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Original Article

Wogonin ameliorates lipotoxicity-induced apoptosis of cultured vascular smooth muscle cells via interfering with DAG-PKC pathway

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Aim: To investigate the effects of wogonin (5,7-dihydroxy-8-methoxyflavone) extracted from *Scutellaria baicalensis* Georgi (*S baicalensis*) on lipotoxicity-induced apoptosis of vascular smooth muscle cells (VSMCs) and the underlying mechanisms.
Methods: Cultured VSMCs were used. Apoptosis of VSMCs was induced by palmitate (0.75 mmol/L), and detected using TUNEL assay. The expression levels of protein and phosphorylated protein were measured using Western blot analysis.
Results: Treatment of VSMCs with wogonin (10, 25 and 50 μmol/L) significantly attenuated the apoptosis and endoplasmic reticulum (ER) stress induced by palmitate in concentration- and time-dependent manners. Wogonin (50 μmol/L) decreased palmitate-induced reactive oxygen species (ROS) generation. The ER stress inhibitor 4-phenyl butyric acid (5 mmol/L) significantly decreased palmitate-induced apoptotic cells, and occluded the anti-apoptotic effect of wogonin (25 μmol/L). Wogonin (10, 25 and 50 μmol/L) significantly reduced the intracellular diacylglycerol (DAG) accumulation and expression levels of phosphorylated PKCs in palmitate-treated VSMCs.
Conclusion: Our results suggest that wogonin inhibits lipotoxicity-induced apoptosis of VSMCs via suppressing the intracellular DAG accumulation and subsequent inhibition of PKC phosphorylation. Wogonin has therapeutic potential for the prevention and treatment of atherosclerosis.

Keywords: wogonin; atherosclerosis; vascular smooth muscle cells; apoptosis; endoplasmic reticulum stress; palmitate; diacylglycerol (DAG); PKC

Introduction

Diabetes mellitus is a major contributor to cerebrovascular and cardiovascular disease morbidity and mortality worldwide. Increased free fatty acid (FFA) levels in the plasma caused lipotoxicity, which is a hallmark of diabetes mellitus and leads to an increased risk of atherosclerosis and cardiovascular diseases[1–3]. Strong evidence has suggested that elevated FFA levels in the plasma enhance the intracellular accumulation of diacylglycerol (DAG), which leads to the activation of protein kinase Cs (PKCs), inhibitor kappaB kinase (IKK), or c-Jun N-terminal kinase (JNK), resulting in atherosclerotic plaque development and instability[4, 5]. Therefore, the plasma FFA and intracellular DAG levels represent selective targets to prevent atherosclerosis.

Vascular smooth muscle cells (VSMCs) play a pivotal role in the initiation and early progression of atherosclerosis and plaque rupture[6, 7]. Due to the important function of synthesizing components of the fibrous cap in plaques, VSMCs are responsible for promoting plaque stability in advanced atherosclerotic lesions. Published evidence has shown that VSMC apoptosis precipitates a number of deleterious consequences in atherosclerosis, such as plaque rupture[7–12]. Therefore, anti-apoptotic therapies for VSMCs may benefit the prevention and treatment of atherosclerosis[8, 10].

Scutellaria baicalensis Georgi (*S baicalensis*) is a traditional Chinese herb (Baikal Skullcap) that is widely used for the treatment of inflammation, infection, cancer, hypertension, and cardiovascular disease[13, 14]. Wogonin (5,7-dihydroxy-8-methoxyflavone) is a major bioactive component of the flavonoids from *S baicalensis*. Pharmacological findings have highlighted the therapeutic potential regarding the use of plant-derived wogonin to modulate endothelial cell and VSMC function for the prevention and treatment of atherosclerosis[15–17]. However, the underlying mechanism is poorly understood.

In this study, our results show that wogonin attenuates lipotoxicity-induced apoptosis and inhibits endoplasmic reticulum (ER) stress by suppressing intracellular DAG accumulation, which perturbs the DAG/PKC pathway in cultured VSMCs. This novel finding supplies evidence for the potential administration of wogonin for the treatment of atherosclerosis.

Materials and methods
Materials and reagents
Biochemical reagents were obtained from the following sources: palmitic acid, tunicamycin, wogonin ($C_{16}H_{12}O_5$) (Figure 1), 4-phenyl butyric acid (4-PBA) from Sigma (St Louis, MO, USA); the anti-CHOP antibody, anti-cleaved caspase-3 antibody, anti-cleaved caspase-6 antibody, anti-eIF2α antibody, anti-phosphorylated-eIF2α Ser51 antibody, anti-Bcl-2 antibody, anti-Bax antibody, PKC isoform antibody sampler kit, and phosphorylated-PKC isoform antibody sampler kit from Cell Signaling Technology; and secondary antibodies that were conjugated to alkaline phosphatase from Promega. The APO-BrdUTM TUNEL Assay Kit was purchased from Invitrogen. The ELISA Kit for DAG was obtained from Uscn Life Science, Inc (Wuhan, China).
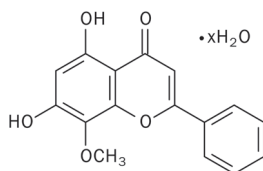
Figure 1. Chemical structure of wogonin.

Cell culture and treatment
Vascular smooth muscle cells (SV40LT-SMC Clone HEP-SA, ATCC CRL-2018™) were cultured in growth medium (Dulbecco's modified Eagle's medium with 4 mmol/L *L*-glutamine, which was adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, 0.2 mg/mL G418, and 10% bovine calf serum). A10 VSMCs (ATCC CRL-1476™) were incubated in DMEM (ATCC 30-2002) containing 10% FBS and 1.0 g/L sodium bicarbonate. The culture conditions were 37.0 °C, 95% O_2, and 5% CO_2. For treatment, the cells were serum-starved for 6 h and then incubated with palmitate and/or wogonin for the desired exposure time. The preparation of palmitate was as described in our previous study[18]. Briefly, palmitic acid was dissolved in ethanol, mixed with 20% BSA and then incubated overnight at 4 °C. The solution was filtered, stored at -20 °C and used within 2 weeks. The same concentration of ethanol was mixed with 20% BSA and used as a control.

Western blot analysis
The cells were lysed with lysis buffer (50 mmol/L Hepes, pH 7.6, 150 mmol/L NaCl, 1% Triton X-100, 10 mmol/L NaF, 20 mmol/L sodium pyrophosphate, 20 mmol/L β-glycerol phosphate, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin,

10 μg/mL aprotinin, and 1 mmol/L phenylmethanesulfonyl fluoride). The cell lysates were incubated on ice for 10 min and then centrifuged at 14 000×*g* for 10 min at 4 °C. The supernatants were mixed with equal volumes of 2×SDS-PAGE sample loading buffer. After heating at 95 °C for 4 min, the proteins were separated using a SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected with the aforementioned antibodies.

Apoptosis determination
Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) was performed to detect cells undergoing apoptosis according to the manufacturer's protocol. Briefly, VSMCs were treated with palmitate and/or wogonin, washed with cold PBS three times and then fixed with 1% of paraformaldehyde on ice for 1 h. After washing three times with PBS, the cells were treated with 70% ethanol and incubated at -20 °C for 24 h. The cells were washed with wash buffer 3 times and incubated in DNA-labeling solution (including terminal deoxynucleotidyl transferase enzyme and BrdU triphosphate) at 37 °C for 1 h. The cells were rinsed with PBS, collected by centrifugation, and incubated in anti-BrdU-staining mix for 45 min at room temperature. The apoptotic nuclei containing nicked DNA were stained brown. To calculate the apoptosis rate, 1000 nuclei were identified in 20 random high power fields per slide.

Extraction and measurement of DAG
Total DAG levels were measured using an ELISA Assay Kit (Uscn Life Science Inc, Wuhan, China) according to the manufacturer's protocol. Briefly, the serum-starved VSMCs (1×10^6 cells/well in 6-well dishes) were incubated with or without different doses of palmitate and/or wogonin for the desired time. Total cell lipids were extracted with chloroform:methanol (1:2, *v/v*) after centrifugation at 5000×*g* for 2 min[19]. The lower chloroform phase was analyzed for DAG contents.

Measurement of reactive oxygen species
Intracellular reactive oxygen species (ROS) production was measured using the method of Shaw *et al*[20]. Briefly, VSMCs were plated in a 24-well plate at a density of 2×10^4 cells/well in DMEM, serum-starved for 6 h, and treated with or without different doses of palmitate and/or wogonin for the desired time. The cells were washed with modified Eagle's medium without phenol red and incubated in the dark for 10 min in Krebs-Ringer solution containing 50 μmol/L DCHF diacetate. Changes in fluorescence intensity were determined using an Flx-800 microplate fluorescence reader (Bio-Tek Instruments) at excitation and emission wavelengths at 485 and 528 nm, respectively[19, 20].

Statistical analysis
Three independent experiments were performed with each sample in triplicate. The data were expressed as the mean±SEM. Statistical analysis was performed using analysis

of variance followed by Student's *t*-test for paired data. $P<0.05$ was considered significant. The figures are representative of at least three independent experiments with similar results.

Results

Effects of wogonin on palmitate-induced apoptosis

Wogonin modulates endothelial cell and VSMC functions to promote its anti-atherosclerotic effects[15–17]. Considering the pivotal roles of VSMC apoptosis[7–12] and the elevated plasma FFA levels[4, 5] in atherosclerotic plaque ruptures, we investigated the potential effects of wogonin on palmitate-induced VSMC apoptosis. The serum-starved VSMCs were treated with different doses of wogonin for the desired time in the presence of 0.75 mmol/L palmitate for 24 h. As shown in Figure 2A and 2B, wogonin significantly attenuated the palmitate-induced apoptosis in a dose- and time-dependent manner. We evaluated the expression levels of cleaved caspase-3 and caspase-6 because activation of the caspase-3 pathway is a hallmark of apoptosis[21–23]. We found that wogonin inhibited the expression of cleaved caspase-3 and caspase-6 in a dose- and time-dependent manner in VSMCs that were pre-treated with 0.75 mmol/L palmitate (Figure 2C and 2D). The anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins are among many key regulators of apoptosis. Therefore, we investigated the effects of wogonin on these regulatory proteins. We treated A10 VSMCs with 0.75 mmol/L palmitate for 12 h followed by treatment with 25 μmol/L wogonin for an additional 12 h. We found that wogonin restored Bcl-2 expression and decreased Bax expression, which consequently restored the ratio of Bcl-2 to Bax (Figure 3). These data suggest that wogonin protects VSMCs from palmitate-induced apoptosis.

Effects of wogonin on palmitate-induced ER stress

To identify the signaling pathway that initialized apoptosis, VSMCs were serum-starved by culturing in serum-free medium. Serum-starved VSMCs were treated with wogonin in a dose- and time-dependent manner in culture medium with 0.75 mmol/L palmitate. The levels of CHOP expression and eIF2α phosphorylation for these cells were analyzed using the Western blot analysis as stated under the Materials and methods. As shown in Figure 4A and 4B, palmitate-induced CHOP expression and eIF2α phosphorylation were suppressed by wogonin in a dose- and time-dependent manner similar to that observed in wogonin-mediated suppression of cleaved caspase-3 and caspase-6 expression and apoptosis. Apoptosis is initialized by extrinsic (activated by death ligands), intrinsic (mitochondrial pathway), or ER stress pathways[21–23]. We treated VSMCs to discover the potential anti-apoptotic effects of wogonin so that we could determine whether ER stress mediated the inhibitory effects of wogonin on apoptosis. 4-PBA (5 mmol/L) was used to inhibit ER stress in A10 VSMCs that were treated with 0.75 mmol/L palmitate and/or 25 mmol/L wogonin. We found that administration of 4-PBA significantly decreased the number of apoptotic cells in A10 VSMCs that were treated with palmitate and that wogonin treatment did not further enhance the inhibitory
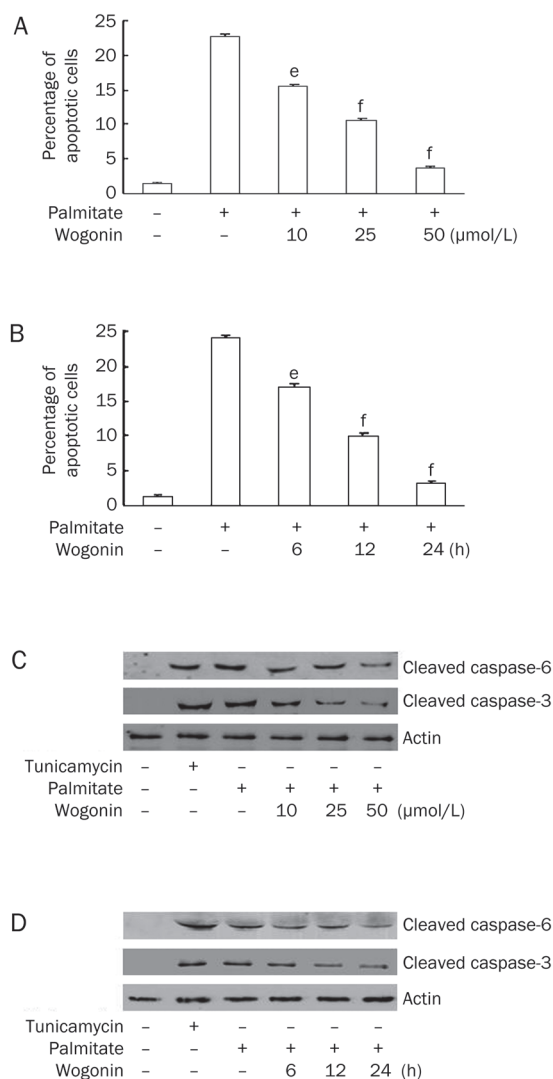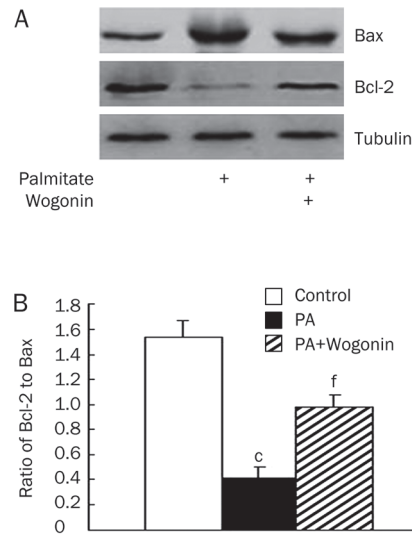

Figure 2. Wogonin prevented palmitate-induced apoptosis. VSMCs were cultured in serum-free medium for 6 h and treated with or without 0.75 mmol/L of palmitate for 12 h, followed by a second treatment with or without 10, 25, and 50 μmol/L of wogonin for an additional 12 h (A and C) or 25 μmol/L of wogonin for 6, 12, and 24 h (B and D). (A and B) The effects of wogonin on the percentage of apoptotic cells. Apoptotic cells were visualized using TUNEL methods. [e]$P<0.05$; [f]$P<0.01$ compared with the palmitate-treated group. (C and D) The effects of wogonin on cleaved caspase-3 and caspase-6 expression. In total, 5 μg/mL of tunicamycin (18 h) was used as a positive control. The expression levels of cleaved caspase-3 and caspase-6 were detected using Western blotting. The figures are representative of at least three independent experiments with similar results.

effects (Figure 5). These data suggest that palmitate-induced apoptosis and wogonin-mediated protection against apoptosis are mediated by ER stress. Taken together, these data indicate that wogonin promotes anti-apoptotic effects via inhibition of ER stress.

### Effects of wogonin on palmitate-induced ROS generation

Over-generation of ROS is responsible for apoptosis in FFA-

Figure 3. Wogonin restored the ratio of Bcl-2 to Bax. A10 VSMCs were cultured in serum-free medium for 6 h and treated with 0.75 mmol/L of palmitate (PA) for 12 h, followed by a second treatment with or without 25 μmol/L of wogonin for an additional 12 h. (A) Bcl-2 and Bax protein expression. (B) Bar graph for the ratio of Bcl-2 to Bax. Western blot was performed to detect the expression levels of Bcl-2 and Bax. The figures are representative of at least three independent experiments with similar results. $^cP<0.01$ compared with the control group. $^fP<0.01$ compared with the palmitate-treated group.
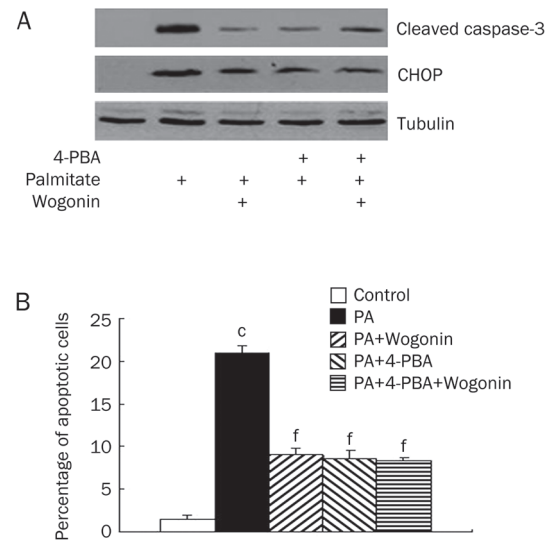
Figure 5. The effects of combined treatment of 4-PBA and wogonin on apoptosis. A10 VSMCs were cultured in serum-free medium for 6 h and treated with or without 4-PBA (5 mmol/L) for 1 h, followed by treatment with or without 25 μmol/L of wogonin for 12 h in the presence of 0.75 mmol/L of palmitate (PA) for 24 h. (A) CHOP and cleaved caspase-3 protein expression. (B) Bar graph for percentage of apoptotic cells. Western blot was performed to detect the expression levels of CHOP and cleaved caspase-3. The figures are representative of at least three independent experiments with similar results. $^cP<0.01$ compared with the control group; $^fP<0.01$ compared with the palmitate-treated group.
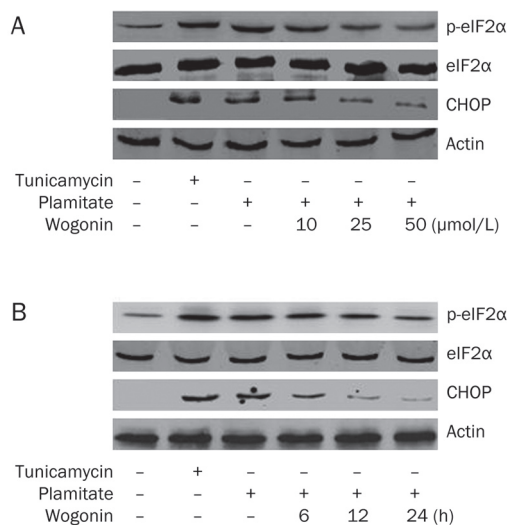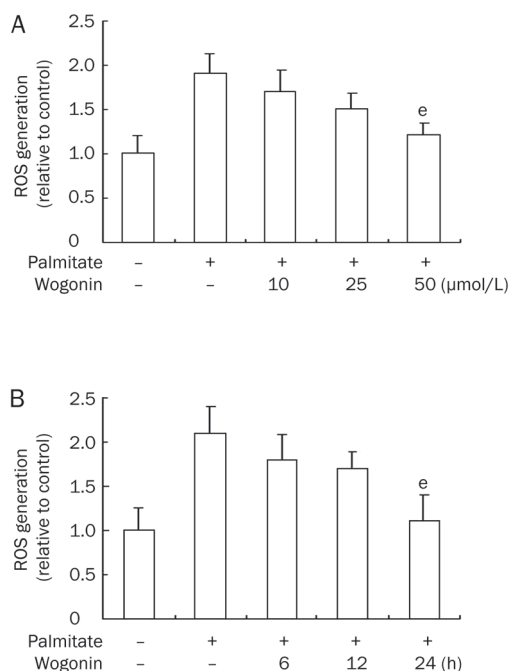

Figure 4. Wogonin inhibited palmitate-induced ER stress. VSMCs were cultured in serum-free medium for 6 h and treated with or without differential doses of wogonin for the indicated time in the presence of 0.75 mmol/L of palmitate or 10 μg/mL of the ER inducer tunicamycin for 24 h. (A) Dose course. In total, 10, 25, or 50 μmol/L of wogonin was added to the culture medium for 24 h. (B) Time course. In total, 50 μmol/L of wogonin was added to the culture medium for 6, 12, or 24 h. Western blot was performed to detect the expression levels of CHOP, eIF2α, and phosphorylated eIF2α. The figures are representative of at least three independent experiments with similar results.

treated cells[24–27]. We addressed whether wogonin has any effects on palmitate-mediated ROS generation. Serum-starved VSMCs were treated with wogonin in a dose- and time-dependent manner in culture medium with 0.75 mmol/L palmitate and evaluated to measure ROS generation. As shown in Figure 6A and 6B, ROS generation was increased in cells that were treated with palmitate and suppressed after a high dose (50 μmol/L) of wogonin treatment ($P<0.05$) and long exposure time (24 h) ($P<0.05$). These results indicate that the inhibition of ROS generation by wogonin is not a major mechanism for its anti-apoptotic effects.

Effects of wogonin on the DAG/PKC pathway

Accumulating evidence suggests that the DAG/PKC pathway contributes to deleterious consequences in cells, including apoptosis in response to FFA administration[28–35]. Based on this evidence, we hypothesized that wogonin-mediated suppression of apoptosis might counter this mechanism and improve cell survival. To confirm this hypothesis, serum-starved VSMCs were treated with wogonin in a dose- and time-dependent manner in culture medium with 0.75 mmol/L of palmitate and evaluated to measure intracellular levels of DAG. As expected, palmitate significantly increased the intracellular contents of DAG, which were suppressed by wogonin administration (Figure 7A and 7B). In addition, the administration of wogonin at a dose of 25 μmol/L for 24 h inhibited palmitate-induced PKC phosphorylation for mul-

Figure 6. Wogonin suppressed palmitate-induced ROS generation. VSMCs were cultured in serum-free medium for 6 h and treated with or without different doses of wogonin for the indicated time in the presence of 0.75 mmol/L of palmitate for 24 h. (A) Dose course. In total, 10, 25, or 50 µmol/L of wogonin was added to the culture medium for 24 h. (B) Time course. In total, 50 µmol/L of wogonin was added to the culture medium for 6, 12, or 24 h. The production of ROS was determined as described in the Materials and methods section. $^{e}P<0.05$, $^{f}P<0.01$ compared with the palmitate-treated group.

tiple PKC-isozymes (Figure 7C). These observations suggest that wogonin inhibits the DAG/PKC pathway, and attenuates apoptosis when combined with palmitate treatment.

Discussion

In this study, we demonstrate a protective effect of wogonin on palmitate-induced VSMC apoptosis. Palmitate-induced apoptosis of VSMCs was attenuated by wogonin administration in a dose- and time-dependent manner. The administration of wogonin inhibits the DAG/PKC pathway by downregulating intracellular DAG accumulation and attenuating ER stress.

It is well documented that obese and diabetic patients have increased FFA levels, which induce atherosclerotic vascular disease. Previous studies have shown that even minute increases in plasma FFA may initiate early vascular abnormalities that promote atherosclerosis and cardiovascular disease (CVD)[1]. The most abundant saturated fatty acid in the plasma is palmitate. Studies have indicated that palmitate is involved in atherogenesis by increasing the extent of plaque formation (or plaque score)[36] and inducing extracellular matrix alterations[37]. In this study, we found that the administration of palmitate induced VSMC apoptosis (Figure 2). VSMCs synthesize components of the fibrous cap in plaques.
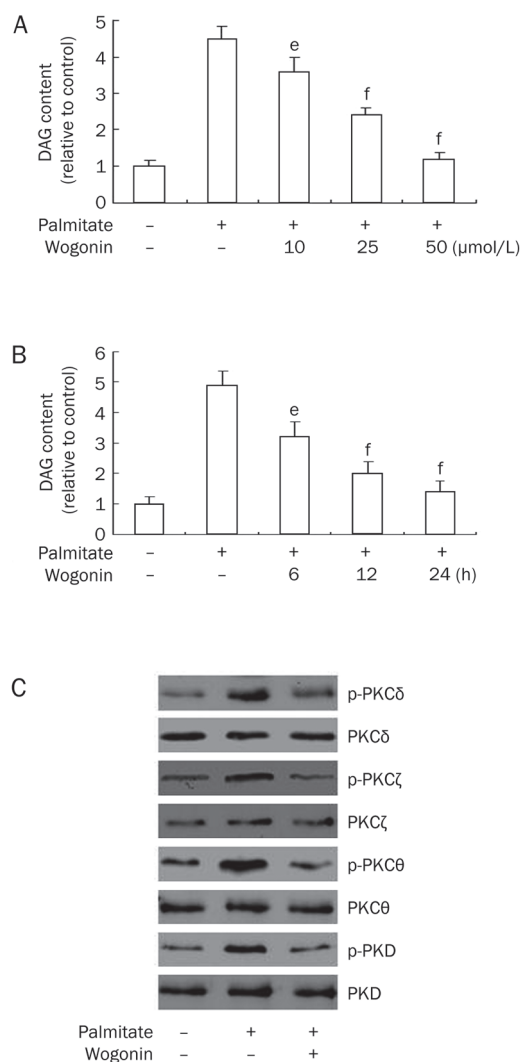
Figure 7. Wogonin ameliorated intracellular DAG accumulation and attenuated PKC phosphorylation. (A and B) VSMCs were cultured in serum-free medium for 6 h and treated with or without 0.75 mmol/L of palmitate for 12 h, followed by a second treatment with or without 10, 25, and 50 µmol/L of wogonin for an additional 12 h (A) or 25 µmol/L of wogonin for 6, 12, and 24 h (B). DAG content in the cells was determined as described in the Materials and methods section. $^{e}P<0.05$, $^{f}P<0.01$ compared with the palmitate-treated group. (C) VSMCs were cultured in serum-free medium for 6 h and treated with or without 25 µmol/L of wogonin for 12 h in the presence of 0.75 mmol/L of palmitate for 24 h. Western blot analysis was performed to detect the expression levels of total and phosphorylated PKC isoforms. The figures are representative of at least three independent experiments with similar results.

Therefore, VSMC apoptosis may result in plaque ruptures[7–12]. Our results indicate a potential mechanism for FFA-induced plaque rupture.

Apoptosis can be initialized through an extrinsic pathway that is activated via death ligands, the intrinsic pathway (mitochondrial pathway), or the ER stress pathway that converges on the activation of caspase-3[21–23]. Our results confirm that palmitate significantly increases CHOP protein expression and

eIF2α phosphorylation (Figure 4).

We detected the increased expression of cleaved caspase-3 and caspase-6 (Figure 2), and the decreased ratio of anti-apoptotic protein Bcl-2 to pro-apoptotic protein Bax (Figure 3). Furthermore, the ER stress inhibitor 4-PBA prevented palmitate-induced apoptosis (Figure 5), which indicated that palmitate-induced apoptosis of VSMCs was mediated by ER stress. These data are congruent with previous studies showing that palmitate is a potent inducer of ER stress[38]. Recent evidence shows that atherosclerosis is associated with ER dysfunction and the accumulation of unfolded proteins[39–43]. Suppressing ER-stress signaling significantly attenuates accelerated atherogenesis[40]. The results of the current study suggest that increased FFA levels enhance ER stress, which may be an important risk factor in the induction of VSMC apoptosis in atherosclerosis.

Wogonin has recently been shown to be toxic in malignant cells but has no or little toxicity in normal cells[44–46]. Wogonin selectively induces apoptosis in tumor cells but has protective effects on glucocorticoid-induced apoptosis in normal rat thymocytes[46]. This selective antitumor function is largely due to its abilities to reduce inflammation, scavenge oxidative radicals, attenuate NF-κB activity, inhibit several genes that are important for regulation of the cell cycle, suppress COX-2 gene expression, block NO, and prevent viral infections[13, 47–49]. Our results demonstrate that wogonin attenuates palmitate-induced apoptosis of VSMCs in a dose- and time-dependent manner (Figure 2), which is accompanied by the suppression of ER stress (Figure 4). When ER stress was inhibited by 4-PBA, the administration of wogonin did not enhance its protective effects on apoptosis (Figure 5). Therefore, these data confirm that the inhibitory effects of wogonin on apoptosis are mediated by the suppression of ER stress.

ER and oxidative stresses are common manifestations in cells that are treated with FFA. The effect of FFA on ROS production has been examined in numerous cell types[50, 51]. ER stress and ROS are proposed to be involved in cell death. Nevertheless, their relative involvements in the processes leading to cell apoptosis are not well elucidated. Research groups have confirmed that over-generation of intracellular ROS might induce apoptosis in endothelial cells, beta cells, and retinal pericytes[24–27]. In contrast, some data shows that apoptosis may not be caused by increased ROS in VSMCs and neonatal cardiomyocytes that are incubated with palmitate[52, 53]. Our results show that palmitate increases ROS generation (Figure 6). Although the administration of wogonin at doses of 10 μmol/L and 25 μmol/L significantly reduced apoptosis (Figure 2), no effects where observed on ROS generation (Figure 6). These results suggest that palmitate-induced ROS generation may not be the major cause of apoptosis in VSMCs.

Furthermore, we found that palmitate increased the intracellular accumulation of DAG and consequently enhanced PKC phosphorylation (Figure 7). This result coincides with previous studies showing that saturated non-esterified fatty acids stimulate an increase in *de novo* intramuscular synthesis and accumulation of DAG[31, 32] and the subsequent activation of PKCs[28–30, 33–35]. Our results show that pre-treatment with wogonin significantly reduces the intracellular DAG levels and attenuates PKC phosphorylation (Figure 7). Because activated PKCs may contribute to altered cellular functions such as regulating signaling function of the ER[54, 55] and enhancing ROS production[51], our data suggest that the DAG/PKC pathway mediates palmitate-induced apoptosis and over-generated ROS production in VSMCs, and that wogonin ameliorates palmitate-induced ER stress by inhibiting the DAG-PKC pathway.

In conclusion, we provide novel evidence that wogonin attenuates lipotoxicity-induced apoptosis by suppressing intracellular DAG accumulation and inhibiting PKC phosphorylation in cultured VSMCs. However, further studies are necessary to determine whether the administration of wogonin will protect *in vivo* VSMCs from apoptosis and the subsequent rupture of advanced atherosclerotic plaques. Additional research into wogonin is also required to investigate the underlying mechanisms that suppress intracellular DAG accumulation in VSMCs.

Acknowledgements

Author contribution

Yu-min LIU, Chang-hua WANG, and Jun-jian ZHANG designed the research; Xiong WANG, Ahmed NAWAZ, and Zhao-hong KONG performed the research; Yan HONG contributed new reagents and analytic tools; Yu-min LIU and Yan HONG analyzed the data; and Chang-hua WANG and Jun-jian ZHANG wrote the paper.

Abbreviations

DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; FFA, free fatty acid; PKC, protein kinase C; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell; PBS, phosphate-buffered saline.

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