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Original Article

Wogonin suppresses osteopontin expression in adipocytes by activating PPARα

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Aim: Wogonin (5,7-dihydroxy-8-methoxyflavone), a major bioactive compound of the flavonoid family, is commonly extracted from the traditional Chinese medicine *Scutellaria baicalensis* and possesses antioxidant and anti-inflammatory activities and is assumed to have anti-diabetes function. Indeed, a current study has shown that it can possibly treat metabolic disorders such as those found in *db/db* mice. However, the underlying molecular mechanism remains largely unclear. The aim of this study was to investigate the impact of wogonin on osteopontin (OPN) expression in adipose tissue from type 1 diabetic mice and in 3T3-L1 adipocytes.
Methods: Type 1 diabetes was induced by streptozotocin (STZ) injection. 3T3-L1 preadipocytes were converted to 3T3-L1 adipocytes through treatment with insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX). Western blot analysis and RT-PCR were performed to detect protein expression and mRNA levels, respectively.
Results: Wogonin treatment suppressed the increase in serum OPN levels and reduced OPN expression in adipose tissue from STZ-induced type 1 diabetic mice. Administration of wogonin enhanced PPARα expression and activity. Silencing of PPARα diminished the inhibitory effects of wogonin on OPN expression in 3T3-L1 adipocytes. Furthermore, the levels of c-Fos and phosphorylated c-Jun were reduced in wogonin-treated adipose tissue and 3T3-L1 adipocytes. In addition, wogonin treatment dramatically mitigated p38 MAPK phosphorylation. Pharmacological inhibition of p38 MAPK by its specific inhibitor SB203580 increased PPARα activity and decreased OPN expression.
Conclusion: Our results suggest that wogonin downregulated OPN expression in adipocytes through the inhibition of p38 MAPK and the sequential activation of the PPARα pathway. Given the adverse effects of high OPN levels on metabolism, our results provide evidence for the potential administration of wogonin as a treatment for diabetes.

Keywords: wogonin; osteopontin; PPARα; p38 MAPK; adipocytes

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Introduction

Osteopontin (OPN) is a multifunctional protein involved in several physiological and pathological events that serves as a biomarker or therapeutic target for such conditions as cancer, cardiovascular diseases, and various intractable inflammatory diseases[1–4]. Currently, OPN has been implicated in the pathogenesis of insulin resistance, obesity, and diabetes[5]. The pharmacological and genetic inhibition of OPN has mitigated hepatic and adipose tissue inflammation, improved obesity-induced insulin resistance in hepatic and adipose tissue, attenuated hepatic steatosis, and prevented the upregulation of gluconeogenic genes in the liver[6–9], suggesting that OPN is an important player in the development of insulin resistance and diabetes.

Adipose tissue and adipocytes are two of the primary targets for OPN action. A high level of OPN is associated with fat storage in adipose tissue, accumulation of adipose tissue macrophages, adipose tissue inflammation, and adipocyte hypertrophy[6–9]. In addition, adipocytes can synthesize OPN. In the early stage of high fat diet (HFD) consumption, adipose tissue showed altered OPN isoform expression, but total OPN protein was unchanged[8]. In agreement with this finding, high levels of OPN mRNA were found in the adipose tissues of obese and insulin-resistant humans and rats[8]. Mice exposed to chronic HFD exhibited dramatically increased plasma OPN levels and OPN protein abundance in adipose tissue[7, 8]. Thus, inhibition of OPN production in adipocytes could be a novel approach for the treatment of insulin resistance and diabetes.

Wogonin (5,7-dihydroxy-8-methoxyflavone) is one of the major bioactive constituents of flavonoids from *Scutellaria baicalensis*, a traditional Chinese herbal medicine for the treatment of infection, inflammation, and cardiovascular diseases. Accumulating evidence suggests the therapeutic potential of wogonin for cancer treatment[10, 11]. Current studies also

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identify wogonin as a functional modulator of endothelial cells[12, 13]. We have recently found that wogonin is a potent antioxidant capable of inhibiting lipotoxicity-induced apoptosis of vascular smooth muscle cells (VSMCs) via suppression of intracellular DAG generation[14]. Intriguingly, wogonin exhibits beneficial effects on glucose and lipid metabolism in *db/db* mice[15], indicating that wogonin is a new candidate for the treatment of diabetes. However, the underlying molecular mechanism of wogonin action remains largely unknown.

Here, we report that wogonin treatment suppressed OPN expression *in vivo* and *in vitro* and blunted OPN secretion in 3T3-L1 adipocytes. We also demonstrated that the inhibitory effect of wogonin on OPN expression is largely due to its ability to activate PPARα. This novel finding adds further evidence in favor of wogonin as a potential diabetes treatment.

Materials and methods

Antibodies and reagents

The biochemical reagents and antibodies used in this study are as following: streptozotocin (STZ), fenofibric acid, and wogonin purchased from Sigma-Aldrich (St Louis, MO, USA); antibodies to OPN, PPARα, p38 MAPK, phospho-p38 MAPK, c-Fos, phospho-c-Jun, and histones purchased from Cell Signaling Technology (Beverly, MA, USA); anti-tubulin antibody, PPARα/shRNA lentiviral particles, and control shRNA lentiviral particles purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase purchased from Promega (Madison, WI, USA).

Animals and treatment

C57BL/6 mice[provided by the Animal Biosafety Level 3 Laboratory (ABSL-3) of Wuhan University]at eight weeks of age were randomized into three groups: a control group, STZ-induced type 1 diabetes group, and wogonin treatment group. Type 1 diabetes was induced by a daily intraperitoneal injection (ip) of 55 mg/kg body weight STZ (dissolved in 0.1 mmol/L citric acid buffer, pH 4.5) for 5 consecutive days[16, 17]. After one week of STZ injection, mice were intraperitoneally administered either 10 mg/kg body weight of wogonin or vehicle control (20% dimethyl sulfoxide and 80% saline) once every three days for 8 weeks. Because the LD_{50} of wogonin administered by intravenous injection in Sprague-Dawley rats was 286.15 mg/kg[18], the doses or dose ranges used in this study were considered safe. At the end of wogonin treatment, the mice were euthanized, and adipose tissue and blood samples were harvested for experimentation. Mice were maintained at 23±2 °C and at 45%±5% humidity with a 12:12 h light-dark cycle. The experiments were conducted according to the protocols approved by the Animal Care and Use Committee of Wuhan University.

3T3-L1 cell culture and differentiation

3T3-L1 cells (ATCC, CL-173) were cultured in complete medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2. To convert 3T3-L1 fibroblasts to adipocytes, the cells were maintained in complete medium until they reached 100% confluence. Three days following confluence, the cells were incubated in complete medium supplemented with 1 μg/mL insulin, 0.25 μmol/L dexamethasone, and 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX) for 3 d, then transferred to complete medium containing 1 μg/mL insulin for another 3 d, and finally maintained in complete medium for an additional 4 d. At that time, approximately 90% of cells were considered to be mature adipocytes as confirmed by Oil Red O staining[19].

RT-PCR

RT-PCR was performed as described previously[19]. Briefly, total RNA was isolated from mouse adipose tissues or 3T3-L1 adipocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The primers used in this study include mouse OPN: forward 5′-AGCCACAAGTTTCACAGCCACAAGG-3′, reverse 5′-CTGAGAAATGAGCAGTTAGTATTCCTGC-3′; mouse PPARα: forward 5′-TCAGGGTACCACTACGGAGT-3′, reverse 5′-CTTGGCATTCTTCCAAAGCG-3′; mouse acyl-CoA-oxidase (ACOX): forward 5′-ACTATATTTGGCCAATTTTGTG-3′, reverse 5′-TGTGGCAGTGGTTTCCAAGCC-3′; mouse β-actin: forward 5′-CGGTTCCGATGCCCTGAGGCTCTT-3′, reverse 5′-CGTCACACTTCATGATGGAATTGA-3′. PCR was performed in a Perkin-Elmer 9600 thermocycler. The cycling parameters were as follows: 50 °C for 10 min and 95 °C for 2 min, followed by 26 cycles of a three-step cycle (95 °C for 15 s, 60 °C for 30 s, and 72 °C for 2 min). PCR products were separated by electrophoresis through 1% agarose, and ethidium bromide stained bands were detected using a UV transilluminator.

Nuclei isolation, Western blot and Pro-Q Diamond phosphoprotein analysis

Nuclei were isolated by commercially available nuclear and cytoplasmic extraction reagents (Bipec Biopharma Corporation, Cambridge, MA, USA) according to the manufacturer's instructions.

Total protein was extracted with lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 20 mmol/L sodium pyrophosphate, 10 mmol/L NaF, 1 mmol/L $NaVO_3$, 2 mmol/L EDTA, 10% glycerol, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10% Nonidet-P40, 2 mmol/L PMSF, and 10% phosphorylase inhibitor cocktail-I, -II, and -III. The protein concentration was determined using the BCA (bicinchoninic acid) protein assay reagent (Thermo, Rockford, IL, USA). For Western blot analysis, the samples in SDS-PAGE loading buffer were heated at 100 °C for 5 min. The proteins were separated by SDS-PAGE gel, transferred onto nitrocellulose membranes, and detected with specific antibodies[20].

Phosphorylation analysis for PPARα was performed using the Pro-Q Diamond phosphoprotein gel stain according to the manufacturer's protocol (Molecular Probes, Invitrogen, Carlsbad, CA, USA). In brief, PPARα was immunoprecipitated and separated by SDS-PAGE gel. The levels of PPARα were deter-

mined by Western blot analysis using a specific anti-PPARα antibody. Phosphoprotein bands were scanned by fluorescence at 532 nm[21].

Biochemical analysis

OPN levels in the serum and culture medium were determined by an osteopontin mouse ELISA Kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. Serum insulin was measured using the rat/mouse insulin ELISA EZRMI-13K from EMD Millipore (Billerica, MA, USA) according to the provided protocol.

Statistical analysis

Each experiment *in vitro* was repeated at least 3 times with similar results. The data are presented as the mean±SEM. Results were analyzed using a one-way ANOVA followed by the Tukey–Kramer *post hoc* test and independent samples *t*-test. The differences were considered statistically significant at $P<0.05$.

Results

Wogonin ameliorated hyperglycemia and reduced OPN expression *in vivo*

To examine the potential impact of wogonin on glucose homeostasis, we compared serum glucose and insulin levels between the STZ-diabetes group and the wogonin treatment group. We found that wogonin-treated mice showed markedly lower glucose levels (Figure 1A) and higher insulin concentrations (Figure 1B) than STZ-diabetic mice.

To observe the impact of wogonin treatment on OPN synthesis and secretion, we first checked the expression of OPN protein and mRNA in perinephric white adipose tissue from STZ-diabetic mice using Western blot analysis and RT-PCR, respectively. As shown in Figure 1C and 1D, the levels of OPN protein and mRNA showed a dramatic upregulation in adipose tissue from the diabetic mice. As expected, wogonin treatment greatly decreased the expression of OPN protein and mRNA (compared to the STZ group: $P<0.05$ and $P<0.01$, respectively). Using a mouse-specific OPN ELISA kit, we found that the diabetic mice had an approximately 4.6-fold increase in serum OPN concentration (Figure 1E), from 64.3 ng/mL to 296.9 ng/mL ($P<0.01$), whereas wogonin treatment significantly decreased serum OPN levels to 143.1 ng/mL ($P<0.05$, compared with the STZ group). These findings indicate that wogonin treatment significantly suppressed adipose tissue expression and secretion of OPN in the STZ-diabetic mice.

Wogonin downregulated OPN expression and secretion in 3T3-L1 adipocytes

To further confirm the effects of wogonin on OPN expression and secretion, 3T3-L1 pre-adipocytes were converted to 3T3-

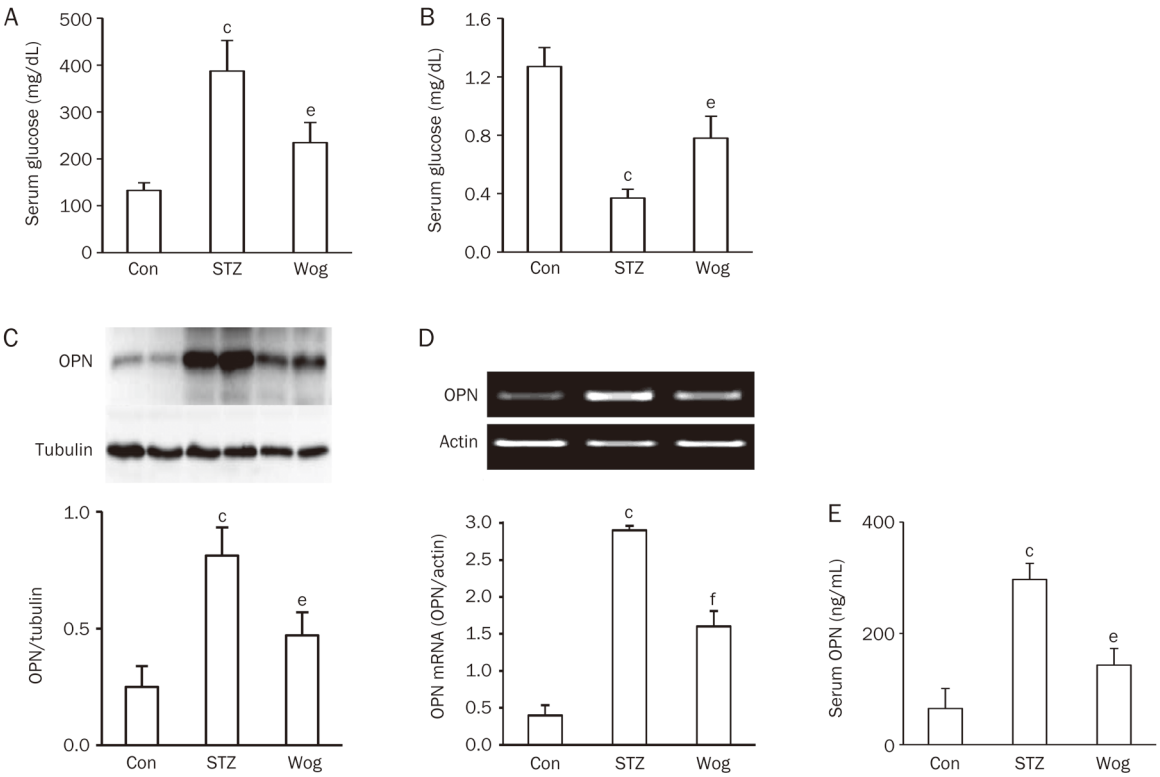

Figure 1. Wogonin improved hyperglycemia and suppressed OPN expression in STZ-induced type 1 diabetic mice. (A) Effect of wogonin on glucose levels. (B) Effect of wogonin on serum insulin concentration. (C and D) Effect of wogonin on the levels of OPN protein (C) and mRNA (D) in adipose tissue. (E) Effect of wogonin on serum OPN concentration. $n=12$. $^{c}P<0.01$ *vs* control group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* STZ group. OPN, osteopontin; Con, control group; STZ, streptozotocin-induced type 1 diabetes group; Wog, wogonin treatment group.

L1 adipocytes as previously described by our laboratory[19]. Then, 3T3-L1 adipocytes were incubated in complete medium containing 25 mmol/L glucose to mimic hyperglycemia. We found that high glucose stimulated the expression of OPN protein and mRNA, as well as OPN secretion (Supplementary Figure 1). When 3T3-L1 adipocytes were exposed to 25 mmol/L glucose in the presence or absence of different concentrations of wogonin for desired time, we found that wogonin inhibited the expression of OPN protein in a dose- and time-dependent manner (Figure 2A and 2B). To observe the impact of wogonin on OPN mRNA and secretion, 3T3-L1 adipocytes exposed to 25 mmol/L glucose were administered with 10 μmol/L wogonin for 12 h. As depicted in Figure 2C and 2D, the levels of OPN mRNA in 3T3-L1 adipocytes (Figure 2C) and OPN protein in cultured medium (Figure 2D) were significantly decreased. These results are consistent with our *in vivo* findings, suggesting that wogonin inhibited OPN synthesis and secretion in 3T3-L1 adipocytes.

Wogonin upregulated PPARα expression and activity

To investigate the underlying mechanism by which wogonin downregulated OPN production, we assessed the expression and activity of PPARα in wogonin-treated 3T3-L1 adipocytes and perinephric white adipose tissue from STZ-induced type 1 diabetic mice. 3T3-L1 adipocytes exposed to 25 mmol/L glucose were treated with 10 μmol/L wogonin for 12 h. As shown in Figure 3A, wogonin greatly enhanced PPARα mRNA expression, but PPARα protein levels remained broadly unchanged (data not shown). Compared with STZ diabetic mice, wogonin treatment recovered STZ-reduced expression of PPARα mRNA (Figure 3B) and protein in both the cytosol (Figure 3C) and nucleus (Figure 3D) of adipose

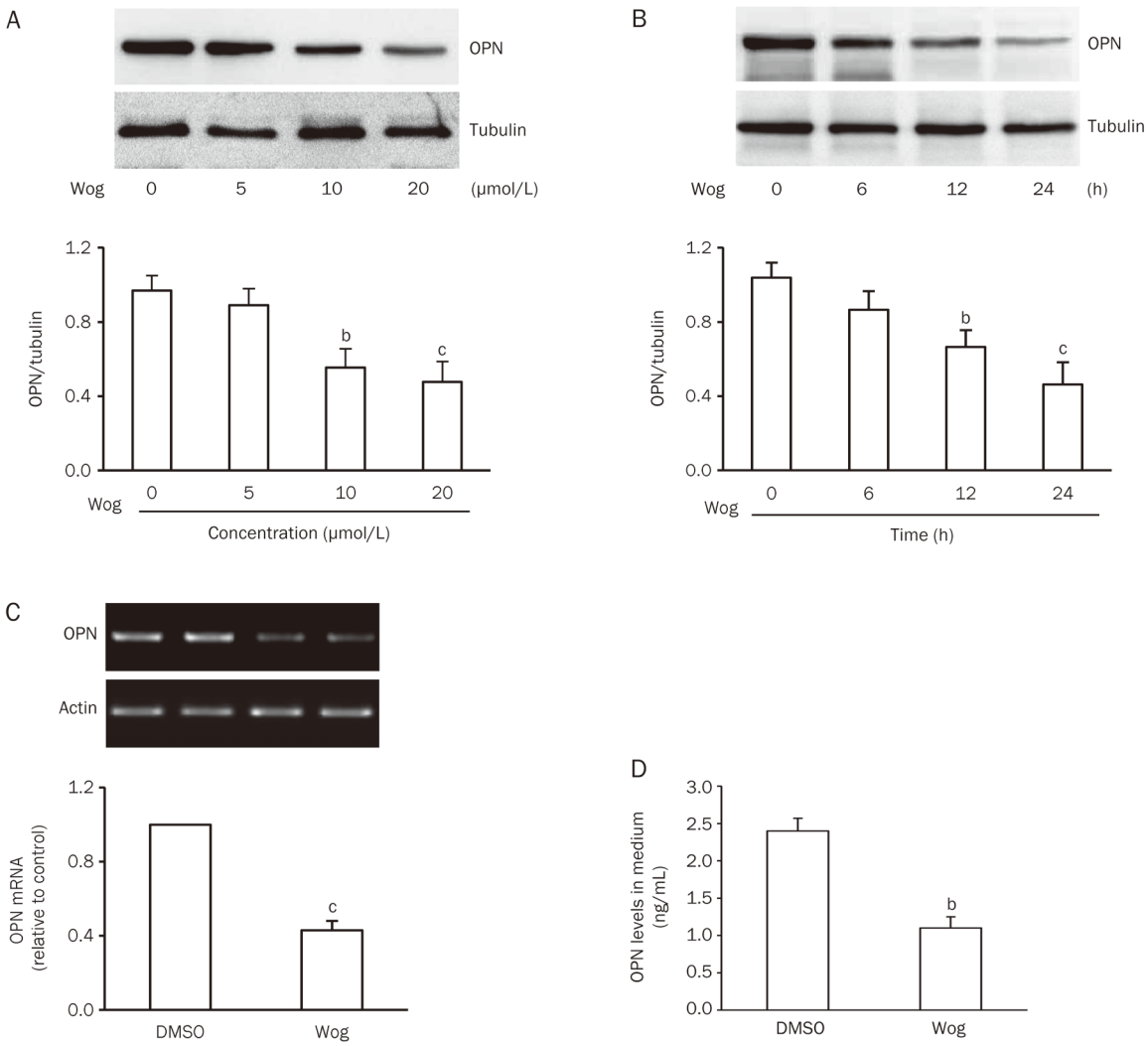

Figure 2. Wogonin inhibited OPN expression and secretion in 3T3-L1 adipocytes. (A and B) Dose-response (A) and time-response (B) of wogonin on OPN protein expression. 3T3-L1 adipocytes were incubated in complete medium containing 25 mmol/L glucose and received indicated concentrations of wogonin for 12 h (A) or 10 μmol/L wogonin for the indicated time (B). (C) Effect of wogonin on the expression of OPN mRNA. (D) Effect of wogonin on OPN concentration in cultured medium. For (C and D), 3T3-L1 adipocytes exposed to 25 mmol/L glucose were treated with or without 10 μmol/L wogonin for 12 h. $^{b}P<0.05$, $^{c}P<0.01$ *vs* DMSO control group. OPN, osteopontin; Wog, wogonin.

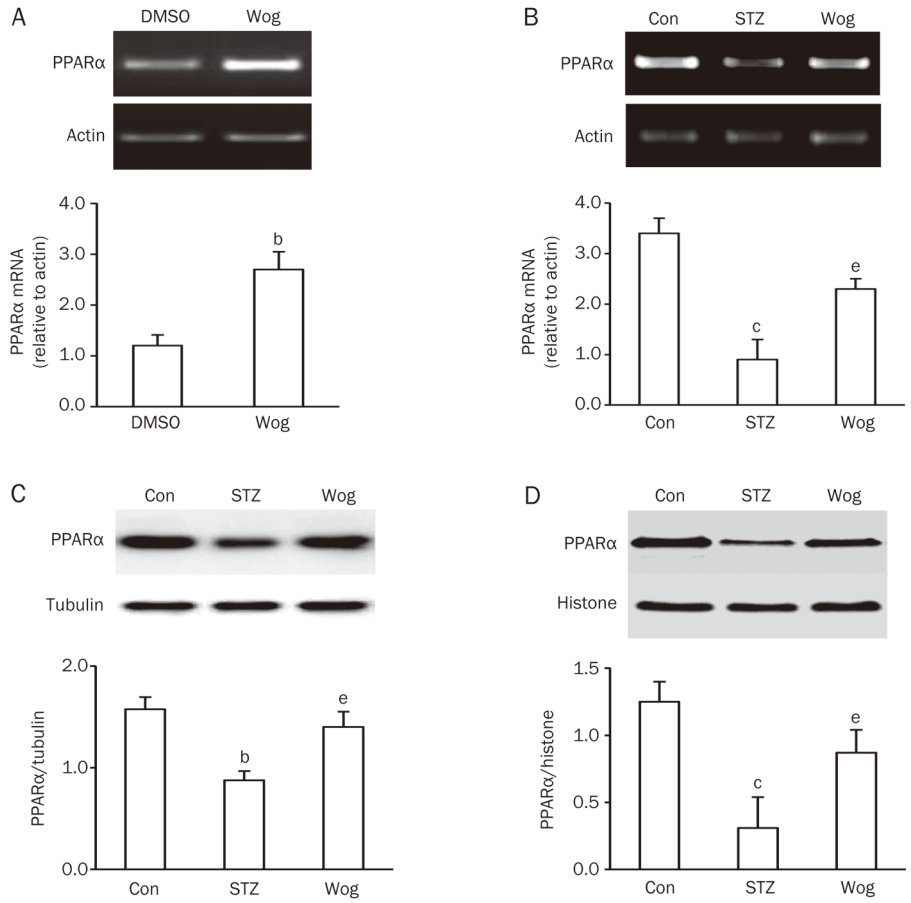

Figure 3. Wogonin increased PPARα expression in adipose tissue and adipocytes. (A) Effect of wogonin on the mRNA expression of PPARα in adipocytes. 3T3-L1 adipocytes exposed to 25 mmol/L glucose were treated with or without 10 μmol/L wogonin for 12 h. (B and C) Effect of wogonin on PPARα mRNA (B) and protein in the cytosol (C) in adipose tissues from STZ-diabetic mice (n=12). (D) Effect of wogonin on PPARα protein in the nucleus of adipose tissue from STZ-diabetic mice (n=6). $^{b}P<0.05$, $^{c}P<0.01$ *vs* DMSO or control group. $^{e}P<0.05$ *vs* STZ-induced diabetic group. Con, control group; STZ, streptozotocin-induced type 1 diabetes group; Wog, wogonin treatment group.

tissue, suggesting that wogonin increased PPARα expression under high glucose conditions.

We next inspected the impact of wogonin on PPARα phosphorylation, which is closely associated with PPARα activity. 3T3-L1 adipocytes were cultured in complete medium containing 25 mmol/L glucose and treated with indicated concentrations of wogonin for 12 h (Figure 4A) or 10 μmol/L of wogonin for varied durations (Figure 4B). PPARα phosphorylation was determined by a Pro-Q Diamond phosphoprotein gel stain. We found that wogonin inhibited PPARα phosphorylation in a dose- and time-dependent manner in 3T3-L1 adipocytes (Figure 4A and 4B). Comparably, wogonin treatment inhibited STZ-stimulated PPARα phosphorylation (Figure 4C) in perinephric white adipose tissue from diabetic mice. Given that phosphorylation events can inhibit PPARα activity[21], these data suggest that wogonin positively regulated PPARα activity in adipocytes. To support this conclusion, we measured PPARα transcriptional activity in a luciferase reporter gene assay. We found that wogonin significantly increased PPARα transactivation (Supplementary Figure 2). In agreement with this result, wogonin treatment reversed STZ-reduced mRNA expression of the PPARα target acyl-CoA oxidase (ACOX) in adipose tissue (Figure 4D).

Silencing of PPARα diminished the inhibitory effect of wogonin on OPN expression in 3T3-L1 adipocytes

To further confirm the role of PPARα in mediating wogonin-reduced OPN expression, 3T3-L1 adipocytes were infected with lentivirus carrying mouse PPARα/shRNA or scrambled sequences and then cultured in complete medium containing 25 mmol/L glucose followed by treatment with or without 10 μmol/L wogonin for 12 h. As expected, PPARα knockdown cells prevented the wogonin-induced reduction of OPN protein (Figure 5A) and mRNA (Figure 5B) and paralleled the restoration of OPN secretion (Figure 5C). In agreement with these results, the PPARα agonist fenofibrate significantly blocked the expression of OPN protein and mRNA, as well as OPN secretion in 3T3-L1 adipocytes (Supplementary Figure 3). However, a combined treatment with wogonin and fenofibrate did not further increase the impact of wogonin on OPN expression in 3T3-L1 adipocytes (Supplementary Figure 4).

It has been suggested that c-Fos and phospho-c-Jun play

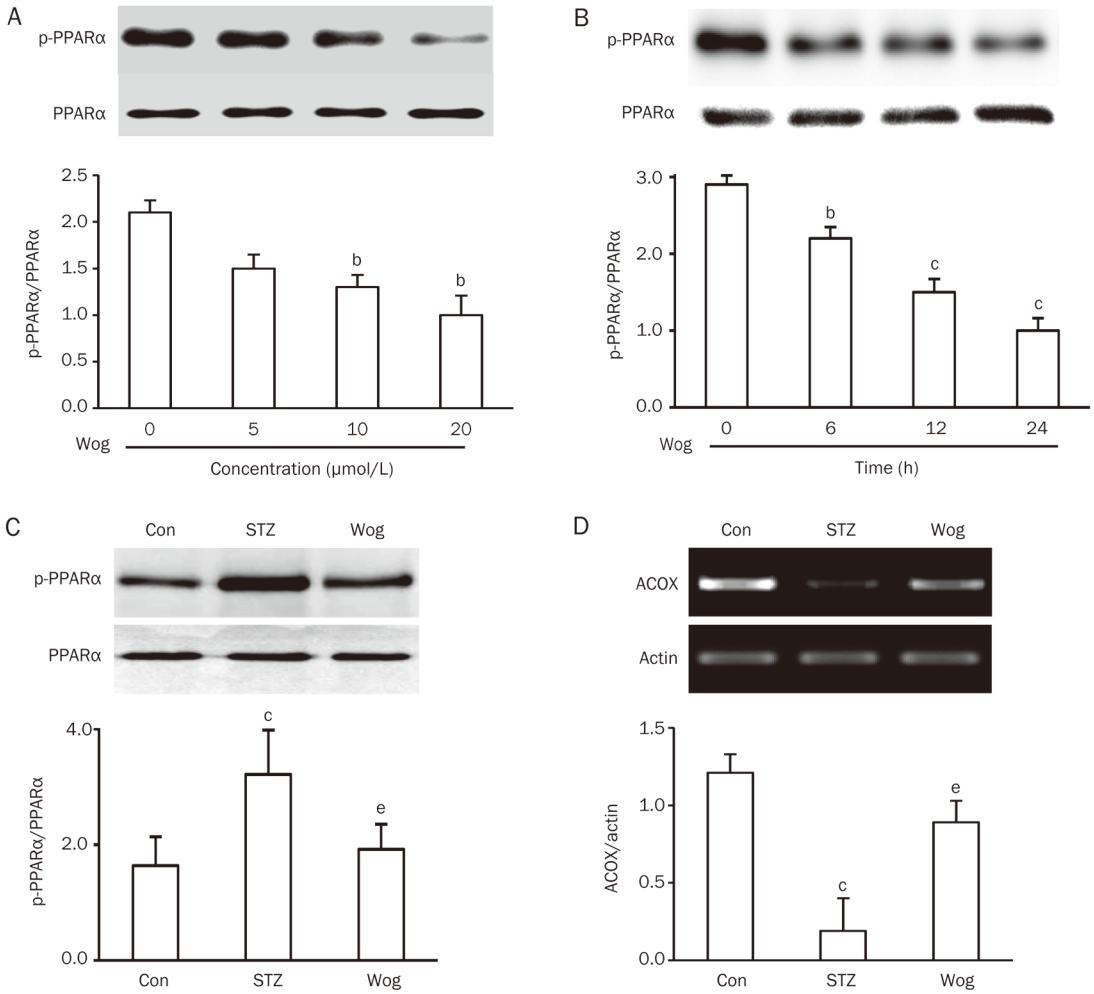

Figure 4. Wogonin inhibited PPARα phosphorylation. (A and B) Dose- and time-response of wogonin on PPARα phosphorylation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated in complete medium containing 25 mmol/L glucose and received indicated concentrations of wogonin for 12 h (A) or 10 μmol/L wogonin for the indicated time (B). (C) Effect of wogonin on PPARα phosphorylation in adipose tissue from STZ-induced type 1 diabetic mice (n=12). Cell extracts or tissues lysates were immunoprecipitated with anti-PPARα antibody. The proteins were separated by SDS-PAGE and phosphoproteins were detected via Pro-Q Diamond phosphoprotein gel stain. (D) Effect of wogonin on the mRNA levels of the PPARα target enzyme acyl-CoA oxidase (ACOX) in adipose tissue from STZ-induced type 1 diabetic mice (n=6). ^{b}P<0.05, ^{c}P<0.01 *vs* control group. ^{e}P<0.05 *vs* STZ group. Con, control group; STZ, streptozotocin-induced type 1 diabetes group; Wog, wogonin treatment group.

a negative role in PPARα-reduced expression of OPN by acting on the proximal OPN promoter[22, 23]. We next detected the expression of c-Fos and phospho-c-Jun in perinephric white adipose tissue and in 3T3-L1 adipocytes using Western blot analysis. As depicted in Figure 6A and 6B, wogonin-treated adipose tissue exhibited lower expression of c-Fos and phospho-c-Jun in the nuclear fraction compared with the STZ group (P<0.05, P<0.05, respectively). Similar results were also found in 3T3-L1 adipocytes, demonstrating that wogonin treatment significantly repressed the expression of c-Fos (Figure 6C) and phospho-c-Jun (Figure 6D) in the nuclear fraction. Together, these results suggest PPARα mediated wogonin action on OPN expression.

Wogonin inhibited p38 MAPK phosphorylation

To investigate the potential role of p38 MAPK in wogonin-reduced OPN expression, we first studied the effect of wogonin on p38 phosphorylation in perinephric white adipose tissue from type 1 diabetic mice and 3T3-L1 adipocytes. As shown in Figure 7A, administration of wogonin inhibited STZ-increased phosphorylation of p38 MAPK in adipose tissue from diabetic mice (P<0.05). When 3T3-L1 adipocytes were cultured in complete medium containing 25 mmol/L glucose and exposed to 10 μmol/L wogonin or 10 μmol/L of the specific p38 MAPK inhibitor SB203580 for 12 h, p38 MAPK phosphorylation was significantly suppressed (Figure 7B). SB203580 was also found to inhibit PPARα phosphorylation (Figure 7C), which was accompanied by decreased OPN expression (Figure 7C and 7D). Additionally, the p38 MAPK inhibitor could not further enhance the inhibitory effects of wogonin on OPN expression in 3T3-L1 adipocytes (Supplementary Figure 5). These results suggest that p38

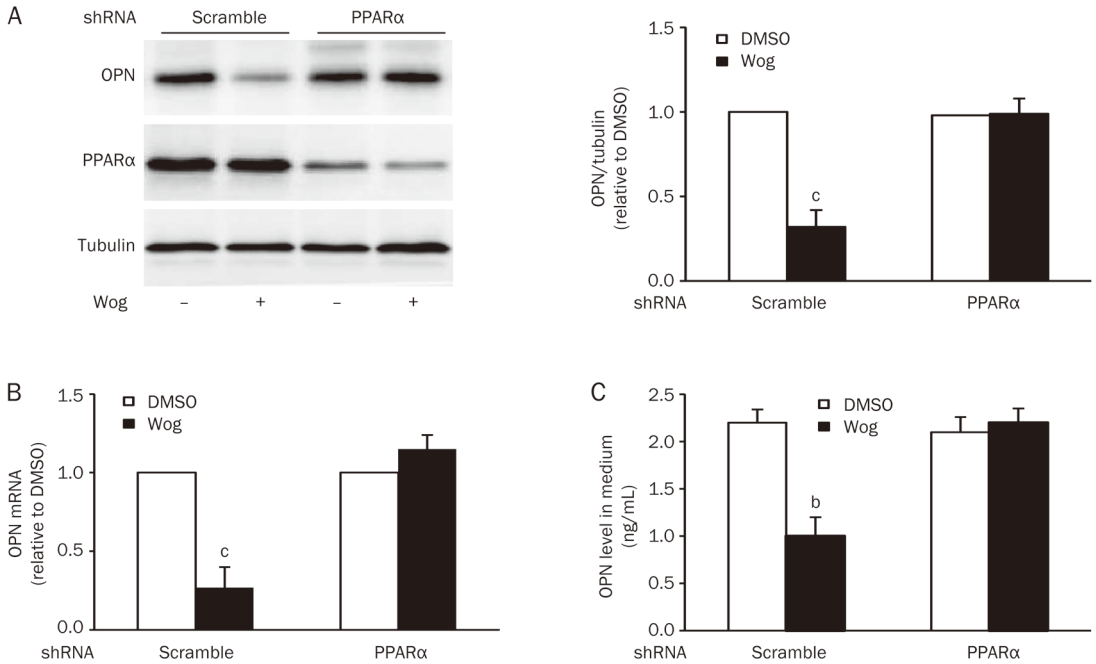

Figure 5. Silencing of PPARα mitigated wogonin action on OPN synthesis and secretion in 3T3-L1 adipocytes. (A and B) Effect of PPARα knockdown on the level of OPN protein (A) and mRNA (B) in 3T3-L1 adipocytes. (C) Effect of PPARα knockdown on OPN concentration in culture medium. 3T3-L1 adipocytes infected with lentivirus carrying PPARα/shRNA and scrambled sequences were cultured in complete medium containing 25 mmol/L glucose and followed by treatment with or without 10 μmol/L wogonin for 12 h. $^{b}P<0.05$, $^{c}P<0.01$ *vs* DMSO control group. OPN, osteopontin; Wog, wogonin.

MAPK is involved in the regulatory mechanism of wogonin for OPN expression.

Discussion

Oxidative stress and inflammation play a pivotal role in the pathogenesis of insulin resistance and diabetes[24, 25]. Wogonin possesses anti-oxidant and anti-inflammatory properties[26, 27] and is assumed to have anti-diabetic activity. Recent studies have indeed shown that wogonin treatment in *db/db* mice prevented weight gain, improved glucose tolerance, decreased serum insulin and cholesterol levels, and suppressed the accumulation of lipid droplets in the liver[15]. These pro-metabolic effects are associated with the enhanced expression of PPARα and adiponectin[15]. In this study, we demonstrate an inhibitory effect of wogonin on OPN expression in adipocytes *in vivo* and *in vitro* (Figure 1 and 2). Given that OPN is responsible for the severe inflammation and insulin resistance in insulin target tissues[6–9], our results present a novel explanation for the anti-diabetic properties of wogonin.

Adipose tissue and adipocytes are two of the primary sites of OPN synthesis[5, 28]. Previous studies have shown that OPN expression is strongly upregulated in adipose tissue from HFD-fed mice[7, 9]. Our results found that STZ-induced type 1 diabetic mice exhibited higher OPN expression in adipose tissue (Figure 1), suggesting that the upregulation of OPN mRNA and protein exists similarly in the adipose tissue of type 1 diabetic mice, which may be due to high blood glucose or hyperglycemia[29, 30]. In fact, high OPN expression and hyperglycemia could influence each other. STZ-induced hyperglycemia lead to the acute upregulation of serum OPN levels and pancreatic OPN mRNA and protein[29]. In cultured rat aortic smooth muscle cells, high glucose has been shown to stimulate OPN expression[30]. In contrast, OPN deficiency prevented glucose production and hyperglycemia in HFD mice[6, 9]. These studies suggest that the interplay between OPN and hyperglycemia may form a vicious cycle, leading to the development of diabetes. Wogonin could interfere with this cycle, thus exhibiting beneficial results for patients with diabetes.

PPARα plays a central role in the regulation of energy metabolism, particularly in lipid and lipoprotein metabolism. Recently, several studies have shown that PPARα is expressed in the adipose tissues of humans and rodents[31, 32]. In both type 1 and type 2 diabetes models, PPARα expression was significantly downregulated in some tissues[15, 33], consistent with our results showing that expression levels of PPARα protein and mRNA in adipose tissues of diabetic mice were statistically significantly decreased compared to those of control mice (Figure 3B–3D). Our results further confirm that wogonin treatment increased PPARα expression (Figure 3), inhibited PPARα phosphorylation (Figure 4A–4C), enhanced PPARα transactivation (Supplementary Figure 2), and restored mRNA expression of the PPARα target ACOX (Figure 4D) in the adipose tissue of diabetic mice and/or in 3T3-L1 adipocytes. PPARα phosphorylation has been suggested to have a negative association with PPARα activity; decreased phosphorylation leads to increased PPARα activity[21]. Hence, our results indicate that wogonin could activate PPARα, in

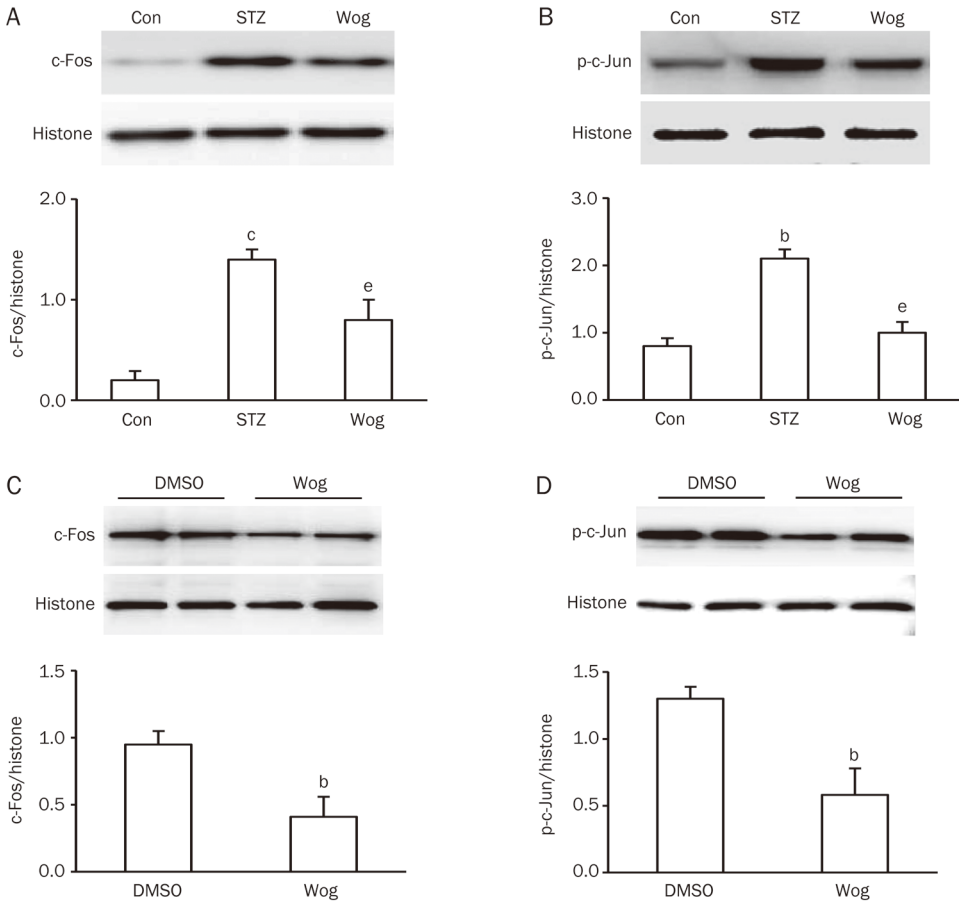

Figure 6. Wogonin inhibited the expressions of c-Fos and phospho-c-Jun in adipose tissue and 3T3-L1 adipocytes. (A and B) Effect of wogonin on the expression of c-Fos (A) and the phosphorylation status of c-Jun (B) in adipose tissue from STZ-induced type 1 diabetic mice (n=12). (C and D) Effect of wogonin on the expression of c-Fos (C) and phosphorylation status of c-Jun (D) in 3T3-L1 adipocytes (n=4). 3T3-L1 adipocytes cultured in high-glucose complete medium were treated with or without 10 μmol/L wogonin for 12 h. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control or DMSO group. $^{e}P<0.05$ *vs* STZ group. Con, control group; STZ, streptozotocin-induced type 1 diabetes group; Wog, wogonin treatment group.

agreement with a previous study showing that wogonin stimulated PPARα expression and activity in the liver and white adipose tissue of *db/db* mice[15]. Furthermore, our results showed that PPARα knockdown abrogated the inhibitory effects of wogonin on OPN expression in 3T3-L1 adipocytes (Figure 5), suggesting that PPARα mediated the OPN lowering effects of wogonin.

Accumulating evidence has suggested that c-Fos and c-Jun are involved in PPARα-induced reduction of OPN expression. A PPARα agonist downregulated the expression of c-Fos and phosphorylated c-Jun and inhibited its association with the OPN promoter, leading to reduced OPN expression. Overexpression of c-Fos and c-Jun abolished the inhibitory effect of PPARα on OPN expression. These results suggest that PPARα negatively regulates OPN expression through negative interference with c-Fos/c-Jun on the proximal OPN promoter[22, 23]. Our results indicated that wogonin inhibited the protein expression of c-Fos and phospho–c-Jun in adipose tissue from diabetic mice and in 3T3-L1 adipocytes (Figure 6). These findings, combined with our observation that wogonin upregulated PPARα expression and activity, further support the notion that PPARα mediates the inhibitory action of wogonin on OPN expression.

The remaining question is how PPARα is activated by wogonin. Previous studies have found convincing evidence that p38 MAPK is a kinase upstream of PPARα. Indeed, the impact of p38 MAPK on PPARα activity is dependent upon cell type and its physiological or pathophysiological condition. Activation of p38 MAPK in cardiomyocytes led to increased PPARα transcriptional activity[34]. Conversely, the p38 activator anisomycin induced a dose-dependent phosphorylation of PPARα and a 50% inhibition of its transcriptional activity in COS-7 cells[21]. Our results indicated that wogonin treatment dramatically mitigated p38 MAPK phosphorylation (Figure 7A and 7B), consistent with early studies showing that the expression of phosphorylated p38 MAPK was significantly attenuated by treatment with wogonin[26, 35]. In addition, the inhibition of p38 MAPK by its specific inhibitor SB203580 decreased PPARα phosphorylation (Figure 7C) and OPN expression (Figure 7C and 7D). These

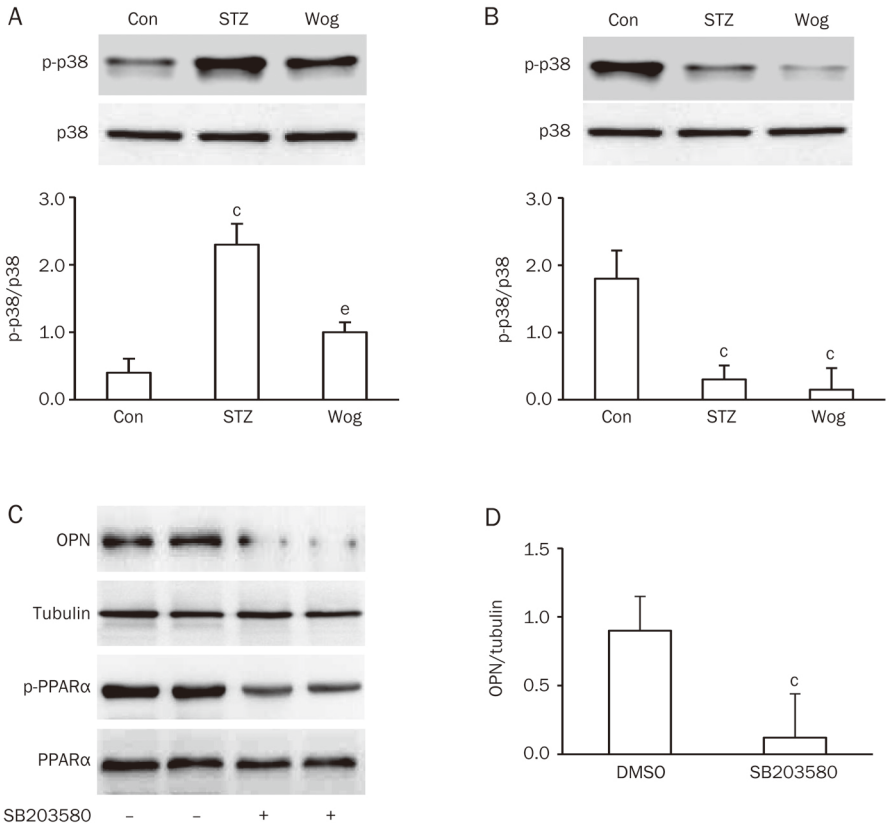

Figure 7. p38 MAPK mediated wogonin action on OPN expression. (A and B) Effect of wogonin on p38 MAPK phosphorylation in adipose tissue from STZ-induced type 1 diabetic mice (A, *n*=12) and 3T3-L1 adipocytes (B, *n*=4). (C) Effect of the specific p38 MAPK inhibitor SB203580 on OPN expression and PPARα phosphorylation. (D) Quantification of OPN levels in SB203580-treated adipocytes in (A). $^{c}P<0.01$ *vs* control or DMSO group. $^{e}P<0.05$ *vs* STZ group. Con, control group; STZ, streptozotocin-induced type 1 diabetes group; Wog, wogonin treatment group.

findings suggest that p38 MAPK negatively impacts PPARα regulation activity in adipocytes. Interestingly, basal p38 MAPK phosphorylation was significantly elevated in type 2 diabetic adipocytes[36]. Activation of p38 MAPK has also been observed in response to such stress stimuli as STZ administration, inflammation, and high glucose[37–40]. Hence, increased p38 MAPK activation may be involved in the development of insulin resistance and diabetes. Our findings implicate p38 MAPK in the regulation of PPARα activity and sequential OPN expression[41].

Traditionally, inflammatory cells are considered a major source of serum OPN in cases of morbid obesity[9, 42]. The contributions of adipose tissue and adipocytes to the circulating levels of OPN remain largely unknown. A previous study has shown a highly significant positive correlation between plasma OPN levels and body fat percentage[43]. In contrast, You *et al* reported that serum OPN concentration was not associated with body fat percentage[44]. However, these two studies have demonstrated that fat loss produced a statistically significant reduction in circulating OPN concentration[43, 44]. In addition, high levels of serum OPN were found in obese individuals[42, 43, 45]. These findings suggest that adipocytes may be one origin of circulating OPN levels.

In the present study, we used 3T3-L1 adipocytes to probe the possibility of induced OPN secretion by adipocytes under high glucose and hyperglycemic conditions. Our results showed that high glucose stimulated OPN secretion in 3T3-L1 adipocytes (Supplementary Figure 1), which was inhibited by wogonin treatment (Figure 2D), suggesting a potential involvement of adipocytes in the regulation of serum OPN concentration. Combined with our findings showing that wogonin attenuated an increase in STZ-induced serum OPN concentration (Figure 1E), our results indicated that adipocytes were at least partially responsible for the increased serum OPN concentration under STZ-induced hyperglycemic status. However, we still cannot conclude that adipocytes are the main source of serum OPN due to the multiple impacts of wogonin on factors related to metabolic disorders. For example, the anti-oxidative, anti-inflammatory, and anti-hyperglycemic activities of wogonin may be directly or indirectly involved in the regulation of OPN secretion by other cell types such as inflammatory cells. Further studies using adipose-specific OPN knockout mice are needed to determine the contribution of adipocytes to the serum OPN concentration. Additionally, we also do not know the detailed mechanism of secretion inhibition by wogonin. One possibility is that diminished OPN generation in adipocytes results in decreased secretion.

Collectively, our results demonstrate that wogonin suppresses OPN expression through a mechanism involving p38 MAPK inhibition and sequential PPARα activation in adipocytes. Thus, targeting OPN by wogonin provides a novel therapeutic strategy for the treatment of insulin resistance and diabetes.

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Author contribution

Chang-hua WANG and Ye-min ZHANG designed the research; Ye-min ZHANG, Ming-xin LI, and Zhao TANG performed the research; Chang-hua WANG analyzed the data and wrote the paper.

Supplementary information

Supplementary figures are available at the Acta Pharmacologica Sinica's website.

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