Grifolic acid induces mitochondrial membrane potential loss and cell death of RAW264.7 macrophages

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Abstract. Grifolic acid is a phenolic compound that was first extracted from the mushroom *Albatrellus confluens*; it acts as an agonist of the free fatty acid receptor (FFAR4). FFAR4 is expressed in macrophages and mediates the anti-inflammatory effects of n-3 unsaturated free fatty acids. In the present study, the effects of grifolic acid on macrophages were observed in mouse RAW264.7 cells. It was demonstrated that grifolic acid (2.5-20 μmol/l) treatment reduced RAW264.7 cell viability in a dose- and time-dependent manner. The number of apoptotic cells significantly increased following grifolic acid treatment compared with the untreated control cells. Grifolic acid treatment resulted in a significant decrease in cellular adenosine 5'-triphosphate (ATP) content in RAW264.7 cells. Mitochondrial membrane potential (MMP), as measured by JC-1 staining, was significantly diminished by grifolic acid treatment in a dose- and time-dependent manner. Treatment with cyclosporine A, a protector of MMP, attenuated grifolic acid-induced reduction of MMP and viability in RAW264.7 cells. FFAR4 knockdown did not significantly influence grifolic acid-induced reduction of cell viability, ATP levels or MMP. In conclusion, grifolic acid may induce macrophage cell death by reducing MMP and by inhibiting ATP production probably in an FFAR4-independent manner.

Introduction

Free fatty acid receptor 4 (FFAR4) is activated by long chain free fatty acids (FFAs), and its activation serves diverse roles in regulating hormone secretion, inflammatory responses and cell survival (1-3). Previous studies that examined the functions of FFAR4 identified a series of non-FFA agonists, and among them was grifolic acid (2,4,5). Grifolic acid is a phenolic compound that was initially isolated from the fruiting bodies of the mushroom *Albatrellus confluens*. The pharmacological actions of grifolic acid have not been fully characterized, except for its ability to activate FFAR4. Grifolic acid was reported to activate extracellular signal-regulated kinase (ERK) responses and to stimulate an increase in intracellular calcium concentrations [(Ca^{2+}_i)] in FFAR4-expressing cells, but not in FFAR1-expressing cells (2). In addition, grifolic acid exhibited stimulatory effects on glucagon-like peptide-1 secretion in mouse enteroendocrine STC-1 cells that express FFAR4 endogenously (2,6). Conversely, grifolic acid inhibited the α-linolenic acid-induced ERK and $[Ca^{2+}]_i$ responses in FFAR4-expressing cells (2). Grifolic acid was also revealed to attenuate lard oil-induced secretion of glucose-dependent insulinotropic polypeptide (GIP) from FFAR4-expressing K cells of the upper small intestine (7).

FFAR4 is expressed in pro-inflammatory $CD11c^+$ macrophages, in mouse RAW264.7 macrophages and in primary intraperitoneal macrophages (8). FFAR4 activation was previously reported to inhibit the lipopolysaccharide (LPS)-stimulated phosphorylation of inhibitor of nuclear factor-κB kinase subunit β and c-Jun N-terminal kinase and subsequently inhibited the secretion of tumor necrosis factor-α and interleukin (IL)-6 in RAW264.7 cells (8). Another previous study demonstrated that FFAR4 activation promoted the actions of cytosolic phospholipase A2 and cyclooxygenase-2 in RAW264.7 cells and in human primary monocyte-derived macrophages, which in turn led to prostaglandin E_2 release, nuclear factor (NF)-κB inhibition and the inhibition of LPS-induced IL-6 secretion (9). Therefore, it is clear that FFAR4 is expressed in macrophages and has anti-inflammatory effects; however, the effects of grifolic acid on the function of macrophages remain unknown. In the present study, the effects of grifolic acid on macrophages were investigated and an FFAR4-independent function was identified. These finding demonstrated a novel action of grifolic acid on cells, and it is recommended that care should be taken when grifolic acid is used as FFAR4 agonist.

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Materials and methods

Chemicals. Grifolic acid was obtained from R&D Systems Inc. (Minneapolis, MN, USA). Rabbit anti-FFAR4 polyclonal antibody (cat. no. SAB4501490) and cellular adenosine 5'-triphosphate (ATP) assay kit were purchased from Sigma-Aldrich (Merck KGaA; Darmstadt, Germany). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining kits were purchased from BD Pharmingen (BD Biosciences, San Jose, CA, USA). Mouse FFAR4 Silencer Select siRNA (cat. no. S200889), Silencer Negative Control siRNA (cat. no. AM4613), Lipofectamine® RNAiMAX reagent, Opti-MEM medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), JC-1 dye and rabbit anti-b-actin polyclonal antibody (cat. no. PA1-183) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture. Mouse RAW264.7 cells were purchased from ATCC (Manassas, VA, USA) and cultured in a humidified incubator with 5% CO_2 at 37˚C in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The medium was changed every 3 days, and RAW264.7 cells were subcultured at 80% confluency and seeded to plates or dishes for each experiment.

Cell viability assay. RAW264.7 cells were cultured in 96-well plates and treated with grifolic acid (1.25-20 μmol/l for 2-24 h at 37˚C) at 90% confluency in serum-free medium. The control cells were treated with the solvent without grifolic acid. Subsequently, MTT was added into medium (0.5 mg/ml) and cells were incubated for 4 h at 37˚C. The medium was discarded and 100 μl isopropanol with 0.01 M HCl was added to each well and plates were agitated to fully dissolve MTT crystals. The absorbance of each well was measured at 560 nm with an ELISA microplate reader (Thermo Fisher Scientific, Inc.). The background absorbance at 690 nm was also measured and subtracted from the values measured at 560 nm. The experiments were repeated three times.

Flow cytometry. The Annexin V-FITC/PI Apoptosis Detection kit was used to measure cell death. Following incubation with 0 μmol/l (control), 10 or 20 μmol/l grifolic acid, RAW264.7 cells were detached from dishes by 0.05% trypsin/EDTA. Cells were washed with cold PBS and resuspended in the binding buffer supplied in the kit at 1x10^6 cells/ml. Annexin V-FITC and PI were added to cell suspension at a dilution of 1:20 and incubated for 15 min at room temperature in the dark. Subsequently, cells were diluted in binding buffer and analyzed by flow cytometry with the CFlow Plus software (BD Biosciences), and the $FITC^+/PI^+$ double-positive cells were counted. The experiments were repeated three times.

Cellular ATP content measurement. RAW264.7 cells (1x10^7 cells per sample) were treated with grifolic acid (10 or 20 μmol/l for 2 h). Subsequently, cells were lysed and intracellular ATP levels were measured using ATP assay kits (Sigma-Aldrich; Merck KGaA). Briefly, cell lysates from different groups were obtained by incubating with detergent and agitation at ~12 Hz for 5 min at room temperature. The constituted substrate solutions given in the kits were added to each sample and incubated for 5 min in the dark. The luminescence of each sample was measured with a luminescence plate reader (Thermo Fisher Scientific, Inc.). ATP levels of each sample were calculated according to the constructed standard curve. The total protein of each sample was extracted using the radioimmunoprecipitation lysis buffer from the Beyotime Institute of Biotechnology (Beijing, China) and quantified by bicinchoninic acid (BCA) assay. The cellular ATP content was corrected to the total protein levels. The experiments were repeated three times.

Measurement of mitochondrial membrane potential (MMP). RAW264.7 cells (5x10^5 cells/well) were plated into 35 mm dishes and stained with JC-1 (5 μg/ml) in serum-free medium for 15 min at 37˚C. Subsequently, cells were washed with serum-free medium, and the fluorescence was recorded prior to (0 min) and following grifolic acid treatment every 10 min using a Leica TCS SP8 confocal microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA) in live cell station at 37˚C. For cyclosporine A treatment, the cells were incubated with 20 μmol/l cyclosporine A for 10 min prior to stimulation with grifolic acid. The red fluorescence (excitation 560 nm; emission 600 nm) and green fluorescence (excitation 488 nm; emission 535 nm) were measured synchronously, and the ratio of fluorescence intensity for each cell was analyzed by LAS LITE version 3.3 (Leica Microsystems, Inc., Buffalo Grove, IL, USA). The experiments were repeated three times.

FFAR4 siRNA transfection. RAW264.7 cells were grown to 80% confluency at the time of transfection. The mouse FFAR4 siRNA (100 pmol, Thermo Fisher Scientific, Inc.) or negative control siRNA (100 pmol; Thermo Fisher Scientific, Inc. was diluted in 50 μl Opti-MEM medium (Thermo Fisher Scientific, Inc.), and Lipofectamine RNAiMAX (1 μl; Thermo Fisher Scientific, Inc.) was diluted in 50 μl Opti-MEM, and they were incubated for 15 min at room temperature. Subsequently, the siRNA and Lipofectamine RNAiMAX solutions were mixed and incubated for 15 min at room temperature to allow complexes to form. The complexes were added to each well at a dilution of 1:4. Cells were incubated at 37˚C in a humidified 5% CO_2 incubator for 24 h to induce gene knockdown. Following 24 h incubation with Opti-MEM medium the cells were used for experiments. The experiments were repeated three times.

Western blot analysis. Total protein was extracted from RAW264.7 cells (1x10^7 cells per sample) using the ReadyPrep Protein Extraction kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified by bicinchoninic acid assay. Proteins (40 μg per sample) were separated using a 12% SDS-PAGE and were transferred to nitrocellulose membranes using a Trans-Blot SD semi-dry electrophoresis transfer cell (Bio-Rad Laboratories, Inc.). Membranes were subsequently blocked at room temperature with 5% skimmed milk for 2 h at room temperature. Then membranes were incubated with a rabbit-anti FFAR4 antibody (1:1,000) or rabbit-anti β-actin antibody (1:1,000) at 4˚C, overnight. Following three washes with TBS, the membranes were incubated with a horseradish

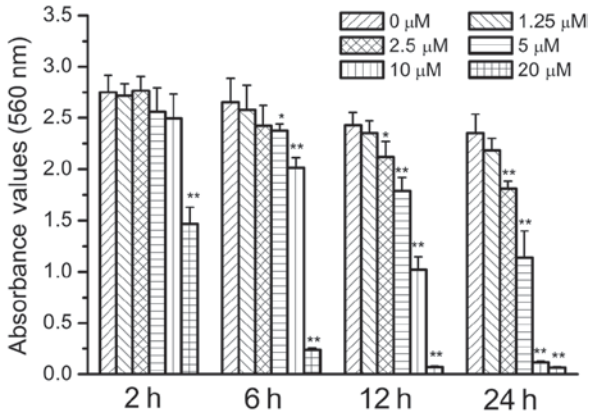

Figure 1. Effects of grifolic acid on RAW264.7 cell viability. Grifolic acid treatment inhibited the viability of RAW264.7 cells, in a dose- and time-dependent manner. n=24; *P<0.05, **P<0.01 vs. Control without grifolic acid.

peroxidase-conjugated goat-anti rabbit immunoglobulin G antibody (1:1,000; cat. no. 31460; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Following three washes with TBS, Clarity Max™ Western enhanced chemiluminescence Substrate (Bio-Rad Laboratories, Inc.) was added and membranes were incubated at room temperature for 4 min. The luminescence from the membranes was imaged by a ChemiDoc MP gel imaging and analysis system (170-8280; Bio-Rad Laboratories, Inc.). The experiments were repeated three times.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Comparisons of means of multiple groups with each other or against one control group were analyzed with one-way analysis of variance followed by Bonferroni post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of grifolic acid on RAW264.7 cell viability. Grifolic acid at 20 μmol/l significantly inhibited RAW264.7 cell viability at 2 h and led to almost total loss of cell viability following 6 h incubation (P<0.01). Observations were obtained at 2, 6, 12 and 24 h for each concentration following preliminary experiments, which demonstrated that these incubation lengths were optimal for use in the present study (data not shown). Grifolic acid at 10 μmol/l exhibited significant inhibition of viability following 6 h incubation and achieved maximal effects at 24 h (P<0.05). Grifolic acid at 5 and 2.5 μmol/l inhibited RAW264.7 cell viability from 6 and 12 h, respectively and demonstrated significantly increased levels with the increased duration of incubation Treatment with grifolic acid at 1.25 μmol/l did not exhibit inhibitory effects at any time points investigated (Fig. 1).

Grifolic acid-treated RAW264.7 cell death. Based on the MTT assay results, at 10 and 20 μmol/l grifolic acid demonstrated more significant effects on RAW264.7 cells and were used in the experiments of Annexin V/PI staining. Observations were obtained at 2 and 6 h for each concentration following preliminary experiments, which demonstrated that longer incubations resulted in cell lysis whereas little effect was observed at shorter incubation times (data not shown). Grifolic acid (20 μmol/l) treatment for 2 or 6 h lead to a significant increase in the number of both Annexin V- and PI-staining positive RAW264.7 cells compared with the control cells that were not treated with grifolic acid (P<0.01; Fig. 2A-D). Similarly, grifolic acid treatment at 10 μmol/l for 6 or 12 h also demonstrated a significant increase in the number of Annexin V-FITC$^+$/PI$^+$ cells compared with the control cells (P<0.01; Fig. 2E).

Inhibition of ATP production by grifolic acid in RAW264.7 cells. The cellular ATP content in untreated control RAW264.7 cells was 32.31±5.32 nmol/mg protein. ATP content significantly reduced to 10.29±4.76 nmol/mg protein when cells were treated with 20 μmol/l grifolic acid for 2 h (P<0.01). Grifolic acid treatment at 10 μmol/l for 2 h also significantly reduced cellular ATP content to 18.26±4.41 nmol/mg protein (P<0.01; Fig. 3).

Effects of FFAR4 knockdown on grifolic acid-induced RAW264.7 cell death. Mouse FFAR4 siRNA transfection resulted in a notable reduction in FFAR4 protein expression level compared with the untransfected control cells (Fig. 4A). FFAR4 knockdown did not exhibit a significant influence on grifolic acid-induced inhibition of cell viability and apoptosis, as indicated by MTT assay and Annexin V-FITC/PI staining (Fig. 4B and C, respectively). Similarly, the decrease in cellular ATP content in grifolic acid-treated RAW264.7 cells was not affected by FFAR4-knockdown (Fig. 4D).

Effects of grifolic acid on MMP of RAW264.7 cells. JC-1 emits green fluorescence in the cytoplasm and exhibits membrane potential-dependent accumulation in mitochondria with a shift of emission wavelength from green to red (10,11); MMP reduction is indicated by a decrease in the red/green fluorescence intensity ratio. In the present study, grifolic acid treatment resulted in a decrease in red/green fluorescence intensity ratio in a dose- and time-dependent manner (Fig. 5). Cells treated with 20 μmol/l grifolic acid exhibited a decrease in MMP at 5 min, with the maximal effects achieved within 20 min (Fig. 5E). Grifolic acid treatment at 10 μmol/l significantly reduced MMP at 10 min and achieved the maximal effects in 40 min (Fig. 5E). MMP was also significantly decreased compared with the control 60 min following treatment with 5 and 2.5 μmol/l grifolic acid at 40 and 50 min, respectively, but not with 1.25 μmol/l treatment (Fig. 5E).

Protective effects of cyclosporine A against grifolic acid-induced MMP loss and Raw264.7 cell death. Cyclosporine A is able to attenuate mitochondrial permeability transition and improve mitochondrial respiratory function (12,13). Therefore, the effects of cyclosporine A on the grifolic acid-induced decrease in MMP were investigated. When RAW264.7 cells were pretreated with cyclosporine A at 20 μmol/l for 10 min, grifolic acid-induced MMP loss was significantly attenuated compared with control cells that were treated with grifolic acid but not with cyclosporine A (P<0.01; Fig. 6A). Untreated control cells demonstrated no changes during measurement (data not shown).

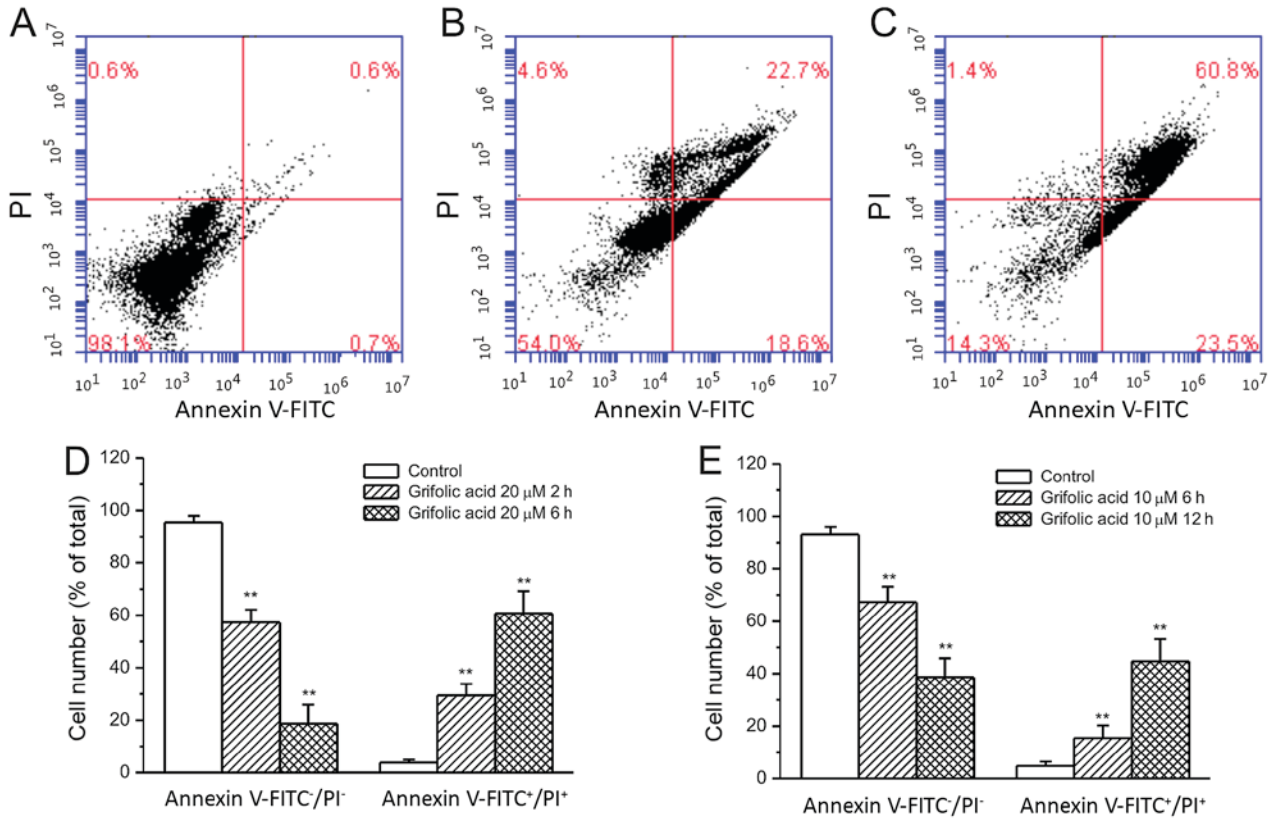

Figure 2. Grifolic acid-induced cell death in RAW264.7 cells. Grifolic acid treatment leads to a significant increase in the number of RAW264.7 cells that are positively stained with both Annexin V-FITC and PI. Representative results of flow cytometric analysis for the (A) control cells, (B) cells treated with 20 μmol/l grifolic acid for 2 h and (C) cells treated with 20 μmol/l grifolic acid for 6 h and (D) their quantification. (E) Cells treated with 10 μmol/l grifolic acid for 6 and 12 h also exhibited a significant increase in Annexin V-FITC$^+$/PI$^+$ RAW264.7 cells compared with the control. n=5; **P<0.01 vs. Control. FITC, fluorescein isothiocyanate; PI, propidium iodide.

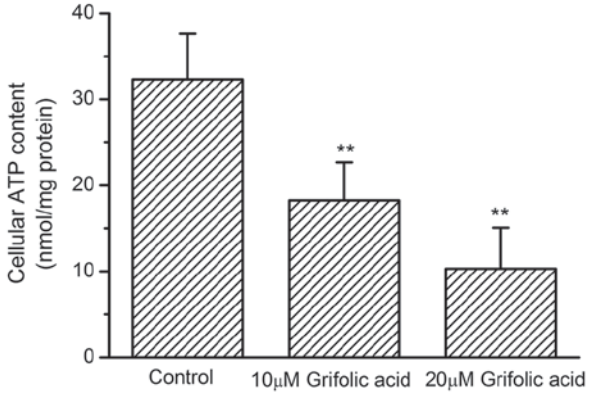

Figure 3. Effects of grifolic acid on cellular ATP content in RAW264.7 cells. Cells treated with either 10 or 20 μmol/l grifolic acid for 2 h exhibited significant reductions in cellular ATP content. n=12; **P<0.01 vs. Control, n=12. ATP, adenosine 5' triphosphate.

In addition, cyclosporine A treatment (10 μmol/l) also inhibited grifolic acid-induced decrease in cell viability of RAW264.7 cells (Fig. 6B).

Discussion

Grifolic acid was first extracted from the fresh fruiting bodies of the mushroom *A. confluens*, but little is known about its actions. Grifolic acid was previously demonstrated to activate ERK and to increase intracellular calcium concentration in FFAR4-expressing cells though FFAR4 signaling (2). A number of other reports also revealed grifolic acid's actions on FFAR4 in FFAR4-expressing mouse intestinal STC-1 cells and K cells that secrete GIP. Grifolic acid was also used to antagonize the effects of FFAs on FFAR4 (7). Therefore, grifolic acid is considered as an agonist or antagonist for FFAR4. FFAR4 is expressed in macrophages including RAW264.7 cells (14). Results from the present study demonstrated that grifolic acid treatment induced mouse RAW264.7 macrophage apoptosis. However, the effects of grifolic acid on cell death were unaltered by the inhibition of FFAR4 expression in RAW264.7 cells. Therefore, the present study suggested that grifolic acid is not a pure FFAR4 agonist or antagonist, and may have additional pharmacological targets to inhibit cell viability.

The effects of grifolic acid to induce RAW264.7 cell death may be due to the decrease in ATP production following damage to mitochondrial functions. Grifolic acid-treated RAW264.7 cells exhibited a significant increase in the percentage of Annexin V-FITC/PI double-positive cells, which indicated necrosis or late stage of apoptosis, but this needs to be confirmed. It is known that cellular ATP levels influence cell viability (15,16). Cellular ATP reduction leads to cell death, and the rate and speed of ATP reduction determines the type of cell death (17). A slow reduction of ATP levels leads to cell apoptosis, whereas rapid reduction of ATP results in cell swelling and necrosis (15,18,19). Grifolic acid induced a

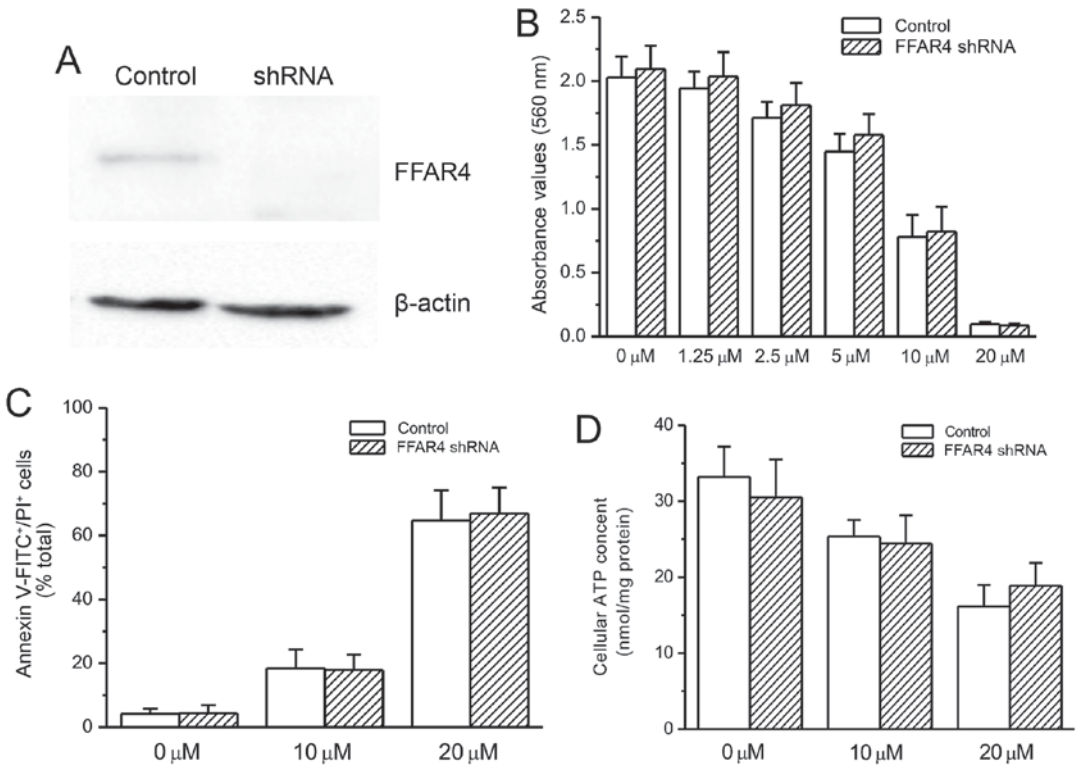

Figure 4. Effects of FFAR4 knockdown on grifolic acid-induced RAW264.7 cell death. (A) Knockdown of FFAR4 expression following siRNA transfection, as indicated by western blotting. (B) FFAR4-siRNA transfection did not affect the grifolic acid treatment-reduced cell viability. (C) Grifolic acid-induced increase in Annexin V-FITC$^+$/PI$^+$ double-staining (late apoptosis/necrosis) was not affected by FFAR4 knockdown. (D) Grifolic acid treatment at either 10 or 20 μmol/l for 2 h reduced cellular ATP content with no significant difference observed between FFAR4 knockdown and control cells. ATP, adenosine triphosphate; FITC, fluorescein isothiocyanate; FFAR4, free fatty acid receptor 4; PI, propidium iodide.

decrease in ATP levels in 2 h in RAW264.7 cells, which may lead to cell necrosis.

Mitochondria are the cell organelles responsible for ATP production (20). To function under physiological conditions, mitochondria need to maintain MMP; a reduction in MMP may lead to functional damage of mitochondria and may result in deficiency of ATP production (21). In the present study, it was demonstrated that grifolic acid significantly reduced MMP of RAW264.7 cells in a dose- and time-dependent manner, which is in accordance with the effects of grifolic acid on RAW264.7 cell death. This is the first study, to the best of the authors' knowledge, to demonstrate the influence of grifolic acid on mitochondria, which indicated that mitochondria may be a target of grifolic acid. The improvement of grifolic acid-induced reduction of MMP and cell death by cyclosporine A further supported the observation that grifolic acid affected mitochondrial function. Mitochondria form permeability transition pores on the membrane under certain pathological conditions, which reduce MMP and lead to a release of cell death regulators (22,23). Cyclosporine A may prevent the formation of permeability transition pores on the mitochondrial membrane by acting on cyclosporine A-binding protein in mitochondria (24-27). Cyclosporine A treatment significantly attenuated the grifolic acid-induced decrease in MMP and protected RAW264.7 cells against grifolic acid-induced cell death. The attenuation of grifolic acid-induced MMP reduction by cyclosporine A, the mitochondria protector, further indicated that mitochondria may be the target of grifolic acid. In conclusion, grifolic acid exposure damaged mitochondrial function, which resulted in MMP reduction and led to the decrease in ATP production causing RAW264.7 cell death.

The effects of grifolic acid on RAW264.7 cell death appear to be independent of FFAR4. FFAR4 is expressed in macrophages such as RAW264.7 cells, and its activation serves an anti-inflammatory role in macrophages (8,28,29). In the present study, FFAR4 expression was successfully inhibited as confirmed by western blot. Knockdown of FFAR4 did not demonstrate any influence on grifolic acid-induced cell death and MMP reduction. Therefore, FFAR4 may not take part in the action of grifolic acid causing RAW264.7 cell death. The molecular mechanism of grifolic acid-induced damage to mitochondria remains unknown and needs to be clarified in the future.

In summary, grifolic acid treatment reduced MMP and ATP production and led to RAW264.7 cell death. Considering that the concentration of grifolic acid used in the present study was within the same range as what was used to activate FFAR4 (2,6,7,30), it was suggested that grifolic acid may not be a pure FFAR4 agonist. Other pharmacological target for grifolic acid that links grifolic acid to cell mitochondria dysfunction remains to be clarified.

Acknowledgements

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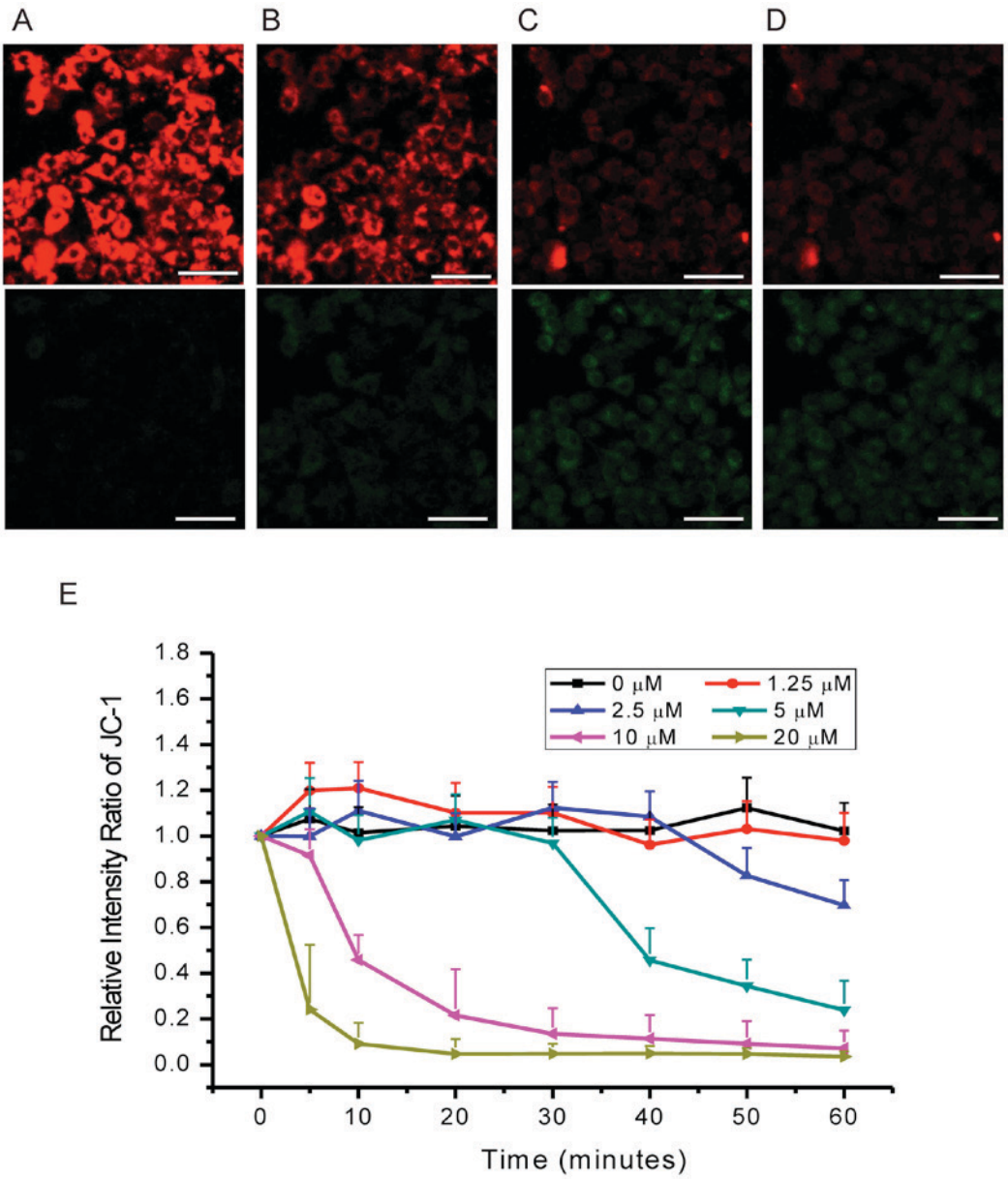

Figure 5. Effects of grifolic acid on MMP in RAW264.7 cells. The cellular intensity of MMP indicator JC-1 in (A) control and following 20 μmol/l grifolic acid treatment for (B) 5, (C) 10 and (D) 20 min in a continuous observation. (E) Statistical analysis of red/green fluorescence intensity ratio in each cell. n=100. MMP, mitochondrial membrane potential.

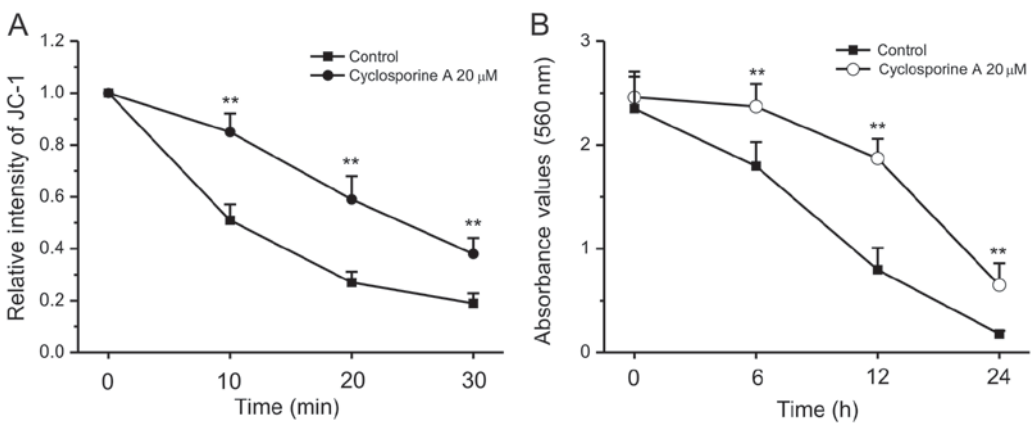

Figure 6. Effects of cyclosporine A treatment on grifolic acid-induced MMP reduction and cell viability in RAW264.7 cells. (A) Cyclosporine A inhibited grifolic acid-induced decreases of MMP of RAW264.7 cells. n=60; **P<0.01 vs. the group treated with grifolic acid alone. (B) Cyclosporine A inhibited grifolic acid-induced reduction of cell viability. n=24; **P<0.01 vs. the group treated with grifolic acid alone. MMP, mitochondrial membrane potential.

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