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Nobiletin improves obesity and insulin resistance in high-fat diet-induced obese mice

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Abstract

Nobiletin (NOB) is a polymethoxylated flavone present in citrus fruits and has been reported to have antitumor and anti-inflammatory effects. However, little is known about the effects of NOB on obesity and insulin resistance. In this study, we examined the effects of NOB on obesity and insulin resistance, and the underlying mechanisms, in high-fat diet (HFD)-induced obese mice. Obese mice were fed a HFD for 8 weeks and then treated without (HFD control group) or with NOB at 10 or 100 mg/kg. NOB decreased body weight gain, white adipose tissue (WAT) weight and plasma triglyceride. Plasma glucose levels tended to decrease compared with the HFD group and improved plasma adiponectin levels and glucose tolerance. Furthermore, NOB altered the expression levels of several lipid metabolism-related and adipokine genes. NOB increased the mRNA expression of peroxisome proliferator-activated receptor (PPAR)-γ, sterol regulatory element-binding protein-1c, fatty acid synthase, stearoyl-CoA desaturase-1, PPAR-α, carnitine palmitoyltransferase-1, uncoupling protein-2 and adiponectin, and decreased the mRNA expression of tumor necrosis factor-α and monocyte chemoattractant protein-1 in WAT. NOB also up-regulated glucose transporter-4 protein expression and Akt phosphorylation and suppressed IκBα degradation in WAT. Taken together, these results suggest that NOB improves adiposity, dyslipidemia, hyperglycemia and insulin resistance. These effects may be elicited by regulating the expression of lipid metabolism-related and adipokine genes, and by regulating the expression of inflammatory makers and activity of the insulin signaling pathway.

Keywords: Nobiletin; High-fat diet-induced obese mice; Obesity; Insulin resistance

1. Introduction

Obesity is a serious health problem and is a risk factor for metabolic diseases such as hypertension, hyperlipidemia and arteriosclerosis. Insulin resistance is also characterized by impaired glucose and lipid metabolism and is strongly associated with obesity [1].

Adipose tissue is a major energy storage organ that accumulates triglycerol (TG) during nutritional excess and releases free fatty acid (FFA) when energy is required. It is recognized as an endocrine organ that synthesizes and secretes biologically active molecules called adipokines, which influence various homeostatic systems [2,3]. In adipose tissue, lipid storage (i.e., lipogenesis) and utilization (i.e., lipolysis) are regulated by several hormones and by the nutritional state [4]. These signals activate various transcription factors [e.g., sterol regulatory element-binding protein (SREBP)-1c and peroxisome proliferator-activated receptor (PPAR)-α/γ] and enzymes [e.g., fatty acid synthase (FAS) and carnitine palmitoyltransferase (CPT)-1] to maintain lipid metabolism. Consequently, an imbalance in lipid metabolism leads to changes in adipose tissue mass [5]. Disorders in lipid metabolism not only increase plasma FFA levels but also alter the production of adipokines, which contribute to the development of obesity-related pathologies, such as insulin resistance [3]. For example, the increased FFA levels impair insulin signaling by reducing glucose uptake in muscle and increasing hepatic glucose production [6,7]. Adiponectin, an adipokine, improves insulin resistance by suppressing hepatic glucose production and enhancing glucose uptake and fatty acid oxidation in muscle [8,9]. On the other hand, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 are associated with obesity-induced chronic low-grade inflammation. These cytokines activate inflammatory pathways, including the inhibitor of nuclear factor-κB kinase-β (IKKβ) and c-Jun NH(2)-terminal kinase (JNK), in adipose tissue, which impairs insulin signaling by targeting the downstream components of the insulin signaling pathway [10–13]. Indeed, nuclear factor-κB (NFκB) activation by cytokines regulates the expression of many genes involved in FFA and glucose uptake, as well as the immune response in adipocytes, which could lead to insulin resistance [14]. Thus, regulation of adipose tissue function is a useful strategy to counter insulin resistance.

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Nobiletin (NOB) is a polymethoxylated flavone found in certain citrus fruits such as *Citrus depressa* (shiikuwasa) and *C. sinensis* (oranges) [15,16] and has anti-inflammatory and antitumor effects (for example, see Refs. [17,18]). It was recently reported that NOB can also regulate lipid and glucose metabolism [19,20]. The anti-inflammatory effects of NOB were achieved by suppressing the production of nitric oxide, the expression of proinflammatory cytokines (TNF-α, IL-6), and by activating NFκB, JNK and p38 mitogen-activated protein kinases in several models of inflammation [17,21–23]. NOB was reported to regulate differentiation and lipolysis by activating the cAMP/cAMP-responsive element binding protein signaling pathways in 3T3-L1 adipocytes and improved insulin resistance by enhancing the insulin signaling pathway and regulating adipokine expression in the WAT of *ob*/*ob* mice [19,20]. These reports suggest that NOB directly affects adipocyte functions, although the underlying mechanisms are unknown.

Because adipocyte dysfunction is closely linked to obesity and insulin resistance, regulation of adipocyte functions is a major target for the prevention and treatment of obesity and insulin resistance. Because NOB has anti-inflammatory effects and can regulate adipocyte functions, we examined its effects on obesity and insulin sensitivity, and the underlying molecular mechanisms in high-fat diet (HFD)-induced obese mice.

2. Materials and methods

2.1. Isolation and identification of NOB

Shiikuwasa (*C. depressa*) was purchased from Teruya nousan (Ohigimison, Okinawa, Japan). The dried peels of shiikuwasa (dry weight, 6 kg) were extracted in methanol (35 L) for 1 week. The evaporated methanol extract (503.81 g) was dissolved in distilled water and fractionated with ethyl acetate. The ethyl acetate-eluted fraction was then fractionated with hexane and 90% methanol. The 90% methanol-eluted fraction (147.11 g) was chromatographed on a silica gel column (BW-820MH, Fuji Silasia Chemical, Nagoya, Japan) using a stepwise gradient with a solvent consisting of hexane and ethyl acetate (90:10, 80:20, 60:40, 40:60, 20:80 and 0:100, v/v). The fractions obtained with 20:80 and 0:100 (v/v; 16 g) hexane and ethyl acetate generated crystals which were identified as NOB by high-performance liquid chromatography and nuclear magnetic resonance (NMR). The ^{1}H and ^{13}C NMR data of purified NOB were consistent with those reported elsewhere [15].

2.2. Animal studies

Male C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan) at 4 weeks of age. The mice were housed in a temperature- (23±3°C) and humidity-controlled room with a 12-h light/dark cycle. Mice were given free access to water and food throughout the experiment. After acclimatization with standard rodent chow diet (CRF-1; Charles River, Japan) for 1 week, the mice were fed either the control normal-fat diet [NFD; $n=8$; CRF-1; protein (casein/L-cystine): 25%; carbohydrate (maltodextrin/sucrose): 61%; fat (lard/soybean oil): 14%; and other, 3.59 kcal/g] or a high-fat diet [HFD; $n=24$; Rodent diet D12492; Research Diets, New Brunswick, NJ, USA; protein (casein/L-cystine): 20%; carbohydrate (maltodextrin/sucrose): 20%; fat (lard/soybean oil): 60%; and other, 5.24 kcal/g] for 8 weeks to induce obesity. Based on body weights and plasma glucose levels, the HFD-fed mice were then divided into three experimental groups ($n=8$/group) and treated without (control HFD group) or with NOB at a dose of 10 mg/kg (HFD+10NOB) or 100 mg/kg (HFD+100NOB). NOB was mixed with 0.3% carboxyl methylcellulose (vehicle) and administered by oral gavage once daily for 5 weeks while continuing the HFD. The NFD and HFD groups were administered with vehicle alone. Body weight and food intake were measured twice per week throughout the study. The experimental design was approved by The Animal Experiment Committee of Chubu University, and the mice were maintained in accordance with the committee's guidelines.

2.3. Collection of plasma, liver, muscle and WAT

At the end of the 5-week treatment period, the mice were fasted for 5 h, blood was collected from the tail vein and the mice were sacrificed by cervical dislocation. Plasma samples were prepared by centrifuging blood samples at 5000×*g* for 15 min at 4°C and stored at −80°C until analysis. Liver and WAT (epididymal, perirenal and mesenteric WAT) were immediately excised, rinsed, weighed, snap-frozen in liquid nitrogen and stored at −80°C until analysis.

2.4. Plasma biochemical assays

Plasma total cholesterol (T-CHO), triglyceride (TG) and glucose levels were determined enzymatically using commercial assay kits (Cholesterol E-Test, Triglyceride E-Test and Glucose C II-Test, respectively; Wako, Osaka, Japan). Plasma adiponectin levels were measured using an enzyme-linked immunosorbent assay (Mouse Adiponectin/Acrp30; R&D Systems, Minneapolis, MN, USA).

2.5. Oral glucose tolerance test

After 4 weeks of treatment with NOB, the mice were fasted overnight and a basal blood sample (0 min) was collected from the tail vein. The mice were then orally administered with glucose (2 g/kg body weight), and additional blood samples were collected at 30, 60 and 120 min. The blood samples were centrifuged at 5000×*g* for 15 min at 4°C, and the plasma samples were stored at −80°C until analysis. Plasma glucose levels were measured as described above, and the area under the curve for blood glucose (AUC_{glu}) over 2 h was calculated.

2.6. Measurement of liver TG levels

Liver total lipids were extracted from mice using the Folch et al. [24] method. The liver TG levels were determined using commercial assay kits (Triglyceride E-Test, Wako). TG levels were related to total protein levels.

2.7. Total RNA isolation and gene expression analyses

Total RNA was isolated from WAT using Isogen reagent (Nippon Gene, Tokyo, Japan), and samples in each group ($n=8$) were pooled for real-time polymerase chain reaction (PCR) analysis. Total RNA (1 μg) was reverse-transcribed to cDNA using a reverse transcription system (a3500, Promega, Madison, WI, USA) with oligo(dT) primers. The mRNA expression levels of adipokines and glucose metabolism-related genes were assessed using gene-specific primers by real-time PCR with the FastStart universal SYBR Green Master PCR kit (Roche, Mannheim, Germany) on an ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA). The following (forward and reverse) primers were used: PPARγ2, 5′-GAG CTG ACC CAA TGG TTG CTG-3′ and 5′-GCT TCA ATC GGA TGG TTC TTC 3′; SREBP-1c, 5′-GTG AGC CTG ACA AGC AAT CA-3′ and 5′-ACC AAG CCA GCA AAT ACA CC-3′; FAS, 5′-CTT CGC CAA CTC TAC CAT GG-3′ and 5′-TTC CAC ACC CAT GAG CGA GT-3′; stearoyl-CoA desaturase-1 (SCD-1), 5′-CCC TCC GGA AAT GAA CGA GAG -3′ and 5′-GCC GGG CTT GTA GTA CCT C-3′; GLUT4, 5′-CAA CGT GGC TGG GTA GGC A-3′ and 5′-ACA ACA TCAG CCC AGC CGG T-3′; adiponectin, 5′-GTT GCA AGC TCT CCT GTT CC-3′ and 5′-CTT GCC AGT GCT GTT GTC AT-3′; TNF-α, 5′-TGT CTC AGC CTC TTC TCA TT-3′ and 5′-AGA TGA TCT GAG TGT GAG GG-3′; MCP-1, 5′-AAG AGA GAG GTC TGT GCT GA-3′ and 5′-TTC ACT GTC ACA CTG GTC AC-3′; IL-6, 5′-CAT GTT CTC TGG GAA ATC GTG G-3′ and 5′-AAC TGA TAT GCT TAG GCA TAA CGC AC-3′; PPARα, 5′-AAG TGC CTG TCT GTC GGG ATG-3′ and 5′-CCA GAG ATT TGAG GTC TGC AGT TTC-3′; CPT-1, 5′-CTC CGC CTG AGC CAT GAA G-3′ and 5′-CAC CAG TGA TGA TGC CAT TCT-3′; uncoupling protein (UCP)-2, 5′-CCT CAG CCC TCG ATC AAC TC-3′ and 5′-CAG TAC ACC GCA GTG TGT CAT-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ATT GGG CGC CTG GTC AC -3′ and 5′-CCA GAG GGG CCA TCC AC-3′. All the samples were normalized for the corresponding expression of GAPDH. The expression level of the gene of interest in the HFD+NOB groups relative to its expression level in the HFD group was calculated using the $2^{-\Delta\Delta Ct}$ formula, where C_t is defined as the cycle number at which the fluorescence was significantly greater than that of the background using the following formulae: $DC_t = DC_{t\ interest} - DC_{t\ GAPDH}$ and $DDC_t = DC_t$ of the HFD-NOB-treated group $- DC_t$ of the HFD group, which was normalized to 1.

2.8. Protein extraction and Western blotting

Tissue samples were homogenized in modified RIPA buffer [50 mmol/L Tris-HCl (pH 7.4), 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% sodium dodecylsulfate (SDS), 1 mM PMSF and 1 mM protease inhibitors], followed by centrifugation at 760×*g* for 5 min at 4°C to remove nuclei and intact cells. The supernatant was then centrifuged at 12,000×*g* for 20 min at 4°C and the resulting supernatant was collected. The protein concentration in the final supernatant was determined by a Bradford protein assay using bovine serum albumin (BSA) as a standard. Aliquots (30 μg protein) of protein were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Cleveland, OH, UK). The membranes were blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated with the following antibodies in TBS-T containing 5% BSA: anti-mouse GLUT4 (AbD SeroTec, Oxford, UK; 1:1000), PPARα (Thermo Scientific, Rockford, IL, USA), actin (Sigma-Aldrich, St. Louis, MO, USA) and anti-rabbit phospho-Akt (ser473), Akt, IκBα and PPARγ (Cell Signaling Technology, Beverley, MA, USA; 1:1000). After incubation, the membranes were washed with TBS-T and then incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% non-fat dried milk in TBS-T. After washing, the immunocomplexes were detected using a chemiluminescence system (Amersham Biosciences, Amersham Biosciences, Buckinghamshire, UK). The exposed films were scanned and the images were subjected to densitometric analysis using the Image J software (http://rsbweb.nih.gov/ij/download.

Table 1
Effects of NOB on body weight and food intake rate in HFD-induced obese mice for 5 weeks

	NFD	HFD	HFD+10NOB	HFD+100NOB
Body weight (g)				
Initial	25.59±0.46	31.53±1.16**	31.14±0.94	30.75±0.52
Final	27.60±0.40	35.98±1.46**	33.96±1.02	32.02±0.62#
Gain	2.01±0.30	4.45±1.38	2.81±0.64	1.27±0.30#
Food intake rate (g/mouse per day)	3.37±0.48	2.61±0.79*	2.58±0.88	2.61±0.67

NFD, Normal-fat diet; HFD, high-fat diet; NOB, nobiletin. Values are expressed as means ±S.E.M. (n=8). *P<.05 and **P<.01 vs. the NFD group; #P<.05 vs. the HFD group.

html). PPARα, γ, GLUT4, p-Akt/Akt, and IκBα expression levels are expressed relative to those in the HFD control group.

2.9. Statistical analysis

Data are expressed as means±S.E.M. Differences in mean values between two groups were analyzed by Student's t test and those between three groups by one-way analysis of variance using Origin 7 Software (MicroCal Software, Northampton, MA, USA). Values of P<.05 were considered to indicate statistical significance.

3. Results

3.1. Effects of NOB on body weight gain and food intake

Body weight gain and food intake are shown in Table 1. Body weight gain in the HFD group was greater than that in the NFD group (P<.01). In contrast, body weight gain was significantly lower in the HFD+100NOB group than in the HFD group (P<.05) and was lower, although not significantly, in the HFD+10NOB group than in the HFD group. Food intake was not significantly different among the HFD and HFD+NOB groups, but was significantly greater in the NFD group than in the HFD group (P<.005).

3.2. Effects of NOB on organ weight and liver TG levels

No significant difference in liver or kidney weights (data not shown) was observed among the four groups (Fig. 1A). Liver TG levels did not change among the four groups. However, liver TG levels tended to decrease in the HFD+100NOB group, although not significantly (Fig. 1B). The epididymal, perirenal, mesenteric and total WAT weights were significantly lower in the HFD+100NOB group than in the HFD group (P<.05), but not in the HFD+10NOB group (Fig. 1C).

3.3. Effects of NOB on plasma biochemical parameters

Table 2 shows the effects of NOB on plasma biochemical parameters. Plasma T-CHO (P<.01), TG (P<.05) and glucose (P<.01) levels were significantly higher in the HFD group than in the NFD group. Plasma T-CHO levels were not significantly different among the HFD and HFD+NOB groups. In contrast, plasma TG levels were significantly lower in the HFD+100NOB group than in the HFD group (P<.05). Plasma glucose levels in the HFD+100NOB group tended to decrease compared with the HFD group, although not in the HFD+10NOB group. The absolute plasma adiponectin level was not significantly different among the four groups (data not shown). However, when plasma adiponectin levels were adjusted for total WAT weight (relative adiponectin levels), they were significantly higher in the NFD group (P<.01) and in the HFD+100NOB group (P<.05) than in the HFD group.

3.4. Effects of NOB on glucose tolerance

To investigate the effects of NOB on glucose tolerance, we performed oral glucose tolerance tests after 4 weeks of treatment with NOB. The plasma glucose levels were significantly higher in the HFD group than in the NFD group at all times after 2 g/kg of glucose administration (all P<.01). HFD+100NOB treatment significantly

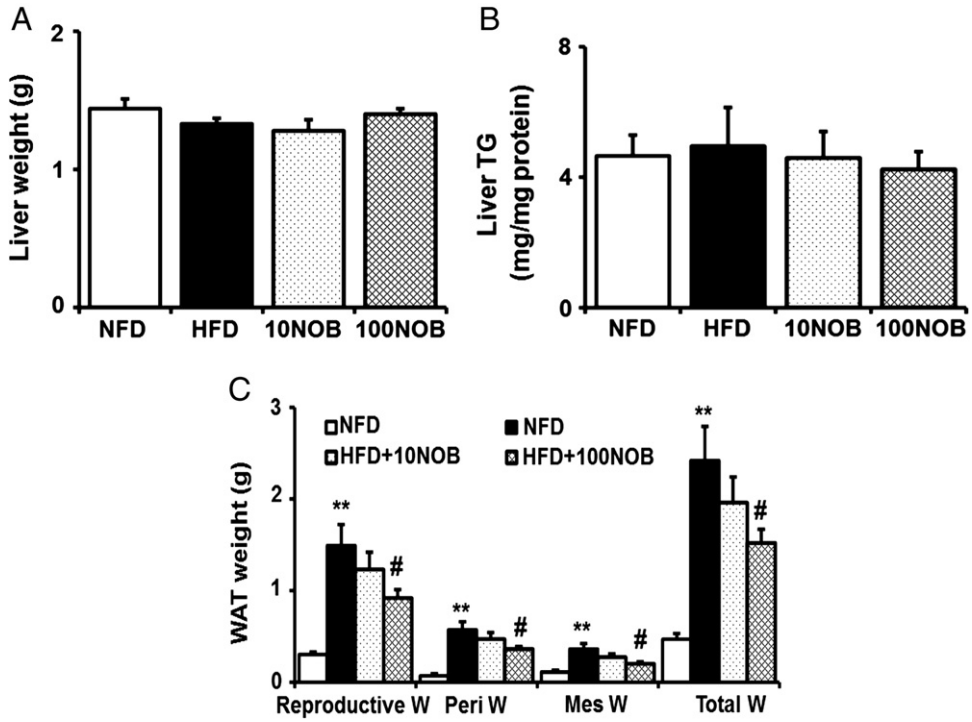

Fig. 1. Effects of NOB on organ weight. (A) Liver weights. (B) Liver TG levels. (C) WAT weights. Reproductive W, Reproductive WAT; Peri W, perirenal WAT; Mes W, mesenteric WAT; Total W, Reproductive W+Peri W+Mes W; NFD, normal-fat diet; HFD, high-fat diet; HFD+10NOB, HFD plus 10 mg/kg NOB; HFD+100NOB, HFD plus 100 mg/kg NOB. Values are expressed as means±S.E.M. (n=8). **P<.01 vs. the NFD group; #P<.05 vs. the HFD group.

Table 2
Effects of NOB on plasma biochemical parameters in HFD-induced obese mice for 5 weeks

	NFD	HFD	HFD+10NOB	HFD+100NOB
T-CHO (mmol/L)	2.67±0.14	4.77±0.3**	4.63±0.2	5.00±0.19
TG (mmol/L)	1.00±0.03	1.17±0.06*	1.11±0.21	0.96±0.05#
Glucose (mmol/L)	11.24±0.51	14.65±0.45**	13.65±0.68	13.10±0.42
Adiponectin (μg/ml per gram of total WAT weights)	12.40±1.62	2.93±0.30**	3.87±0.86	5.34±0.97#

NFD, Normal-fat diet; HFD, high-fat diet; NOB, nobiletin; T-CHO, total cholesterol; TG, triglyceride. Values are expressed as means±S.E.M. ($n=8$). *$P<.05$ and **$P<.01$ vs. the NFD group; #$P<.05$ vs. the HFD group.

reduced glucose levels at 0, 15 and 60 min after glucose administration (all $P<.05$) compared with those in the HFD group (Fig. 2A). The AUC_{glu} was also lower in the HFD+100NOB group than in the HFD group ($P<.01$) (Fig. 2B). In contrast, there were no significant differences in plasma glucose levels or AUC_{glu} between the HFD+10NOB and HFD groups. These results indicate that NOB improves glucose intolerance in HFD-induced obese mice.

3.5. Effects of NOB on mRNA expression of lipid metabolism-related genes and proteins

To investigate the anti-obesity effect of NOB, we analyzed the mRNA levels of lipid metabolism-related genes in WAT. As shown in Fig. 3A, PPARγ mRNA levels in WAT tended to decrease in the NFD group and were increased significantly in the HDF+10NOB and in the HFD+100NOB groups compared with the HFD group (both $P<.01$). SREBP-1C (vs. HFD; HFD+10NOB: $P<.01$; HFD+100NOB: $P<.05$), FAS (NFD: $P<.005$; HFD+10NOB: $P<.005$; HFD+100NOB: $P<.005$) and SCD-1 (NFD: $P<.05$; HFD+10NOB: $P<.005$; HFD+100NOB: $P<.005$) mRNA expression levels in WAT were also significantly increased in the NFD and HFD+NOB groups compared with those in the HFD group; however, these effects were not dose-dependent. The mRNA expression levels of PPARα ($P<.01$), CTP-1 ($P<.05$) and UCP2 ($P<.05$) in WAT were significantly higher in the HFD+100NOB group than in the HFD group, but not in the HFD+10NOB group (Fig. 3B). We also examined PPARα and PPARγ protein expression. PPARα protein levels in the HFD+100NOB group tended to increase compared with the HFD group but did not change in the HFD+10NOB group. PPARγ protein expression levels were significantly increased in the HFD+100NOB groups compared with the HFD group. In the HFD+10NOB group, PPARγ protein expression levels tended to increase (Fig. 3C).

3.6. Effects of NOB on adipokine gene expression in WAT

It is known that adipokines play critical roles in obesity-related insulin resistance [3]. Therefore, to explore the effects of NOB on adipokine expression, we analyzed the mRNA expression levels of adiponectin, TNF-α, IL-6 and MCP-1 (Fig. 4). The mRNA levels of adiponectin, TNF-α and MCP-1 in WAT were significantly decreased in the NFD group than in the HFD group ($P<.05$, $P<.05$ and $P<.01$, respectively). Adiponectin mRNA levels were increased in both HFD+NOB groups compared with the HFD group. Meanwhile, TNF-α mRNA levels were significantly decreased in the HFD+100NOB group compared with the HFD group ($P<.05$), but not in the HFD+10NOB group. MCP-1 mRNA levels were significantly decreased in the HFD+NOB groups compared with the HFD group (both $P<.01$). IL-6 mRNA expression levels did not differ significantly among the four groups.

3.7. Effects of NOB on components of the insulin signaling pathway and inflammatory makers in WAT

It has been reported that the inflammatory pathway, activated by TNF-α, IL-6 and MCP-1, for example, has influence on the insulin signaling pathway [14]. Therefore, to determine the effects of NOB on crosstalk between the insulin signaling pathway and the inflammation pathway, we examined the protein expression of the components of both pathways (Fig. 5). Phosphorylated Akt (p-Akt) levels were significantly greater in the NFD group ($P<.05$) and in the HFD+100NOB group ($P<.05$) compared with the HFD group. GLUT4 protein expression levels were significantly greater in the NFD group ($P<.05$) and in the HFD+100NOB group ($P<.05$) than in the HFD group. However, p-Akt levels and GLUT4 protein expression levels did not change in the HFD+10NOB group. IκBα protein expression in WAT was also significantly increased in the HFD+10NOB and HFD+100NOB (both $P<.05$) groups compared with the HFD group, but not in the NFD group.

4. Discussion

In the present study, we investigated the effects of NOB on obesity and insulin sensitivity and analyzed the potential underlying mechanisms using HFD-induced obese mice. We found that NOB significantly reduced body weight gain, WAT weight and plasma TG and glucose levels. Furthermore, NOB increased relative

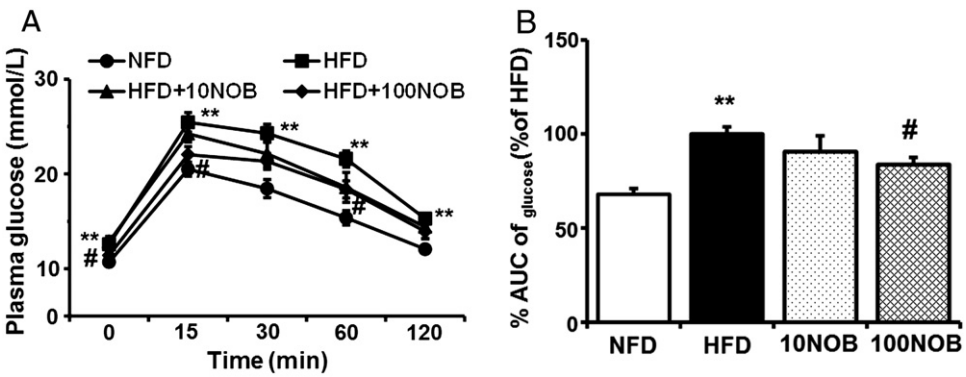

Fig. 2. Effects of NOB on glucose tolerance. (A) Plasma glucose levels during an oral glucose tolerance test (OGTT). (B) Area under the curve for plasma glucose. NFD, Normal-fat diet; HFD, high-fat diet; HFD+10NOB, HFD plus 10 mg/kg NOB; HFD+100NOB, HFD plus 100 mg/kg NOB. Values are expressed as means±S.E.M. ($n=8$). **$P<.01$ vs. the NFD group; #$P<.05$ vs. the HFD group.

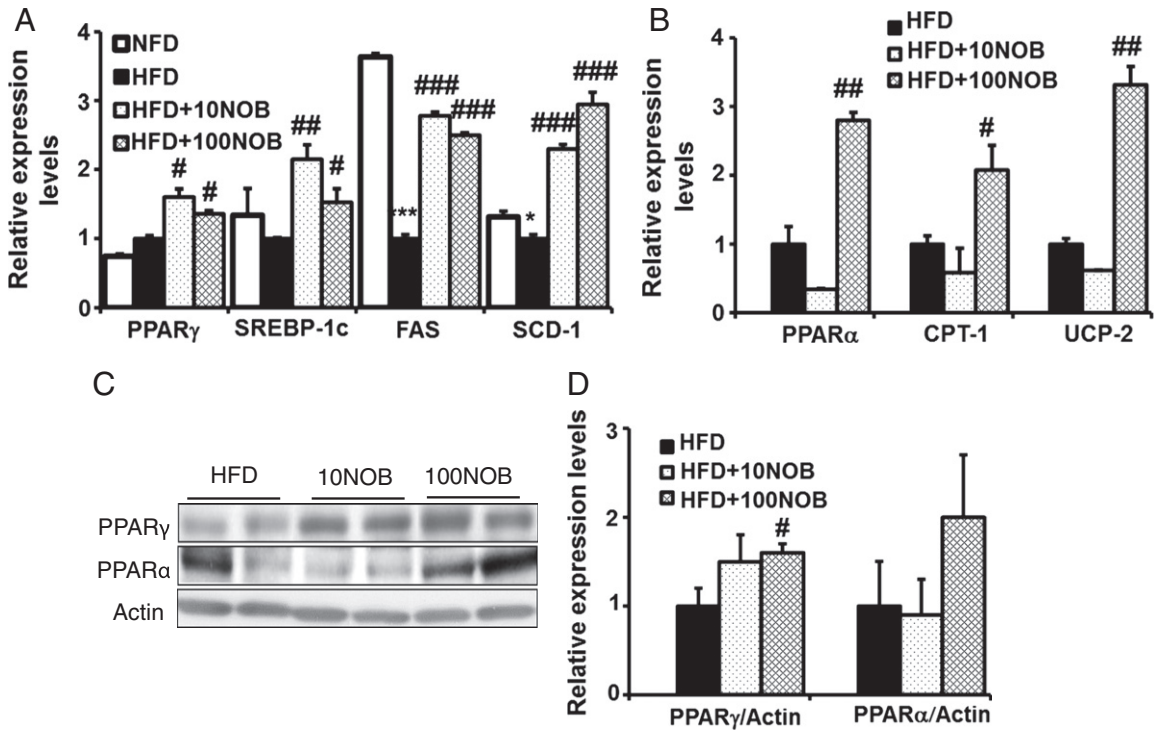

Fig. 3. Effects of NOB on the expression levels of lipid metabolism-related genes and proteins in WAT. (A) The mRNA levels of lipogenesis-related genes. (B) Energy expenditure-related genes. (C) PPARα and γ protein expression levels. (D) Densitometric analysis of protein expression. The mRNA expression levels are expressed as the fold increase relative to the HFD group after normalization for the GAPDH mRNA level. The protein expression levels are expressed as the fold increase relative to the HFD group after normalization for the actin expression level. NFD, Normal-fat diet; HFD, high-fat diet; HFD+10NOB, HFD plus 10 mg/kg NOB; HFD+100NOB, HFD plus 100 mg/kg NOB. Values are expressed as means±S.E.M. $^{*}P<.05$ and $^{***}P<.005$ vs. the NFD group; $^{\#}P<.05$, $^{\#\#}P<.01$ and $^{\#\#\#}P<.005$ vs. the HFD group.

adiponectin levels and glucose tolerance compared with those in HFD-induced obese mice. These observations are consistent with our previous study showing that NOB improves insulin resistance in *ob*/*ob* mice [20]. These results suggest that NOB ameliorates adiposity, hyperlipidemia, hyperglycemia and insulin resistance in obese mice.

In our study, NOB reduced body weight gain and WAT weights in mice with HFD-induced obesity. These results indicate that NOB inhibits lipid accumulation in adipose tissues in mice with HFD-induced obesity.

Adipose tissue performs two reciprocal biochemical processes, lipogenesis and lipolysis, to maintain lipid homeostasis. These two processes are regulated by hormones and nutritional conditions, as

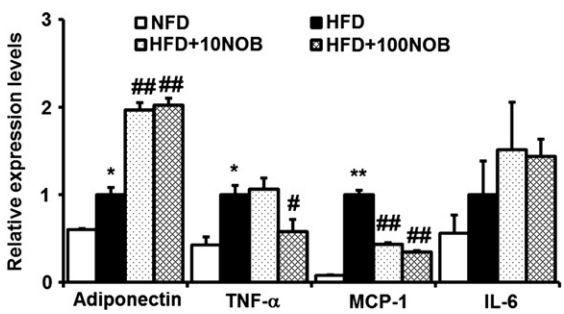

Fig. 4. Effects of NOB on the mRNA expression of adipokine gene in WAT. The mRNA levels are expressed as the fold increase relative to the HFD group after normalization for the GAPDH mRNA level. NFD, Normal-fat diet; HFD, high-fat diet; HFD+10NOB, HFD plus 10 mg/kg NOB; HFD+100NOB, HFD plus 100 mg/kg NOB. Values are expressed as means±S.E.M. ($n=8$). $^{*}P<.05$ and $^{**}P<.01$ vs. the NFD group; $^{\#}P<.05$ and $^{\#\#}P<.01$ vs. the HFD group.

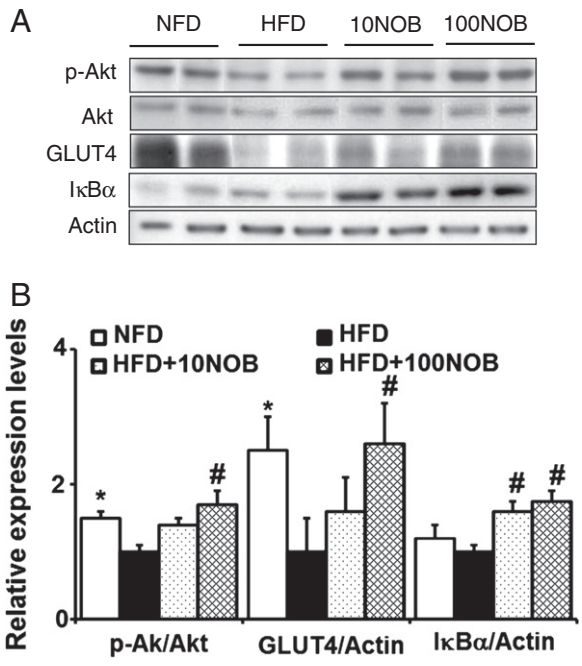

Fig. 5. Effects of NOB on the insulin signaling pathway and inflammatory markers in WAT. (A) p-Akt, Akt, GLUT4 and IκBα protein expression levels. (B) Densitometric analysis of protein expression. The relative p-Akt, GLUT4 and IκBα protein expression levels were normalized for Akt protein or actin, respectively, and compared with the corresponding expression level in the HFD group, which was assigned a value of 1.0. NFD, Normal-fat diet; HFD, high-fat diet; HFD+10NOB, HFD plus 10 mg/kg NOB; HFD+100NOB, HFD plus 100 mg/kg NOB. Values are expressed as means±S.E.M. ($n=8$). $^{*}P<.05$ vs. the NFD group; $^{\#}P<.05$ vs. the HFD group.

well as by transcription factors and enzymes [4]. Among the transcription factors, PPARγ regulates the expression of genes involved in adipocyte differentiation, and the lipid and glucose metabolism in WAT. SREBP-1c is involved in *de novo* fatty acid synthesis by regulating the gene expression of downstream factors, including FAS and SCD-1 [5]. PPARα regulates lipid metabolism through stimulating fatty acid uptake and utilization [25]. CPT1, the rate-limiting enzyme in mitochondrial fatty oxidation, mediates the transport of long-chain fatty acid into the mitochondria for β-oxidation [26]. UCP2 is involved in maintaining the energy balance and thermogenesis, although its precise biological role is less clear [27]. In our study, NOB up-regulated PPARγ, SREBP-1c, FAS and SCD-1 mRNA levels and PPARγ protein levels in WAT compared with the HFD feeding. Expression of PPARγ was increased in WAT in the HFD group compared with the NFD groups, which might be associated with increased adipose cell differentiation and lipid storage [28]. Nevertheless, NOB increased greater PPARγ expression in WAT compared with the HFD group, which is consistent with a previous report that NOB enhances adipocytes differentiation through PPARγ expression [19]. These results suggest that NOB may lead to more FFA uptake/lipid accumulation. In addition, PPARγ regulates the expression of adiponectin gene. Reportedly, adiponectin enhances fatty oxidation via AMPK activation in muscle and liver [9]. In our study, NOB increased the gene expression and plasma levels of adiponectin, which might enhance fatty oxidation in peripheral tissues. Furthermore, NOB increased PPARα, CPT-1 and UCP-2 mRNA levels, and PPARα protein levels tended to increase in WAT compared with the HFD-fed group. Several reporters have demonstrated that HFD feeding decreases the expression of energy expenditure-related genes, PPARα and CTP-1, in WAT from obese humans and animals [29,30]. These results indicate that NOB can induce both lipid storage (FFA uptake) and fatty acid oxidation at the same time. FFA uptake and lipid storage increased by NOB can become glycerol and FFA, which is used for fatty acid oxidation based on increased expression of energy expenditure-related genes and lipolysis; this would result in reduction of body weight gain and WAT weight. Furthermore, increase of expression of lipid metabolism-related genes might explain the lower plasma TG levels in the NOB-treated group. However, unlike lipogenic gene expression, energy expenditure-related gene expression varied between 10 mg/kg and 100 mg/kg doses of NOB; 10 mg/kg NOB doses decreased them compared with HFD group, whereas 100 mg/kg NOB doses increased them. Effects of intermediate doses of NOB on expression of energy expenditure-related genes still need to be confirmed.

It is known that disordered adipokine secretion can cause obesity-induced inflammation, which links obesity to the pathogenesis of insulin resistance and Type 2 diabetes [3]. Adiponectin increases glucose uptake by increasing GLUT4 gene expression and GLUT4 translocation to the plasma membrane in adipocytes, which ultimately enhance insulin sensitivity [31]. Adiponectin-deficient mice exhibit insulin resistance and glucose intolerance, whereas overexpression of adiponectin in adipose tissue improves insulin sensitivity in mice [32,33]. In contrast, inflammatory cytokines such as TNF-α, IL-6 and MCP-1 impair insulin signaling. TNF-α and IL-6 disrupt insulin signaling by suppressing the expression/translocation of GLUT4 and increasing IRS-1 phosphorylation at inhibitory sites [10,11]. TNF and TNF receptor knockout mice show enhanced insulin sensitivity [34]. Meanwhile, increased MCP-1 expression in adipose tissue induces macrophage infiltration into the adipose tissue and contributes to the development of insulin resistance [12]. In our study, NOB increased adiponectin mRNA levels and the relative plasma adiponectin levels, and decreased TNF-α and MCP-1 mRNA levels. Reportedly, adiponectin expression and plasma levels were down-regulated in obese or diabetic states. In our study, relative plasma adiponectin levels significantly increased in NFD feeding compared with HFD feeding, but adiponectin gene expression levels did not. It is needed to clarify the difference between gene expression and plasma levels of adiponectin. These findings are consistent with those of our previous report showing that NOB regulates the expression of adipokine genes in *ob*/*ob* mice [20]. These effects of NOB may help to correct the adipokine expression profiles. In particular, PPARγ plays an important role in adipokine regulation [35], including adiponectin. Thiazolidinediones, which are synthetic PPARγ ligands, increase the expression of adiponectin and suppress the expression of inflammatory cytokines such as TNF-α, by activating PPARγ in adipocytes [3,35]. As mentioned above, in our study, NOB regulated PPARγ and its target genes, including FAS, adiponectin and inflammatory genes, although NOB did not stimulate PPARγ ligand activity (data not shown). Based on these results and other report [3,35], NOB may correct adipokine release by targeting PPARγ and hence improve insulin sensitivity.

In general, it has been established that the phosphatidylinositol-3-kinase/Akt pathway, a component of the insulin signaling pathway, regulates glucose uptake by stimulating the expression and translocation of GLUT4 [36]. The inflammatory cytokines such as TNF-α, IL-6 and MCP-1 activate IKK/NFκB and JNK, two components of the inflammatory pathway, and suppress the insulin signaling pathway by regulating IRS-1 serine phosphorylation [11,13]. IKK phosphorylates IκBα and, hence, activates NFκB, which suppresses the expression of genes associated with FFA and glucose uptake and regulates the expression of genes involved in multiple inflammatory responses that are ultimately associated with insulin resistance [14]. We did not examine the phosphorylation of IKK and JNK levels and failed to detect the phosphorylation of IRS-1 at ser307, which inactivates insulin signaling. However, we found that NOB increased p-Akt levels, as well as GLUT4 and IκBα expression. These findings are consistent with those of a previous report showing that NOB activates the insulin signaling pathway in *ob*/*ob* mice [20]. Indeed, previous studies have shown that NOB can inhibit the production of nitric oxide and proinflammatory cytokines, IκBα degradation and NFκB transcriptional activation in various cell types [17,21–23]. Based on these results and previous reports [17,20–23], NOB may reduce the inflammatory cytokine-induced activation of the IKK/NFκB pathway and, hence, improve insulin resistance by activating the IRS-1/Akt signal pathway in WAT. To clarify this hypothesis, we needed to investigate whether NOB can inhibit IKK/JNK activity using *in vitro* assays and measurement of phosphorylation levels in further study.

The average human dietary intake of NOB is not known; however, extrapolation of animal doses to human doses can be calculated by using the following formula. The 100 mg/kg dose used in the present study would equal 486.5 mg/days in a 60-kg human based on body surface area [37]. NOB is particularly abundant in citrus fruits, such as shiikuwasa, which contains an average of 30.3 mg NOB per 100 g of fresh shiikuwasa fruit [16]. By this calculation, a human would have to eat 160.5 g of shiikuwasa fruit juices. In a safety test, six volunteers took shiikuwasa juices containing 650 mg of powdered shiikuwasa hydrated in ethanol extracts daily for 4 weeks and detected no subjective symptoms (http://ebn.arkray.co.jp). Taken together, we expect that NOB improves lipid and glucose metabolism without side effects in humans.

In conclusion, we found that NOB reduces WAT weight, plasma TG and glucose levels, and insulin resistance in HFD-induced obese mice. These effects were accompanied by changes in the expression of lipid metabolism-related genes, regulation of adipokine gene expression and activation of the insulin signaling pathway in WAT. These results confirm that NOB can regulate adipose tissue function and has beneficial effects for the prevention and treatment of obesity and insulin resistance.

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