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Chicoric Acid Induces Apoptosis in 3T3-L1 Preadipocytes through ROS-Mediated PI3K/Akt and MAPK Signaling Pathways

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Supporting Information

ABSTRACT: Chicoric acid has been reported to possess various bioactivities. However, the antiobesity effects of chicoric acid remain poorly understood. In this study, we investigated the effects of chicoric acid on 3T3-L1 preadipocytes and its molecular mechanisms of apoptosis. Chicoric acid inhibited cell viability and induced apoptosis in 3T3-L1 preadipocytes which was characterized by chromatin condensation and poly ADP-ribose-polymerase (PARP) cleavage. Mitochondrial membrane potential (MMP) loss, Bax/Bcl-2 dysregulation, cytochrome *c* release, and caspase-3 activation were observed, indicating mitochondria-dependent apoptosis induced by chicoric acid. Furthermore, PI3K/Akt and MAPK (p38 MAPK, JNK, and ERK1/2) signaling pathways were involved in chicoric acid-induced apoptosis. The employment of protein kinase inhibitors LY294002, SB203580, SP600125, and U0126 revealed that PI3K/Akt signaling pathway interplayed with MAPK signaling pathways. Moreover, chicoric acid induced reactive oxygen species (ROS) generation. Pretreatment with the antioxidant N-acetylcysteine (NAC) significantly blocked cell death and changes of Akt and MAPK signalings induced by chicoric acid. In addition, chicoric acid down regulated HO-1 and COX-2 via the PI3K/Akt pathway.

KEYWORDS: *chicoric acid, 3T3-L1 preadipocytes, apoptosis, PI3K/Akt, MAPK, reactive oxygen species*

INTRODUCTION

Obesity has become the leading public health problem due to high-calorie diets and lack of physical activity. It increases the risk of various diseases, including type 2 diabetes mellitus, coronary heart disease, hypertension, cancer, respiratory complications, and osteoarthritis. Hyperplasia (cell number increase) and hypertrophy (cell size increase) are two possible growth mechanisms of adipose tissue.[1] Hyperplasia of adipocytes appears only at early stages in adipose tissue development and due to increased proliferation and differentiation of preadipocytes.[2,3] Hypertrophy of adipocytes occurs prior to hyperplasia to meet the need for additional fat storage capacity in the progression of obesity and due to increased storage of triacylglycerol in fully differentiated adipocytes.[4] Therefore, the cell death of preadipocytes and the inhibition of adipogenesis in adipocytes play key roles in mediating the antiobesity effects.

Much attention has been focused on the role of apoptosis in cell death. Apoptosis, which is programmed cell death, can be initiated via extrinsic and intrinsic pathways. The intrinsic pathway of apoptosis is driven by a mitochondria-mediated death signaling cascade. Translocation of Bax to mitochondria can change the mitochondrial membrane permeabilization (MMP). Subsequently, the decreased MMP triggers the release of cytochrome *c* from the mitochondria into the cytosol followed by the activation of caspase cascades and eventually results in poly ADP-ribose-polymerase (PARP) cleavage and apoptosis.[5] Additionally, several signaling pathways including phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and mitogen-activated protein kinases (MAPKs) play critical roles in controlling cell survival and apoptosis through altering the level of Bcl-2 family proteins.[6,7]

Chicoric acid, a natural dicaffeyltartaric acid, has been found in large number of edible plants and vegetables including *Echinacea purpurea*, dandelion, basil, iceberg lettuce, *Cichorium intybus* L., and *Orthosiphon stamineus* Benth.[8−13] Some of them are being used in folk medicine. Chicoric acid exhibits antioxidant activities and has a stimulatory effect upon phagocytes.[14,15] It has also been reported to inhibit HIV integrase which catalyzes the integration of HIV DNA copy into the host cell DNA.[16] In addition, chicoric acid is well documented as a potent stress busting agent because of its ability to regulate behavioral and biochemical alterations and restore diminished immune response in chronically stressed mice.[17,18] Moreover, recent research reported that chicoric acid was able to enhance glucose uptake in muscle cells and stimulate insulin secretion in Langerhans islets.[19] The chemical structure of chicoric acid is shown in Figure 1A. Despite so many beneficial bioactivities of chicoric acid reported in the literature, few studies have regarded the effects of chicoric acid on 3T3-L1 preadipocytes.

In the present study, the murine 3T3-L1 cell line was used because of its wide use as a cell model for adipose cell biology research for several decades.[20] The aim of this work was to investigate the molecular mechanisms underlying the proapoptotic effect of chicoric acid on 3T3-L1 preadipocytes. Our results suggest that chicoric acid induces intracellular reactive oxygen species (ROS) generation, subsequently regulated

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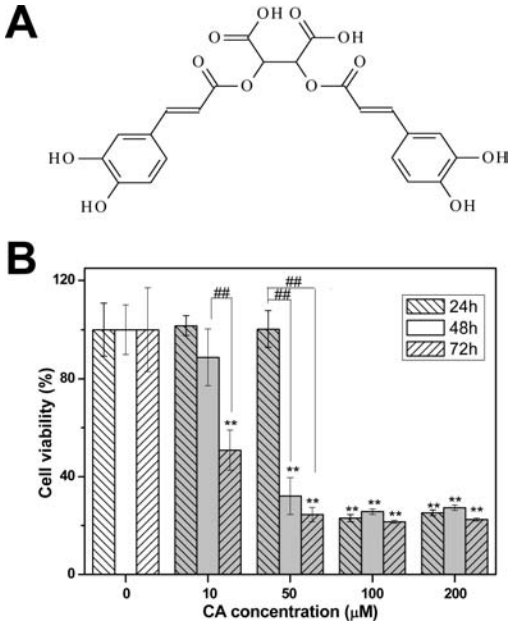

Figure 1. Chicoric acid inhibits cell viability in a dose- and time-dependent manner. (A) Chemical structure of chicoric acid. (B) 3T3-L1 preadipocytes were exposed to increasing concentrations of chicoric acid and cell viability was determined using MTT assay. "CA" means chicoric acid. Values are presented as the mean ± SD of three independent experiments. *$p < 0.05$ and **$p < 0.01$, compared with serum deprived cells (control groups).

PI3K/Akt and MAPK signaling pathways which lead to mitochondria-dependent apoptosis in 3T3-L1 preadipocytes.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle medium (DMEM), calf serum, and ECL chemiluminescence kit were purchased from Thermo Fisher Scientific Inc. (Shanghai, PR China). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), MAPKs inhibitors (SB203580, SP600125, and U0126), 2,7-dichloridihydrofluorescein (H_2DCFDA), NAC, and chicoric acid were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Chicoric acid was dissolved in dimethyl sulfoxide (DMSO) to 100 mM and stored at −20 °C. The concentrations used here were diluted with DMEM to final concentrations prior to use. 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), PI3K inhibitor (LY294002), 4′,6-diamidino-2-phenylindole (DAPI), acridine orange (AO), ethidium bromide (EB), cell mitochondria isolation kit, and antibody against COX IV were obtained from Beyotime Institute of Biotechnology (Nantong, Jiangsu, china). Antibodies against active caspase-3, cytochrome *c*, PARP, Bax, Bcl-2, heme oxygenase-1 (HO-1), cyclooxygenase-2 (COX-2), *β*-actin, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against Akt, p-Akt, extracellular signal-related kinase (ERK1/2), p-ERK1/2, p38 MAPK, p-p38 MAPK, c-jun NH2-terminal kinase (JNK), and p-JNK were supplied by Cell Signaling Technology Inc. (Danvers, MA, USA). All other chemicals were reagent grade.

Cell Culture. The cell line of murine 3T3-L1 preadipocyte was obtained from the Center of Cells, Peking Union Medical College, Chinese Academy of Medical Sciences (Beijing, China). The cells were routinely grown in DMEM, supplemented with 10% calf serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C in a humidified atmosphere with 5% CO_2, and medium was replaced every 2−3 days.

Cell Viability Assay. Cell viability was determined by the MTT assay. Briefly, cells were seeded onto 96-well plates at a seeding density of 4000 cells/well and cultured with 100 μL DMEM overnight. After experimental treatment, media were removed and cells were incubated in MTT solution (0.5 mg/mL in serum-free DMEM) for 4 h at 37 °C. The unreacted dye was removed, then the insoluble formazan crystals were dissolved by the addition 100 μL dimethyl sulfoxide (DMSO) to each well, and the plates were shaken for 10 min. The absorption, which was proportional to the number of live cells, was measured using a microplate reader (Bio-Rad, USA) at 490 nm. Control cells were arbitrarily assigned 100% viability. Six replicate wells were used for each data point in the experiments.

DAPI and AO/EB Staining. Cell apoptosis was evaluated by DAPI or AO/EB staining, respectively. In brief, the 3T3-L1 preadipocytes were incubated with 0, 10, 50, and 100 μM chicoric acid for 48 h. Then cells were washed with PBS twice and stained with DAPI (5 μg/mL) or AO/EB (50 μg/mL each in PBS) solutions in the dark at room temperature, respectively. After 30 min, the cells were observed under an inverted fluorescence microscope (Olympus LX71 Microscope) and photographed.

Measurement of Mitochondrial Membrane Potential (MMP). MMP was assessed using the fluorescent carbocyanine dye JC-1. Briefly, cells were treated with 0, 10, 50, and 100 μM chicoric acid for 48 h, washed with PBS, and then incubated with JC-1 (10 μg/mL) at 37 °C for 30 min. After washing out the excess probe, the fluorescence of the stained cells was observed using the inverted fluorescence microscope and photographed. Quantitation of the MMP was determined using SpectraMax M2 microplate reader (Molecular Devices, Menlo Park, California, USA) and presented as the relative ratio of aggregate to monomer (red/green) fluorescence intensity values.

Measurement of Intracellular ROS. Generation of intracellular ROS was monitored by fluorescence microscopy or flow cytometer using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent probe. This nonfluorescent compound can be oxidized by cellular oxidants into the highly fluorescent compound 2′,7′-dichlorofluorescin (DCF) which is trapped inside the cells.[21] Thus, the fluorescence intensity is proportional to the level of peroxide produced by the cells. Briefly, after treatment by different concentrations of chicoric acid for 24 h, 3T3-L1 preadipocytes were washed with PBS and then stained by 10 μM H_2DCFDA at 37 °C for 30 min. After the cells were washed twice with PBS, the fluorescence of DCF was detected by the inverted fluorescence microscope or CyFlow Cube flow cytometer (Partec GmbH, Münster, Germany).

Protein Extraction and Western Blot Analysis. After treatment for the time indicated, the cells were washed twice with ice-cold PBS and then lysed with lysis buffer (Beyotime, Jiangsu, China) containing 1% phenylmethylsulfonyl fluoride (PMSF) and 20 mM NaF. The whole-cell lysates were collected by scraping from the plates and then were centrifuged at 15 000 rpm for 10 min at 4 °C. Isolation of cytosol and mitochondria was performed using a Cell Mitochondria Isolation kit (Beyotime Institute of Biotechnology, Nantong, China) according to the standard procedure. Protein concentrations were determined using the BCA Protein Assay kit (Thermo Fisher, Shanghai, China). The protein samples (20−50 μg) were boiled with loading buffer for 10 min, subjected to SDS-PAGE, and transferred onto a polyvinylidene fluoride membrane (0.45 μm, Millipore) using a semidry electroblotting system (Bio-Rad, Shanghai, China). The membrane was blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris, 166 mM NaCl, and 0.05% Tween 20, pH 7.5) at room temperature for 2 h and washed three times with TBST buffer prior to incubation with primary specific antibodies (1:1000) in TBST buffer at 4 °C overnight. The membranes were then washed three times with TBST for 10 min each, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:2000) for an additional 2 h at 25 °C. Protein bands were detected using an ECL chemiluminescence kit (Thermo Fisher, Shanghai, China) on a Chemi Doc XRS Imaging system (Bio-Rad, Shanghai, China).

Statistical Analysis. Unless otherwise indicated, all data are expressed as means ± standard deviation (SD). Statistical differences were evaluated using ANOVA, and significant difference between the tested groups was determined using Duncan's posthoc test (SPSS 16.0). *P* values lower than 0.05 were considered significant.

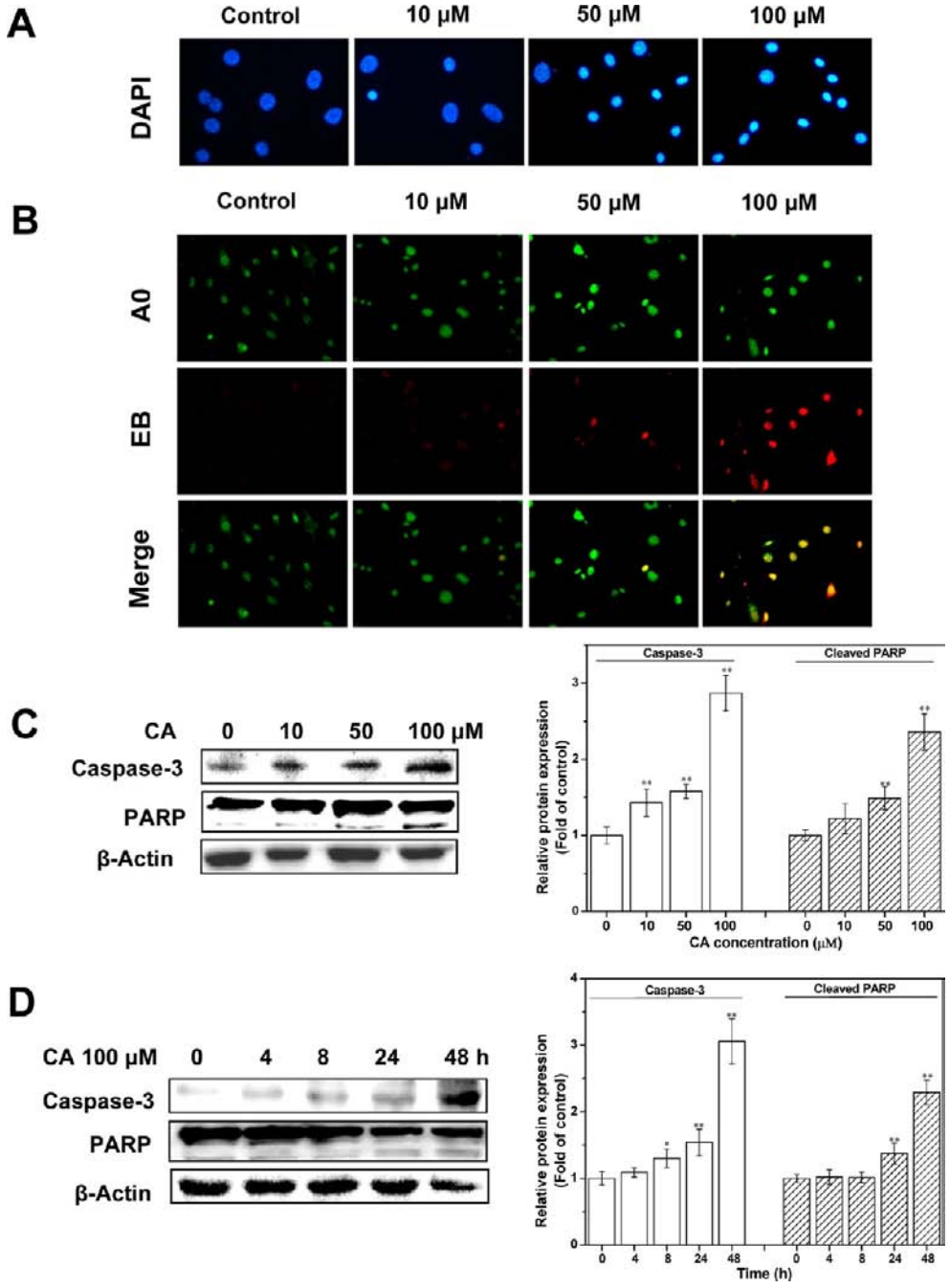

Figure 2. Chicoric acid induces caspase-3-dependent apoptosis. (A, B) Representative images of fluorescent microscopic analysis stained with DAPI (A) and AO/EB (B). After incubation with indicated concentrations of chicoric acid for 48 h, cells were stained with DAPI and AO/EB, respectively, and analyzed morphology characteristic of apoptosis. (C, D) Cells were treated with 0−100 μM chicoric acid for 48 h (C) or 100 μM chicoric acid for 0−48 h (D). Aliquots of cell lysates were separated by SDS-PAGE and analyzed for caspase-3 or PARP protein expression by Western blot as described under Materials and Methods, and the intensity of each band was quantified by densitometry analysis. β-Actin was employed as a loading control. "CA" means chicoric acid. The results shown here are representative of three independent experiments. *$p < 0.05$ and **$p < 0.01$, compared with control groups.

RESULTS

Chicoric Acid Effectively Reduces Viability of 3T3-L1 Preadipocytes. We first determined the effect of chicoric acid on cell proliferation using the MTT assay. As shown in Figure 1B, exposure to chicoric acid (10−200 μM) for 24, 48, and 72 h caused a dose- and time-dependent decrease in cell viability. There was no notable change in the viability of 3T3-L1 preadipocytes treated with 10−50 μM of chicoric acid for 24 h, but high concentrations of chicoric acid (100 μM and 200 μM) significantly decreased cell viability. The viability of 3T3-L1 preadipocytes treated with 10, 50, and 100 μM of chicoric acid for 48 h were approximately 88.81%, 32.08%, and 25.69% compared to control, respectively. No significant difference was observed in cell viability between the 100 μM and 200 μM chicoric acid treatment groups. Additionally, there were no significant changes in the growth of BRL-3A rat liver cells after treatment of 100 μM chicoric acid for 72h (data not shown).

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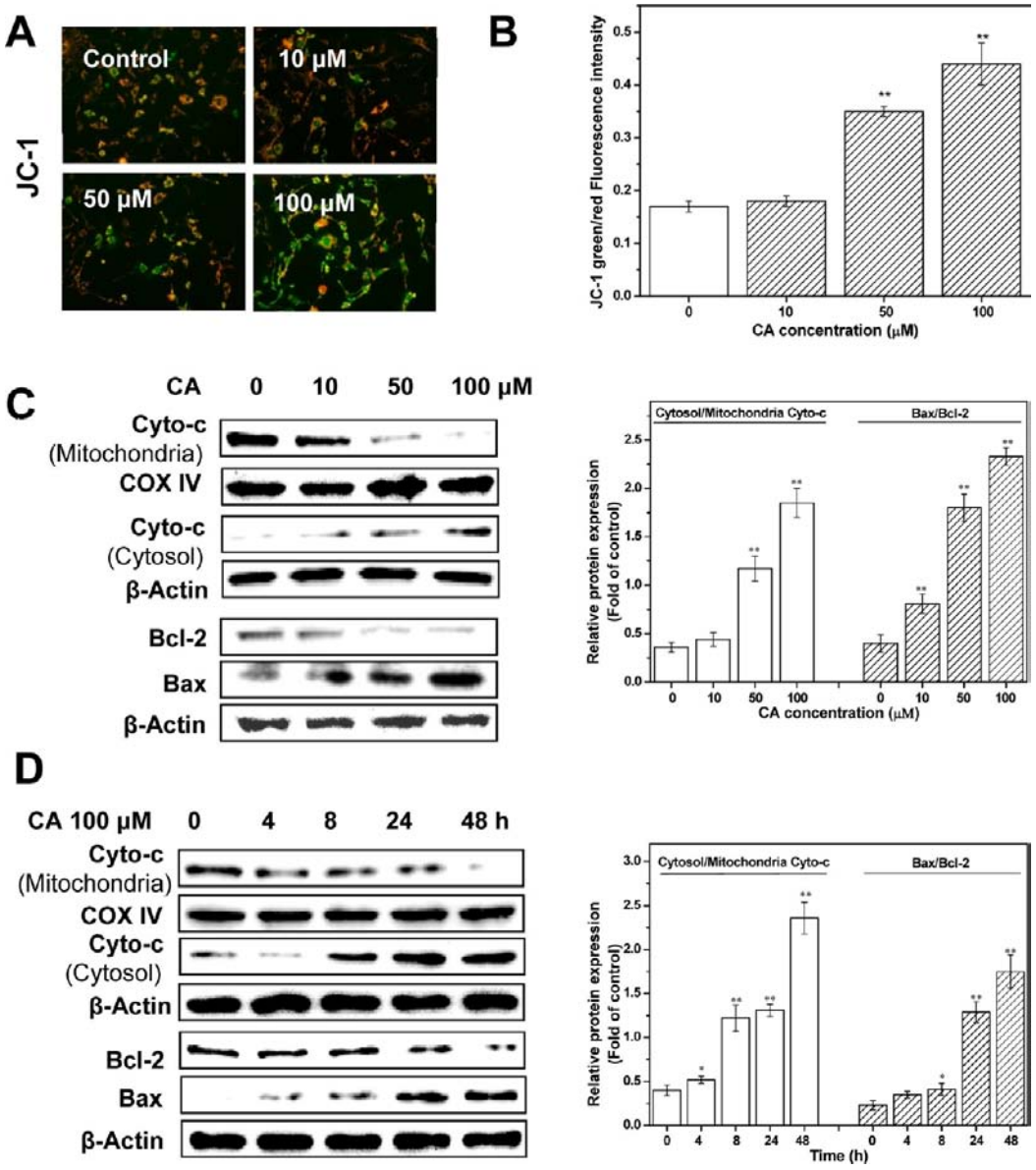

Figure 3. Chicoric acid induces mitochondria-dependent apoptosis. (A, B) Cells were seeded in 96-well plates overnight and then treated with 0−100 μM chicoric acid for 48 h. Mitochondrial membrane potential (MMP) in 3T3-L1 preadipocytes were determined by JC-1 staining. The graphs were obtained using an inverted fluorescence microscope (A) and quantitation of the MMP was determined using a SpectraMax M2 microplate reader (B). (C−F) Cells were treated with 0−100 μM chicoric acid for 48 h (C) or 100 μM chicoric acid for 0−48 h (D). Aliquots of cell lysates were separated by SDS-PAGE and analyzed for cytochrome *c*, Bcl-2, or Bax level by Western blot. β-Actin and COX IV were used as an internal control to monitor for equal loading. "CA" means chicoric acid. "Cyto-c" means cytochrome *c*. The results shown here are representative of three independent experiments. *$p < 0.05$ and **$p < 0.01$, compared with control groups.

According to the above results, the concentrations of chicoric acid used in the present work were no more than 100 μM.

Chicoric Acid Induces Apoptosis through Caspase-3-dependent Pathway. To investigate whether the reduction in cell number by chicoric acid involved apoptosis, chicoric acid-treated cells were stained using the fluorescent DNA-binding agents DAPI or AO/EB, respectively. Results showed that cells treated with 100 μM of chicoric acid for 48 h demonstrated typical characteristics of apoptosis such as cell shrinkage, chromatin condensation, and the increased permeability of cell membranes after DAPI and AO/EB staining (Figure 2A,B). These morphological changes suggest that apoptosis occurs in chicoric acid-treated 3T3-L1 preadipocytes, which was confirmed by PARP cleavage induced by chicoric acid (Figure 2C,D). Meanwhile, a dose- and time-dependent increase of cleaved caspase-3 (17 kDa) expression level was observed in chicoric acid-treated cells (Figure 2C,D). These results suggest that chicoric acid induces caspase-3-dependent apoptosis in 3T3-L1 preadipocytes.

Chicoric Acid Triggers Mitochondria-Dependent Apoptosis. To further investigate the molecular mechanism of chicoric acid-induced apoptosis, we next examined the effect of chicoric acid on mitochondrial function in 3T3-L1 preadipocytes. As illustrated in Figure 3A,B, treatment with increasing concentrations of chicoric acid induced a dose-dependent loss of MMP, measured by JC-1 fluorescence. In addition, we also found the dose- and time-dependent release of cytochrome *c* from the mitochondria into the cytosol, which

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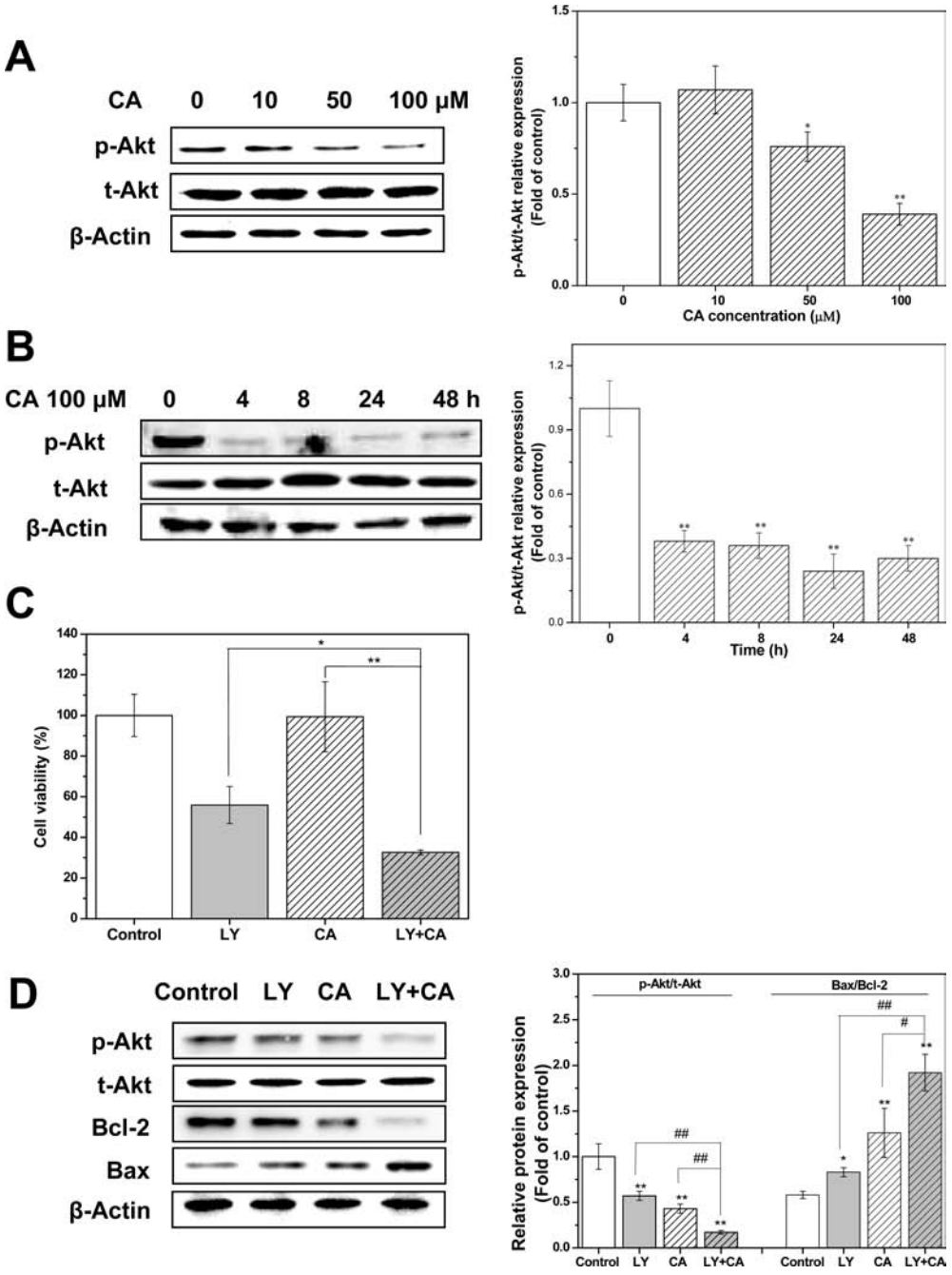

Figure 4. Chicoric acid induces Akt inactivation in 3T3-L1 preadipocytes. (A, B) Cells were treated with 0−100 μM chicoric acid for 48 h (A) or 100 μM chicoric acid for 0−48 h (B). Aliquots of cell lysates were separated by SDS-PAGE and analyzed for phosphorylated Akt (p-Akt) or total Akt (t-Akt) protein expression by Western blot. β-Actin was employed as a loading control. (C) Cells were treated with LY294002 (20 μM), chicoric acid (50 μM), or LY294002 (20 μM) plus chicoric acid (50 μM) for 24 h. Then the cell viability was determined using the MTT assay. The data are expressed as the means ± SD of three independent experiments. (D) Cells were incubated with LY294002 (20 μM) for 30 min prior to chicoric acid (100 μM) treatment for another 24 h. Aliquots of cell lysates were separated by SDS-PAGE and analyzed for p-Akt, t-Akt, Bcl-2, and Bax level by Western blot. β-Actin was used as an internal control to monitor for equal loading. "LY" means LY294002, "CA" means chicoric acid. The results shown here are representative of three independent experiments. *p < 0.05 and **p < 0.01, significantly differs the control groups.

was associated with the increase of the Bax/Bcl-2 ratio (Figure 3C,D). These data reveal that chicoric acid causes mitochondrial dysfunction in 3T3-L1 preadipocytes.

Akt Inactivation Mediates Chicoric Acid-Induced Apoptotsis. Several studies have demonstrated that the PI3K/Akt pathway plays a role in the regulation of apoptosis in tumor cells and 3T3-L1 preadipocytes.[22−24] To determine the role of PI3K/Akt pathway in chicoric acid-induced apoptosis, we measured the total and phosphorylated Akt levels in 3T3-L1 preadipocytes. Western blot analysis revealed the protein level of p-Akt was decreased by chicoric acid in a dose- and time-dependent manner. However, the protein level of total Akt was not affected by the presence of chicoric acid (Figure 4A,B). To further confirm the involvement of PI3K/Akt signaling in chicoric acid-induced cell apoptosis, cells were pretreated with LY294002 (20 μM), a representative PI3K

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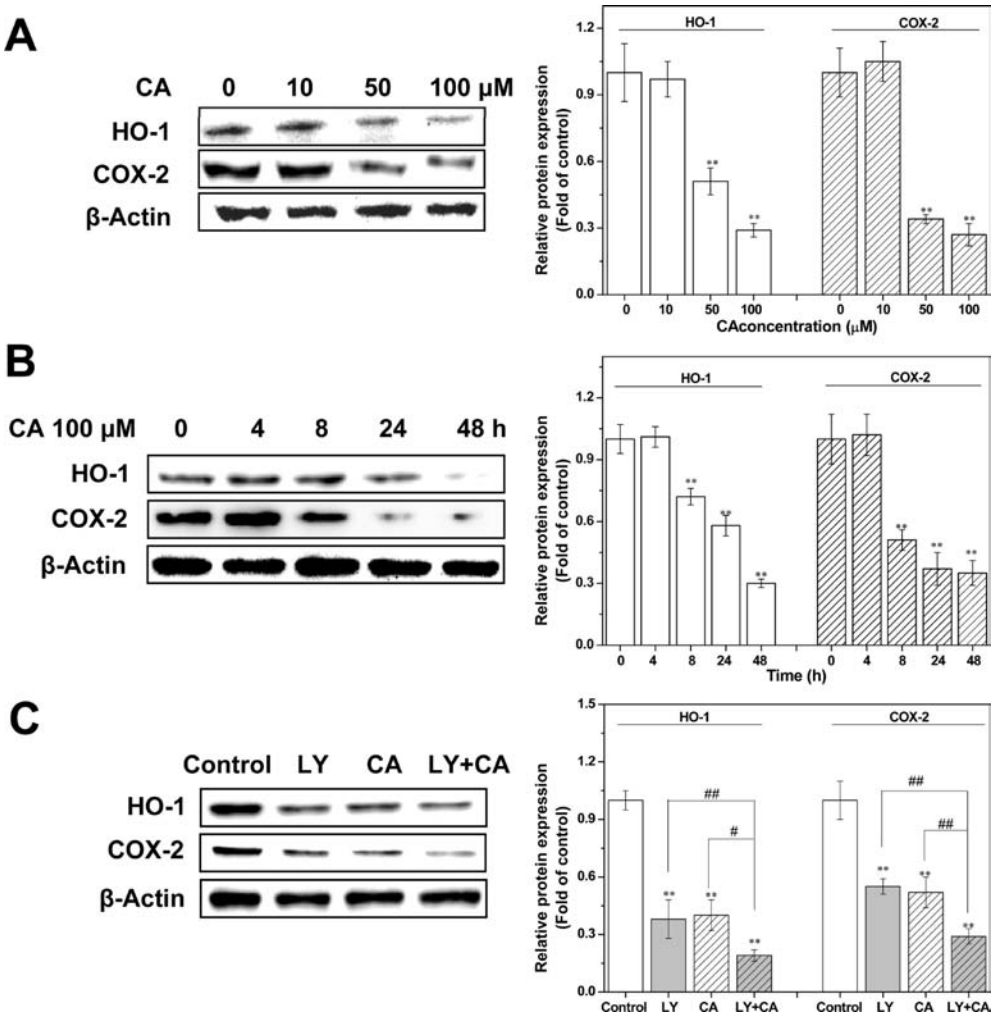

Figure 5. The down-regulation of HO-1 and COX-2 induced by chicoric acid is dependent on the PI3K/Akt pathway. (A, B) Cells were treated with 0−100 μM chicoric acid for 48 h (A) or 100 μM chicoric acid for 0−48 h (B). Aliquots of cell lysates were separated by SDS-PAGE and analyzed for HO-1 and COX-2 protein expression by Western blot. β-Actin was used as an internal control to monitor for equal loading. (C) Cells were incubated with LY294002 (20 μM) for 30 min prior to chicoric acid (100 μM) treatment for another 24 h. Aliquots of cell lysates were separated by SDS-PAGE and analyzed for HO-1 and COX-2 levels by Western blot. β-Actin was employed as a loading control. "LY" means LY294002, "CA" means chicoric acid. Representative blots of three independent experiments are shown. *p < 0.05 and **p < 0.01, significantly differs from the control groups.

inhibitor, for 30 min prior to the addition of chicoric acid (50 μM) that induced cell death slightly. As shown in Figure 4C,D, LY294002 significantly enhanced chicoric acid-induced decrease in cell viability and markedly increased Akt dephosphorylation in 3T3-L1 preadipocytes.

Activated Akt kinase has been proposed as a central role in suppressing apoptosis by modulating the activities of Bcl-2 family proteins. Thus, to ascertain the linkage between Akt inactivation and changes of Bcl-2 family proteins caused by chicoric acid, we assessed the effects of LY294002 on the levels of Bcl-2 and Bax. As shown in Figure 4D, LY294002 remarkably enhanced down-regulation of Bcl-2 and up-regulation of Bax induced by chicoric acid. These results indicate that chicoric acid induces mitochondria-dependent apoptosis through inhibiting Akt activation.

Chicoric Acid Induces HO-1 and COX-2 Down-Regulations through PI3K/Akt Pathway. HO-1 and COX-2 proteins have been reported to be expressed in 3T3-L1 preadipocytes and proved to be associated with apoptosis in other cells.[25,26] Thus, we examined the role of HO-1 and COX-2 in chicoric acid-induced apoptosis. Chicoric acid reduced the expression of HO-1 and COX-2 in a dose- and time-dependent manner (Figure 5A,B), and this effect was enhanced by pretreatment with LY294002 (Figure 5C). These results suggest that chicoric acid down-regulates the levels of HO-1 and COX-2 via PI3K/Akt pathway.

MAPK Signaling Pathways Are Involved in Chicoric Acid-Induced Apoptosis. In this study, we examined whether the phosphorylation of MAPKs was involved in chicoric acid-induced cell apoptosis and the relationship between p38 MAPK, JNK, and ERK1/2. As shown in Figure 6A,B, chicoric acid stimulated p38 MAPK phosphorylation as well as inhibited JNK and ERK1/2 phosphorylation in a dose- and time-dependent manner. Furthermore, the p38 MAPK inhibitor, SB203580, the JNK inhibitor, SP600125, and the ERK1/2 inhibitor, U0126, were applied to confirm the role of MAPKs in chicoric acid-induced cell apoptosis. As illustrated in Figure 6C−G, the decrease in cell viability and the increase of Bax/Bcl-2 ratio induced by chicoric acid was significantly blocked by SB203580 and enhanced by SP600125 and U0126,

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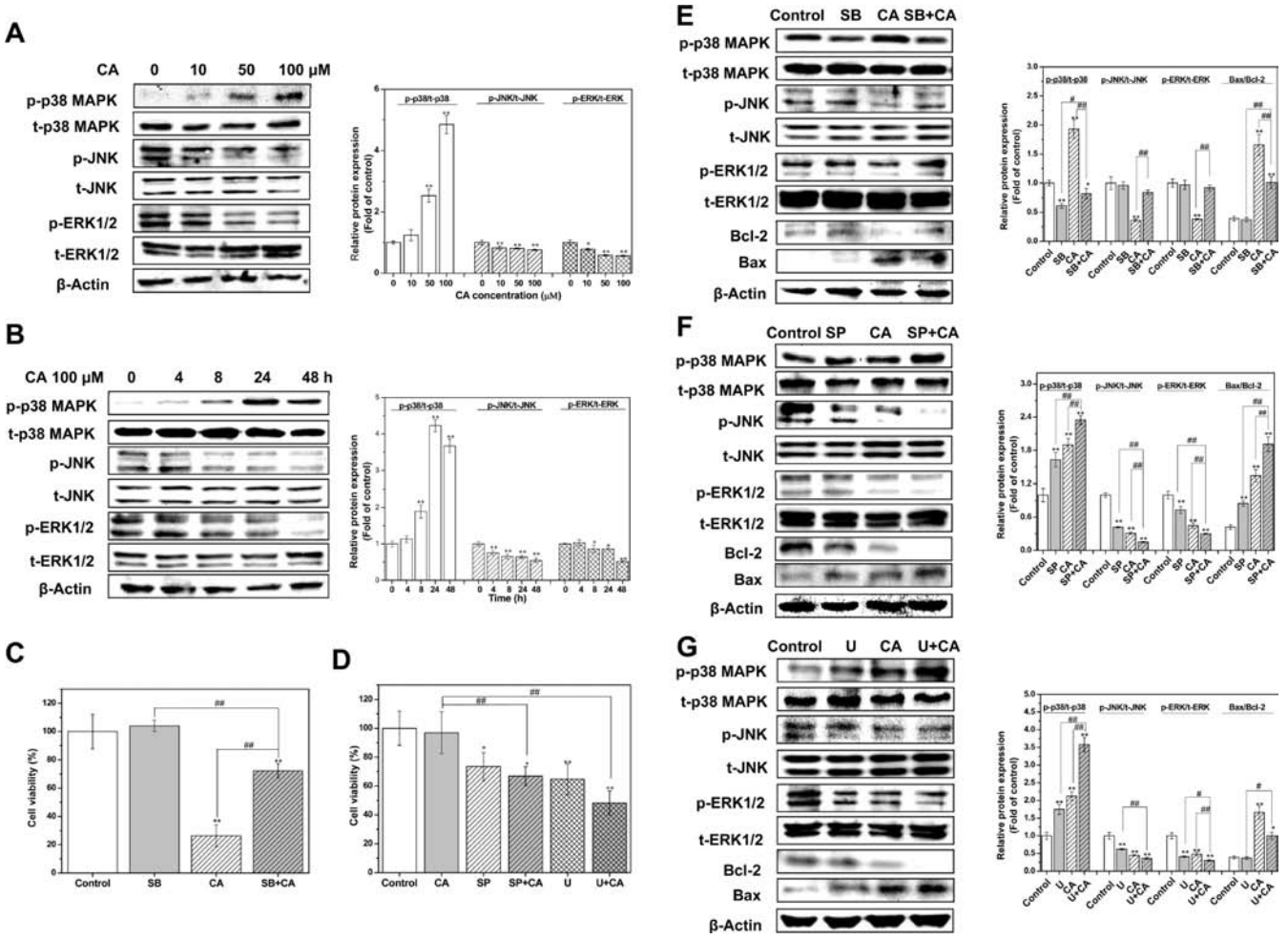

Figure 6. MAPK signaling pathways are involved in chicoric acid-induced apoptosis. (A, B) Cells were treated with 0−100 μM chicoric acid for 48 h (A) or 100 μM chicoric acid for 0−48 h (B). Aliquots of cell lysates were separated by SDS-PAGE. Phosphorylated p38 MAPK (p-p38 MAPK), total p38 MAPK (t-p38 MAPK), phosphorylated JNK (p-JNK), total JNK (t-JNK), phosphorylated ERK (p-ERK), and total ERK (t-ERK) were analyzed by Western blot. β-Actin was used as an internal control. (C) Cells were treated with SB253580 (20 μM), chicoric acid (100 μM), or SB253580 (20 μM) plus chicoric acid (100 μM) for 48 h. Then the cell viability was determined using the MTT assay. (D) Cell viability was measured by the MTT assay, 24 h after treatment with 50 μM chicoric acid in the presence or absence of 10 μM SP600125 or 20 μM U0126. The data are expressed as the means ± SD of three independent experiments. (E−G) Cells were incubated with 20 μM SB253580 (E), 10 μM SP600125 (F), or 20 μM U0126 (G) for 30 min prior to chicoric acid (100 μM) treatment for another 24 h. Aliquots of cell lysates were separated by SDS-PAGE and Western blot analysis was performed with antibodies against p-p38 MAPK, t-p38 MAPK, p-JNK, t-JNK, p-ERK, t-ERK, Bcl-2, and Bax. β-Actin was used as a loading control. "CA" means chicoric acid, "SB" means SB253580, "SP" means SP600125, and "U" means U0126. Representative blots of three independent experiments are shown. *p < 0.05 and **p < 0.01, significantly differs from the control groups.

respectively. Additionally, the effects of chicoric acid on MAPKs activation were also reduced by SB203580 and intensified by SP600125 and U0126, respectively (Figure 6E−G). Taken together, these results suggest that MAPKs which mutually interact are involved in the action of chicoric acid on 3T3-L1 preadipocytes.

Interplay between PI3K/Akt and MAPK Pathways in Chicoric Acid-Induced Apoptosis. To expand on our observations, we next measured the relationship between PI3K/Akt and MAPK pathways using several kinase inhibitors, including LY294002, SB203580, SP600125, and U0126. As shown in Figure 7A, treatment of SB203580 or chicoric acid individually decreased the level of p-Akt. Unexpectedly, the addition of chicoric acid after pretreatment of SB203580 significantly elevated the activation of Akt. Moreover, SP600125 and U0126 enhanced this effect of chicoric acid on Akt activation, respectively (Figure 7B,C). Reversely, treatment with LY294002 resulted in significant increases in chicoric acid-induced activation of p38 and inactivation of JNK and ERK (Figure 7D). These results imply the interplay between PI3K/Akt and MAPK signaling pathways in chicoric acid-induced apoptosis.

ROS Generation Is Responsible for PI3K/Akt and MAPK Signaling Pathways in Chicoric Acid-Induced Apoptosis. ROS play a central role in the regulation of cell apoptosis. In this work, we tested whether chicoric acid induced intracellular ROS generation and the potential role of ROS in mediating PI3K/Akt and MAPK signaling pathways. The results showed that in the cells treated with different concentrations of chicoric acid for 24 h, ROS generation increased in a dose-dependent manner (Figure 8A,B). To verify the role of ROS in chicoric acid-induced apoptosis, an ROS scavenger NAC (1 mM) was used. Data of the MTT assay showed that the decrease in the viability of 3T3-L1 preadipocytes by chicoric acid was significantly prevented by the addition of NAC (Figure 8C). We also validated that

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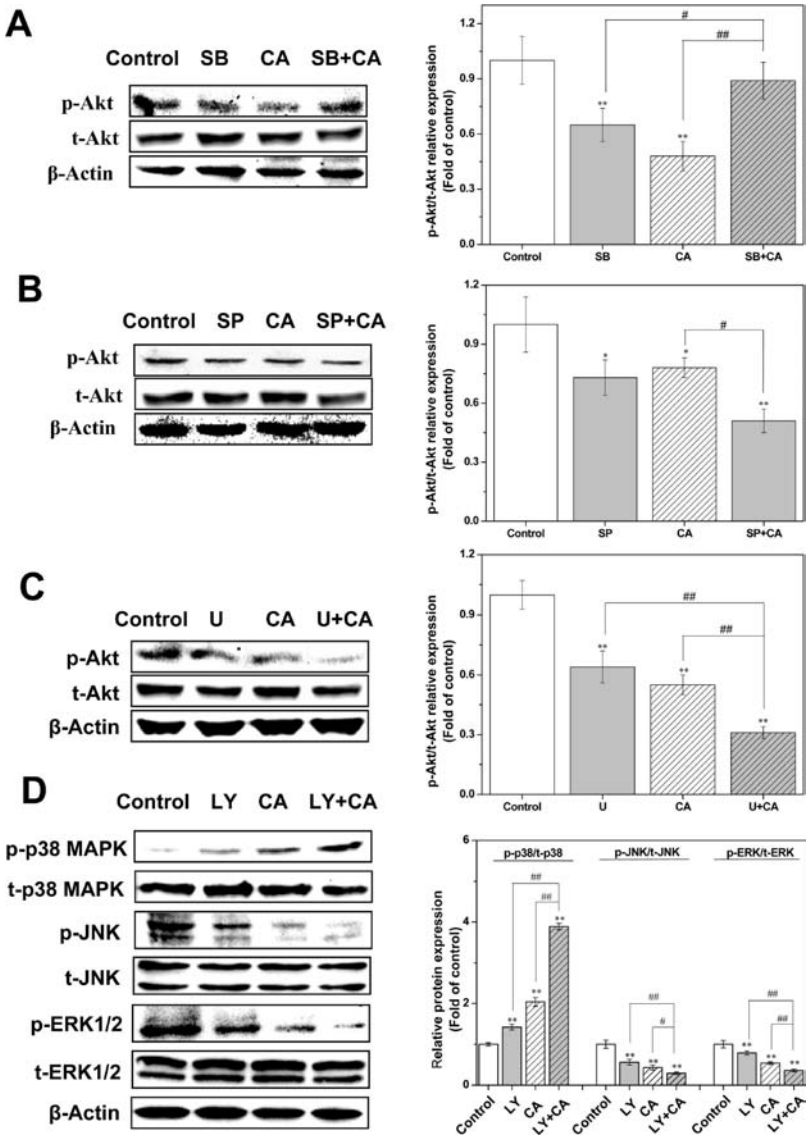

Figure 7. PI3K/Akt pathway interplays with MAPK pathways in chicoric acid-induced apoptosis. (A–C) Cells were incubated with 20 μM SB253580 (A), 10 μM SP600125 (B), or 20 μM U0126 (C) for 30 min prior to chicoric acid (100 μM) treatment for another 24 h. Thereafter, aliquots of cell lysates were analyzed by Western blot using antibodies against p-Akt and t-Akt. (D) Cells were incubated with LY294002 (20 μM) for 30 min prior to chicoric acid (100 μM) treatment for another 24 h. Aliquots of cell lysates were separated by SDS-PAGE and analyzed for p-p38 MAPK, t-p38 MAPK, p-JNK, t-JNK, p-ERK, and t-ERK levels by Western blot. β-Actin was used as a loading control. Representative blots of three independent experiments are shown. "CA" means chicoric acid, "SB" means SB253580, "SP" means SP600125, "U" means U0126, and "LY" means LY294002. *$p < 0.05$ and **$p < 0.01$, significantly differs from the control groups.

pretreatment with NAC attenuated the effects of chicoric acid on PI3K/Akt and MAPK signaling pathways (Figure 8D). These data suggest that ROS generation is required for PI3K/Akt and MAPK signaling pathways in chicoric acid-induced 3T3-L1 preadipocytes.

DISCUSSION

Phenolic compounds, widely distributed in the plant kingdom, have been reported to induce the apoptosis in 3T3-L1 preadipocytes and been used for the treatment of obesity.[27−30]

In this work, we first found that chicoric acid reduced cell viability in 3T3-L1 preadipocytes in a concentration- and time-dependent manner. Characteristic events in apoptosis including morphological changes and PARP cleavage were observed in chicoric acid-treated 3T3-L1 preadipocytes.

Apoptosis is triggered by two different death signaling pathways, such as the extrinsic pathway and the intrinsic pathway.[31] The intrinsic mitochondrial-dependent pathway which involves the disruption of outer mitochondrial membrane integrity was followed by the decrease of MMP, the release of cytochrome *c* and other proapoptotic molecules from the mitochondria to the cytosol.[32] Additionally, the Bcl-2 family proteins are the essential regulators of apoptosis through controlling mitochondrial permeability.[33] Mitochondria-dependent apoptosis has been determined in phenolic acids-treated 3T3-L1 preadipocytes and other cells.[29,34] Therefore, a possible mitochondrion-dependent apoptosis may reside in chicoric acid-treated 3T3-L1 preadipocytes. Further exploration of the inhibitory effects of chicoric acid in 3T3-L1 preadipocytes provided several evidence to confirm a role for a mitochondria-dependent apoptosis pathway, involving the

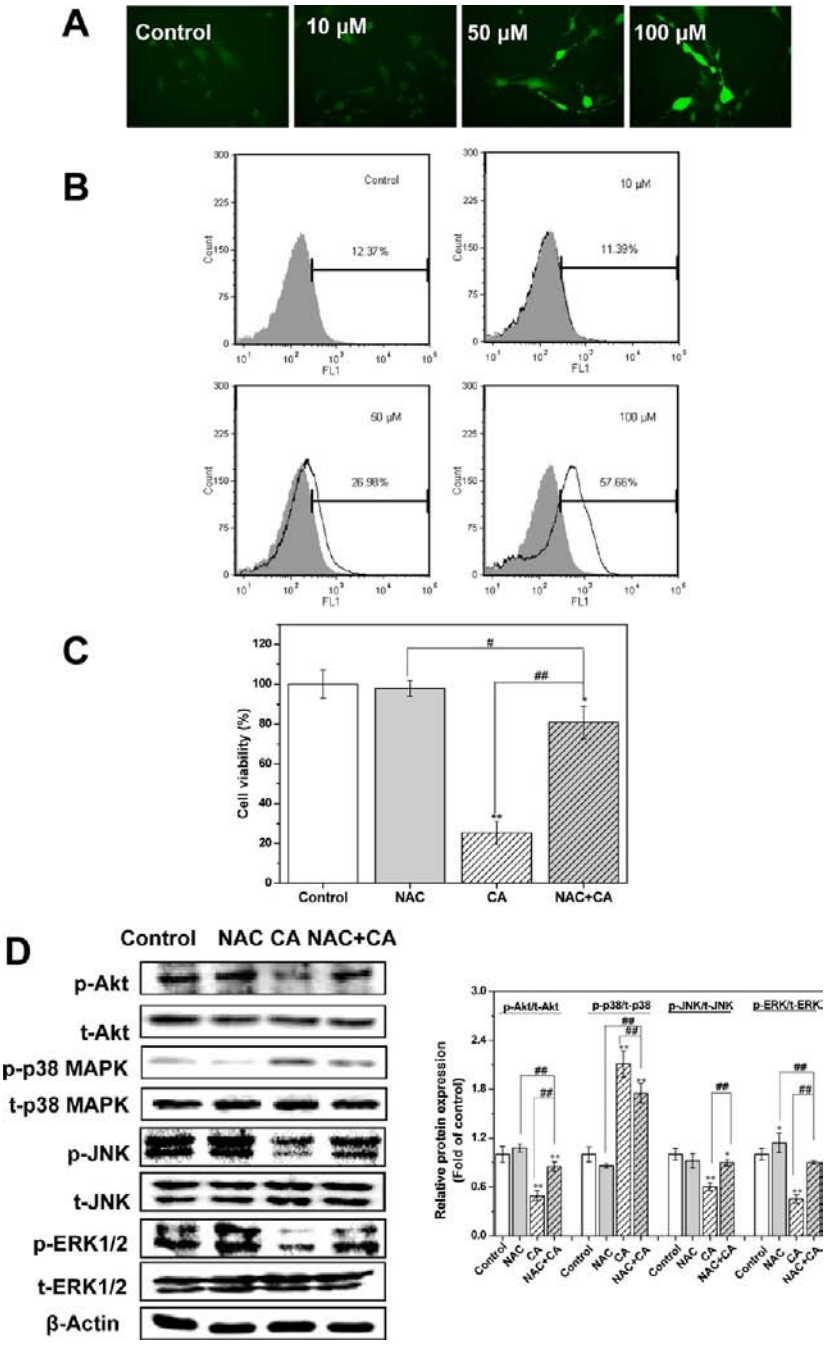

Figure 8. ROS mediate PI3K/Akt and MAPK signaling pathways in chicoric acid-induced apoptosis. (A, B) Cells were treated with different concentrations of chicoric acid for 24 h and then incubated with 10 μM H_2DCFDA for 30 min at 37 °C. The ROS were detected using inverted fluorescence microscope (A) or flow cytometer (B). (C) Cells were treated with or without chicoric acid (100 μM) in the presence or absence of NAC (1 mM) for 24 h. Then the cell viability was determined using the MTT assay. The data are expressed as the means ± SD of three independent experiments. *p < 0.05 and **p < 0.01, significantly differs from the indicated groups. (D) Cells were incubated with 1 mM NAC for 30 min prior to chicoric acid (100 μM) treatment for another 24 h. Then equal amounts of cell lysates were analyzed by Western blot using antibodies against p-Akt, t-Akt, p-p38 MAPK, t-p38 MAPK, p-JNK, t-JNK, p-ERK, and t-ERK. β-Actin was used as a loading control. "CA" means chicoric acid. Representative blots of three independent experiments are shown. *p < 0.05 and **p < 0.01, significantly differs from the control groups.

loss of MMP, the release of cytochrome *c* into the cytosol, the increase of Bax/Bcl-2 ratio, and the accumulation of active caspase-3.

Another goal of this study was to identify the upstream elements of mitochondria that are involved in chicoric acid-induced apoptosis. Akt, the major effector of the PI3K/Akt signal pathway, can inhibit the conformational change of the pro-apoptotic Bax protein and its translocation to mitochondria.[35] Moreover, the inhibition of PI3K/Akt pathway stimulates apoptosis in various cells.[22,23] A recent finding has shed light on the role of Akt in resveratrol-induced 3T3-L1 preadipocytes apoptosis.[24] In the current study, we found that chicoric acid inhibited Akt activation dose- and time-dependently. To confirm the role for Akt signaling in chicoric acid-induced apoptosis, the PI3K inhibitor LY294002 was used. LY294002 markedly promoted chicoric acid-induced cell death,

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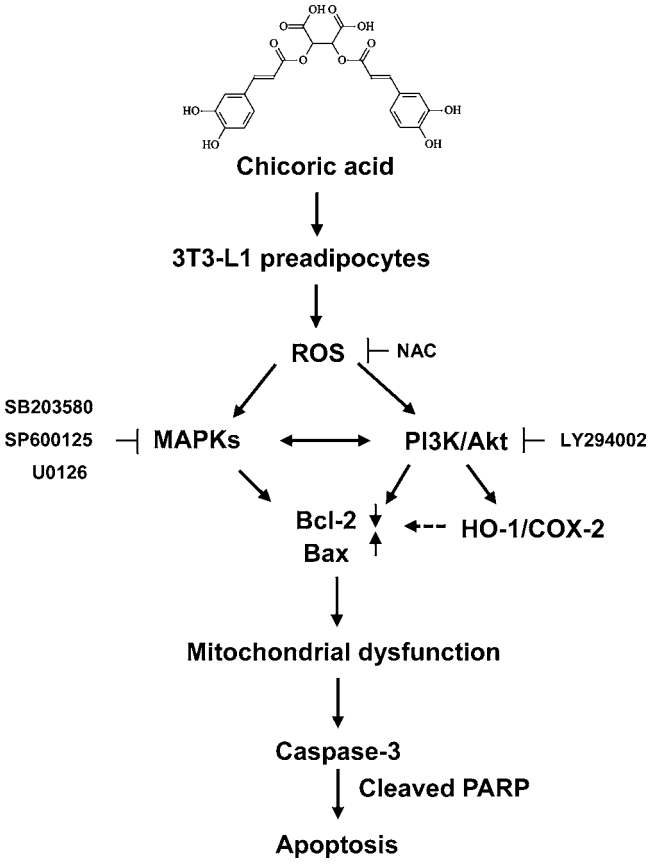

Figure 9. Proposed schematic diagram of chicoric acid-mediated apoptosis in 3T3-L1 preadipocytes. Chicoric acid induces ROS generation which regulates PI3K/Akt and MAPK signaling pathways and, in turn, causes the increase of the Bax/Bcl-2 ratio and promotes mitochondrial dysfunction followed caspase-3 activation. Finally chicoric acid induces PARP cleavage and apoptosis in 3T3-L1 preadipocytes. Chicoric acid also down-regulates the levels of HO-1 and COX-2 via PI3K/Akt pathway.

Akt inactivation, and the increase of the Bax/Bcl-2 ratio, implying that Akt signaling mediates the mitochondria-dependent apoptosis induced by chicoric acid.

HO-1 is a stress response protein and exerts antiproliferative and antiapoptotic effects.[36,37] The inducible enzyme COX-2 is overexpressed in many cancer cells, and the inhibition of COX-2 is associated with cancer cell apoptosis.[38,39] Furthermore, the expression of HO-1 and COX-2 can be regulated through the PI3K/Akt pathway.[40,41] However, it was unclear whether HO-1 and COX-2 played roles in chicoric acid-induced apoptosis and were regulated by the PI3K/Akt pathway. In the present study, we observed that chicoric acid dose- and time-dependently down-regulated the expression of HO-1 and COX-2. Moreover, LY294002 enhanced the effect of chicoric acid on HO-1 and COX-2. These results indicate that chicoric acid down-regulates HO-1 and COX-2 via PI3K/Akt signaling pathway. However, the downstream signal of HO-1 and COX-2 in this system remained to be investigated. It has been reported that HO-1 and COX-2 regulated the expression of Bcl-2 family members.[42,43] Therefore, it is speculated that HO-1 and COX-2 mediate chicoric acid-induced apoptosis through regulating the level of Bcl-2 family proteins. Further work should be performed to clarify this issue.

The members of MAPKs, p38 MAPK, ERK1/2, and JNK were also reported to participate in cell apoptosis.[44] ERK1/2 is generally associated with cell survival, whereas JNK and p38 MAPK are induced by stress responses and cytokines mediate differentiation and cell apoptosis.[45] Numerous research studies have revealed that MAPK signaling are involved in phenolic compound-induced cell apoptosis. In a recent study, carnosic acid, a rosemary phenolic compound, induced apoptosis in human neuroblastoma IMR-32 cells by activating p38 MAPK.[46] Rosmarinic acid induced apoptosis in human colorectal HCT15 cells via inhibiting ERK1/2 phosphorylation.[47] The proapoptotic effects of ursolic acid on human leukemia cells were associated with the activation of JNK.[48] Additionally, the treatment of (−)-epigallocatechin gallate (EGCG) in 3T3-L1 preadipocytes decreased the level of p-ERK1/2, but the expression of p-p38 MAPK and p-JNK was not changed.[49] Our results showed that chicoric acid treatment also caused activation of p38 MAPK in 3T3-L1 preadipocytes. However, unlike EGCG, the inactivation of JNK and ERK1/2 was observed in chicoric acid-induced cells. The effects of chociric acid on cell death and MAPKs were suppressed by inhibitor of p38 MAPK (SB203580) and promoted by inhibitors of JNK (SP600125) and ERK1/2 (U0126), respectively. In addition, the interaction between MAPKs was found in this work.

Previous research reported that p38 MAPK inhibitor SB203580 blocked Akt-associated signaling pathways and then inactivated Akt.[50−52] A similar effect of SB203580 was observed in our study. Meanwhile, our results showed that the treatment of chicoric acid also inhibited the activation of Akt. However, we found that the cotreatment of SB203580 and chicoric acid significant elevated the level of p-Akt. The potential explanation of this unusual outcome might be that chicoric acid achieved its effects on p-Akt through different ways. One was that chicoric acid inhibits Akt activation based on our results. The other was that chicoric acid reversed the inhibitory effect of SB203580 on p-p38, and in turn, the activation of p38 resulted in the concurrent activation of Akt which had been proved in a previous study.[53] However, the later pathway played a dominant role in this process. Further investigations are needed to verify these speculations. Moreover, chicoric acid-stimulated Akt inactivation was enhanced by SP600125 and U0126, respectively. Interestingly, we also observed that the inhibition of Akt activation resulted in a significant increase in chicoric acid-stimulated phosphorylation of p38 and dephosphorylation of JNK and ERK. These findings support the conclusion that MAPK signaling pathways interplay with the PI3K/Akt signaling pathway in chicoric acid-induced apoptosis.

Low concentration of ROS is important in keeping redox balance and cell proliferation.[54] However, excessive ROS accumulation induces protein oxidation, lipid peroxidation, and DNA damage in cells, followed by cell death or apoptosis.[55,56] Consistent with previous reports, which have revealed that phenolic acids provoke ROS generation in HepG2 cells and IMR-32 cells,[46,57] our results showed that chicoric acid induced ROS generation in 3T3-L1 preadipocytes. Furthermore, pretreatment of ROS scavenger, NAC, significantly blocked chicoric acid-induced cell death, confirming the involvement of ROS in this process. It has been demonstrated that ROS-mediated apoptosis is modulated by Akt and MAPK signaling pathways in HepG2 cells and U-937 cells, respectively.[6,58] Therefore, we hypothesized that ROS might play a key role in regulating PI3K/Akt and MAPK signaling pathways in chicoric acid-induced apoptosis, which was verified using NAC in the present study.

In conclusion, our findings suggest that chicoric acid could inhibit proliferation and induce apoptosis in 3T3 preadipocytes. Chicoric acid induces ROS generation which modulates PI3K/Akt and MAPK signaling pathways, and in turn, up-regulates the Bax/Bcl-2 ratio, activates mitochondrion-mediated pathway including loss of MMP, release of cytochrome *c* and activation of caspase-3, and finally results in cleavage of PARP and cell apoptosis (summarized in Figure 9). This study clarifies a previously unknown mechanism for chicoric acid-induced apoptosis in 3T3-L1 preadipocytes which provides evidence that chicoric acid might be a potential therapeutic tool to treat or prevent obesity. Further research needs to be carried out to explore the binding sites and binding mechanism of chicoric acid in 3T3-L1 preadipocytes.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods; Supplementary Figure 1: The cytotoxicity of chicoric acid on BRL-3A cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DAPI, 4′,6-diamidino-2-phenylindole; AO/EB, acridine orange/ethidium bromide; PARP, poly ADP-ribose-polymerase; MMP, mitochondrial membrane potential; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; MAPKs, mitogen-activated protein kinases; JNK, c-jun N-terminal kinase; ERK1/2, extracellular signal-related kinase; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; HO-1, heme oxygenase-1; COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; PMSF, phenylmethylsulfonyl fluoride

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