Wogonin promotes cholesterol efflux by increasing protein phosphatase 2B-dependent dephosphorylation at ATP-binding cassette transporter-A1 in macrophages☆

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Abstract

Wogonin, one component in Scutellaria baicalensis Georgi extracts, has several beneficial properties for cancers and inflammatory diseases. However, the efficacy of wogonin in cholesterol metabolism of macrophages remains unknown. In macrophages, cholesterol uptake is controlled by scavenger receptors (SR-A and CD36) and cholesterol efflux by SR-BL, ATP-binding cassette transporter-A1 (ABCA1) and ABCG1. In the present study, we investigated the effect and underlying molecular mechanism of wogonin on the formation of macrophage foam cells by murine J774.A1 macrophages. Wogonin attenuated oxidized low-density lipoprotein (oxLDL)-induced cholesterol accumulation in macrophages. The binding of oxLDL to macrophages and protein expression of SR-A and CD36 were not affected by wogonin. Wogonin enhanced cholesterol efflux and increased the protein level of ABCA1 without affecting the protein expression of SR-BL or ABCG1. Inhibition of ABCA1 by pharmacological inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt or neutralizing antibody abolished this suppressive effect of wogonin on lipid accumulation. Moreover, the up-regulation of ABCA1 protein by wogonin resulted from a decrease in degradation rate of ABCA1 protein, with no effect on ABCA1 mRNA expression. This reduction in ABCA1 degradation was due to increased protein phosphatase 2B (PP2B)-mediated ABCA1 dephosphorylation, as evidenced by increased interaction between ABCA1 and PP2B; pharmacological inhibition of PP2B would prevent wogonin-induced ABCA1 protein expression, dephosphorylation and attenuation of lipid accumulation. Collectively, wogonin increases the protein stability of ABCA1 via PP2B-mediated dephosphorylation, thus leading to reduced cholesterol accumulation in macrophage foam cells.

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1. Introduction

Oxidized low-density lipoprotein (oxLDL) promotes inflammation to recruit monocytes to the vascular intima, where monocytes differentiate into macrophages to engulf the oxLDL [1,2]. The internalized oxLDL is processed, stored and progressively accumulated in cytoplasmic lipid droplets, thus leading to the formation of foam cells. Regulation of cholesterol metabolism and inflammatory response by these lipid-laden macrophages is a critical step for the initiation and progression of atherosclerosis [2–4]. The formation of foam cells is mainly due to uncontrolled uptake of oxLDL or impaired cholesterol efflux in macrophages, which results in excessive lipoprotein-derived cholesterol accumulation inside macrophages. 41 Scavenger receptors (SRs), class A SR (SR-A) and CD36, are responsible for the internalization of oxLDL [5–7]. In contrast, the efflux of accumulated cholesterol in macrophages is mediated through reverse cholesterol transporters (RCTs) including SR-BL, ATP-binding cassette transporter A1 and G1 (ABCA1 and ABCG1) [8–10]. Therefore, the cellular lipid content in foam cells is dynamically regulated by these SRs and cholesterol efflux transporters. Ample evidence has demonstrated that dietary supplementation with flavonoids decreases the expression of SRs or increases that of 50 cholesterol efflux transporters, which leads to the attenuation of 51 cholesterol accumulation in macrophages and retards atherosclerotic progression [11–14].
Over the past decade, Chinese herbs have received increasing attention and been extensively used for preventing or attenuating human diseases, particularly cancers and inflammatory diseases [15–17]. Baicalin, baicain and wogonin, the most abundant flavonoids in the root of Scutellaria baicalensis Georgi, are known to be beneficial for various cellular functions. For example, these flavonoids exhibit potent anti-tumor effects by inducing cell-cycle arrest or apoptosis in cancer cells [18–20]. Moreover, these flavonoids also exert excellent anti-inflammatory and anti-oxidative properties in vascular cells [21–24]. These lines of evidence strongly imply that these flavonoids may have therapeutic efficacy for cardiovascular diseases. However, effects and underlying molecular mechanisms of baicalin, baicain and wogonin in the formation of foam cells have never been demonstrated. In the present study, we investigated the effects and the involved molecular mechanisms of flavonoids, especially wogonin, on cholesterol metabolism in macrophages. Only wogonin suppressed the formation of foam cells and increased the ABCA1-dependent cholesterol efflux. This beneficial effect resulted from an increase in protein phosphatase 2B (PP2B)-mediated dephosphorylation of ABCA1 protein. Our findings provide a novel explanation for the anti-atherogenic action of wogonin and suggest a potential molecular target in the treatment or prevention of atherosclerosis.

2. Materials and methods

2.1. Reagents

- Rabbit anti-CD36, anti-PPAR-β, anti-ABCA1, goat anti-SR-A antibodies (Abs) and protein A/G-Sepharose obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).
- Mouse anti-ABC1 and rabbit anti-SR-BI Abs were from Abcam (Cambridge, MA, USA).
- Rabbit anti-phospho-serine/threonine (Ser/Thr) Ab was purchased from Cell Signaling Technology (Beverly, MA, USA).
- Tri reagent, mouse anti-β-tubulin Ab, 4,4′-disothiocyanatostilbene-2-2′-disulfonic acid disodium salt (DIDS) and human LDL powder were from Sigma (St. Louis, MO, USA).
- Dil-labeled oxLDL was from Biomedical Technologies (Stoughton, MA, USA).
- 3-Hexanoyl-NBD-cholesterol was from Cayman Chemical (Ann Arbor, MI, USA).
- Cyclohexamide (CHX) and fenvalerate were from Calbiochem (Merck Biosciences, Germany).

2.2. Cell culture

Murine J774.A1 macrophages (ATCC, TIB-67) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

2.3. Modification of low-density lipoprotein modification

The oxLDL was prepared as described previously [25]. LDL was exposed to 5 μM CuSO4 for 24 h at 37°C and Cu2+ was then removed by extensive dialysis. The extent of modification was determined by measuring thiobarbituric acid-reactive substances (TBARS) defined as malondialdehyde equivalent per milligrams LDL protein was used for experiments.

2.4. Oil red O staining

Oil red O staining was performed as described previously [26]. Briefly, cells were fixed with 4% paraformaldehyde and then stained with 0.5% oil red O. Hematoxylin was used as counterstaining. The density of lipid content was evaluated by alcohol extraction after oil red O staining. The absorbance at 540 nm was measured by use of a microplate reader (BioTek Instrument, Winooski, VT, USA).

2.5. Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from cells by Trizol reagent and converted into cDNA by use of reverse transcriptase (New England Biolabs, Ipswich, MA, USA) with oligo-dT as the primer. The obtained cDNA was then used as the template for semiquantitative polymerase chain reaction (PCR). PCR was performed in a DNA Thermal Cycler (Biometra T Personal, Göttingen, Germany). The PCR program was 94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min; and then one cycle of 72°C for 10 min. The nucleotide sequences of the primers were as follows: ABCA1: sense, 5′-TGT TCC AGT ATG ACT CCA CTC-3′; anti-sense, 5′-TGT GCT ATG ACT CCA CTC-3′; GAPDH: sense, 5′-TGT TCC AGT ATG ACT CCA CTC-3′; anti-sense, 5′-TCT ACC ACC CTG TTG CTC TA-3′.

2.6. Western blot analysis

Cells were lysed with PBS containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride on ice. After sonication, crude extracts underwent centrifugation at 12,000×g for 5 min at 4°C. The supernatants were collected as cell lysates. All protein concentrations were determined by a protein assay. Aliquots (50 μg) of cell lysates were separated on 8% SDS-PAGE and then transblotted on an Immobilon-P membrane (Millipore, Bedford, MA, USA). After being blocked with 5% skim milk, blots were incubated with primary Abs and then with secondary Abs. The protein bands were detected by an enhanced chemiluminescence kit (PerkinElmer, Boston, MA, USA).

2.7. Cholesterol and triglyceride measurement

Measurement of cellular cholesterol and triglyceride was performed as described previously [26]. Cellular cholesterol and triglyceride were extracted by use of hexane/isopropanol (3:2, v/v). The extracts were dried, and then reagent from the assay kit was added to measure the level of cholesterol and triglyceride.

2.8. Dil-oxLDL binding assay

Dil-oxLDL, copper-oxidized LDL labeled with green fluorescent, has been used for experiments of oxLDL binding to scavenger receptors of macrophages [27]. Macrophages were treated with various concentrations of wogonin (10, 20, 40 μM) for 12 h, then with 10 μg/ml Dil-oxLDL at 4°C for 4 h. Cells were washed and lysates were analyzed by fluoreometry (Molecular Devices) with a 540-nm excitation laser line and 590-nm emission filters.

2.9. Cholesterol efflux assay

We have previously used NBD-cholesterol to study the cholesterol efflux in macrophages [26]. Macrophages were treated with various concentrations of wogonin (10, 20, 40 μM) for 12 h, then equilibrated with NBD-cholesterol (1 μg/ml) for an additional 6 h in the presence of wogonin. NBD-cholesterol-labeled cells were washed with PBS and incubated in RPMI 1640 medium for 6 h. The fluorescence-labeled cholesterol released from the cells into the medium was measured by use of a multilabel counter (PerkinElmer, Waltham, MA, USA).

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Fig. 1. Wogonin alleviates oxLDL-induced lipid accumulation in macrophages. (A) J774A1 macrophages were incubated with vehicle (DMSO), wogonin (40 μM), oxLDL (25 μg/ml) alone or with wogonin for 24 h. After fixation, cells were stained with oil red O, and hematoxylin was used as counterstaining. The magnification of each panel is 400×. (B) Lipid-bound stain was extracted with alcohol to measure the lipid content in macrophages. The intracellular level of cholesterol (C) and triglyceride (D) was evaluated by alcoholsolubilization. The absorbance at 540 nm was measured by use of a microplate reader (BioTek Instrument, Winooski, VT, USA).
2.10. Immunoprecipitation

To identify the protein–protein interaction between ABCA1 and PP2B, cells were lysed with an immunoprecipitation (IP) buffer [25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 0.1% NP-40] supplemented with 1% Triton X-100, 0.1 mM Na3VO4 and protease inhibitors. Cells were sheared by brief sonication on ice, and cellular debris was removed by centrifugation at 10,000×g for 10 min. Aliquots (1000 μg) of lysates were incubated with protein A/G-Sepharose for 1 h at 4°C and then incubated with anti-ABCA1 Ab, anti-PP2B polyclonal Ab or preimmune IgG at a final concentration of 1 μg/ml overnight at 4°C. Protein A/G-Sepharose was then added for 2 h at 4°C. Immune complexes were collected by centrifugation, washed three times with cold PBS, disrupted by boiling in 5× SDS loading dye and then subjected to Western blot analysis.

2.11. Statistical analysis

Data represent the mean±S.E.M. values from at least four independent experiments. Statistical analyses involved one-way ANOVA with post hoc Fisher LSD test. A P value <.05 was considered statistically significant.

3. Results

3.1. Wogonin reduces the oxLDL-induced lipid accumulation in foam cells

The formation of lipid-laden macrophage foam cells is critical for the initiation and progression of atherosclerosis [2,4]. To delineate the effects of S. baikalesis Georgi extracts on lipid accumulation in macrophages, macrophages were treated with wogonin, baicalein, baicalin or in combination with oxLDL. Compared with oxLDL-only treatment, wogonin plus oxLDL significantly ameliorated oxLDL-induced lipid accumulation (Fig. 1A and B), whereas baicalein and baicalin had no effect on lipid accumulation (Supplementary Fig. 1). This inhibitory effect of wogonin was due to a decrease in intracellular cholesterol content (Fig. 1C) but not in triglyceride content (Fig. 1D). These data suggest that wogonin suppresses the oxLDL-induced formation of macrophage foam cells by decreasing the accumulation of cholesterol.

3.2. Wogonin promotes ABCA1-dependent cholesterol efflux in macrophages

The formation of foam cells is mainly due to uncontrolled uptake of oxLDL or impaired cholesterol efflux in macrophages [2,4]. We assessed the effect of wogonin on cholesterol uptake and efflux. Incubation with wogonin did not affect the Dil-oxLDL binding to macrophages (Fig. 2A) or the protein expression of SR-A and CD36, two key receptors for oxLDL uptake (Fig. 2B–D). We next investigated the effect of wogonin on cholesterol efflux. By using fluorescence-conjugated cholesterol, we found that wogonin dose-dependently increased the efficiency of cholesterol efflux in macrophages (Fig. 3A).

Fig. 2. Wogonin has no effect on oxLDL uptake and protein expression of SR-A and CD36. (A) J774.A1 macrophages were treated with the indicated concentrations of wogonin for 12 h and then incubated with DiO-oxLDL (10 μg/ml) for an additional 4 h at 4°C. After PBS washes, cell lysates were collected for calculation of fluorescence. (B–D) Cells were incubated with the indicated concentrations of wogonin for 24 h, then lysed and subjected to Western blot analysis to determine the protein expression of SR-A, CD36 and α-tubulin. Data are mean±S.E.M. from four independent experiments.

Fig. 3. Wogonin promotes the ABCA1-dependent cholesterol efflux. (A) J774.A1 macrophages were treated with the indicated concentrations of wogonin for 12 h, then with NBD-cholesterol (1 μg/ml) for another 6 h. The medium and cell lysates were collected for the measurement of fluorescence. The calculation of cholesterol efflux was defined as fluorescence in the medium relative to the total amount of fluorescence. (B–E) Macrophages were incubated with the indicated concentrations of wogonin for 24 h. The protein level of ABCA1, ABCG1, SR-BI and α-tubulin was evaluated by Western blot analysis. (F) Macrophages were preincubated with ABCA1 inhibitor, DIDS or ABCA1 neutralizing Ab for 1 h, then treated with wogonin (40 μM) for 12 h. Then cells were incubated with oxLDL (25 μg/ml) for an additional 6 h. After fixation, cells were stained with oil red O. Alcohol was used to extract the lipid-bound stain from cells to detect absorbance at 540 nm. Data are mean±S.E.M. from four independent experiments. *P<.05 vs. vehicle-treated group; $P<.05 vs. oxLDL-treated group; #P<.05 vs. wogonin/oxLDL-treated group.
Additionally, the protein level of ABCA1, but not of ABCG1 or SR-BI, was significantly elevated with wogonin treatment (Fig. 3B–E). We further demonstrated that functional inhibition of ABCA1 with DIDS, a pharmacological inhibitor of ABCA1 or ABCA1 neutralizing Ab abrogated the suppressive effect of wogonin on lipid accumulation (Fig. 3F). These results suggest that wogonin reduced lipid accumulation in foam cells by increasing ABCA1-dependent cholesterol efflux. Moreover, activation of sterol regulatory element binding protein 2 (SREBP2), a key transcription factor regulating multiple genes involved in the synthesis of cholesterol, was not altered by wogonin (data not shown).

3.3. Wogonin increases the protein stability of ABCA1 by PP2B-mediated dephosphorylation

We further delineated the molecular mechanisms underlying the effect of wogonin on ABCA1-dependent cholesterol efflux by examining the mRNA expression and the protein stability of ABCA1 in response to wogonin. RT-PCR revealed that wogonin at various concentrations did not alter the mRNA level of ABCA1 (Fig. 4A). However, the rate of ABCA1 degradation was significantly attenuated in the presence of wogonin (Fig. 4B). The phosphorylation status of ABCA1 protein is crucial for its protein stability[28–33]. However, PP2B, a protein phosphatase, can also regulate the protein expression of ABCA1 and cholesterol homeostasis[34,35]. Since our results demonstrated that treatment with wogonin increased the protein stability of ABCA1, we further defined the phosphorylation status of ABCA1. Indeed, the phosphorylation level at Ser/Thr residues of ABCA1 was decreased in a time-dependent manner by wogonin (Fig. 5A). To confirm whether PP2B mediates the dephosphorylation of ABCA1 by wogonin, we examined the interaction between PP2B and ABCA1. Immunoprecipitation assays revealed that wogonin increased the interaction between PP2B and ABCA1 (vehicle up to 60 min, Fig. 5B). Preincubation with fenvalerate, an inhibitor of PP2B, significantly reversed the wogonin-induced dephosphorylation (Fig. 5C) and increased the protein stability of ABCA1 (Fig. 5D), which suggests a key role of PP2B in ABCA1-dependent cholesterol efflux by wogonin.
3.4. PP2B is essential for the suppressive effect of wogonin on oxLDL-induced cholesterol accumulation

According to our findings of the importance of PP2B in the protein stability of ABCA1, we next determined the functional significance of PP2B in the wogonin-mediated modulation of lipid accumulation and cholesterol content in foam cells. Pharmacological inhibition of PP2B abrogated the inhibitory effect of wogonin on the intracellular content of lipid and cholesterol (Fig. 6A–C). These data imply that PP2B plays a crucial role in the inhibitory effect of wogonin on the formation of macrophage-foam cells.

4. Discussion

In this study, we characterized the molecular mechanism underlying the beneficial effect of the flavonoid wogonin on macrophage foam cells. In macrophages incubated with the extracts of S. baicalensis Georgi, only wogonin attenuated the oxLDL-induced lipid accumulation in macrophages, whereas baicalein or baicalin had no such inhibitory effect. We further demonstrated that PP2B-mediated dephosphorylation of ABCA1 protein is an important regulation of this anti-atherogenic action of wogonin in foam cells. Over the past decade, in vitro and in vivo experiments have shown that wogonin has anti-oxidative, anti-tumor or anti-inflammatory effects [17,19,22]. Additionally, emerging research has demonstrated that wogonin profoundly inhibits the expression of pro-atherogenic molecules in endothelial cells and smooth muscle cells [22,23].

However, the effect of wogonin and its underlying molecular mechanism on cholesterol metabolism of macrophage foam cells have never been defined.

Accumulation of macrophage-derived foam cells in the subendothelial space is a critical event in the development of atherosclerotic lesions. Several lines of evidence suggest that inhibition of foam cell formation retards the progression of atherosclerosis in rodent models [36–38]. Indeed, the results of the first part of this study show that wogonin treatment markedly ameliorated the oxLDL-induced cholesterol accumulation in macrophages. From this observation, we further delineated the potential molecular mechanisms underlying the wogonin-invoked suppression on the formation of foam cells.

The formation of foam cells is mainly due to the dysregulation of intracellular lipid homeostasis, including uncontrolled internalization of oxLDL or impaired cholesterol efflux in macrophages, two processes that are tightly controlled by SRs and RCTs, respectively [5–10]. The importance of SR-A and CD36 in oxLDL uptake has been well established [5–7]. Macrophages lacking both SR-A and/or CD36 are defective in oxLDL internalization and are less prone to foam cell formation [39,40]. However, ABCA1, ABCG1 and SR-BI, the three key regulators of cholesterol efflux and lipid clearance from foam cells, are critical in the cholesterol homeostasis of macrophages [8–10]. Foam cell accumulation and atherosclerotic lesions are markedly increased in individual transporter null mice [41–43]. Increasing evidence points to reduced expression of SRs or promoted function of RCTs in macrophages leading to reduced cholesterol accumulation in macrophages by dietary flavonoids with anti-atherogenic actions such as wogonin.
procyandin and resveratrol [44,45]. Therefore, we hypothesized that wogonin might regulate the expression of SRs or RCTs to modulate the formation of foam cells. Our data show that wogonin did not affect the protein level of SR-A, CD36, SR-B1 or ABCG1. In contrast, wogonin increased the protein expression of ABCA1 but had no effect on its mRNA expression, which suggests that the up-regulation of ABCA1 by wogonin may be regulated at the post-translational level. Furthermore, wogonin increased cholesterol efflux, and the inhibition of ABCA1 function by a pharmacological inhibitor or neutralizing Ab reversed the inhibitory action of wogonin on lipid accumulation. These findings suggest that wogonin attenuates cholesterol accumulation by escalating ABCA1-dependent cholesterol efflux during the transformation of foam cells, which is in agreement with previous studies finding that induction of ABCA1 contributes to the suppression of lipid accumulation in foam cells [45,46].

More importantly, we found that the half-life of ABCA1 protein was significantly prolonged, from 6 to 9 h, with wogonin, which implies that treatment with wogonin could stabilize ABCA1 protein. This result is consistent with previous findings that stabilization of ABCA1 protein enhances cholesterol efflux and leads to reduced lipid accumulation in foam cells [47–49]. In contrast, destabilization of ABCA1 protein impairs lipid clearance and results in an augmentation of cholesterol accumulation in macrophages [29,50]. Collectively, our findings suggest that the up-regulation of ABCA1 by wogonin likely contributes to a suppressive effect on foam cell formation. Moreover, recent studies have reported that the phosphorylation at Ser/Thr residues of ABCA1 protein is a key determinant in the protein stability of ABCA1 [28,32]. Indeed, our data showed that wogonin time-dependent decreased the phosphorylated level of ABCA1 protein. PP2B, a protein Ser/Thr phosphatase, has been linked to the regulation of ABCA1 stability [35]. Epidemiological trials indicate that long-term treatment with a PP2B inhibitor causes hyperlipidemia, hypertension and diabetes, and increases the risk of cardiovascular disease-related morbidity and mortality [34,35,51,52]. Our data clearly showed the involvement of PP2B in the wogonin-mediated promotion in ABCA1-dependent cholesterol efflux, as evidenced by an increase in the interaction between PP2B and ABCA1 and in the prevention of the wogonin-mediated phosphorylation and up-regulation of ABCA1 by the PP2B inhibitor, fenvalerate, in macrophages. This notion is further confirmed by the suppressive effect of wogonin on cholesterol accumulation also being diminished by treatment with fenvalerate, which confirms the critical role of PP2B in the anti-atherogenic property of wogonin in macrophages.

In conclusion, this study demonstrates a unique protective effect of wogonin in reducing cholesterol accumulation in foam cells via up-regulating ABCA1 and is modulated by PP2B-dependent dephosphorylation in macrophages. Our findings suggest that wogonin may have therapeutic value in inhibiting the progression of atherosclerosis.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.08.014.

References


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