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Nobiletin improves hyperglycemia and insulin resistance in obese diabetic *ob*/*ob* mice

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ABSTRACT

Nobiletin is a polymethoxylated flavone found in certain citrus fruits that exhibits various pharmacological effects including anti-inflammatory, antitumor and neuroprotective properties. The present study investigated the effects of nobiletin on insulin sensitivity in obese diabetic *ob*/*ob* mice, and the possible mechanisms involved. The *ob*/*ob* mice were treated with nobiletin (200 mg/kg) for 5 weeks. Nobiletin significantly improved the plasma glucose levels, homeostasis model assessment index, glucose tolerance in an oral glucose tolerance test and plasma adiponectin levels. In white adipose tissue (WAT), nobiletin significantly decreased the mRNA expression levels of inflammatory adipokines such as interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 and increased the mRNA expression levels of adiponectin, peroxisome proliferator-activated receptor (PPAR)-γ and its target genes. At the same time, nobiletin increased the glucose transporter (Glut) 4 expression levels in the whole plasma membrane, and Glut1 and phospho-Akt expression in the whole cell lysates in WAT and muscle. Nobiletin also increased Glut4 protein expression level in the whole cell lysates of the muscle. Taken together, the present results suggest that nobiletin improved the hyperglycemia and insulin resistance in obese diabetic *ob*/*ob* mice by regulating expression of Glut1 and Glut4 in WAT and muscle, and expression of adipokines in WAT.

1. Introduction

Insulin resistance has been proposed as the major underlying cause of type 2 diabetes, hypertension, dyslipidemia and atherosclerosis. Insulin resistance is defined as reduced effects of insulin in its target tissues, mainly muscle, liver and adipose tissues [1–3].

Glucose uptake is regulated by insulin thorough activating a complex cascade of signaling pathway in skeletal muscle and adipose tissue. Phosphorylation of insulin receptor (IR) by insulin activates phosphatidylinositol 3-kinase (PI3K), of which increases the activation of protein kinase B (PKB/Akt) and atypical protein kinase C (aPKC), of which proteins of downstream signaling. And it results in Glut4 translocation to the plasma membrane [4]. It has been reported that overexpression of Akt in 3T3-L1 adipocytes activated the Glut4 translocation to the plasma membrane, whereas inhibition of Akt by interfering antibodies attenuated the insulin-stimulated glucose uptake [5,6].

In adipose tissue, insulin resistance is manifested as reduced glucose uptake and increased lipolysis and free fatty acid release [1]. In addition, dysfunctions of adipokines, which are bioactive substances secreted by adipocytes, play important roles in the development of obesity and insulin resistance [7–10]. Among the adipokines, adiponectin is downregulated in the obese and diabetic state [11,12]. Adiponectin enhances glucose uptake and fatty acid oxidation in muscle [13,14] and improves insulin resistance by suppressing hepatic glucose production [15,16]. Furthermore, adiponectin-deficient mice exhibit insulin resistance and glucose intolerance [17]. On the other hand, inflammatory molecules, including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1, are upregulated in adipose tissue in the obese and insulin-resistant state [18]. TNF-α and IL-6 impair insulin signaling by suppressing the expressions of proteins such as insulin receptor substrate-1 and glucose transporter (Glut) 4 [18–20]. In addition, TNF-α causes insulin resistance by regulating the expressions of genes associated with adipogenesis, lipolysis and adipokines in adipose tissue [7,21]. MCP-1, a member of the CC chemokine family, decreases insulin-stimulated glucose uptake in adipocytes [7,22]. MCP-1-deficient mice show improved insulin resistance [23]. Recent studies have demonstrated that increased MCP-1 expression in

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adipose tissue induces macrophage infiltration into the adipose tissue and contributes to the development of insulin resistance [22–24]. Taken together, these observations suggest that dysfunctions of adipokines play critical roles in the development of insulin resistance. Therefore, regulation of adipokine functions is a useful strategy for improving insulin resistance.

Liver also play important roles in maintaining glucose homeostasis. In insulin resistance state, it is shown that hepatic glucose production is upreglated by increased gluconeogenesis [25,26]. This gluconeogenesis is regulated by rate-controlling gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [26]. The mRNA expression levels of hepatic PEPCK and G6Pase are increased in several animal models of type 2 diabetes [27].

Recently, much attention has been focused on screening of products from natural sources, such as flavonoids, that may be beneficial for reducing the risk for metabolic syndrome [25,28], because products from plant sources are usually considered to be less toxic and have fewer side effects than products from synthetic sources.

Nobiletin is a polymethoxylated flavone (PMF) found in citrus fruits such as *Citrus depressa* (shiikuwasa), *Citrus sinensis* (oranges) and *Citrus limon* (lemons). The amount of nobiletin (w/w) was contained from the peels of shiikuwasa more than oranges and lemons [29,30]. It has been reported to exhibit biological effects including anti-inflammatory, antitumor and neuroprotective properties [31–33]. Lin et al. [34] reported that nobiletin suppressed the expressions of proinflammatory cytokines, including IL-1, IL-6 and TNF-α in mouse macrophages. In addition, it has been reported that a mixture of nobiletin and tangeretin, another PMF, regulated glucose metabolism in hamsters with fructose-induced insulin resistance by modulating adipokines [35]. Recently, Saito et al. [36] demonstrated that nobiletin enhances the differentiation and lipolysis of 3T3-L1 adipocytes, but does not act as a peroxisome proliferator-activated receptor (PPAR) γ ligand. These observations help to provide an increased understanding of how nobiletin influences insulin sensitivity. However, the effects of nobiletin alone on the enhancement of insulin sensitivity are not fully understood in vivo. In the present study, we examined the effects of nobiletin on glucose metabolism and insulin sensitivity, and analyzed the molecular mechanisms involved in these effects using obese diabetic *ob*/*ob* mice.

2. Materials and methods

2.1. Isolation and identification of nobiletin

Shiikuwasa (*C. depressa*) were purchased from Okinawa, Japan. The peels of shiikuwasa (dry weight, 8 kg) were extracted with methanol (40 l) for 2 weeks. The evaporated methanol extract (581.4 g) was dissolved in distilled water, and successively fractionated with ethyl acetate, hexane and 90% methanol. The 90% methanol-eluted fraction (245 g) was chromatographed on an octadecylsilyl silica gel open column (Cosmosil $_{75}C_{18}$-OPN; Cosmosil, Japan). The column was eluted with a stepwise gradient of water and methanol (60:40, 40:60, 20:80 and 0:100, v/v). The fraction obtained with water and methanol at 20:80 (v/v, 67 g) was fractionated in fractions 1–7 using a reverse-phase preparative high-performance liquid chromatography (HPLC) system equipped with a Cosmosil $_{5}C_{18}$-AR-II column (mobile phase, water and methanol at 20:80 (v/v); size, 50 mm × 500 mm; flow rate of mobile phase, 50 ml/min). Fraction 5 was subsequently identified as nobiletin based a comparative study with standard nobiletin (Wako Pure Chemical Industries, Japan) by HPLC analysis. And the analysis of purified nobiletin was determined by nuclear magnetic resonance (NMR) and mass spectrometry (MS). The analytic HPLC was carried out on Tosho UV-8011 HPLC system with a Cosmosil Cholester Waters column. The mobile phase for the HPLC system was 25% water, 75% methanol and 0.1% Trifluoroacetic acid (TFA) with flow rate of 1.0 ml/min. The eluent was detected with UV wavelength at 260 nm. The ^{1}H NMR (400 MHz, CD_3COCD_3) and ^{13}C NMR (100 MHz, CD_3COCD_3) spectra were obtained on a FT NMR (JEOL, Japan). ESI-MS was carried out using an Esquire 3000 plus mass spectrometer (Bruker Daltonics, Germany) equipped with a gas nebulizer probe, capable of analyzing ions up to m/z 6000. The mass spectrometer was operated in the full-scan mode analyzing positive.

2.2. Experimental animals

C57BL/6J-*ob*/*ob* (*ob*/*ob*) mice were purchased from Charles River Japan at 6 weeks of age. The mice were individually housed under temperature (23 ± 3 °C)—and humidity-controlled conditions with a 12-h light/dark cycle, and given free access to water and food. The mice were allowed to adapt to these conditions for 1 week before the beginning of the experimental protocol. The experimental design was approved by The Animal Experiment Committee of Chubu University, and the mice were maintained in accordance with their guidelines.

2.3. Nobiletin treatment study

The *ob*/*ob* mice were divided into two groups (n = 10) based on their body weights and plasma glucose levels. One group of mice were administered a daily dose of nobiletin (200 mg/kg body weight) mixed with the vehicle (0.3% carboxyl methyl cellulose) while the other group of mice were administered the vehicle alone by oral gavage once daily for 5 weeks. During the experimental period, the mice had free access to water and food, and their body weights and food intakes were measured twice per week.

2.4. Collection of plasma, liver, muscle and white adipose tissue (WAT)

Before, during and at the end of the experimental period, blood samples were taken from the tails of the mice for biochemical plasma analyses in the non-fasted and fasted states. At the end of the 5-week period, the mice were sacrificed by cervical dislocation following blood collection. Plasma samples were obtained by centrifugation at 10,000 × g for 10 min at 4 °C. The separated plasma samples were stored at −80 °C until analysis. Liver, muscle and WAT (epididymal and periperal WAT) were excised immediately, rinsed, weighed, frozen in liquid nitrogen and stored at −80 °C until analysis.

2.5. Biochemical analyses of plasma

Plasma glucose, triglyceride (TG) and total cholesterol (T-CHO) levels were determined by enzymatic methods using commercial assay kits (Glucose C II-Test, Triglyceride E-Test and Cholesterol E-Test; Wako Pure Chemical Industries, Japan) according to the manufacturer's protocols. Plasma insulin and adiponectin levels were measured by immunoassays using ELISA kits (Mouse Insulin ELISA Kit (U-type); Shibayagi, Japan; and Mouse Adiponectin/Acrp30; R&D Systems, USA) according to the manufacturers' protocols. The homeostasis model assessment (HOMA) index was calculated from the fasting plasma glucose and insulin levels as a measure of insulin resistance as follows [37]: HOMA = fasting glucose level (mg/dl) × fasting insulin level (ng/ml)/405.

2.6. Oral glucose tolerance test (OGTT)

After 4 weeks of treatment with nobiletin, the mice were fasted overnight and a basal blood sample (0 min) was collected from the tail vein. The mice were then orally administered glucose (2 g/kg

body weight) and additional blood samples were collected at 30, 60 and 120 min. The blood samples were centrifuged at 10,000 × *g* for 10 min at 4 °C, and the plasma samples were stored at −80 °C until analysis. The plasma glucose levels were measured with the above-described Glucose C II-Test.

2.7. Total RNA isolation and gene expression analyses

Total RNA was isolated from tissues (WAT and liver) using the Isogen reagent (Nippon Gene, Japan) according to the manufacturer's protocol and pooled for real-time PCR analysis of each group (n = 10). Total RNA (1 μg) was reverse-transcribed to cDNA in a final volume of 20 μl using a reverse transcription system (a3500; Promega, USA) according to the manufacturer's protocol. Real-time PCR was performed in a final volume of 20 μl containing 50 ng of cDNA template and primers using a FastStart Universal SYBR Green Master PCR Kit (Roche, Germany) and an ABI Prism 7700 system (Applied Biosystems, USA) according to the their manufacturer's protocol, respectively. All reactions were carried out using the following protocol: 95 °C for 2 min; 40 cycles of 95 °C for 20 s, 60 °C for 20 s, 72 °C for 40 s and 72 °C for 30 s; and a 5-min extension at 72 °C with a melting curve. The sequences of the primers used for the quantitative real-time PCR are shown in Table 1. All the

Table 1
Primer sequences used for real-time PCR.

Gene	Primer sequences
Adiponectin	5′-GTTGCAAGCTCTCCTGTTCC-3′
NM_009605	5′-CTTGCCAGTGCTGTTGTCAT-3′
TNF-α	5′-ACGGCATGGATCTCAAAGAC-3′
NM_031168	5′-CGGACTCCGCAAAGTCTAAG-3′
IL-6	5′-CATGTTCTCTGGGAAATCGTGG-3′
NM_031168	5′-AACTGATATGCTTAGGCATAACGCAC-3′
MCP-1	5′-AAGAGAGAGGTCTGTGCTGA-3′
NM_011333	5′-TTCACTGTCACACTGGTCAC-3′
Glut4	5′-CAACGTGGCTGGGTAGGCA-3′
NM_009204	5′-ACAACATCAGCCCAGCCGGT-3′
PPARγ	5′-GCTGTTATGGGTGAAACTCTG-3′
NM_011146	5′-ATAAGGTGGAGATGCAGGTTC-3′
aP2	5′-CAACCTGTGTGATGCCTTTGTG-3′
NM_024406	5′-CTCTTCCTTTGGCTCATGCC-3′
LPL	5′-GCATTTGAGAAAGGGCTCTG-3′
NM_008509	5′-CTGACCAGCGGAAGTAGGAG-3′
Perilipin	5′-GATCGCCTCTGAACTGAAGG-3′
NM_175640	5′-GATCCACATGGCCAGAGAGT-3′
PEPCK	5′-AGCGGATATGGTGGGAAC-3′
NM_011044	5′-GGTCTCCACTCCTTGTTC-3′
G6Pase	5′-CGACTCGCTATCTCCAATTGA-3′
NM_008061	5′-GTTGAACCAGTCTCCGACCA-3′
GAPDH	5′-ATTGGGCGCCTGGTCAC-3′
XM_001473623	5′-CCAGAGGGGCCATCCAC-3′

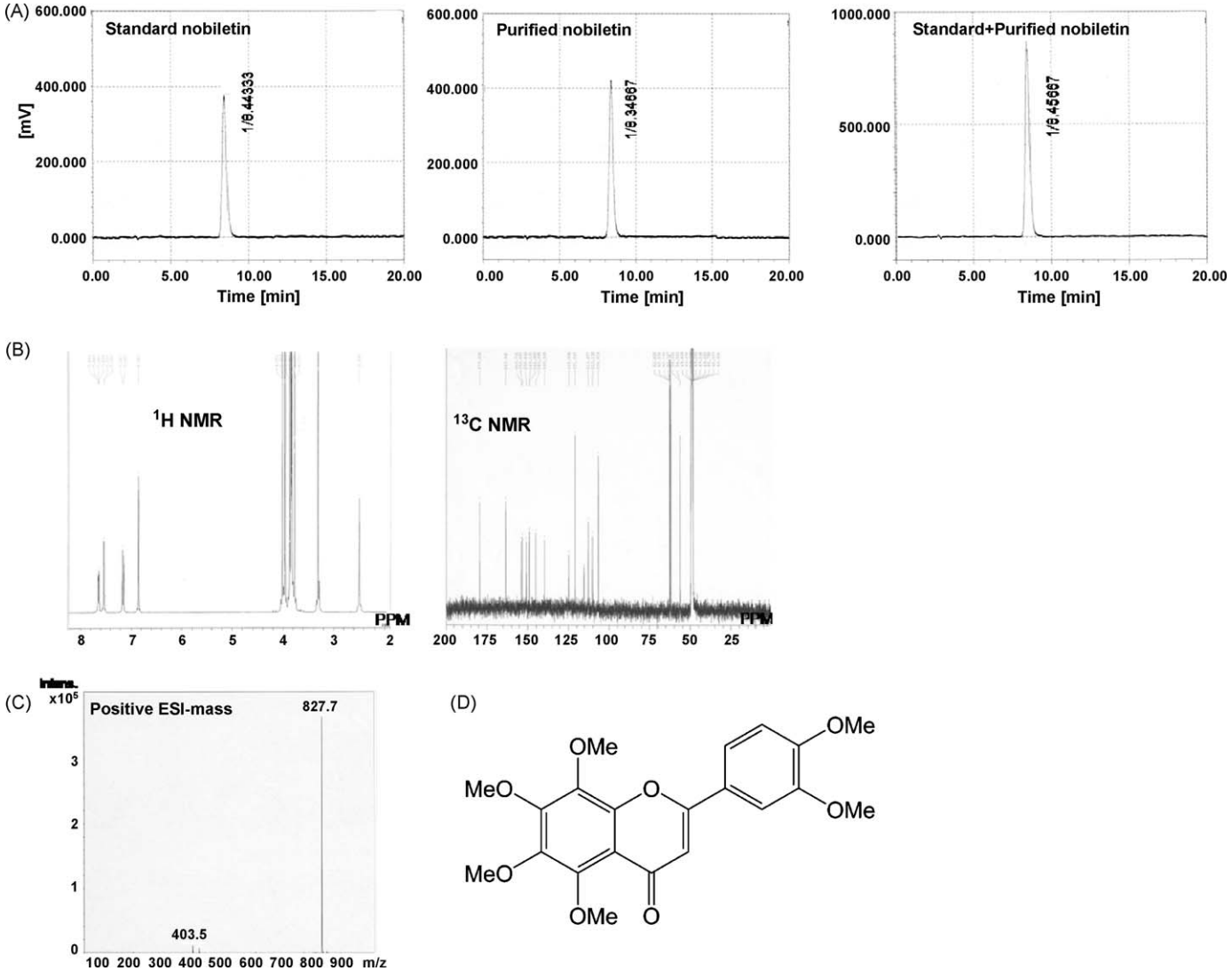

Fig. 1. Identification of purified nobiletin. (A) HPLC spectrums, (B) ^{1}H NMR (400 MHz, CD_3COCD_3) and ^{13}C NMR (100 MHz, CD_3COCD_3) spectrums, (C) MS spectrum and (D) the structure of nobiletin (molecular weight 402.4).

samples were normalized by the corresponding expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression level of the gene of interest in the nobiletin-treated group relative to its expression level in the vehicle-treated group was calculated using the $2^{-\Delta\Delta C_t}$ formula, where C_t is defined as the cycle number at which the fluorescence became significantly higher than the background. Specifically, $\Delta C_t = \Delta C_{t\ \text{interest}} - \Delta C_{t\ \text{GAPDH}}$ and $\Delta\Delta C_t = \Delta C_t$ of the nobiletin-treated group $- \Delta C_t$ of the vehicle-treated group, which was normalized to 1.

2.8. Protein extraction and Western blotting

The subcellular fractionation of WAT and muscle are carried out according to the method of Rampal et al. [38] with slight modification. Tissues were homogenized in buffer (0.25 M sucrose, 10 mM Tris–HCl pH 7.4, 2 mM EDTA, 1 mM PMSF). The homogenate was centrifuged at 760 × *g* for 10 min to remove nuclei and unbroken cell. The supernatant was centrifuged at 12,000 × *g* for 20 min to pellet the whole plasma membrane. The whole plasma membrane pellets were suspended in buffer and frozen at −80 °C. To prepare the whole cell lysate, tissues were homogenized in modified RIPPA buffer (50 mmol/l Tris–HCl, pH7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% sodium dodecylsulfate (SDS), 1 mM PMSF). The homogenate was centrifuged at 760 × *g* for 5 min to remove nuclei and unbroken cell. The supernatant was centrifuged at 12,000 × *g* for 20 min, supernatant was collected and the protein concentrations were determined by protein assays (Bradford, USA) using BSA as a standard (Wako Pure Chemical Industries, Japan). Aliquots (30 μg protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% gels and transferred to polyvinylidene difluoride membranes (Amersham, USA). The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated with anti-rabbit Glut4, phospho-Akt (ser473), Akt, PPARγ, perilipin (Cell Signaling Technology, USA), anti-rabbit Glut1, PEPCK-C, G6Pase (Santa Cruz Biotechnology, USA) or anti-mouse Na^+/K^+ ATPase α-1 (Upstate, USA) antibodies in 5% BSA in TBS-T. Following the incubation, the membranes were washed three times with TBS-T for 15 min each, and then incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, USA) in 5% non-fat dry milk in TBS-T. After washing, the immunocomplexes were visualized by enhanced chemiluminescence (Amersham, UK). The exposed films were scanned and the obtained images were subjected to densitometric analysis using Scion Image Release Beta 4.02 software (http://www.scioncorp.com). The Glut4 expression levels were expressed relative to those in the vehicle-treated group.

Table 2
Body weight gains, food intake rates, organ weights, and plasma TG, T-CHO and insulin levels in the vehicle-treated and 200 mg/kg nobiletin-treated *ob*/*ob* mice after 5 weeks.

Experimental groups	Vehicle	Nobiletin
Body weight gain (g)	9.37 ± 0.74	9.33 ± 0.49
Food intake rate (g/day/mouse)	5.36 ± 0.16	5.00 ± 0.11
WAT weight (g)	5.94 ± 0.12	5.69 ± 0.17
Liver weight (g)	3.17 ± 0.13	3.07 ± 0.17
Plasma TG (mg/dl)	83.88 ± 11.32	69.15 ± 2.17
Plasma T-CHO (mg/dl)	166.20 ± 6.26	164.13 ± 6.93
Plasma insulin (ng/ml)	9.05 ± 1.20	7.16 ± 0.97
Plasma glucose (mg/dl)[a]	182.14 ± 9.56	145.26 ± 7.56*
Plasma insulin (ng/ml)[a]	14.35 ± 1.60	12.39 ± 1.70
HOMA	7.13 ± 0.88	4.39 ± 0.67*

WAT: white adipose tissue; TG: triglyceride; T-CHO: total cholesterol.
[a] Glucose and insulin levels were measured after fasting for 16 h after 4 weeks of nobiletin treatment. Values are expressed as means ± SEM ($n = 10$).
* $P < 0.05$, vs. the vehicle-treated group.

2.9. Statistical analysis

Data are expressed as means ± SEM. Differences between the mean values in the two groups were analyzed by Student's *t*-test and one-way ANOVA using the Origin 7 Software (MicroCal Software, USA). Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Isolation and identification of nobiletin

Nobiletin from the peels of shiikuwasa was analyzed by HPLC and the results are shown in Fig. 1. Compared with standard nobiletin, purified nobiletin showed same retention time (8.34 min) by HPLC analysis (Fig. 1A). The ^{1}H NMR, ^{13}C NMR and the MS spectrograms of purified nobiletin are shown in Fig. 1B and C. These spectral data of purified nobiletin were in agreement with published data [39,40]. The structure of nobiletin is shown in Fig. 1D.

3.2. Physical and metabolic characteristics

Table 2 shows the effects of the treatment with nobiletin (200 mg/kg) on the body weight gain, food intake rate, tissue weight, and TG, T-CHO and insulin levels of the *ob*/*ob* mice. The body weight gains did not differ between the vehicle-treated and nobiletin-treated groups, and there were no significant differences in the mean daily food intakes throughout the experimental

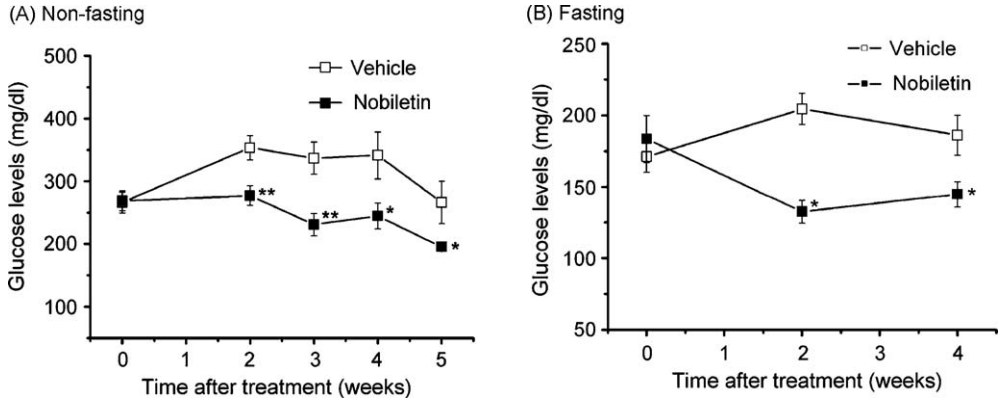

Fig. 2. Plasma glucose levels in the non-fasting (A) and fasting (B) states in the vehicle-treated and 200 mg/kg nobiletin-treated *ob*/*ob* mice during 5 weeks of treatment. Values are expressed as means ± SEM ($n = 10$). $^*P < 0.05$, $^{**}P < 0.01$ vs. the vehicle-treated group.

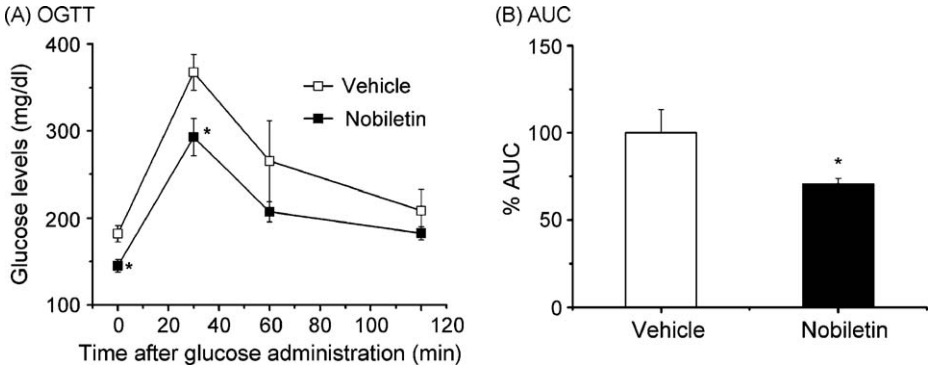

Fig. 3. Oral glucose tolerance tests in the vehicle-treated and 200 mg/kg nobiletin-treated *ob*/*ob* mice after 4 weeks. Values are expressed as means ± SEM (n = 10). $^{*}P < 0.05$ vs. the vehicle-treated group.

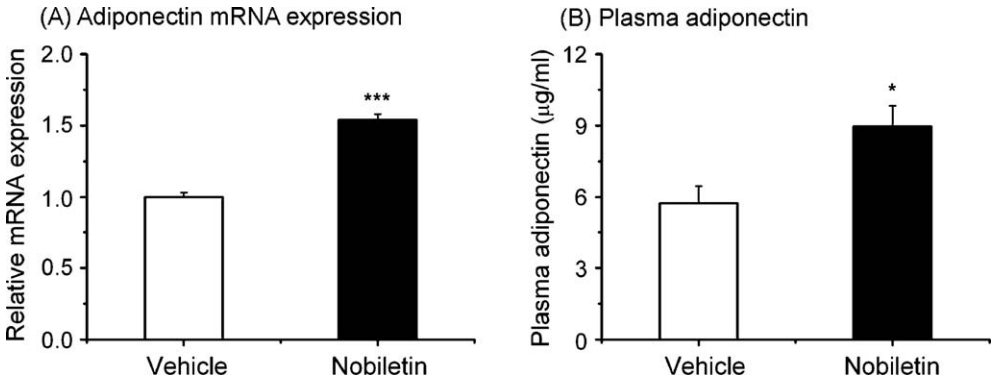

Fig. 4. Effects of nobiletin on adiponectin levels. (A) Adiponectin mRNA level in the WAT. The mRNA level was expressed as the fold increase relative to the vehicle-treated group after normalization by the GAPDH mRNA expression. Values are expressed as means ± SEM of triplicate analyses of pooled RNA samples for each group (n = 10). (B) Plasma adiponectin levels. Values are expressed as means ± SEM (n = 10). $^{*}P < 0.05$, $^{***}P < 0.005$ vs. the vehicle-treated group.

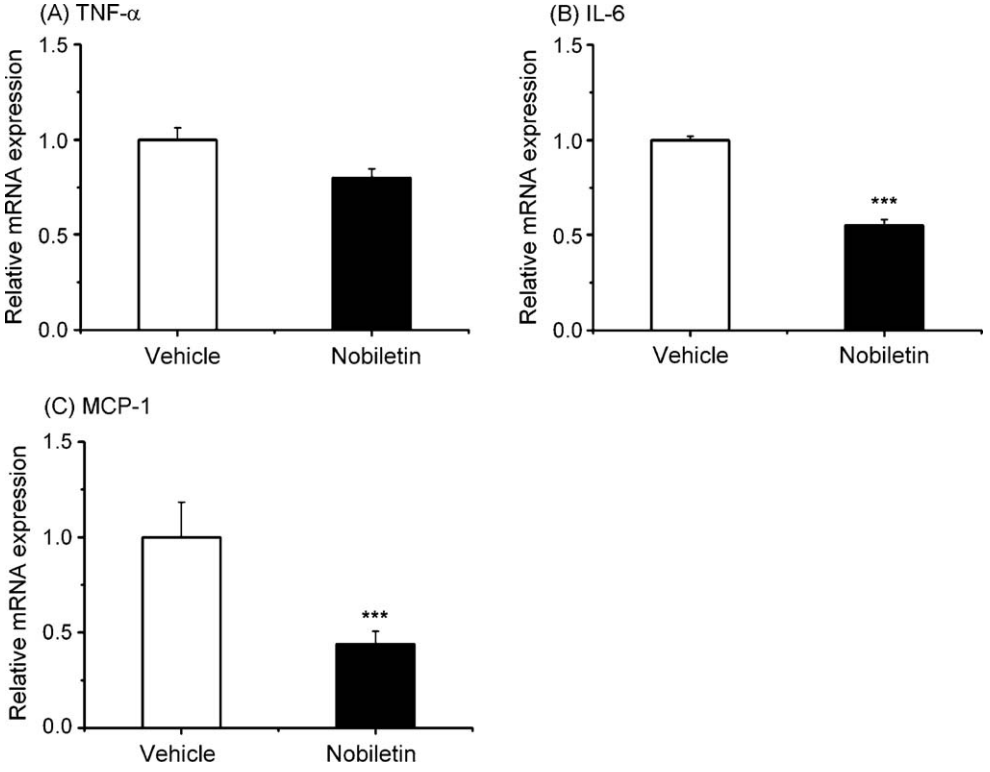

Fig. 5. Effects of nobiletin on inflammatory adipokine gene expressions in WAT. (A) TNF-α, (B) IL-6 and (C) MCP-1 mRNA expression levels in WAT. The mRNA expression levels were expressed as the fold increase relative to the vehicle-treated group after normalization by the GAPDH mRNA expression. Values are expressed as means ± SEM of triplicate analyses of pooled RNA samples for each group (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. the vehicle-treated group.

period. The liver and WAT weights did not differ significantly between the vehicle-treated and nobiletin-treated groups. The plasma insulin, TG and T-CHO levels also did not differ significantly between the vehicle-treated and nobiletin-treated groups. However, the HOMA index, as an indicator of insulin resistance, was significantly improved in the nobiletin-treated group.

3.3. Effects of nobiletin on plasma glucose levels

The non-fasting and fasting plasma glucose levels were measured during the experimental period. After 2 weeks of treatment, the non-fasting and fasting plasma glucose levels were significantly lower in the nobiletin-treated group than in the vehicle-treated group (Fig. 2A and B). This pattern persisted for the 5-week duration of the nobiletin treatment.

3.4. Effects of nobiletin on glucose tolerance in OGTTs

To analyze the glucose tolerance, we carried out OGTTs after 4 weeks of nobiletin treatment. The plasma glucose levels were significantly lower in the nobiletin-treated group than in the vehicle-treated group at 30 min after glucose administration at 2 g/kg (Fig. 3A). The area under the curve (AUC) was also lower in the nobiletin-treated group than in the vehicle-treated group (Fig. 3B). These data indicate improved glucose tolerance in the nobiletin-treated group.

3.5. Effects of nobiletin on adiponectin levels

The adiponectin mRNA expression levels in WAT and the plasma adiponectin levels are shown in Fig. 4. The adiponectin mRNA level in WAT was significantly increased by 1.5-fold in the nobiletin-treated group compared with the vehicle-treated group (Fig. 4A). The plasma adiponectin level was also significantly increased in the nobiletin-treated group compared with the vehicle-treated group (Fig. 4B).

3.6. Effects of nobiletin on inflammatory adipokine gene expressions in WAT

Although the difference was not statistically significant, the mRNA level of TNF-α tended to be lower in the nobiletin-treated group than in the vehicle-treated group (Fig. 5A). The mRNA levels of IL-6 (Fig. 5B) and MCP-1 (Fig. 5C) in WAT were significantly decreased by 45% and 56% in the nobiletin-treated group compared with the vehicle-treated group, respectively.

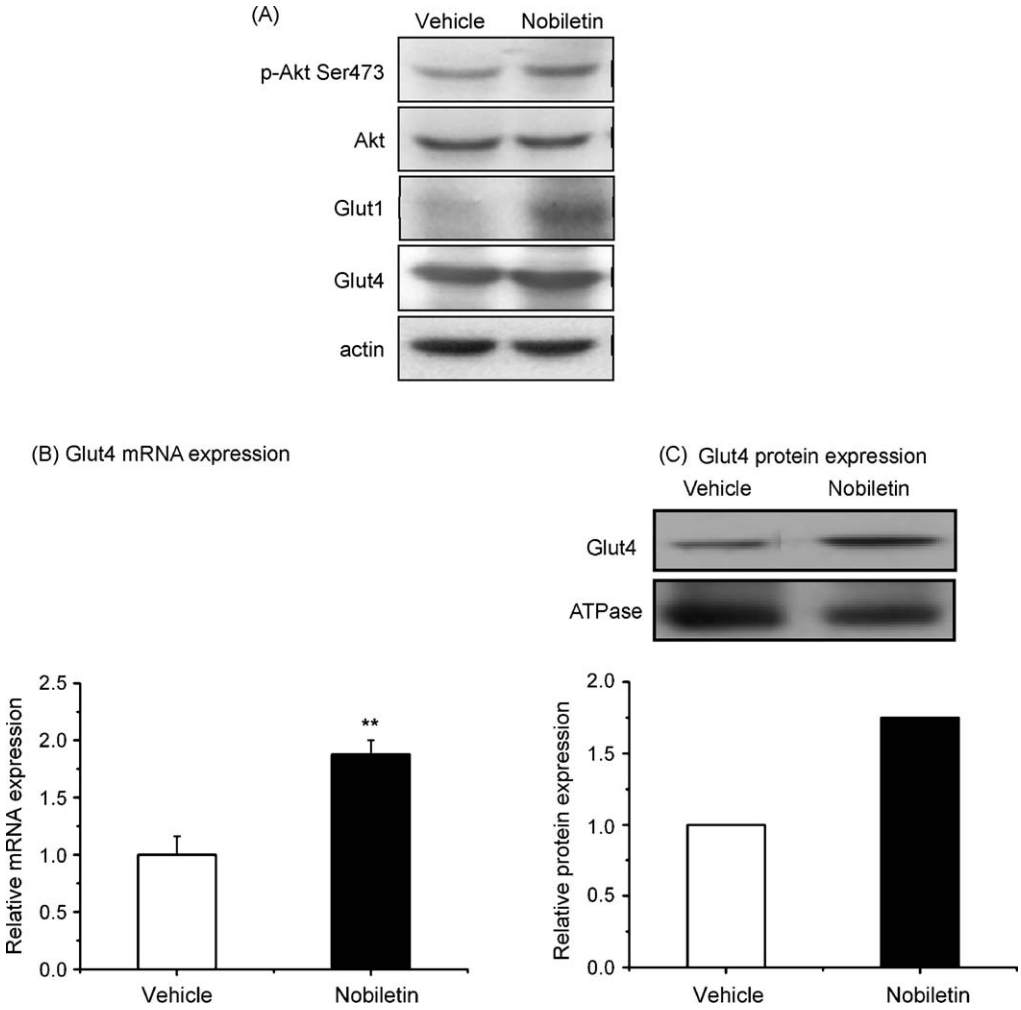

Fig. 6. Effects of nobiletin on p-Akt, Glut1 and Glut4 expression in WAT. (A) The p-Akt, Glut1 and Glut4 protein levels in WAT. The protein samples from 10 mice were pooled and the whole cell lysates p-Akt, Akt, Glut1 and Glut4 protein levels were detected using Western blotting. (B) The Glut4 mRNA expression level in WAT. The mRNA expression level was expressed as the fold increase relative to the vehicle-treated group after normalization by the GAPDH mRNA expression. Values are expressed as means ± SEM of triplicate analyses of pooled RNA samples for each group (n = 10). (C) The whole plasma membrane Glut4 protein level in WAT. The protein samples from 10 mice were pooled and whole plasma Glut4 protein level was expressed as the fold increase relative to the vehicle-treated group after normalization by the Na^+/K^+ ATPase α-1 (ATPase) level. $^*P < 0.05$, $^{**}P < 0.01$ vs. the vehicle-treated group.

3.7. Effects of nobiletin on Glut1, Glut4 and phosphorylation of Akt in WAT

To understand the molecular mechanism of nobiletin on decreasing glucose levels, we examined the effect of nobiletin on insulin signaling in WAT. In WAT, phosphorylation of Akt and Glut1 protein expression levels in the whole cell lysates are increased in the nobiletin-treated group compared with the vehicle-treated group (Fig. 6A). In WAT, the Glut4 mRNA level was increased (Fig. 6B), and the Glut4 protein expression levels in the whole plasma membrane (Fig. 6C) was also significantly increased without changes in the whole cell lysates (Fig. 6A) in the nobiletin-treated group compared with the vehicle-treated group.

3.8. Effects of nobiletin on the expressions of PPARγ and its target genes in WAT

PPARγ induces adipocyte differentiation and regulates the expressions of key genes involved in lipid and glucose metabolisms [41–43]. It has been reported that the mRNA expression levels of PPARγ2 are significantly increased by nobiletin in adipogenesis [36]. Therefore, we examined the expressions of PPARγ and its target genes. The mRNA level of PPARγ in WAT was significantly increased by 1.8-fold in the nobiletin-treated group compared with the vehicle-treated group (Fig. 7A). Moreover, the mRNA level of the PPARγ target gene perilipin was increased by 1.6-fold in the nobiletin-treated group compared with the vehicle-treated group (Fig. 7B). There was no significant difference in the mRNA expression levels of adipocyte fatty acid-binding protein (aP2) between the two groups (Fig. 7C). The mRNA expression level of lipoprotein lipase (LPL) tended to increase in the nobiletin-treated group compared with the vehicle-treated group, but the difference was not significant (Fig. 7D). Protein expression levels of PPARγ and perilipin are also increased in WAT of the nobiletin-treated group compared with the vehicle-treated group (Fig. 7E).

3.9. Effects of nobiletin on Glut1, Glut4 and phosphorylation of Akt in muscle and gluconeogenesis-related gene and protein expressions in liver

Muscle and liver tissues are known to play important roles in maintaining glucose homeostasis. In muscle, phosphorylation of Akt, Glut1 and Glut4 protein expression of the whole cell lysates were increased in the nobiletin-treated group compared with the vehicle-treated group. In addition, Glut 4 expression levels in the whole membrane fractions were significantly increased in the

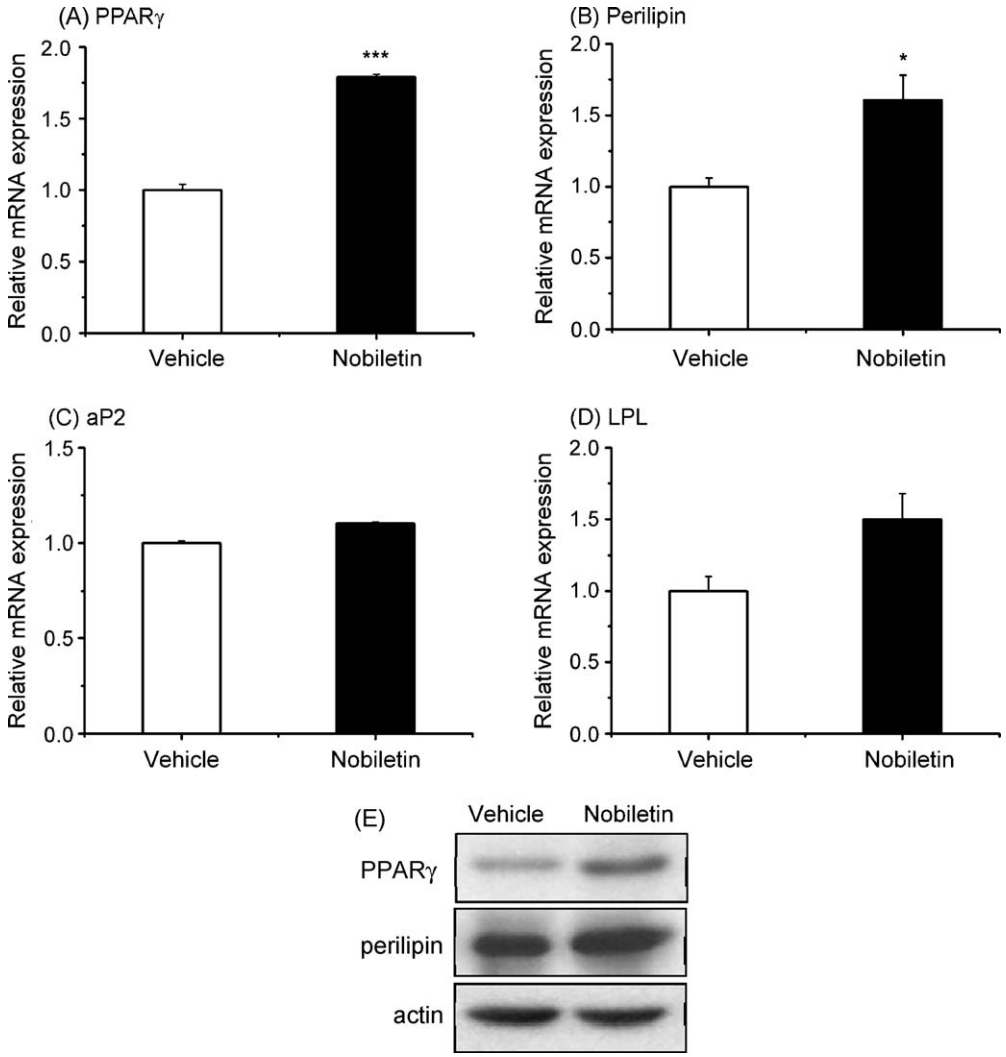

Fig. 7. Effects of nobiletin on the expressions of PPARγ and its target genes in WAT. (A) PPARγ, (B) perilipin, (C) aP2 and (D) LPL mRNA expression levels in WAT. The mRNA expression levels were expressed as the fold increases relative to the vehicle-treated group after normalization by the GAPDH mRNA expression. Values are expressed as means ± SEM of triplicate analyses of pooled RNA samples for each group (n = 10). (E) The PPARγ and perilipin protein levels in WAT. The protein samples from 10 mice were pooled and the whole cell lysates PPARγ and perilipin protein level were detected using Western blotting.

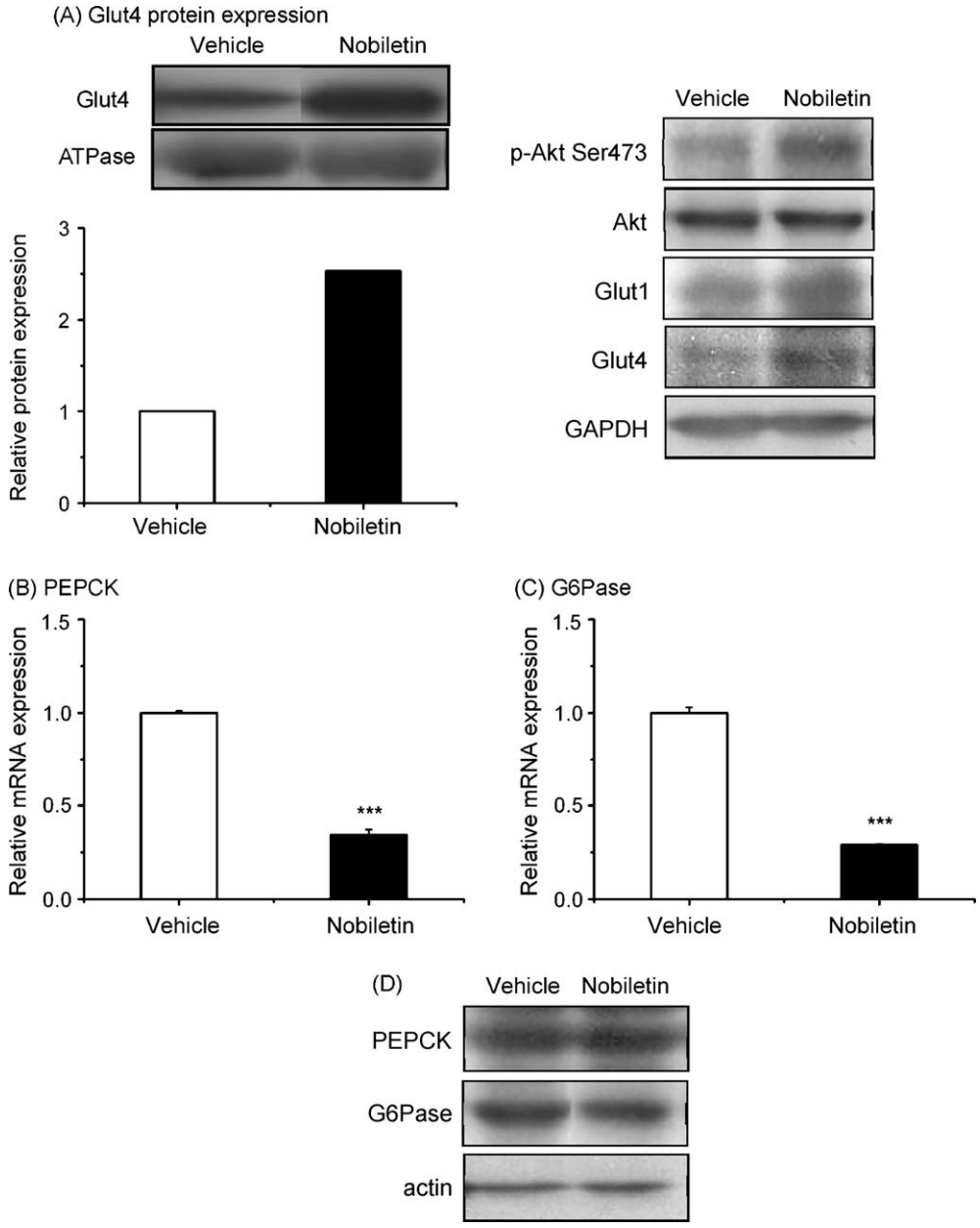

Fig. 8. Effects of nobiletin on p-Akt, Glut1 and Glut4 expression in muscle and gluconeogenesis-related expressions in liver. (A) The p-Akt, Glut1 and Glut4 protein levels in muscle. The protein samples from 10 mice were pooled and the whole cell lysates p-Akt, Akt, Glut1 and Glut4 protein levels and whole plasma Glut4 protein level were detected Western blotting. Whole plasma membrane Glut4 protein levels was expressed as the fold increase relative to the vehicle-treated group after normalization by the Na^+/K^+ ATPase α-1 (ATPase) level. (B) PEPCK and (C) G6Pase mRNA expression levels in liver. The mRNA levels were expressed as the fold increases relative to the vehicle-treated group after normalization by the GAPDH expression. Values are expressed as means ± SEM of triplicate analyses of pooled RNA samples for each group (n = 10). (D) The PEPCK and G6Pase protein levels in liver. The protein samples from 10 mice were pooled and the whole cell lysates PEPCK and G6Pase protein levels were detected using Western blotting.

nobiletin-treated group compared with the vehicle-treated group, respectively (Fig. 8A). The mRNA levels of PEPCK (Fig. 8B) and G6Pase (Fig. 8C) in liver were significantly decreased by 66% and 71% in the nobiletin-treated group compared with the vehicle-treated group, respectively. However, PEPCK and G6Pase protein expression levels showed no changes in liver of the nobiletin-treated group (Fig. 8D).

4. Discussion

The present study has demonstrated that nobiletin has positive effects on glucose metabolism. A previous study demonstrated that dietary supplementation with PMFs, comprising a mixture of nobiletin and tangeretin, improved glucose homeostasis in hamsters with fructose-induced insulin resistance [33]. Although several reports have demonstrated that the citrus flavonoids hesperidin and naringin have hypoglycemic effects [25], there are no reports of treatment with the citrus flavonoid nobiletin alone. In the present study, we investigated the effects of nobiletin on glucose metabolism and insulin sensitivity, and analyzed the mechanisms involved in its effects in obese diabetic *ob*/*ob* mice. Treatment with nobiletin for 5 weeks significantly reduced the glucose levels and HOMA indexes, and improved the glucose tolerance. These results suggest that nobiletin ameliorates hyperglycemia and insulin resistance.

We have been tested the effect of nobiletin from shiikuwasa on plasma glucose level in C57BL/6J mice fed with high-fat diet (HFD). Plasma glucose levels were significantly reduced in 200 mg/kg

nobiletin-treated group, however, were no changes in 20 mg/kg nobiletin-treated group compared with the HFD group (data not shown). Based on this result, we selected to the dose (200 mg/kg) for this study. Unfortunately, we did not invest the pharmacokinetic parameters after oral administration of nobiletin in vivo. Although metabolite studies were established in vivo, there is no study on pharmacokinetic parameters of nobiletin [44]. It needs to the further investigation.

Insulin resistance is associated with abnormal insulin signaling though reduction in the IRS-1, PI3K and Akt pathway [4]. Increased serine phosophorlation or reduced tyrosine phosphorylation caused the reduced phosphorylation of PI3K and its downstream signaling mediators such as Akt/PKB, leading to reduction of Glut4 translocation and glucose uptake [4,45]. Glut1, a member of Glut family, plays minor role in glucose transport in insulin-sensitive tissues and overexpression of it improves glucose tolerance [46]. In the present study, nobiletin increased phosphorylation of Akt and expression of Glut1 in the whole cell lysates and Glut4 in whole plasma membrane in WAT and muscle. These findings suggest that effect of nobiletin on inducing Glut4 translocation via activating Akt is associated with improving glucose intolerance and insulin resistance. At the same time, we have been shown that improving glucose intolerance and insulin resistance by nobiletin are mediated by several possible mechanisms.

One possibility is associated with the regulation of adipokine expressions. We found that nobiletin increased the mRNA expression and plasma levels of adiponectin and decreased the mRNA expression levels of IL-6 and MCP-1. Nobiletin also showed a tendency to decrease TNF-α gene expression although the difference was not significant. These effects of nobiletin may be favorable for improving the dysregulation of adipokine expressions. It is well known that dysregulation of the functions of adipokines in adipose tissue can result in chronic inflammatory responses in the obese state and trigger the development of insulin resistance [8,10]. Several reports have demonstrated that adiponectin increases insulin-stimulated glucose uptake through increased Glut4 gene expression and increased Glut4 translocation to the plasma membrane in 3T3-L1 adipocytes and rat skeletal muscle cells [47,48]. Furthermore, TNF-α, IL-6 and MCP-1 reduce the expression of Glut4 and insulin-stimulated glucose uptake in 3T3-L1 adipocytes [20,22,49]. Consequently, the beneficial effects of nobiletin on insulin sensitivity observed in the present study may be partially explained by its regulatory effects on adipokines. The other possibility is increasing expression of PPARγ. PPARγ is a nuclear receptor that regulates the expressions of key genes involved in adipocyte differentiation, and lipid and glucose metabolisms [41–43]. Several studies have shown that Suppression of PPARγ decreases Glut4 expression, while PPARγ ligands, thiazolidinediones, increase expression of adiponecin and Glut4 [50–52]. In addition, PPARγ ligands suppress the expression and secretion of inflammatory adipokines in monocytes and humans [53]. Our data revealed that nobiletin increased the mRNA and protein expression levels of PPARγ and its target genes perilipin in WAT. Based on these reports and our results, it is suggested that the regulation of adipokines and PPARγ expression by nobiletin may contribute to increased levels of Glut4 mRNA, protein expression and translocation in WAT and muscle.

In general, PEPCK and G6Pase are involved in gluconeogenesis. Changes in the expressions of these genes can alter the plasma glucose levels. It is possible that plasma glucose levels are altered by regulating expression of these genes [25,26]. The mRNA expression levels of hepatic PEPCK and G6Pase are increased in several animal models of type 2 diabetes [27]. We found that nobiletin decreased the mRNA expression levels of PEPCK and G6Pase, however, it unfortunately failed to change protein levels of them in liver of the *ob*/*ob* mice. Based on these result, we think that nobiletin does not affect gluconeogenesis in liver.

In conclusion, the present study has shown for the first time that daily chronic administration of nobiletin improves insulin resistance in the animal model of type 2 diabetic *ob*/*ob* mice, and that this effect was accompanied by increased glucose utilization in WAT and muscle, and regulation of adipokine expressions in WAT. Our results provide important implications for preventing insulin resistance.

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