JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 4191−4197 **4191**

Inhibitory Effect of Phenolic Acids on the Proliferation of 3T3-L1 Preadipocytes in Relation to Their Antioxidant Activity

CHIN-LIN HSU,† SHIH-LI HUANG,‡ AND GOW-CHIN YEN*,†

Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan, and Department of Baking Technology and Management, Kaohsiung Hospitality College, 1 Sungho Road, Kaohsiung 81271, Taiwan

Obesity is an important topic in the world of public health and preventive medicine. Inhibition of preadipocyte proliferation plays an important role in the mechanisms of proposed antiobesity. In this in vitro study, the inhibitory effect of phenolic acids on 3T3-L1 preadipocytes was evaluated, and a relationship analysis was then conducted. The results showed that the addition of phenolic acids to the growth medium decreased the cell population growth of 3T3-L1 preadipocytes. The IC_{50} values of chlorogenic acid, gallic acid, *o*-coumaric acid and *m*-coumaric acid on 3T3-L1 preadipocytes were 72.3, 43.3, 48.2, and 49.2 μM, respectively. A relationship analysis indicated that there is a significant linear correlation between the influence of phenolic acids on cell population growth and their antioxidant activity ($r = 0.77$, $p < 0.01$). The cell cycle assay indicated that the treatment of 3T3-L1 preadipocytes with chlorogenic acid, *o*-coumaric acid, and *m*-coumaric acid caused cell cycle arrest in the G_1 phase. Gallic acid did not affect the cell cycle profile; however, it increased the number of apoptotic cells (sub-G_1 phase) in a time- and dose-dependent manner. Annexin V-fluorescein isothiocyanate (FITC)-propidium iodide (PI) apoptosis flow cytometric assay showed that gallic acid increased the number of early apoptotic (annexin V-FITC+/PI-) and late apoptotic cells (annexin V-FITC+/PI+) but not necrotic cells (annexin V-FITC-/PI+). The treatment of cells with gallic acid caused the loss of mitochondrial membrane potential ($\Delta\Psi_m$). These results indicate that the inhibition of preadipocyte population growth by some phenolic acids might have further implication in in vivo antiobesity effects.

KEYWORDS: Phenolic acid; preadipocytes; proliferation; antiobesity; antioxidant activity

INTRODUCTION

Many studies have focused on the anticarcinogenic, antimutagenic, or chemopreventive activities of phytochemicals, particularly those included in the human diet (*1*, *2*). Phenolic compounds constitute a group of substances that are widely present in the plant kingdom, where more than 8000 are known, with different chemical structures and activities. Phenolic acids have pharmacological properties such as antioxidant, antithrombosis, antiinflammatory and anti-HIV-1 (*3*−*5*). They are found in fruits, vegetables, nuts, and seeds as well as in tea, red wine, citrus fruits, and other food sources. Dietary tea catechins rich in (−)-epicatechin gallate and (−)-epigallocatechin gallate have antioxidant activity and reduce the deposition of visceral fat in mice fed a high-fat diet for 11 months (*6*, *7*). Moreover, the past 15 years of SAR research has evidence supporting the role of specific structural components as requisites for radical scavenging, chelating, and oxidant activity (*8*). Both the configuration and total number of hydroxyl groups of many polyphenolic antioxidants substantially influence antioxidant activity (*9*). The correlation between the influence of phenolic acids on cell population growth and their antioxidant activity is limited.

Over the last few decades, obesity has become a global epidemic in both developed and developing countries. Obesity causes or exacerbates many health problems, both independently and in association with other diseases (*10*). Obesity has been associated with the development of type II diabetes mellitus, coronary heart disease, an increased incidence of certain forms of cancer, and respiratory complications. Obesity is characterized at the cell biological level by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes in adipose tissues (*11*). There are several mechanisms of proposed antiobesity including decreased energy/food intake and increased energy expenditure, decreased preadipocyte differentiation and proliferation, decreased lipogenesis, and increased lipolysis and fat oxidation (*12*).

Phenolic acids, especially hydroxycinnamic acids and hydroxybenzoic acids, are secondary plant products and are commonly found in plant-derived foodstuffs. They are consumed regularly as part of the human diet (*13*, *14*). Many phenolic compounds, such as gallic acid (3,4,5-trihydroxybenzoic acid) and its derivatives, show selective cytotoxicity against a variety

* To whom correspondence should be addressed. Tel: 886-4-22879755. Fax: 886-4-22854378. E-mail: gcyen@nchu.edu.tw.
† National Chung Hsing University.
‡ Kaohsiung Hospitality College.

10.1021/jf0609882 CCC: $33.50 © 2006 American Chemical Society
Published on Web 05/12/2006

of tumor cells with a higher activity than that shown against normal cells (*15*−*18*). Many epidemiological studies indicated that consumption of some foods and drinks with high phenolic content is associated with the prevention of some diseases (*19*−*21*). Wu et al. (*22*) reported that the induction of apoptosis by green tea (−)-epigallocatechin gallate in 3T3-L1 preadipocytes was via the Cdk2 pathway, and the apoptotic effect of EGCG on 3T3-L1 preadipocytes might be related to the mechanism by which EGCG modulates body weight. However, because the decreased proliferation of preadipocytes by phenolic acids for the mechanisms of proposed antiobesity is limited, we focused on the inhibitory effect of phenolic acids on 3T3-L1 preadipocytes. In the present study, we studied the antiobesity potential of phenolic acids using mouse embryo 3T3-L1 preadipocytes because the mouse embryo 3T3-L1 cell line was also used as an in vitro evaluation of antiobesity (*23*−*25*). The objective of this study was focused on the inhibition of preadipocyte proliferation by phenolic acids for the mechanisms of proposed antiobesity. Moreover, a relationship analysis was also conducted.

MATERIALS AND METHODS

Materials. Phenolic acids (caffeic acid, chlorogenic acid, gallic acid, protocatechuic acid, gentisic acid, vanillic acid, *p*-hydroxybenzoic acid, 3,4-dimethoxybenzoic acid, sinapinic acid, salicylic acid, *m*-coumaric acid, *o*-coumaric acid, *p*-coumaric acid, syringic acid, and ferulic acid), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT dye), β-phycoerythnin (β-PE) from *Porphydium cruentum*, propidium iodide (PI), and RNase were purchased from Sigma Chemical (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM-High glucose), bovine calf serum, and the antibiotic mixture (penicillin−streptomycin) were purchased from Gibco BRL (Grand Island, NY). 2,2′-Azobis (2-amidinopropane) hydrochloride (AAPH) was purchased from Wako Chemicals (Osaka, Japan). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich (Milwaukee, WI). All other chemicals were reagent grade.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The automated ORAC assay was carried out on a Fluostar Galaxy plate reader (BMG LabTechologies, GmbH, Offenburg, Germany) with a fluorescent filter (ex 540 nm; em 565 nm). The procedure was based on the previous report of Cao et al. (*26*) with a slight modification. Briefly, in the final assay mixture, β-PE (16.7 nM) was used as a target of free radical (or oxidant) attack with AAPH (40 mM) as a peroxyl radical generator. Trolox (1 μM) was used as a standard and prepared fresh daily. The analyzer was programmed to record the fluorensence of β-PE, every 5 min after AAPH was added. All fluorescence measurements were expressed relative to the initial reading. Final results of $ORAC_{ROO}$. value were calculated using the differences of area under the β-PE decay curves between the blank and a sample and expressed as micromoles of Trolox equivalents to per micromole of sample.

Cell Culture. The mouse embryo 3T3-L1 cell line (BCRC 60159) was obtained from Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). The culture medium included Dulbecco's modified Eagle's medium, 10% calf serum, 1.5 g/L of sodium bicarbonate, and 100 U/mL of penicillin−streptomycin. The cell culture condition was 37 °C in a humidified 5% CO_2 incubator.

Cell Population Growth by MTT Assay. The MTT assay was performed according to the method of Mosmann (*27*). The 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 (0−1000 μM) of the phenolic acids, and the cells were incubated for 24, 48, and 72 h. Salicylic acid and gentisic acid were dissolved in PBS, and the other phenolic acids were dissolved in DMSO. The final concentration of solvent was less than 0.1% in the cell culture medium. The culture medium was removed and replaced by 90 μL of fresh culture medium. Ten microliters of sterile filtered MTT solution (5 mg/mL) in phosphate buffered saline (PBS, pH 7.4) was added to each well, reaching 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 200 μL/well dimethyl sulfoxide and measured spectrophotometrically in a FLUOstar galaxy spectrophotometer (BMG Labtechnologies Ltd., Offenburg, Germany) at 570 nm. The inhibition (%) was expressed as the percentage of cell growth compared to control (nontreated cells, at which growth is considered 0%), and it was calculated by $100 - (A_{570}$ nm (phenolic acid)$/A_{570\ nm}$ (control) $\times$ 100). The IC_{50} value was calculated as phenolic acid concentration, under which 50% inhibition of cell growth occurred compared to nontreated controls.

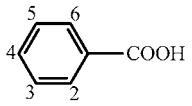
LDH Leakage Assay. LDH leakage activity was performed using a commercial kit (St. Louis, MO). The cells were incubated with 0−250 μM of gallic acid for 24−72 h and then analyzed for LDH leakage into the culture medium. The total LDH leakage was determined after the cells were thoroughly disrupted by sonication. The percentage of LDH leakage was then calculated to determine membrane integrity. The LDH leakage was expressed as a percentage of total activity: (activity in the medium)/(activity in the medium + activity of the cells) $\times$ 100.

Analyses of Cell Cycle and Cell Apoptosis (PI Staining Method) by Flow Cytometry. The 3T3-L1 preadipocytes stimulated with various concentrations (0, 50, 100, and 250 μM) of phenolic acids for 24, 48, and 72 h were assayed for cell cycle progression and/or apoptosis by the PI staining method, as previously described (*28*). Briefly, cells were harvested by a 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 of RNase for 30 min at 37 °C. The cells were then stained with 40 mg/mL of PI for 15 min at room temperature and subjected to a flow cytometric analysis of DNA content using a FACScan flow cytometer (Becton-Dickinson Immunocytometery Systems USA, San Jose, CA). Approximately 10 000 counts were made for each sample. The percentage of distribution of cell cycle phase and apoptosis were calculated by CELL Quest software.

Mitochondrial Membrane Potential ($\Delta\Psi$m) Analysis. The mitochondrial membrane potential was determined using the MitoPT 100 Test Kit (Immunochemistry Technologies, LLC). JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria and indicates a fluorescence emission shift from green to red. The cells were seeded in 12-well plates. After 24 h, the cells were treated with various concentrations of gallic acid (0, 50, 100, and 250 μM) for 6, 12, and 24 h, respectively. The routine passage consisted of rinsing cells in 12-well plates once with PBS, followed by harvesting with 0.1 mL of TE solution, the addition of 1 mL of fresh culture medium, and thorough dispersion. Aliquots of the resultant cell suspensions were placed in eppendorf, 1×10^6 cells/eppendorf containing 1 mL of culture medium. After centrifugation, the cells were incubated with 10 μg/mL of JC-1 at 37 °C for 15 min in a humidified 5% CO_2 incubator. The cells were collected and washed with 1× assay buffer (MitoPT 100 Test kit). The cells were resuspended in adequate amount of the same solution and analyzed by a FLUOstar galaxy fluorescence plate reader with an excitation wavelength of 485 nm and emission wavelengths of 590 nm for red fluorescence. Apoptotic cells will generate a lower reading of red fluorescence, and the changes in the mitochondrial membrane potential ($\Delta\Psi$m) can most accurately be assessed by comparing the red fluorescence of untreated cells with those of the cells treated with phenolic acids.

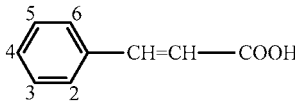
Annexin V-FITC/PI Double Staining Assay. Annexin V-FITC/PI double staining of the cells was determined using the Annexin V-FITC kit (ANNEX100F, SEROTEC, U.K.). This test employs the property of Annexin V-FITC to bind to the membrane phospholipid phosphatidylserine (PS) in the presence of Ca^{2+}. To detect early apoptosis, late apoptosis, and necrosis induced by gallic acid, 3T3-L1 preadipocytes (1×10^6 cells/dish) were added to each well of a 6 cm dish and treated for 48 h at 37 °C in 1 mL of culture medium containing test agents at suitable concentrations to give the final concentrations of 0, 50, 100, and 250 μM. Approximately 1×10^5 cells were then stained for 10 min at room temperature with Annexin V-FITC and PI in a Ca^{2+}

Hydroxybenzoic acids

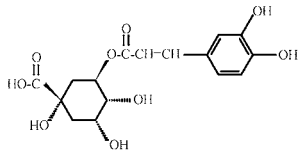


Salicylic acid 2 = OH
p-Hydroxybenzoic acid 4 = OH
Protocatechuic acid 3 = 4 = OH
Gentisic acid 3 = 6 = OH
Gallic acid 3 = 4 = 5 = OH
Vanillic acid 3 = OCH_3, 4 = OH
3,4-Dimethoxybenzoic acid 3 = 4 = OCH_3
Syringic acid 3 = 5 = OCH_3, 4 = OH

Hydroxycinnamic acids



o-Coumaric acid 2 = OH
m-Coumaric acid 3 = OH
p-Coumaric acid 4 = OH
Caffeic acid 3 = 4 = OH
Ferulic acid 3 = OCH_3, 4 = OH
Sinapic acid 3 = 4 = OCH_3, 4 = OH



Chlorogenic acid

Figure 1. Chemical structures of phenolic acids.

enriched binding buffer (Annexin V-FITC kit) and analyzed by an 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, whereas 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 10 000 counts were made for each sample. The percentages of distribution of normal, early apoptosis, late apoptosis, and necrosis cells were calculated by CELL Quest software.

Statistical Analysis. A statistical analysis was performed using SAS software. Analyses of variance were performed using ANOVA procedures. Correlation and regression analyses and a principal component analysis were performed using the SigmaPlot scientific graph system. Significant differences ($p < 0.05$) between the means were determined by Duncan's multiple range tests.

RESULTS

Cell Population Growth of 3T3-L1 Preadipocytes. The chemical structures of phenolic acids tested in the present study are shown in **Figure 1**. The inhibitory effects of 15 phenolic acids on cell population growth in 3T3-L1 preadipocytes were determined by MTT assay. As shown in **Table 1**, some phenolic acids caused the inhibition of cell growth in the 3T3-L1 preadipocytes. The data indicated that gallic acid had the highest inhibition on cell population growth (63.9%) among the 15 phenolic acids tested. Moreover, the 50% inhibitory concentration (IC_{50}) was measured by an MTT assay after 72 h of incubation with phenolic acids. Gallic acid, *o*-coumaric acid, *m*-coumaric acid, and chlorogenic acid showed relatively higher inhibitory activities with IC_{50} values of 43.3, 48.2, 49.2, and 72.3 μM, respectively, in 3T3-L1 preadipocytes.

Antioxidant Activity of Phenolic Acids Evaluated by the ORAC Assay. Figure 2 shows the antioxidant activity of phenolic acids against peroxyl radical ($ORAC_{ROO\cdot}$ activity). The data indicated that gallic acid had the highest $ORAC_{ROO\cdot}$ value (Trolox equiv, 16.3 μM) among the 15 phenolic acids tested. *p*-Hydroxybenzoic acid, gentisic acid, vanillic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, caffeic acid, and chlorogenic acid had $ORAC_{ROO\cdot}$ values within the range of 10.8−14.1 μM trolox equiv. However, salicylic acid, protocatechuic acid, 3,4-dimethoxybenzoic acid, syringic acid, ferulic acid, and sinapinic acid had lower $ORAC_{ROO\cdot}$ values (Trolox equiv, 6.0−9.8 μM). In general, phenolic acids that inhibited cell population growth were found to have higher antioxidant capacities.

Table 1. Effect of Phenolic Acids on Cell Population Growth in 3T3-L1 Preadipocytes

phenolic acids	inhibition (%)[a]	IC_{50} (μM)[b]
salicylic acid	3.6 ± 0.4	[c]
p-hydroxybenzoic acid	37.4 ± 0.2	443.9 ± 37.4
protocatechuic acid	1.6 ± 0.5	[c]
gentisic acid	25.5 ± 1.0	[c]
gallic acid	63.9 ± 8.1	43.3 ± 10.9
vanillic acid	46.5 ± 2.8	188.2 ± 8.2
3,4-dimethoxybenzoic acid	2.8 ± 0.3	[c]
syringic acid	5.1 ± 0.4	[c]
o-coumaric acid	55.6 ± 2.6	48.2 ± 4.6
m-coumaric acid	57.8 ± 2.5	49.2 ± 5.8
p-coumaric acid	20.4 ± 3.6	471.7 ± 69.2
caffeic acid	6.2 ± 1.9	[c]
ferulic acid	14.2 ± 2.3	[c]
sinapinic acid	5.7 ± 0.6	[c]
chlorogenic acid	45.4 ± 4.5	72.3 ± 10.1

[a] The cells were incubated with 100 μM phenolic acids for 72 h. [b] The cells were incubated with phenolic acids for 72 h. [c] The IC_{50} value was over 500 μM. The reported values are the means ± SD ($n = 3$).

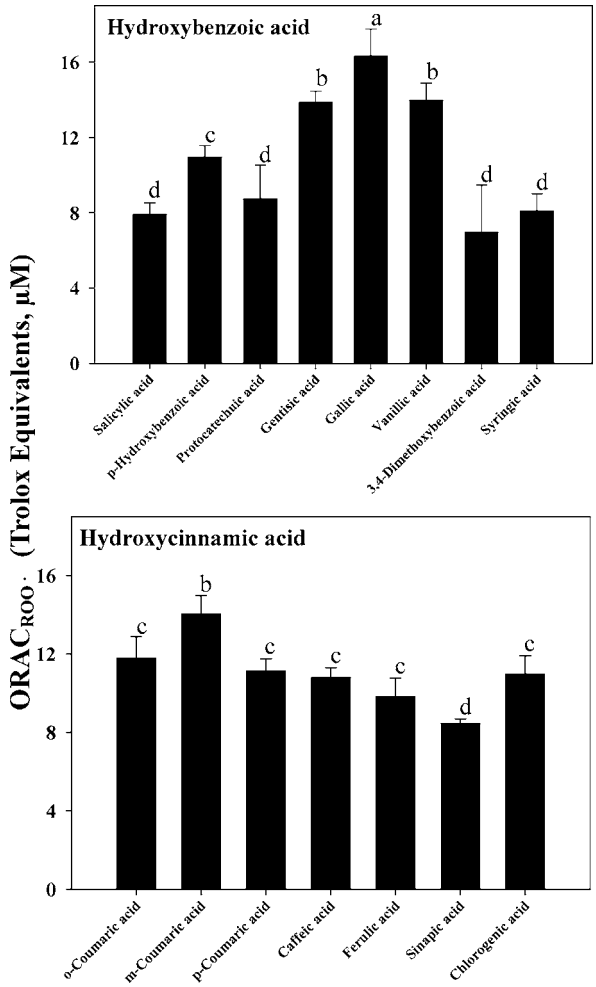


Figure 2. $ORAC_{ROO\cdot}$ activity (Trolox equiv, μM) of phenolic acids. Data are expressed as mean ± SD from three experiments. Bars topped by different letters are significantly different at $p < 0.05$.

Relationship between the Influence of Phenolic Acids on Cell Population Growth and Their Antioxidant Activity. The influence of phenolic acids on cell population growth in relation

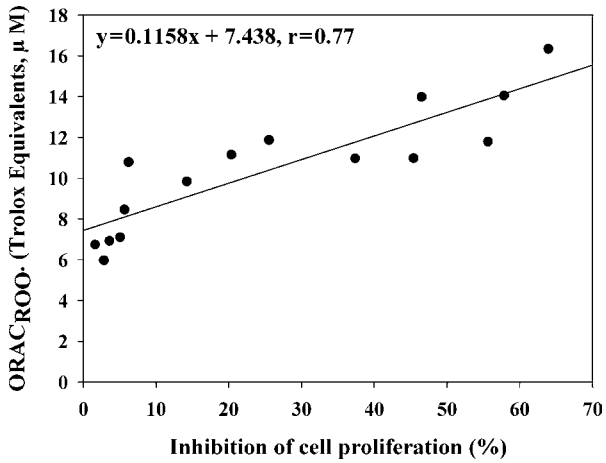


Figure 3. Effect of phenolic acids on the inhibition of cell population growth$_{(X)(\%)}$ in relation to their ORAC$_{ROO^{\bullet}(Y)(\text{Trolox equivalents, }\mu M)}$. Each value is the mean of three experiments.

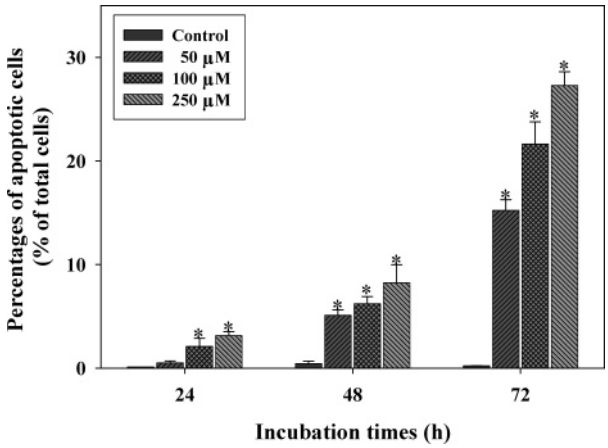


Figure 4. Flow cytometric analysis of gallic acid-mediated cell apoptosis percentage of 3T3-L1 preadipocytes. The ratio of apoptotic cells was calculated by CELL Quest software (mean ± SD, $n = 3$); *$p < 0.05$ is significantly different to that of the control.

to their antioxidant activity is presented in **Figure 3**. It was found that there is a significant linear correlation between the influence of phenolic acids on cell population growth of 3T3-L1 preadipocytes and their antioxidant activity. A correlation coefficient ($r = 0.77$, $p < 0.01$) was observed between the influence of phenolic acid on cell population growth and their ORAC values. These results suggest that the phenolic acids tested on the cell population growth were well correlated to their antioxidant activity.

Phenolic Acid Induced Apoptosis and Cell Cycle Arrest. The inhibition of cell population growth might be going through cell cytotoxic effect or cell cycle arrest. Cell cycle analysis of 3T3-L1 preadipocytes was performed by flow cytometry after the exposure of the cells to the phenolic acids (including gallic acid, *o*-coumaric acid, *m*-coumaric acid, and chlorogenic acid) for 0−72 h. The presence of gallic acid in the medium did not affect the cell cycle profile of 3T3-L1 preadipocytes. However, the addition of gallic acid to 3T3-L1 preadipocytes resulted in a markedly increased accumulation of the sub-G_1 phase (apoptotic cells) in a time- and dose-dependent manner (**Figure 4**). Moreover, a quantitative analysis of LDH activity can determine the percentage of cells that are dead to double check the results. As shown in **Figure 5**, the treatment with gallic acid caused a significant increase ($p < 0.05$) in LDH leakage compared to

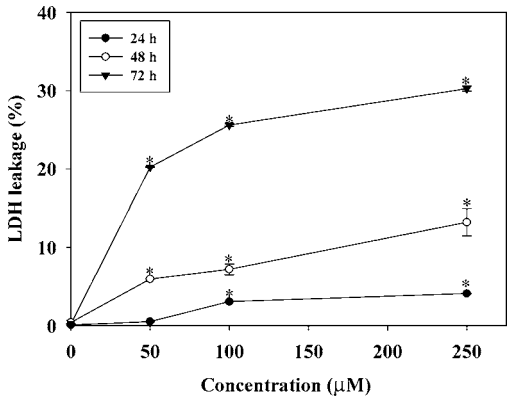


Figure 5. Effect of gallic acid on LDH leakage in 3T3-L1 preadipocytes. The reported values are the means ± SD ($n = 3$); *$p < 0.05$ is significantly different to that of the control.

that of the control. In cell death, the result of LDH leakage was similar to that shown in **Figure** 4.

o-Coumaric acid (250 μM) resulted in a significant ($p < 0.05$) increase in cell population in the G1 phase from 79.3% (control) to 89.9% at 48−72 h (**Figure 6A**). As shown in **Figure 6B**, the exposure of 3T3-L1 preadipocytes to *m*-coumaric acid resulted in a significant ($p < 0.05$) increase in the population of cells in the G_1 phase from 79.3% (control) to 85.0% (250 μM) at 24−72 h. In chlorogenic acid treated cells, a significant ($p < 0.05$) increase in the number of cells in the G_1 phase at 24−72 h was observed (**Figure 6C**). These results indicate that some phenolic acids (including *o*-coumaric acid, *m*-coumaric acid, and chlorogenic acid) led to cell cycle arrest at the G_1 phase in a time- and dose-dependent manner. However, gallic acid showed the greatest antiproliferative activity in 3T3-L1 preadipocytes.

Disruption of Mitochondria Membrane Potential in Cells Induced by Gallic Acid. Alterations in mitochondrial function have been shown to play a crucial role in apoptosis, and thus, the effect of gallic acid on $\Delta\Psi$m was investiged. 3T3-L1 preadipocytes showed a significant increase in fluorescence intensity when treated with gallic acid for 6−24 h (**Figure 7**). These results reflected the collapse of mitochondrial membrane potential.

Apoptosis/Necrosis Induced by Gallic Acid. Annexin V-FITC binding analysis was performed to identify apoptosis. Propidium iodide (PI) staining was performed for the purpose of excluding necrotic cells. 3T3-L1 preadipocytes were treated with 0−250 μM of gallic acid for 72 h and then stained with Annexin V-FITC/PI. As shown in **Figure 8**, gallic acid increased the number of early apoptotic and late apoptotic cells in a dose-dependent manner.

DISCUSSION

Obesity can be defined as a disease in which excess body fat has accumulated such that health may be adversely affected. Obesity is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes in adipose tissues (*11*). Wang and Jones (*12*) indicated that the decreased energy/food intake and increased energy expenditure, decreased differentiation and proliferation of preadipocytes, decreased lipogenesis and increased lipolysis, and fat oxidation are mechanisms of proposed antiobesity.

Some phenolic acids (gallic acid, *o*-coumaric acid, *m*-coumaric acid, and chlorogenic acid) had lower IC_{50} values on the

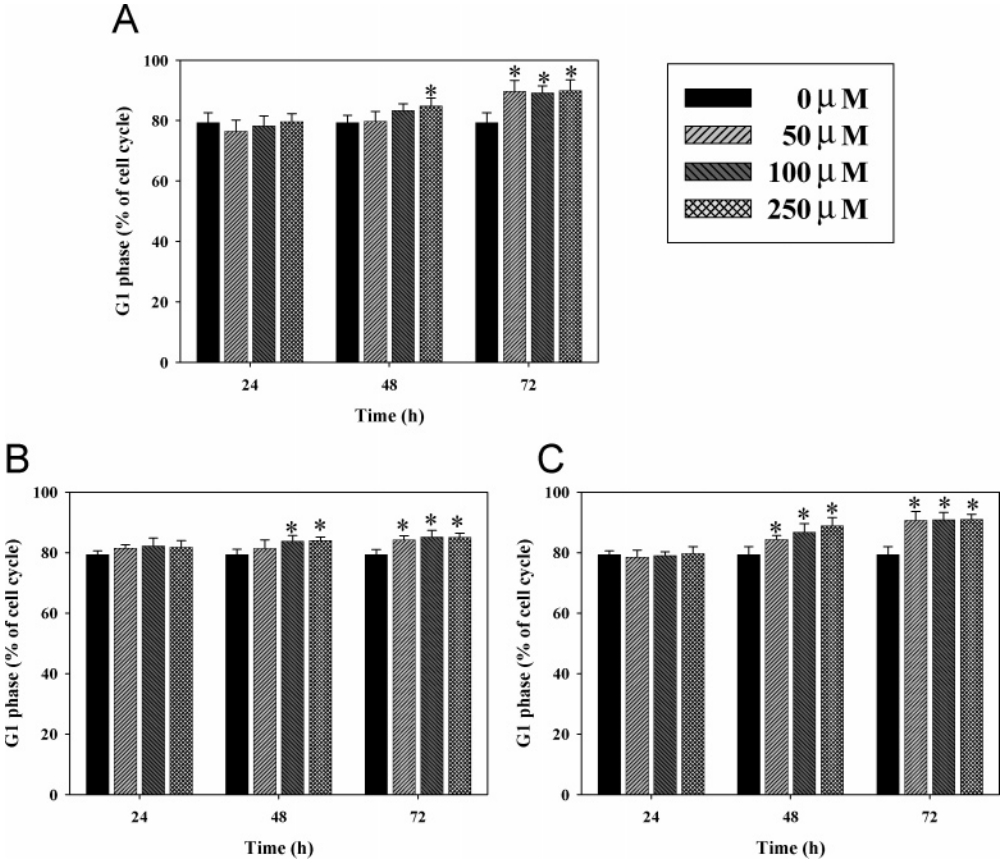


Figure 6. Effect of phenolic acid-mediated G_1 phase of cell cycle distribution in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were treated with 0–250 μM of (A) *o*-coumaric acid, (B) *m*-coumaric acid, and (C) chlorogenic acid for 24, 48, and 72 h at 37 °C in a humidified 5% CO_2 incubator. The reported values are the means ± SD ($n = 3$); *p <0 .05 is significantly different to that of the control.

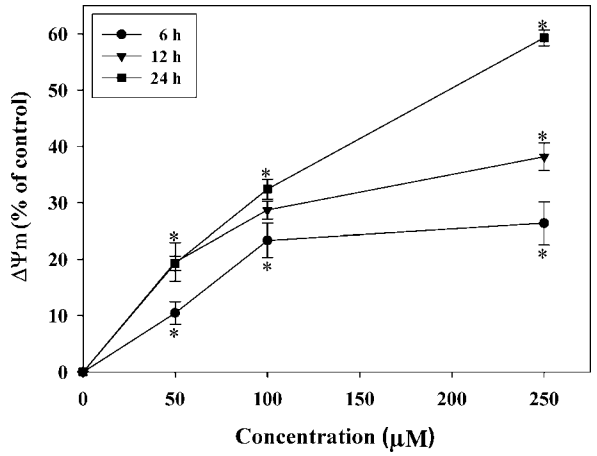


Figure 7. Effect of gallic acid on mitochondrial membrane potential ($\Delta\Psi$m) in 3T3-L1 preadipocytes. The results are expressed as percentages of mitochondrial membrane potential compared with that of the untreated control (mean ± SD, $n = 3$); *$p < 0.05$ is significantly different to that of the control.

proliferative inhibition of 3T3-L1 preadipocytes than other phenolic acids tested (**Table 1**). The ORAC assay is one of the methods used to evaluate the antioxidant capacity of various biological substrates, ranging from pure compounds, such as melatonin (*29*) and flavonoids, (*30*) to complex matrixes, such as vegetables (*31*) and animal tissues (*32*). In the present study, it was found that there was a significant correlation between the influence of phenolic acids on both cell population growth and their ORAC values ($r = 0.77$, $p < 0.01$) (**Figure 3**).

Our results showed that phenolic acids (*o*-coumaric acid, *m*-coumaric acid, and chlorogenic acid) could lead to cell cycle arrest at the G_1 phase in a time- and dose-dependent manner (**Figure 6**). Nyska et al. (*33*) indicated that natural antioxidants could cause G_1 phase arrest in prostatic carcinoma cells. Moreover, a quantitative analysis of LDH activity can determine the percentage of cells that are dead. It is a stable cytoplasmic enzyme, which is present in most cells. Our results also indicated that gallic acid had cytotoxicity against 3T3-L1 preadipocytes (**Figure 5**). Wu et al. (*22*) indicated that EGCG decreased the cell viability of preadipocytes by 15−30% at concentrations of 100−400 μM upon 48 h of treatment. Other reports indicated that the observed decrease in the number of preadipocytes by EGCG could be attributed to its inhibition of cell mitogenesis (*34*, *35*). In cell apoptosis, the results showed that the exposure of 3T3-L1 preadipocytes to gallic acid caused a significant increase ($p < 0.05$) in the accumulation of the sub-G_1 phase (apoptotic cells) (**Figure 4**). Thus, our results suggest that gallic acid has the potential to inhibit cell population growth and induce apoptosis in 3T3-L1 preadipocytes.

Mitochondria play an essential role in death signal transduction such that the permeability of the transition pore opening and the collapse of the $\Delta\Psi$m resulted in the rapid release of caspasse activators (*36*, *37*). In the current study, 3T3-L1 preadipocyte treatment with gallic acid increased the mitochondrial membrane potential (**Figure 7**). These results demonstrated that the early damage to the mitochondrial membrane potential might further activate the intrinsic pathway of apoptosis.

It is well-known that cell death can be categorized into apoptosis and necrosis. To quantify the modes of cell death (apoptosis or necrosis) induced by gallic acid, treated preadi-

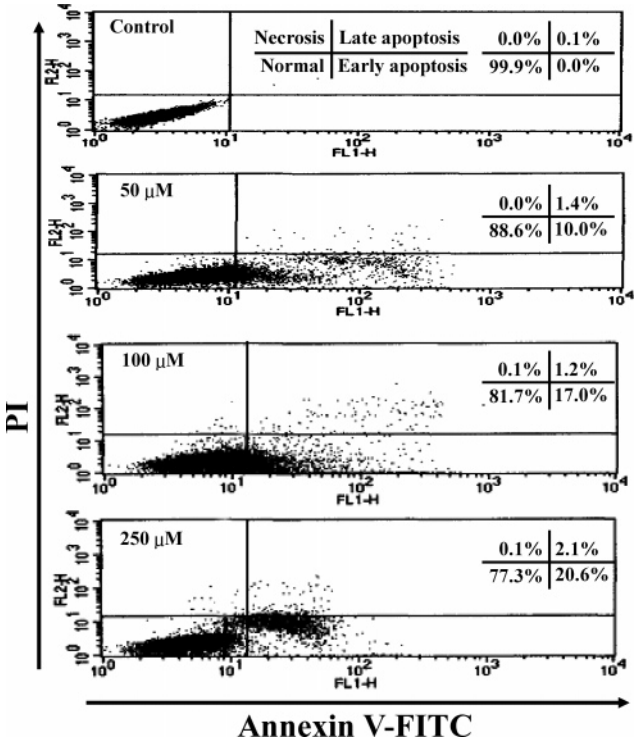


Figure 8. Apoptosis and necrosis induced by gallic acid in 3T3-L1 preadipocytes. The **Figure** shows the flow cytometric analysis of annexin V-FITC/PI double stained cells. The cells were untreated or treated with 0, 50, 100, and 250 μM for 72 h. The percentages of cells were calculated by CELL Quest software (mean, n=3).

pocytes were double stained with Annexin V-FITC and PI and analyzed by flow cytometry. The exposure of the membrane phospholipid and phosphatidylserine to the external cellular environment is one of the earliest markers of apoptotic death. Annexin V-FITC binds to phosphatidylserine and can be used to detect the early stages of apoptosis (*38*). Exposing the 3T3-L1 preadipocytes to gallic acid resulted in an increase in the early apoptotic and late apoptotic cells (**Figure 8**). Some studies indicated that apoptosis was induced in 3T3-L1 preadipocytes by EGCG (*22*), TNF-α (*39*), and interleukin-4 (*40*). These results demonstrated that gallic acid could induce apoptotic cell death in 3T3-L1 preadipocytes.

Recent studies concerning the bioavailability of polyphenols are in agreement with their potential therapeutic effects. These health promoting effects have been mainly attributed to the content of polyphenols and plant secondary metabolites (*41*−*43*). In the present study, the concentrations of phenolic acids were 0−250 μM. However, data presented here indicated that phenolic acids inhibited cell viability and induced apoptosis in preadipocytes at concentrations below 100 μM. The range of concentrations used in the present study was consistent with those in many other studies on the growth inhibitory effect of preadipocytes (*22*, *44*−*47*). The IC_{50} values (43−72 μM) of some effective phenolic acids adopted in this study were within the concentration range of phenolic acids used in a study regarding the absorption and bioavailability of phenolic acids (artepillin c and coumaric acid) in rats (*48*). They indicated that the serum concentration of phenolic acid after the administration of coumaric acid (100 μmol/kg rat) might reach a maximum concentration of 74.8 μmol/L.

In conclusion, the relationship analysis of the present study indicated that phenolic acids tested on the cell population growth were well correlated to their antioxidant activity. The results also demonstrated that some phenolic acids (including *o*-coumaric acid, *m*-coumaric acid, and chlorogenic acid) caused better inhibition of cell growth and induction of apoptosis in 3T3-L1 preadipocytes. Inhibitory effects of phenolic acids on 3T3-L1 preadipocytes may provide a proposed mechanism for antiobesity and have further implication in in vivo antiobesity effects.

ABBREVIATIONS USED

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IC_{50}, 50% growth inhibitory concentrations; PBS, phosphate buffered saline; TE, trypsin-EDTA; PI, propidium iodide; RNase, ribonuclease; $\Delta\Psi$m, mitochondrial membrane potential; Annexin V-FITC, annexin V-fluorescein isothiocyanate.

LITERATURE CITED

(1) Ferguson, L. R. Antimutagens as cancer chemopreventive agents in the diet. *Mutat. Res.* **1994**, *307*, 395−410.
(2) Stavric, B. Role of chemopreventers in human diet. *Clin. Biochem.* **1994**, *27*, 319−332.
(3) Son, S.; Lewis, B. A. Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: structure−activity relationship. *J. Agric. Food Chem.* **2002**, *50*, 468−472.
(4) Artico, M.; Di Santo, R.; Costi, R.; Novellino, E.; Greco, G.; Massa, S.; Tramontano, E.; Marongiu, M. E.; De Montis, A.; La Colla, P. Geometrically and conformationally restrained cinnamoyl compounds as inhibitors of HIV-1 integrase: synthesis, biological evaluation, and molecular modeling. *J. Med. Chem.* **1998**, *41*, 3948−3960.
(5) Montpied, P.; de Bock, F.; Rondouin, G.; Niel, G.; Briant, L.; Courseau, A. S.; Lerner-Natoli, M.; Bockaert, J. Caffeic acid phenethyl ester (CAPE) prevents inflammatory stress in organotypic hippocampal slice cultures. *Brain Res. Mol. Brain Res.* **2003**, *115*, 111−120.
(6) Muramatsu, K.; Fukuyo, M.; Hara, Y. Effect of green tea catechins on plasma cholesterol level in cholesterol-fed rats. *J. Nutr. Sci. Vitaminol.* **1986**, *32*, 613−622.
(7) Murase, T.; Nagasawa, A.; Suzuki, J.; Hase, T.; Tokimitsu, I. Beneficial effects of tea catechins on dietinduced obesity: stimulation of lipid catabolism in the liver. *Int. J. Obes.* **2002**, *26*, 1459−1464.
(8) Heim, K. E.; Tagliaferro, A. R.; Bobilya, D. J. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem.* **2002**, *13*, 572−584.
(9) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Biol. Med.* **1997**, *22*, 749−760.
(10) Kopelman, P. G. Causes and consequences of obesity. *Med. Int.* **1994**, *22*, 385−388.
(11) 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.
(12) 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.
(13) Benavente-Garcia, O.; Castillo, J.; Marin, F. R.; Del Rio, J. A. Uses and properties of citrus flavonoids. *J. Agric. Food Chem.* **1997**, *45*, 4505−4515.
(14) Middleton, E., Jr.; Kandaswami, C.; Theoharides, T. C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, *52*, 673−751.
(15) Serrano, A.; Palacios, C.; Roy, G.; Cespon, C.; Villar, M. L.; Nocito, M.; Gonzales-Porque, P. Derivatives of gallic acid induce apoptosis in tumoural cell lines and inhibit lymphocyte proliferation. *Arch. Biochem. Biophys.* **1998**, *350*, 49−54.

(16) Ohno, Y.; Fukuda, K.; Takemura, G.; Toyota, M.; Watanabe, M.; Yasuda, N.; Xinbin, O.; Maruyama, R.; Akao, S.; Gotou, K.; Fujivara, T.; Fujivara, H. Induction of apoptosis by gallic acid in lung cancer cells. *Anticancer Drugs* **1999**, *10*, 845−851.
(17) Yoshioka, K.; Kataoka, T.; Hayashi, T.; Hasegawa, M.; Ishi, Y.; Hibasami, H. Induction of apoptosis by gallic acid in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines. *Oncol. Rep*. **2000**, *6*, 1221−1223.
(18) Salucci, M.; Stivala, L. A.; Maiani, G.; Bugianesi, R.; Vannini, V. Flavonoids uptake and their effect on cell cycle of human colon adenocarcinoma cells (CaCo2). *Br. J. Cancer* **2002**, *86*, 1645−1651.
(19) Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P.; Rice-Evans, C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys.* **1995**, *322*, 339−346.
(20) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr*. **2000**, *130*, 2073S-85S.
(21) Hertog, M. G.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch. Intern. Med*. **1995**, *155*, 381−386.
(22) Wu, B. T.; Hung, P. F.; Chen, H. C.; Huang, R. N.; Chang, H. H.; Kao, Y. H. The apoptotic effect of green tea (−)-epigallocatechin gallate on 3T3-L1 preadipocytes depends on the Cdk2 pathway, *J. Agric. Food Chem.* **2005**, *53*, 5695−5701.
(23) Green, H.; Kehinde, O. Sublines of mouse 3T3 cells that accumulate lipid. *Cell* **1974**, *1*, 113−116.
(24) Spiegelman, B. M.; Choy, L.; Hotamisligil, G. S.; Graves, R. A.; Tontonoz, P. Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes. *J. Biol. Chem*. **1993**, *268*, 6823−6826.
(25) Ross, S. E.; Hemati, N.; Longo, K. A.; Bennett, C. N.; Lucas, P. C.; Erickson, R. L.; MacDougald, O. A. Inhibition of adipogenesis by Wnt signaling. *Science* **2000**, *289*, 950−953.
(26) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant and pro-oxidant behavior of flavonoids: structure−activity relationships. *Free Radical Biol. Med.* **1993**, *22*, 749−760.
(27) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(28) 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.
(29) Aejmelaeus, R.; Holm, P.; Kaukinen, U.; Metsä-Ketelä,T. J. A.; Laippala, P.; Hervonen, A. J. L.; Alho, H. R. O. Age-related changes in the peroxyl radical scavenging capacity of human plama. *Free Radical Biol. Med.* **1997**, *23*, 69−75.
(30) Cao, G.; Verdon, C. P.; Wu, A. H. B.; Wang, H.; Prior, R. L. Automated oxygen radical absorbance capacity assay using the COBAS FARA II. *Clin. Chem.* **1997**, *41*, 1738−1744.
(31) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant capacity of tea and common vegetables. *J. Agric. Food Chem*. **1996**, *44*, 3426−3431.
(32) Kohen, R.; Beit-Yannai, E.; Berry, E. M.; Tirosh, O. Overall low molecular weight antioxidant activity of biological fluids and tissues by cyclic voltammetry. *Methods Enzymol*. **1999**, *300*, 285−296.
(33) Nyska, A.; Suttie, A.; Bakshi, S.; Lomnitski, L.; Grossman, S.; Bergman, M.; Ben-Shaul, V.; Crocket, P.; Haseman, J.; Moser, G.; Goldsworthy, T. L.; Maronpot, R. R. Slowing tumorigenic progression in TRAMP mice and prostatic carcinoma cell lines using natural anti-oxidant from spinach, NAO−A comparative study of three anti-oxidants. *Toxicol. Pathol.* **2003**, *31*, 39−51.
(34) Kao, Y. H.; Hiipakka, R. A.; Liao, S. Modulation of obesity by a green tea catechin. *Am. J. Clin. Nutr*. **2000**, *72*, 1232−1234.
(35) Hung, P. F.; Wu, B. T.; Chen, H. C.; Chen, Y. H.; Chen, C. L.; Wu, M. H.; Liu, H. C.; Lee, M. J.; Kao, Y. H. Antimitogenic effect of green tea (−)-epigallocatechin gallate on 3T3-L1 preadipocytes depends on the ERK and Cdk2 pathways. *Am. J. Physiol.: Cell. Physiol*. **2005**, *288*, C1094−108.
(36) Li, P.; Nijhawan, D.; Budihardjo, J.; Srinivasula, S. M.; Ahmad, M.; Alnewri, E. S.; Wang, X. Cytochrome *c* and ATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic proteasome cascade. *Cell* **1997**, *91*, 479−489.
(37) Green, D. R.; Reed, J. C. Mitochondria and apoptosis. *Science* **1998**, *281*, 1309−1312.
(38) 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 labeled Annexin V. *J. Immunol. Methods* **1995**, *184*, 39−51.
(39) Niesler, C. U.; Urso, B.; Prins, J. B.; Siddle, K. IGF-I inhibits apoptosis induced by serum withdrawal, but potentiates TNF-alpha-induced apoptosis, in 3T3-L1 preadipocytes. *J. Endocrinol.* **2000**, *167*, 165−174.
(40) Hua, K.; Deng, J.; Harp, J. B. Interleukin-4 inhibits platelet-derived growth factor-induced preadipocyte proliferation. *Cytokine* **2004**, *25*, 61−67.
(41) Arai, Y.; Watanabe, S.; Kimira, M.; Simio, K.; Mochizuki, R.; Kinae, N. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol. *J. Nutr*. **2001**, *130*, 2378−2383.
(42) Halliwell, B.; Rafter, J.; Jenner, A. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *Am. J. Clin. Nutr*. **2005**, *81*, 268S-276S.
(43) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr*. **2004**, *79*, 727−747.
(44) Evans, M.; Geigerman, C.; Cook, J.; Curtis, L.; Kuebler, B.; McIntosh, M. Conjugated linoleic acid suppresses triglyceride accumulation and induces apoptosis in 3T3-L1 preadipocytes. *Lipids* **2000**, *35*, 899−910.
(45) Carrière, A.; Fernandez, Y.; Rigoulet, M.; Pènicaud, L.; Casteilla, L. Inhibition of preadipocyte proliferation by mitochondrial reactive oxygen species. *FEBS Lett.* **2003**, *550*, 163−167.
(46) Jensen, B.; Farach-Carson, M. C.; Kenaley, E.; Akanbi, K. A. High extracellular calcium attenuates adipogenesis in 3T3-L1 preadipocytes. *Exp. Cell Res.* **2004**, *301*, 280−292.
(47) Lin, Y. T.; Tang, C. H.; Chuang, W. J.; Wang, S. M.; Huang, T. F.; Fu, W. M. Inhibition of adipogenesis by RGD-dependent disintegrin. *Biochem. Pharmacol.* **2005**, *70*, 1469−1478.
(48) Konishi, Y.; Hitomi, Y.; Yoshida, M.; Yoshioka, E. Absorption and bioavailability of artepillin C in rats after oral administration. *J. Agric. Food Chem.* **2005**, *53*, 9928−9933.

Received for review April 8, 2006. Accepted April 20, 2006. This research work was partially supported by the Department of Health, the Republic of China, under grant DOH94-TD-F-113-013.

JF0609882