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Gallic Acid Induces Apoptosis in 3T3-L1 Pre-adipocytes via a Fas- and Mitochondrial-Mediated Pathway

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Gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally abundant plant phenolic compound. Our previous studies have shown that some phenolic acids such as gallic acid inhibit cell growth and induce apoptosis in 3T3-L1 pre-adipocytes. However, the molecular mechanism of gallic acid in the induction of cell apoptosis is still unclear. In this study, we investigated the effect of gallic acid on the apoptotic pathway in 3T3-L1 pre-adipocytes. Western blot data revealed that gallic acid stimulated an increase in the protein expression of Fas, FasL, and p53. The ratio of expression levels of pro- and anti-apoptotic Bcl-2 family members was changed by gallic acid treatment. Gallic acid released mitochondrial cytochrome *c* into the cytosol and subsequently induced the activation of caspase-9 and caspase-3, which were followed by the cleavage of poly(ADP-ribose) polymerase. Pretreatment with a general caspase-9 inhibitor (Z-LEHD-FMK) and caspase-3 inhibitor (Z-DEVD-FMK) prevented gallic acid from inhibiting cell viability in 3T3-L1 pre-adipocytes. The data also indicated that treatment with gallic acid inhibited histone deacetylase activity in 3T3-L1 pre-adipocytes. These results demonstrate that gallic acid induces apoptosis in 3T3-L1 pre-adipocytes through the Fas and mitochondrial pathway. The induction of cell apoptosis by gallic acid may prove to be a pivotal mechanism for decreased pre-adipocyte proliferation.

KEYWORDS: Gallic acid; apoptosis; 3T3-L1 pre-adipocytes

INTRODUCTION

Over the past few decades, obesity has become one of the main public health problems in developed countries. Obesity can be defined as a disease in which excess body fat has accumulated such that health may be adversely affected. Obesity is considered 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*). Obesity is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from fibroblastic pre-adipocytes in adipose tissues (*2*). The prevalence of obesity has doubled in the past 25 years; today, two-thirds of adults are overweight in the U.S. (*3*). Obesity seems to decrease antioxidant defense by lowering the levels of antioxidant enzymes such as catalase, glutathione peroxidase, and glutathione reductase (*4*, *5*). Therefore, the use of antioxidants may be important in the prevention of obesity.

Many naturally occurring agents have shown chemopreventive potential in a variety of bioassay systems and animal models. Phenolic acids, especially hydroxycinnamic acids and hydroxybenzoic acids, are secondary plant products and are commonly found in plant-derived foodstuffs. They are widely distributed in higher plants and form part of the human diet (*6*). Phenolic compounds are reported to have antioxidant, antimutagenic, and anticarcinogenic activity and are expected to reduce the risk of disease and bring health benefits on daily intake (*7*). Many phenolic compounds such as gallic acid (3,4,5-trihydroxybenzoic acid) and its derivatives show selective cytotoxicity against a variety of tumor cells with a higher activity than that shown against normal cells (*8, 9*).

Apoptosis is characterized by the activation of the caspases family of cysteine proteases, followed by caspase-mediated specific morphological changes including cell shrinkage, chromatin condensation, unclear DNA fragmentation, membrane blebbing, and breakdown of the cell into apoptotic bodies (*10*). In cells responsive to apoptotic stimuli, there are two major apoptotic pathways, including intrinsic and extrinsic apoptosis signaling (*11*). Apoptosis may be initiated through the regulation of death receptors located on the cell surface or through an intrinsic pathway including the release of apoptotic signals from the mitochondria (*12*). Many models of apoptosis show a loss of the mitochondrial transmembrane potential (MTP) mediated by the opening of the megachannel (permeability transition pore), which precedes caspase activation (*13*).

The results suggest mechanisms of anti-obesity including decreased energy intake and increased energy expenditure,

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decreased pre-adipocyte differentiation and proliferation, decreased lipogenesis and increased lipolysis, and fat oxidation (*14*). Among these mechanisms, pre-adipocytes play a key role in differentiation into mature adipocytes and increased fat mass. Our previous study established that some phenolic acids (such as gallic acid) caused better inhibition of cell growth and induction of apoptosis in 3T3-L1 pre-adipocytes (*15*); however, the molecular mechanism remained unclear. The aim of this study was to investigate the effect of gallic acid on the intrinsic pathway of apoptosis in 3T3-L1 pre-adipocytes.

MATERIALS AND METHODS

Materials. Gallic acid, MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], PI (propidium iodide), and DAPI (4,6-diamidine-2-phenylindole) were purchased from the Sigma Chemical Co. (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from the Merck Co. (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum, and the antibiotic mixture (penicillin−streptomycin) were purchased from the Invitrogen Co. (Carlsbad, CA). Anti-β-actin, anti-Bad, anti-Bak, anti-Bax, anti-Bcl-2, anti-caspase-3, anti-cytochrome *c*, anti-Fas, anti-FasL, anti-PARP [poly-(ADP-ribose) polymerase], and anti-p53 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-Bcl-X_L antibody was obtained from Biosource (Camarillo, CA). Anti-caspase-9 antibody, caspase-3/CPP32 inhibitor (Z-DEVD-FMK), and caspase-9/Mch6 inhibitor (Z-LEHD-FMK) were obtained from BioVision (Mountain View, CA). Anti-rabbit or anti-mouse secondary horseradish peroxidase antibodies were purchased from Bethyl Laboratories (Montgomery, TX). Protein molecular mass markers 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 pre-adipocytes were incubated in culture medium including DMEM, 10% calf serum, 1.5 g/L sodium bicarbonate, and 100 U/mL penicillin−streptomycin. Cells were cultured at 37 °C in a humidified 5% CO_2 incubator.

MTT Assay. The MTT assay was performed according to the method of Mosmann (*16*). 3T3-L1 pre-adipocytes 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 gallic acid, 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) were 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., Offenburg, Germany) at 570 nm. The cell population growth percentage (%) was expressed as the percentage of cell growth as compared to control, and it was calculated by $A_{570\ nm}$ [gallic acid]/$A_{570\ nm}$ [control] $\times$ 100.

Nuclear Staining with PI and DAPI. Apoptosis was evaluated by staining with PI and DAPI. 3T3-L1 pre-adipocytes were stimulated with 100 μM gallic acid for 48 h. PI stained cells were fixed with 80% ethanol for 30 min and incubated with a 40 μg/mL PI solution for 30 min in the dark. DAPI stained cells were fixed with 4% paraformaldehyde for 30 min and incubated with a 1 μg/mL DAPI solution for 30 min in the dark. The nuclear morphology of the cells was examined by fluorescence microscopy (Olympus, Tokyo, Japan). Typical apoptotic changes included chromatin condensation, chromatin compaction along the periphery of the nucleus, and segmentation of the nucleus.

Measurement of Caspase-3 and -9 Activity. Cells were treated with 0−50 μM gallic acid for 6 and 12 h. Caspase-3 and -9 activities were assayed using commercial kits as specified by the manufacturers (Upstate Biotechnology, Lake Placid, NY and BioVision, Mountain View, CA). Fluorescence was measured with a FLUOstar galaxy fluorescence plate reader (BMG Labtechnologies Ltd., Offenburg, Germany).

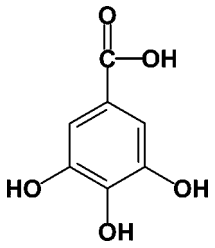

Figure 1. Chemical structure of gallic acid.

Western Blot Analysis. 3T3-L1 pre-adipocytes (1×10^7 cells/10 cm dish) were incubated with 0−50 μM gallic acid for 6 and 12 h at 37 °C in 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 Fas, FasL, p53, Bcl-2, Bax, Bak, Bad, Bcl-X_L, caspase-9, caspase-3, and PARP proteins were assessed in 3T3-L1 pre-adipocytes. The release of mitochondrial cytochrome *c* was determined by Western blotting. After the various treatments, the cytosolic fraction of cells was isolated using a Mitochondria/Cytosol Fractionation kit (BioVision, Mountain View, CA) as specified by the manufacturer. The protein concentration of the extracts was estimated with the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. 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 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 3 times in PBST for 10 min between each step. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). Relative protein expression was quantified densitometrically using the LabWorks 4.5 software and calculated according to the β-actin reference bands.

Measurement of HDAC Activity. Cells were treated with 0−50 μM gallic acid for 6 and 12 h. The HDAC (histone deacetylase) activity assay was performed using a commercial kit (BioVision, Mountain View, CA) as specified by the manufacturer. Fluorescence was measured with a FLUOstar galaxy fluorescence plate reader (BMG Labtechnologies Ltd., Offenburg, Germany).

Statistical Analysis. Statistical analysis was performed using the SAS software. Analyses of variance were performed using ANOVA procedures. Significant differences ($p < 0.05$) between the means were determined by Duncan's multiple range tests. Each treatment was performed in triplicate.

RESULTS

Effect of Gallic Acid on Cell Population Growth and Morphology. To reveal the molecular mechanism of cell growth inhibition and induction of apoptosis in 3T3-L1 pre-adipocytes by gallic acid, the effect of gallic acid on the apoptotic pathway in 3T3-L1 pre-adipocytes was investigated. **Figure 1** shows the chemical structure of gallic acid tested in the present study. As shown in **Figure 2**, gallic acid had a clear inhibitory effect on cell population growth in 3T3-L1 pre-adipocytes. The results showed that gallic acid caused a significant decrease ($p < 0.05$) in cell population growth of 3T3-L1 pre-adipocytes at 48 and 72 h. Classical apoptotic cells were observed in gallic acid-mediated cell death by cell shrinkage and membrane blebbing to form apoptotic bodies (**Figure 3A**). The nuclear morphology of untreated and treated cells is shown in **Figure 3B**,**C** by PI

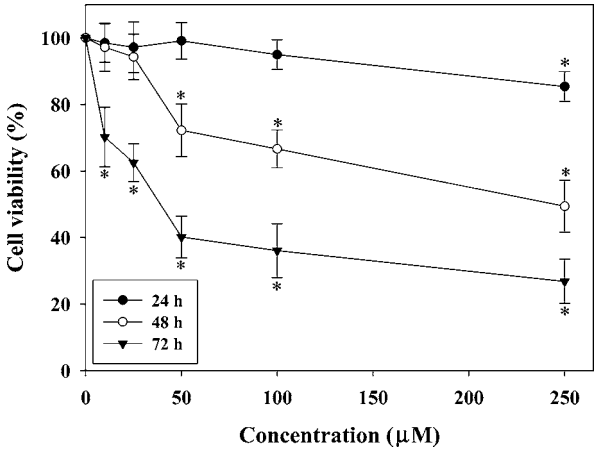

Figure 2. Effect of gallic acid on cell population growth in 3T3-L1 pre-adipocytes. Cells were treated with 0–250 μM gallic acid for 24, 48, and 72 h. Reported values are the means ± SD ($n = 3$). *Significantly different from control ($p < 0.05$).

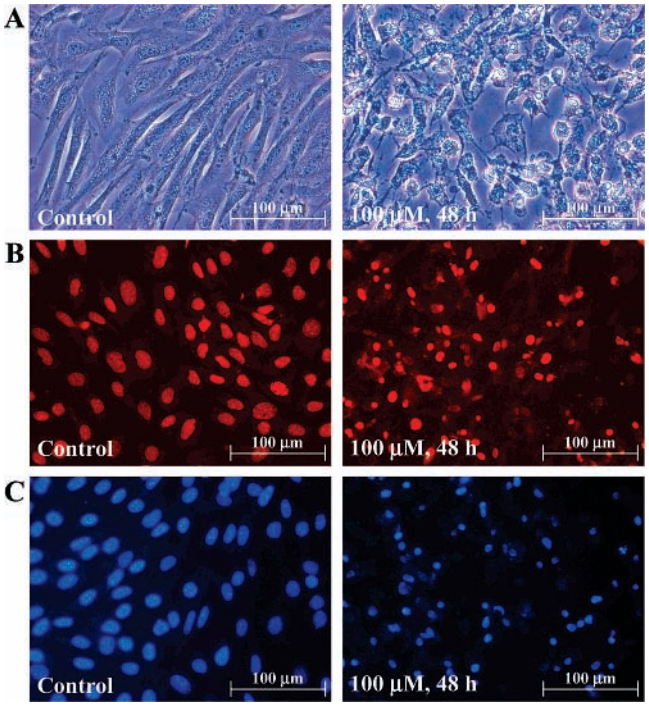

Figure 3. Effect of gallic acid on cell morphology in 3T3-L1 pre-adipocytes. (**A**) Unstained, (**B**) stained with PI, and (**C**) stained with DAPI. Cells were treated with 100 μM gallic acid for 48 h.

and DAPI staining, respectively. PI and DAPI staining revealed that apoptotic bodies appeared when the cells were treated with 100 μM gallic acid for 48 h.

Effect of Gallic Acid on Protein Expression of Fas, FasL, and p53. The Fas (CD95)/Fas Ligand (FasL; CD95L) system is an important extracellular pathway in apoptotic signaling. As shown in **Figure 4**, the protein expression of Fas and FasL was increased in a time- and dose-dependent manner in 3T3-L1 pre-adipocytes. Gallic acid (50 μM, 12 h) resulted in a significant ($p < 0.05$) increase in Fas and FasL expression from 1.00 (control) to 3.02- and 1.47-fold, respectively. Cellular functions modulated by the p53 protein include DNA synthesis, DNA repair, gene transcription, cell cycle arrest, and apoptosis. Gallic acid (50 μM) significantly ($p < 0.05$) stimulated p53 expression

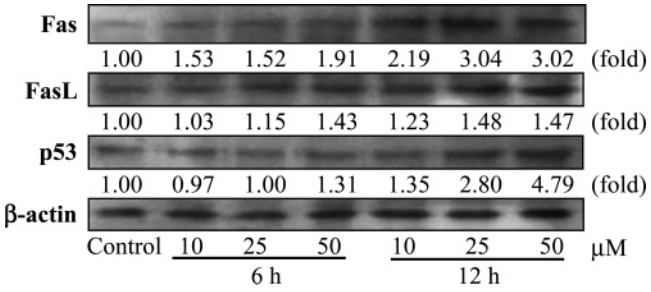

Figure 4. Effect of gallic acid on expression of Fas, Fas-L, and p53 in 3T3-L1 pre-adipocytes. Cells were treated with 0–50 μM gallic acid for 6 and 12 h. Relative protein expression was quantified densitometrically using the LabWorks 4.5 software and calculated according to the β-actin reference bands.

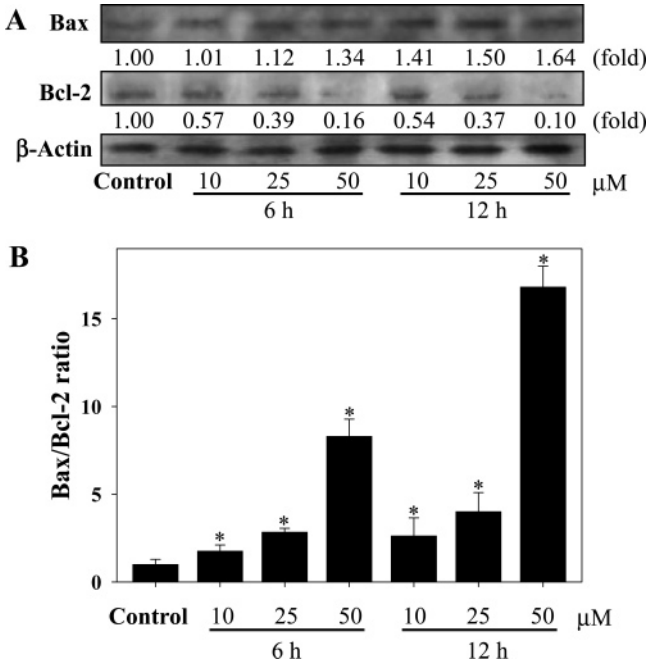

Figure 5. Effect of gallic acid on expression of Bax and Bcl-2 (**A**) and Bax/Bcl-2 ratio (**B**) in 3T3-L1 pre-adipocytes. Cells were treated with 0–50 μM gallic acid for 6 and 12 h. Relative expression was quantified densitometrically using the LabWorks 4.5 software and calculated according to the β-actin reference bands. Reported values are the means ± SD ($n = 3$). *Significantly different from control ($p < 0.05$).

in a time- and dose-dependent manner, with a maximal increase of 4.79-fold after 12 h.

Effect of Gallic Acid on Protein Expression of Bcl-2 Family and Cytochrome *c*. The Bcl-2 family plays a crucial role in apoptosis since it includes both anti-apoptotic members such as Bcl-2 and Bcl-X_L and pro-apoptotic members such as Bax, Bad, and Bak (*17*). Therefore, we next studied the effects of gallic acid on the constitutive protein levels of Bax, Bcl-2, Bak, Bad, and Bcl-X_L in 3T3-L1 pre-adipocytes. As shown in **Figure 5A**, gallic acid (50 μM, 12 h) resulted in a significant ($p < 0.05$) increase in Bax expression from 1.00 (control) to 1.64-fold. Treatment of cells with gallic acid (50 μM, 12 h) significantly ($p < 0.05$) decreased Bcl-2 expression from 1.00 (control) to 0.10-fold. A significant time- and dose-dependent shift in the ratio of Bax and Bcl-2 was observed after gallic acid treatment, indicating the induction of the apoptotic process (**Figure 5B**). The effects of gallic acid on the expression of Bak, Bad, Bcl-X_L, and cytochrome *c* in 3T3-L1 pre-adipocytes

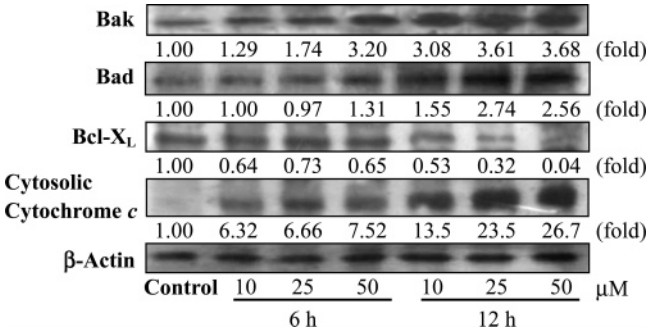

Figure 6. Effect of gallic acid on expression of Bak, Bad, Bcl-X_L, and cytochrome *c* in 3T3-L1 pre-adipocytes. Cells were treated with 0–50 μM gallic acid for 6 and 12 h. Relative protein expression was quantified densitometrically using the LabWorks 4.5 software and calculated according to the β-actin reference bands.

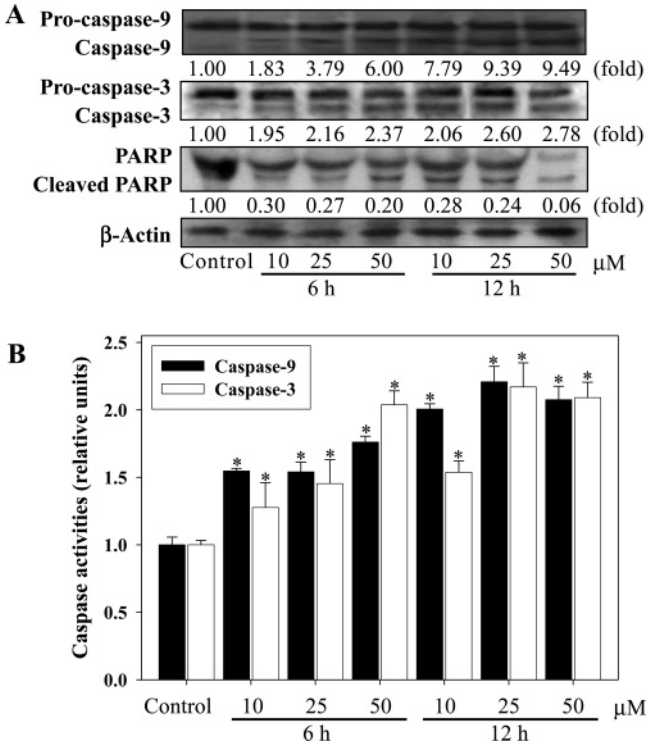

Figure 7. Effect of gallic acid on expression of caspase-9, caspase-3, and PARP (**A**) and activities of caspase-9 and caspase-3 (**B**) in 3T3-L1 pre-adipocytes. Cells were treated with 0–50 μM gallic acid for 6 and 12 h. Reported values are the means ± SD (n = 3). Relative protein expression was quantified densitometrically using the LabWorks 4.5 software and calculated according to the β-actin reference bands. *Significantly different from control (p < 0.05).

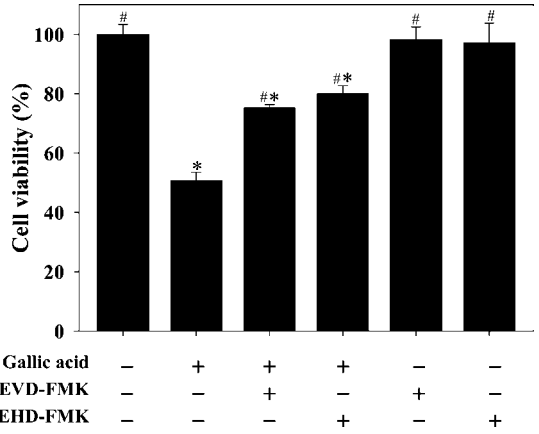

Figure 8. Suppression of gallic acid-induced cell death by caspase-3 and caspase-9 inhibitors. Cells were pretreated with 25 μM caspase-3 (Z-DEVD-FMK) and caspase-9 (Z-LEHD-FMK) inhibitors for 3 h and then treated with 100 μM gallic acid for 48 h. Reported values are the means ± SD (n = 3). *p < 0.05 as compared to control. #p < 0.05 as compared to gallic acid alone.

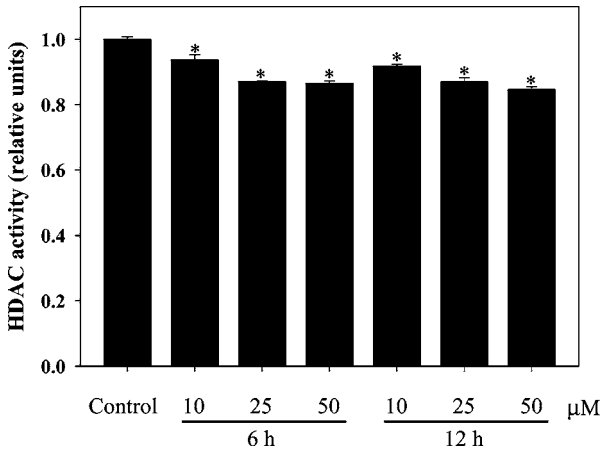

Figure 9. Effect of gallic acid on HDAC activity in 3T3-L1 pre-adipocytes. Cells were treated with 0–50 μM gallic acid for 6 and 12 h. Reported values are the means ± SD (n = 3). *Significantly different from control (p < 0.05).

are shown in **Figure 6**. Gallic acid stimulated Bak and Bad expression in a time- and dose-dependent manner with statistical significance (p < 0.05) and maximal increases of 3.68- and 2.56-fold, respectively, after treatment with 50 μM gallic acid for 12 h. Gallic acid (50 μM, 12 h) resulted in a significant (p < 0.05) decrease in Bcl-X_L expression from 1.00 (control) to 0.04-fold. Cytochrome *c* release in the cytosolic fraction following gallic acid treatment was then investigated. Gallic acid (50 μM, 12 h) resulted in a significant (p < 0.05) increase in cytosolic cytochrome *c* expression from 1.00 (control) to 26.7-fold.

Effect of Gallic Acid on Protein Expression and Caspase Activity. Figure 7A shows the effect of gallic acid on the expression of caspase-9, caspase-3, and PARP in 3T3-L1 pre-adipocytes. Gallic acid stimulated caspase-9 and -3 expression in a time- and dose-dependent manner with a significant effect (p < 0.05) and maximal increase of 9.49- and 2.78-fold, respectively, after treatment with 50 μM gallic acid for 12 h. The proteolysis of pro-caspase-3 into small active fragments and the cleavage of PARP present additional evidence of caspase-3 activation. Treatment of cells with gallic acid induced PARP cleavage in a time- and dose-dependent manner with statistical significance (p < 0.05). The maximal degradation was 0.06-fold, which occurred after treatment with 50 μM gallic acid for 12 h. To monitor the enzymatic activities of caspase-3 and -9, the specific caspase-3 and -9 fluorogenic substrates, Ac-DEVD-MCA and LEHD-AFC, respectively, were used. The results indicated that gallic acid treatment caused a significant time- and dose-dependent increase in caspase-3 and -9 activities (p < 0.05) (**Figure 7B**).

Effect of Caspase Inhibitor on Cell Population Growth. In this study, data indicated that gallic acid induced cell apoptosis in 3T3-L1 pre-adipocytes via expression of the apoptotic proteins Fas, FasL, p53, the Bcl-2 family, and caspase-3 and -9. The caspase-3/CPP32 inhibitor (Z-DEVD-FMK) and caspase-9/Mch6 inhibitor (Z-LEHD-FMK) were used

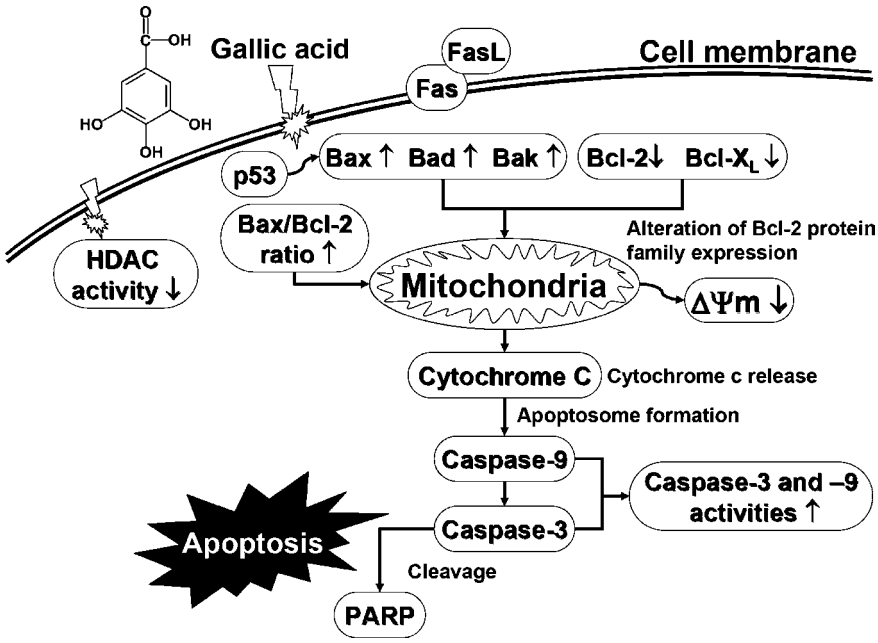

Figure 10. Schematic representation of the mechanism by which gallic acid induces apoptosis in 3T3-L1 pre-adipocytes. Gallic acid can mediate the apoptotic pathway through the induction of stress proteins. Gallic acid leads to death receptor (Fas and FasL) and p53 signaling, loss of the mitochondrial transmembrane potential ($\Delta\Psi$m), release of cytochrome *c* from the mitochondria into the cytosol, and subsequent activation of caspase-9 and caspase-3, followed by the cleavage of PARP. Ratio of expression levels of pro- and anti-apoptotic Bcl-2 family members is also changed by gallic acid treatment.

to block intracellular proteases, and the inhibition of cell viability by gallic acid was analyzed by the MTT assay. The results shown in **Figure 8** indicate that both caspase-3 and -9 inhibitors significantly reduced the inhibition of cell viability caused by gallic acid in the 3T3-L1 pre-adipocytes ($p < 0.05$). Protection from caspase-3 and -9 inhibitors indicated that the death receptor signaling pathway might be involved in this process.

Effect of Gallic Acid on HDAC Activity. HDAC is a key regulatory enzyme involved in the control of signal transduction. The inhibition of this enzyme by specific compounds has been widely correlated to cell cycle regulation and apoptosis. **Figure 9** shows the effect of gallic acid on HDAC activity in 3T3-L1 pre-adipocytes. The results indicate that gallic acid treatment caused a significant time- and dose-dependent decrease in HDAC activity ($p < 0.05$).

DISCUSSION

Our previous study established that some phenolic acids (such as gallic acid) inhibited cell growth and induced apoptosis in 3T3-L1 pre-adipocytes (*15*). In the present study, we found that gallic acid caused a significant decrease ($p < 0.05$) in the cell population growth of 3T3-L1 pre-adipocytes at 48 and 72 h (**Figure 2**). Apoptotic cells are characterized by distinct morphological features such as cell shrinkage, chromatin condensation, membrane blebbing, and formation of apoptotic bodies. In this study, data indicated that the characteristic change in nuclear morphology is the most accurate indicator of the involvement of apoptosis in the death of a cell (**Figure 3**). However, the molecular mechanism of apoptosis induction remains unclear. The present study further investigated the effect of gallic acid on the intrinsic pathway of apoptosis in 3T3-L1 pre-adipocytes. Apoptosis or programmed cell death can be activated through two main pathways, ultimately classified into the mitochondria-dependent pathway (intrinsic pathway) and the death receptor-dependent pathway (extrinsic pathway) (*18*). Fas and its receptor Fas ligand (FasL) are part of an important cellular pathway regulating the induction of apoptosis in diverse cell types and tissues (*19*). In this study, data indicated that treatment of gallic acid markedly activated Fas, FasL, and p53 proteins (**Figure 4**). Shen and White (*20*) indicated that the tumor suppressor gene p53 leads either to the induction of cell cycle arrest or to apoptosis.

The mitochondria-dependent pathway and death receptor-dependent pathway are both regulated by Bcl-2 family proteins. Bcl-2 family proteins involved in apoptosis have been identified, including the pro-apoptoic proteins Bax, Bak, Bad, and Bcl-X_S and the anti-apoptoic proteins Bcl-2, Bcl-X_L, and Mcl-1. Moreover, the ratio of Bax/Bcl-2 is a decisive factor that plays an important role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death (*21*). In this study, data indicated that the up-regulation of Bax and the down-regulation of Bcl-2 could be another molecular mechanism through which gallic acid induced apoptosis in 3T3-L1 pre-adipocytes (**Figure 5**). Cory and Adams (*22*) indicated that mitochondrial release of cytochrome *c* can be controlled by the Bcl-2 family of proteins and may be activated by proteolytic cleavage and heterodimerization. Treatment of cells with gallic acid markedly down-regulated the Bcl-X_L protein and activated the Bak and Bad proteins (**Figure 6**). Gallic acid also caused the release of mitochondrial cytochrome *c* into the cytosol. Bax induces cell apoptosis through mitochondrial membrane permeabilization (MMP), which leads to the release of cytochrome *c* (*22*).

Among the apoptotic processes, caspase-3 has been shown to play a pivotal role in the terminal execution phase of apoptosis and can be induced by diverse stimuli (*23*). Previous reports indicated that the disruption of the mitochondrial membrane potential is defined as an early stage of apoptosis, where release of cytochrome *c* from the mitochondria is followed by caspase-3/caspase-9 cascade activation (*24*). The data in this report

clearly show that gallic acid induced caspase-3 and -9 in association with the degradation of PARP (**Figure 7A**). These results were further confirmed upon monitoring the cleavage of PARP, which is targeted by active caspase-3 (*25*). Moreover, 3T3-L1 pre-adipocytes showed increasing caspase-3 and -9 activities after gallic acid treatment (**Figure 7B**). We next examined whether caspase-3 and -9 inhibitors significantly reduced the inhibition of cell viability caused by gallic acid in 3T3-L1 pre-adipocytes (**Figure 8**). Pretreatment with the caspase-9 and -3 inhibitors inhibited gallic acid-induced apoptosis, suggesting that apoptosis induced by gallic acid also involves caspaes-9- and -3-mediated mechanisms.

HDAC is the most promising target in drug development for cancer therapy. This enzyme is involved in the control of gene expression, and its inhibition by specific drugs has been widely correlated to cell cycle arrest and apoptosis (*26*, *27*). Reports have indicated that HDAC is overexpressed in various tumor cells (*28*). Inhibition of HDAC also leads to the acetylation of non-histone proteins such as p53 tumor suppressors (*29*). The present study showed that treatment of gallic acid also inhibited HDAC activity in 3T3-L1 pre-adipocytes (**Figure 9**). Other inhibitors of HDAC (such as sodium butyrate, trichostatin A, and *Helminthosporium carbonum* toxin) also exhibit antiproliferative properties (*30*, *31*).

In conclusion, we propose that the signaling pathway of gallic acid-induced apoptosis in 3T3-L1 pre-adipocytes is as shown in **Figure 10**. The present results show that gallic acid triggers apoptosis through the regulation of Fas/FasL, p53, and Bcl-2 family members (Bad, Bak, Bax, Bcl-2, and Bcl-X_L) and through activation of the caspase cascade (caspase-3 and -9). These results provide a potential molecular mechanism for gallic acid-induced apoptosis in 3T3-L1 pre-adipocytes.

ABBREVIATIONS USED

DAPI, 4,6-diamidine-2-phenylindole; DMSO, dimethylsulfoxide; HDAC, histone deacetylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MTP, mitochondrial transmembrane potential; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PVDF, polyvinyldifluoride; PI, propidium iodide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

LITERATURE CITED

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

(2) 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.

(3) Evans, W. D.; Finkelstein, E. A.; Kamerow, D. B.; Renaud, J. M. Public perceptions of childhood obesity. *Am. J. Prev. Med.* **2005**, *28*, 26−32.

(4) Asayama, K.; Nakane, T.; Dobashi, K.; Kodera, K.; Hayashibe, H.; Uchida, N.; Nakazawa, S. Effect of obesity and troglitazone on expression of two glutathione peroxidases: Cellular and extracellular types in serum, kidney, and adipose tissue. *Free Radical Res.* **2001**, *34*, 337−347.

(5) Carmiel-Haggai, M.; Cederbaum, A. I.; Nieto, N. A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats. *FASEB J.* **2005**, *19*, 136−138.

(6) 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.

(7) Galati, G.; O'Brien, P. J. Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. *Free Radical Biol. Med.* **2004**, *37*, 287−303.

(8) 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.

(9) 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.

(10) Johnstone, R. W.; Ruefli, A. A.; Lowe, S. W. Apoptosis: A link between cancer genetics and chemotherapy. *Cell* **2002**, *108*, 153−164.

(11) Alvarez, S.; Drane, P.; Meiller, A.; Bras, M.; Deguin-Chambon, V.; Bouvard, V.; May, E. A comprehensive study of p53 transcriptional activity in thymus and spleen of γ-irradiated mouse: High sensitivity of genes involved in the two main apoptotic pathways. *Int. J. Radiat. Biol.* **2006**, *82*, 761−770.

(12) Vermeulen, K.; Van Bockstaele, D. R.; Berneman, Z. N. Apoptosis: Mechanisms and relevance in cancer. *Ann. Hematol.* **2005**, *84*, 627−639.

(13) Zamzami, N.; Metivier, D.; Kroemer, G. Quantitation of mitochondrial transmembrane potential in cells and isolated mitochondria. *Methods Enzymol.* **2000**, *322*, 208−213.

(14) 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.

(15) 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.

(16) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.

(17) Hunt, A.; Evan, G. Apoptosis. Till death us do part. *Science (Washington, DC, U.S.)* **2001**, *293*, 1784−1785.

(18) Thornberry, N. A.; Rano, T. A.; Peterson, E. P.; Rasper, D. M.; Timkey, T.; Garcia-Calvo, M.; Houtzager, V. M.; Nordstrom, P. A.; Roy, S.; Vaillancourt, J. P.; Chapman, K. T.; Nicholson, D. W. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* **1997**, *272*, 17907−17911.

(19) Nagata, S.; Golstein, P. The Fas death factor. *Science (Washington, DC, U.S.)* **1995**, *267*, 1449−1456.

(20) Shen, Y.; White, E. p53-Dependent apoptosis pathways. *Adv. Cancer Res.* **2001**, *82*, 55−84.

(21) Merry, D. E.; Korsmeyer, S. J. Bcl-2 gene family in the nervous system. *Ann. Rev. Neurosci.* **1997**, *20*, 245−267.

(22) Cory, S.; Adams, J. M. The Bcl2 family: Regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* **2002**, *2*, 647−656.

(23) Thornberry, N. A.; Lazebnik, Y. Caspases: Enemies within. *Science (Washington, DC, U.S.)* **1998**, *281*, 1312−1316.

(24) Green, D. R.; Reed, J. C. Mitochondria and apoptosis. *Science (Washington, DC, U.S.)* **1998**, *281*, 1309−1312.

(25) Debatin, K. M.; Krammer, P. H. Death receptors in chemotherapy and cancer. *Oncogene* **2004**, *23*, 2950−2966.

(26) Rosato, R. R.; Grant, S. Histone deacetylase inhibitors in cancer therapy. *Cancer Biol. Ther.* **2003**, *2*, 30−37.

(27) Mork, C. N.; Faller, D. V.; Spanjaard, R. A. A mechanistic approach to anticancer therapy: Targeting the cell cycle with histone deacetylase inhibitors. *Curr. Pharm. Des.* **2005**, *11*, 1091−1104.

(28) Yang, W. M.; Yao, Y. L.; Sun, J. M.; Davie, J. R.; Seto, E. Isolation and characterization of cDNAs corresponding to an additional member of the human histone deacetylase gene family. *J. Biol. Chem.* **1997**, *272*, 28001−28007.

(29) Gu, W.; Roeder, R. G. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **1997**, *90*, 595−606.

(30) Archer, S. Y.; Meng, S.; Shei, A.; Hodin, R. A. $p21^{WAF1}$ is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6791−6796.

(31) Joung, K. E.; Kim, D. K.; Sheen, Y. Y. Antiproliferative effect of trichostatin A and HC-toxin in T47D human breast cancer cells. *Arch. Pharm. Res.* **2004**, *27*, 640−645.

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