26) Bray, G. A.; Tartaglia, L. A. Medicinal strategies in the treatment of obesity. *Nature* **2000**, *404*, 672−677.
(27) Cowherd, R. M.; Lyle, R. E.; McGehee, R. E., Jr. Molecular regulation of adipocyte differentiation. *Cell Dev. Biol.* **1999**, *10*, 3−10.
(28) Saltiel, A. R.; Kahn, C. R. Insulin signaling and the regulation of glucose and lipid metabolism. *Nature* **2001**, *414*, 799−806.
(29) Wang, Y. W.; Jones, P. J. Conjugated linolic acid and obesity control: efficacy and mechanisms. *Int. J. Obes. Relat. Metab. Disord.* **2004**, *28*, 941−955.
(30) Vermes, I.; Haanen, C.; Steffens-Nakken, H.; Reutelingsperger, C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* **1995**, *184*, 39−51.
(31) Raghuvar Gopal, D. V.; Narkar, A. A.; Badrinath, Y.; Mishra, K. P.; Joshi, D. S. Protection of Ewing's sarcoma family tumor (ESFT) cell line SK-N-MC from betulinic acid induced apoptosis by α-DL-tocopherol. *Toxicol. Lett.* **2004**, *153*, 201−212.
(32) Li, P.; Nijhawan, D.; Budihardjo, I.; Srinivasula, S. M.; Ahmad, M.; Alnemri, E. S.; Wang, X. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **1997**, *91*, 479−489.
(33) Lazebnik, Y. A.; Kaufmann, S. H.; Desnoyers, S.; Poirer, G. G.; Earnshaw, W. C. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **1994**, *371*, 346−347.
(34) Jung, M. Y.; Kang, H. J.; Moon, A. Capsaicin-induced apoptosis in SK-Hep-1 hepatocarcinoma cells involves Bcl-2 downregulation and caspase-3 activation. *Cancer Lett.* **2001**, *165*, 139−145.
(35) Yang, J. Y.; Della-Fera, M. A.; Baile C. A. Esculetin induces mitochondria-mediated apoptosis in 3T3-L1 adipocytes. *Apoptosis* **2006**, *11*, 1371−1378.
(36) Poulos, S. P.; Sisk, M.; Hausman, D. B.; Azain, M. J.; Hausman, G. J. Pre- and postnatal dietary conjugated linoleic acid alters adipose development, body weight gain and body composition in sprague−dawley rats. *J. Nutr.* **2001**, *131*, 2722−2731.
(37) Mochizuki, M.; Hasegawa, N. Effects of green tea catechin-induced lipolysis on cytosol glycerol content in differentiated 3T3-L1 cells. *Phytother. Res.* **2004**, *18*, 945−946.
(38) Mori, M.; Hasegawa, N. Superoxide dismutase activity enhanced by green tea inhibits lipid accumulation in 3T3-L1 cells. *Phytother. Res.* **2003**, *17*, 566−567.
(39) Takahata, T.; Kumano, T.; Ookawa, K.; Hayakari, M.; Kakizaki, I.; Tsuchida, S. Inhibition of 3T3-L1 adipocyte differentiation by 6-ethoxyzolamide: repressed peroxisome proliferator-activated receptor γ mRNA and enhanced CCAAT/enhancer binding protein β mRNA levels. *Biochem. Pharmacol.* **2004**, *67*, 1667−1675.
(40) Kersten, S.; Desvergne, B.; Wahli, W. Roles of PPARs in health and disease. *Nature* **2000**, *405*, 421−424.
(41) Pajvani, U. B.; Du, X.; Combs, T. P.; Berg, A. H.; Rajala, M. W.; Schulthess, T.; Engel, J.; Brownlee, M.; Scherer, P. E. Structure−function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. *J. Biol. Chem.* **2003**, *278*, 9073−9085.
(42) Ahima, R. S.; Flier, J. S. Leptin. *Annu. Rev. Physiol.* **2000**, *62*, 413−437.
(43) Jeon, T.; Hwang, S. G.; Hirai, S.; Matsui, T.; Yano, H.; Kawada, T.; Lim, B. O.; Park, D. K. Red yeast rice extracts suppress adipogenesis by down-regulating adipogenic transcription factors and gene expression in 3T3-L1 cells. *Life Sci.* **2004**, *75*, 3195−3203.
(44) Rumsfield, J. A.; West, D. P. Topical capsaicin in dermatologic and peripheral pain disorders. *Ann. Pharmacother.* **1991**, *125*, 381−387.
(45) Hwang, J. T.; Park, I. J.; Shin, J. I.; Lee, Y. K.; Lee, S. K.; Baik, H. W.; Ha, J.; Park, O. J. Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 694−699.

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