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8-Prenylnaringenin suppresses obesity in high-fat diet-fed C57BL/6J mice via adiponectin secretion

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8-Prenylnaringenin (8-PN) is a prenylflavonoid found in hops (*Humulus lupulus L.*). It has several beneficial functions, which include the inhibition of bone loss and muscle atrophy. 8-PN is a metabolite of xanthohumol, which can prevent obesity in mice; however, the effect of 8-PN on obesity is still unknown. In the present study, we found that 8-PN prevented obesity in high-fat diet-fed mice. When C57BL6/J male mice were fed 8-PN at 0.0005% or 0.005% with a high-fat diet for 8 weeks, body weight gain, fat accumulation in adipose tissue, and fatty liver induced by the high-fat diet were prevented. In mice fed a high-fat diet and 8-PN, adenosine monophosphate-activated protein kinase (AMPK) was activated in visceral adipose tissue, which was accompanied by decreased expression of a fatty acid synthesis-related factor and increased expression of a mitochondrial biosynthesis-related factor downstream of AMPK. AMPK appeared to be activated by adiponectin secretion, which was associated with increased expression of adipocyte differentiation markers in mice fed a high-fat diet and 8-PN. For the first time, this study shows that 8-PN can prevent obesity in mice and that it is effective at low concentrations that humans could consume in their daily diet.

***Key Words*: prenylflavonoid, anti-obesity, adiponectin, AMPK, visceral fat**

Obesity is a state of excessive fat accumulation in the body, especially in abdominal adipose tissue, that results from a positive energy balance. Biological pathways such as appetite regulation, metabolism, and adipogenesis are important factors in obesity etiology.[1] Obesity can cause many diseases, including diabetes, hypertension, stroke, dementia, and cardiovascular disease; it also increases the risk of various disease-associated complications.[2] Many studies on obesity prevention have been conducted[3–5]; these show that obesity can be prevented through the intake of particularly food components such as polyphenols. Targets for obesity prevention include the inhibition of lipid absorption and fat accumulation and the promotion of lipolysis and energy metabolism. Among these targets, this study focuses on food components that promote energy metabolism.

Adenosine 5'-monophosphate-activated protein kinase (AMPK) is a ubiquitously expressed enzyme that plays an important role in the energy metabolism of skeletal muscle, liver, fat, myocardium, and other tissues.[6] AMPK inhibits fatty acid synthesis by directly phosphorylating acetyl-CoA carboxylase and promotes lipolysis by activating lipases such as hormone-sensitive lipase and adipose triglyceride lipase. It also plays a role as the main regulator of lipid metabolism by inhibiting sterol regulatory element-binding protein-1 (SREBP-1), a major transcription factor in lipogenesis, and activating peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), a transcriptional coactivator that regulates mitochondrial biosynthesis.[6] Several physiological processes stimulate AMPK, including alterations of the intracellular ATP/AMP ratio (induced by hypoxia and glucose deprivation) and elevations in calcium concentration, as well as the action of various hormones, cytokines, and adipokines.[6,7]

Adiponectin, an adipokine, is one factor that activates AMPK.[7,8] It also improves energy metabolism and suppresses obesity and hyperglycemia. Proteins that regulate the synthesis of adiponectin include peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein (C/EBP), and liver receptor homolog-1.[8] Therefore, food factors that promote adiponectin secretion or are adiponectin receptor agonists may prevent obesity. For example, lemon verbena extract stimulates adiponectin secretion and AMPK phosphorylation, which suppresses obesity *in vivo* and inhibits lipid accumulation at a dose of 0.02 mg/ml in 3T3-L1 cells.[9] Other studies report that both cinnamon and *Petasites japonicus* extract suppress lipid storage via adiponectin in 3T3-L1 cells.[10,11] However, most reports have studied food extracts; there have been few studies on the effects of specific compounds on adiponectin secretion and lipid accumulation to date.

8-Prenylnaringenin (8-PN) is a prenylflavonoid contained in hops (*Humulus lupulus L.*). 8-PN is generated when xanthohumol, also contained in hops, is converted to isoxanthohumol during the beer production process; this is then demethylated by cytochrome P450 in the liver and intestinal bacteria to yield 8-PN.[12] The structure of the 8-PN is shown in Fig. 1. Prenylflavonoids are more bioactive than non-prenylated form because of their increased hydrophobicity and affinity for biological membranes.[13] Thus, 8-PN has attracted attention as a food constituent that exerts biological effects at low concentrations. The effects of 8-PN intake have already been reported in mice,

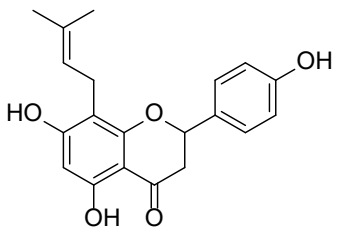

Fig. 1. Chemical structure of 8-prenylnaringenin.

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where it suppresses bone loss at a dose of 0.67–18 mg/kg body weight/day for 28 days and ameliorates disuse muscle atrophy in mice fed a control diet with 0.0005% 8-PN.[14,15] However, whether 8-PN prevents obesity is unknown. Xanthohumol, a precursor of 8-PN, suppresses obesity by suppressing SREBP-1 activation[16]; as xanthohumol is metabolized in the body to produce 8-PN, 8-PN may also prevent obesity. Therefore, in the present study, we investigated 8-PN as an anti-obesity agent and explored the mechanisms underlying its effects.

Materials and Methods

Materials. 8-PN was provided by Daicel Corporation (Tokyo, Japan). Primary antibodies for western blotting [PGC-1α (#2178S), AMPK (#2532S), p-liver kinase B1 (LKB1) (#3055S), LKB1 (#3047S), p-calcium/calmodulin-activated kinase kinase β (CaMKKβ) (#12818S), PPARγ (#2443S), and β-actin (#4967S)], and secondary antibody horseradish peroxidase-conjugated anti-rabbit immunoglobin G (#7074), were purchased from Cell Signaling Technology (Beverly, MA). The antibody for p-AMPK (#07-681) and insulin receptor substrate-1 (IRS-1) (#06-248) were purchased from Merck Millipore (Darmstadt, Germany). Antibodies for CaMKKβ (#sc-50341) and C/EBPα (#sc-61) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). The antibodies for SREBP-1 (#ab3259) and carnitine palmitoyl transferase 1a (CPT-1a) (#ab128568) were purchased from Abcam Co., Ltd. (Cambridge, UK). The antibody for uncoupling protein 2 (UCP-2) (#615902) was purchased from BioLegend (San Diego, CA). LabAssay glucose (LABGLUC-M1), triglyceride (LABTRIG-M1), cholesterol (LABCHO-M1), and non-esterified fatty acid (NEFA) (LABNEFA-M1) kits, as well as an LBIS Mouse/Rat HMW Adiponectin ELISA Kit (AKMAN-011), were purchased from FUJIFILM Wako Pure Chemical Co., Ltd. (Osaka, Japan). All other reagents were of sufficient grade for each experiment.

Animal treatments. All animal experiments were approved by the Institutional Animal Care and Use Committee of Kobe University (Kobe, Japan) (#2020-10-3) and carried out according to the guidelines set by this institution. Male C57BL/6J mice (5 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and maintained at 23 ± 2°C with a 12-h light–dark cycle (lights on at 09:00). The mice were allowed free access to water and food, and were acclimatized to the study environment for 7 days before the start of the experiments. To examine the effect of 8-PN on obesity, mice were subjected to one of the following conditions: a control diet (AIN-93M), a high-fat diet containing 30% lard, or the control or the high-fat diet plus 8-PN; all diets were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Specifically, mice were randomly divided into six groups of 6–7 and fed a control or high-fat diet supplemented with 0% (C-0, HF-0), 0.0005% (C-0.0005, HF-0.0005), or 0.005% (C-0.005, HF-0.005) 8-PN for 8 weeks. At the end of the experimental feeding period, mice were fasted for 16 h and sacrificed by cardiac blood collection using a heparinized syringe under mixed anesthesia with sodium pentobarbital and medetomidine hydrochloride. At dissection, plasma, liver, epididymal white adipose tissue (eWAT), perirenal WAT, mesenteric WAT, subcutaneous WAT, and brown adipose tissue were collected. The liver and each WAT were weighed and immediately frozen using liquid N_2. Blood was centrifuged at 3,000 × *g* at 4°C for 10 min, and the supernatant was collected as plasma. Obtained plasma and organs were kept at −80°C until use.

Oral glucose tolerance tests (OGTTs). OGTTs were performed at week 7. Mice were fasted for 13 h before a glucose solution (1.0 g/kg body weight) was administered orally. After administration, blood was collected from the tail vein at 0, 15, 30, 60, and 120 min into heparinized microtubes. Blood was centrifuged at 3,000 × *g* for 10 min at 4°C, and the supernatant was collected. Plasma glucose concentration was then measured using the already-mentioned commercial assay kits.

Measurement of plasma parameters. Plasma concentrations of cholesterol, triglycerides, free fatty acids (FFAs), and adiponectin were measured using the commercial assay kits mentioned above according to the manufacturer's instructions. Adiponectin index was calculated as plasma adiponectin level (μg/ml)/body fat percentage (%). Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated using the following formula. Plasma glucose level (mg/dl) × plasma insulin level (μU/ml)/405.

Measurement of lipid levels in the liver. Seventy milligrams of mouse liver and 500 μl of water were taken in microtubes and homogenized using an ultrasonic homogenizer. Two milliliters of chloroform:methanol 2:1 mixture was added to the tissue solution and stirred for 3 min. The lower chloroform layer was collected after centrifugation at 3,000 × *g* for 10 min at 4°C. To the upper water layer, 0.5 ml of the chloroform:methanol mixture was added, stirred for 1 min, and centrifuged at 3,000 × *g* for 10 min at 4°C before the lower layer was collected. To the obtained organic layer, 1/4 volume of 0.88% KCl solution was added, stirred for 1 min, and centrifuged at 3,000 × *g* for 10 min at 4°C. The lower layer was collected and air-dried. The weight of the remaining substance was measured as total liver lipids. The remaining substance was dissolved in 1 ml of isopropanol with 10% (v/v) Triton-X solution and triglyceride, cholesterol, and FFA concentrations were determined using LabAssay triglyceride (LABTRIG-M1), cholesterol (LABCHO-M1), and NEFA (LABNEFA-M1) kits, respectively.

Measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities. For AST activity, Reaction Mix for AST activity [β-NADH 0.53 mg, 625 U/ml lactate dehydrogenase (LDH) 4 μl in solution A [bovine serum albumin (BSA) 30.6 mg, glycerol 500 μl, 88 mM Tris-HCl (pH 7.8) 500 μl], 625 U/ml malate dehydrogenase in solution A 4 μl, 88 mM Tris-HCl (pH 7.8) 1.99 ml] 400 μl, 100 mM aspartic acid in 176 mM Tris-HCl (pH 7.8) 800 μl, plasma 100 μl were mixed and incubated for 2 min at 30°C. Then, it was mixed with 100 mM 2-oxoglutaric acid in 176 mM Tris-HCl (pH 7.8) 100 μl for 30 s and 340 nm absorbance was measured every 5 s for 2 min. 88 mM Tris-HCl (pH 7.8) was used for blank. AST activity was calculated using the following formula: AST activity (U/L) = $(\Delta Abs_{sample}/min - \Delta Abs_{blank}/min)/(6.3 \times 10^{-3}) \times 1.4/0.1$.

For ALT activity, Reaction Mix for ALT activity [β-NADH 0.56 mg, 500 U/ml LDH 17.08 μl in solution B [BSA 29.7 mg, glycerol 500 μl, 111 mM Tris-HCl (pH 7.5) 500 μl], 111 mM Tris-HCl (pH 7.5) 1.58 ml] 300 μl, 1.0 M L-alanine in 222 mM Tris-HCl (pH 7.5) 500 μl, plasma 100 μl were mixed and incubated for 2 min at 30°C. Then, it was mixed with 150 mM 2-oxoglutaric acid in 222 mM Tris-HCl (pH 7.5) 100 μl for 30 s and 340 nm absorbance was measured every 5 s for 2 min. 111 mM Tris-HCl (pH 7.5) was used for blank. ALT activity was calculated using the following formula: ALT activity (U/L) = $(\Delta Abs_{sample}/min - \Delta Abs_{blank}/min)/(6.3 \times 10^{-3}) \times 1.0/0.1$.

Preparation of eWAT lysate. eWAT was chopped and added to RIPA buffer [1% (v/v) NP-40, 150 mM NaCl, 0.5% (w/w) sodium deoxycholate, 0.1% (w/w) sodium dodecyl sulfate, 50 mM Tris-HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 mM sodium fluoride, 1 mM Na_3VO_4, and 500 μM dithiothreitol] and homogenized with a polytronhomogenizer. Then, the samples were left on ice for 1 h and centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was collected and stored at −80°C. The protein concentration of each tissue lysate was quantified using the Lowry method. Obtained tissue lysates were used for the following immunoblotting.

Immunoblotting. Proteins in the tissue lysate of eWAT were separated by sodium dodecyl sulfate–polyacrylamide gel

electrophoresis and transferred to a polyvinylidene difluoride membrane. After treatment with commercial Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 30 min, the membrane was incubated overnight at 4°C with each primary antibody (1:5,000 dilution), followed by the corresponding horseradish peroxidase-conjugated secondary antibody (1:50,000 dilution) for 3 h at 4°C. Specific bands were visualized using the ImmunoStar LD luminescence system (FUJIFILM Wako Pure Chemical, Co., Ltd.) and detected using the Light-Capture II imaging system (ATTO, Co., Ltd, Tokyo, Japan). The density of specific bands was calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from eWAT using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA); cDNA was synthesized by using the ReverTra Ace RT-qPCR Master Mix (TOYOBO, Osaka, Japan). cDNA was amplified using TB Green Premix Ex Taq II (Takara Bio Co., Ltd., Shiga, Japan) in a Thermal Cycler Dice real-time system III (Takara Bio Co., Ltd.). The following primers were used: *AdiporR1* (5'-ACGTTGGAGAGTCATCCCGTAT-3', 5'-CTCTGTGTGGATGCGGAAGAT-3'), *AdipoR2* (5'-TCCCAGGAAGATGAAGGGTTTAT-3', 5'-TTCCATTCGTTCCATAGCATGA-3'), and *β-actin* (5'-GGTCATCACTATTGGCAACG-3', 5'-TCCATACCCAAGAAGGAAGG-3'). *β-actin* mRNA was used as a control.

Statistical analyses. Statistical analyses were performed with JMP statistical software ver. 11.2.0 (SAS Institute, Cary, NC). Data are presented as mean ± SE (n = 4–7). Means were compared among the six groups using the Tukey–Kramer multiple comparison test. The significance level was set as p<0.05.

Results

The effect of 8-PN on body and tissue weight gain in high-fat diet-fed mice. To examine the effect of 8-PN on high-fat diet-induced obesity, C57BL/6J mice were fed a control or high-fat diet alone or with 0.0005% or 0.005% 8-PN for 8 weeks. The composition of the diets is shown in Table 1. Body and tissue weights at the end of feeding are shown in Table 2. The body, liver, and WAT depot weights of HF-0 mice were significantly higher than those of C-0 mice. 8-PN significantly suppressed this increase in a dose-dependent manner. There were no differences in body weight or the weight of each WAT depot between the C-0, C-0.0005, and C-0.005 mice. These results suggest that 8-PN have an anti-obesity effect.

The effect of 8-PN on lipid metabolism-related factors in eWAT. As intake of 8-PN prevented high-fat diet-induced body weight gain and fat accumulation in WAT, we attempted to elucidate the mechanisms underlying this effect. Expression levels of lipid metabolism-related factors associated with metabolic dysfunction were measured in eWAT by western blotting.[17] The expression level of cleaved SREBP-1, a transcription factor for fatty acid synthesis-related factors, was significantly higher in HF-0 mice than in C-0 mice; 8-PN suppressed this increase (Fig. 2A). 8-PN administration resulted in significantly higher expression level of PGC-1α, a transcriptional coactivator for thermogenesis-related factors, in HF-0.005 (Fig. 2B). Expression of CPT-1a, the rate-limiting enzyme for fatty acid β-oxidation, and UCP-2 were unaffected by diet or 8-PN administration (Fig. 2C and D). These results indicate that 8-PN may suppress obesity by inhibiting fatty acid synthesis and increasing energy expenditure by promoting mitochondrial production.

The effect of 8-PN on AMPK activity. The expression and phosphorylation level of AMPK, which regulates lipid metabolism upstream of SREBP-1 and PGC-1α, was measured. As shown in Fig. 3, 8-PN promoted AMPK phosphorylation in high-fat diet-fed mice, resulting in significantly higher levels in HF-0.005 mice than in HF-0 mice. These results suggest that 8-PN regulates SREBP-1 and PGC-1α expression via AMPK activation in high-fat diet-fed mice. To investigate the mechanism underlying the effect of 8-PN on AMPK phosphorylation, the protein and phosphorylation levels of LKB1 and CaMKKβ, the major kinases upstream of AMPK, were measured. However, there were so significant differences between groups (Fig. 4).

The effect of 8-PN on AMPK activity via adiponectin. To find the upstream factor by which 8-PN activated AMPK, we investigated adipocytokines. Among adipokines, adiponectin is

Table 1. Composition of diets

Ingredient	g/100 g diet	
	Control (AIN-93M)	High-fat
Casein	14	14
L-cysteine	0.2	0.2
Cornstarch	46.6	16.6
Dextrin	15.5	15.5
Sucrose	10	10
Soybean oil	4	4
Cellulose	5	5
Mineral mixture	3.5	3.5
Vitamin mixture	1	1
Choline bitartate	0.3	0.3
tert-Butylhydroxyquinone	0.0008	0.0008
Lard	0	30
Energy density	348 kcal	518 kcal

Table 2. Body and tissue weight (g) of mice fed a control or high-fat diet with or without 8-PN

8-PN (%)	Control			High-fat		
	0	0.0005	0.005	0	0.0005	0.005
Body weight	24.35 ± 0.84^{a}	25.22 ± 0.40^{a}	24.22 ± 0.26^{a}	30.92 ± 1.19^{b}	26.30 ± 0.54^{a}	26.26 ± 0.45^{a}
Liver	1.16 ± 0.05^{ab}	1.22 ± 0.05^{a}	1.11 ± 0.04^{abc}	1.20 ± 0.05^{a}	0.99 ± 0.03^{bc}	0.95 ± 0.02^{c}
Mesenteric WAT	0.21 ± 0.02^{a}	0.23 ± 0.03^{a}	0.16 ± 0.03^{a}	0.54 ± 0.11^{b}	0.26 ± 0.06^{a}	0.25 ± 0.04^{a}
Perirenal WAT	0.14 ± 0.03^{a}	0.18 ± 0.03^{a}	0.10 ± 0.02^{a}	0.58 ± 0.11^{b}	0.21 ± 0.05^{a}	0.22 ± 0.04^{a}
Epididymal WAT	0.43 ± 0.04^{a}	0.51 ± 0.07^{a}	0.29 ± 0.04^{a}	1.28 ± 0.24^{b}	0.59 ± 0.12^{a}	0.58 ± 0.09^{a}
Subcutaneous WAT	0.7 ± 0.09^{a}	0.79 ± 0.11^{a}	0.36 ± 0.06^{a}	1.94 ± 0.42^{b}	0.76 ± 0.22^{a}	0.79 ± 0.15^{a}
Total WAT	1.48 ± 0.18^{a}	1.71 ± 0.23^{a}	0.89 ± 0.12^{a}	4.34 ± 0.87^{b}	1.81 ± 0.44^{a}	1.85 ± 0.31^{ab}
Brown adipose tissue	0.10 ± 0.01^{a}	0.1 ± 0.01^{a}	0.08 ± 0.01^{a}	0.15 ± 0.02^{b}	0.09 ± 0.01^{a}	0.10 ± 0.01^{a}

Data are presented as mean ± SE (n = 6–7). Means with the same letter(s) are not significantly different from each other according to the Tukey–Kramer multiple comparison test (p<0.05). 8-PN, 8-prenylnaringenin; WAT, white adipose tissue.

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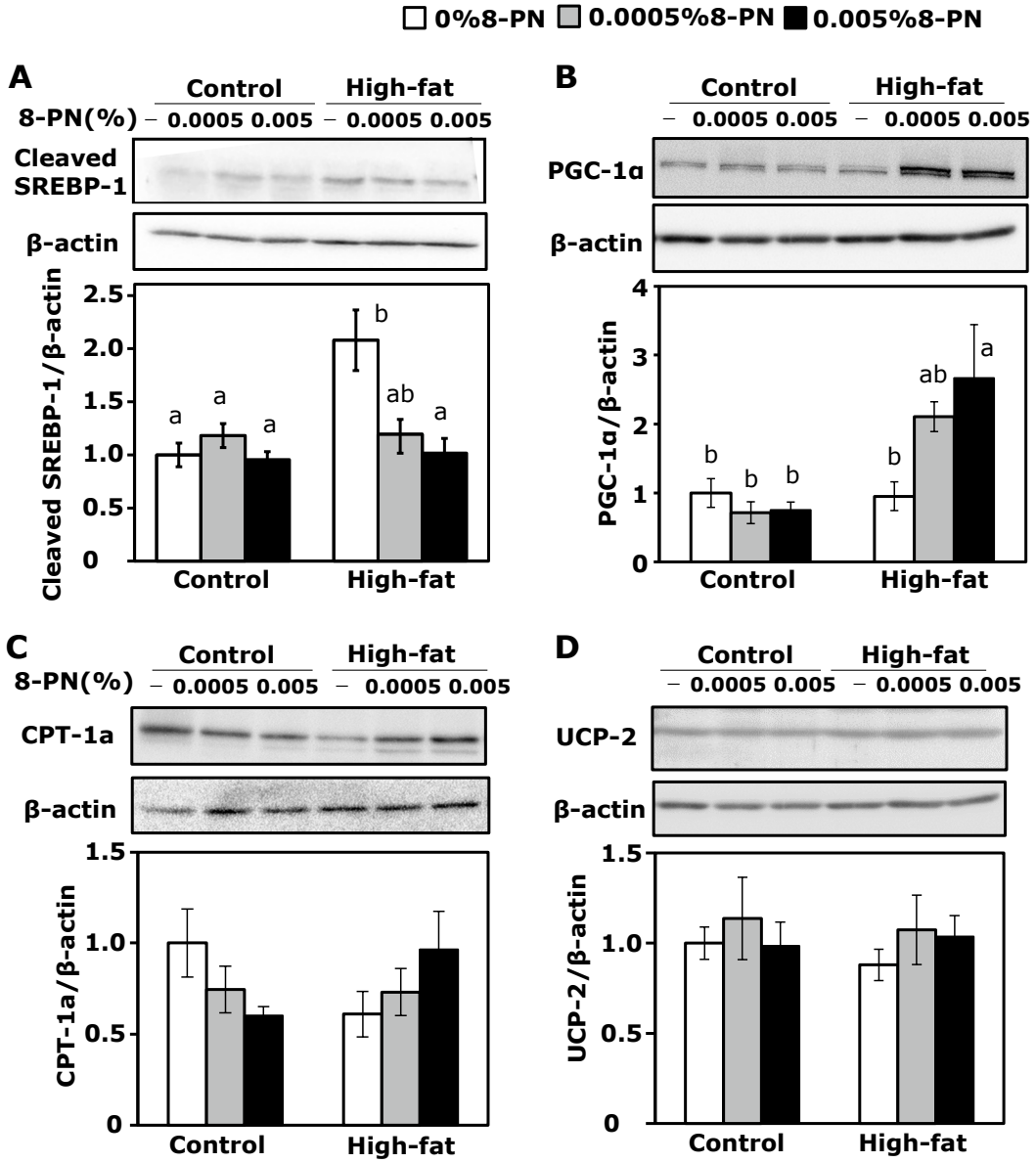

Fig. 2. Effects of 8-PN on protein levels of lipid metabolism-related factors. (A–D) Protein levels of (A) SREBP-1, (B) PGC-1α, (C) CPT-1a, and (D) UCP-2 are shown. Tissue lysate was extracted from the epididymal white adipose tissue of mice fed a control or high-fat diet with or without 8-PN. Data are presented as mean ± SE (n = 5–7). Means were compared among the six groups using the Tukey–Kramer multiple comparison test; means with the same letter(s) are not significantly different from each other. The significance level was set as $p<0.05$. 8-PN, 8-prenylnaringenin; SREBP-1, sterol regulatory element-binding protein-1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; CPT-1a, carnitine palmitoyl transferase 1α; UCP-2, uncoupling protein 2.

closely related to AMPK activation; therefore, adiponectin concentrations in the plasma were measured. Plasma adiponectin levels were significantly higher in HF-0.005 mice than in HF-0 mice (Fig. 5A). The adiponectin index was also significantly higher in HF-0.0005 and HF-0.005 mice than in HF-0 mice (Fig. 5B). These results suggest that 8-PN promotes adiponectin secretion from adipocytes to activate AMPK. We next measured *AdipoR1/2* mRNA levels in eWAT (Fig. 5C and D). However, there were no significant effects of diet or 8-PN supplementation on *AdipoR1/2* expression. To clarify whether 8-PN directly stimulated adiponectin secretion, we examined the expression of factors involved in adiponectin secretion. As adiponectin secretion is lower in hypertrophic adipocytes than in normal adipocytes, the involvement of 8-PN as a marker for adipocyte miniaturization was determined. The expression levels of PPARγ and C/EBPα, markers of adipocyte differentiation, in eWAT (Fig. 5E and F) were measured. HF-0.005 mice had significantly higher expression levels of these proteins than HF-0 mice. These results strongly suggest that 8-PN increases adiponectin secretion via adipocyte differentiation factors and AMPK activation.

The effect of 8-PN on hyperglycemia. As AMPK activation and PPARγ signaling improve insulin resistance, an OGTT was conducted at week 7.[8] Blood samples were taken 0, 15, 30, 60, and 120 min after glucose administration and plasma glucose levels were measured (Fig. 6). At 0, 15, 30, and 60 min after glucose administration, the glucose levels of HF-0 mice were significantly higher than those of C-0 mice. However, 8-PN significantly suppressed high-fat diet-induced hyperglycemia dose-dependently so that differences between the control and high-fat diet-fed groups were not significantly different (Fig.

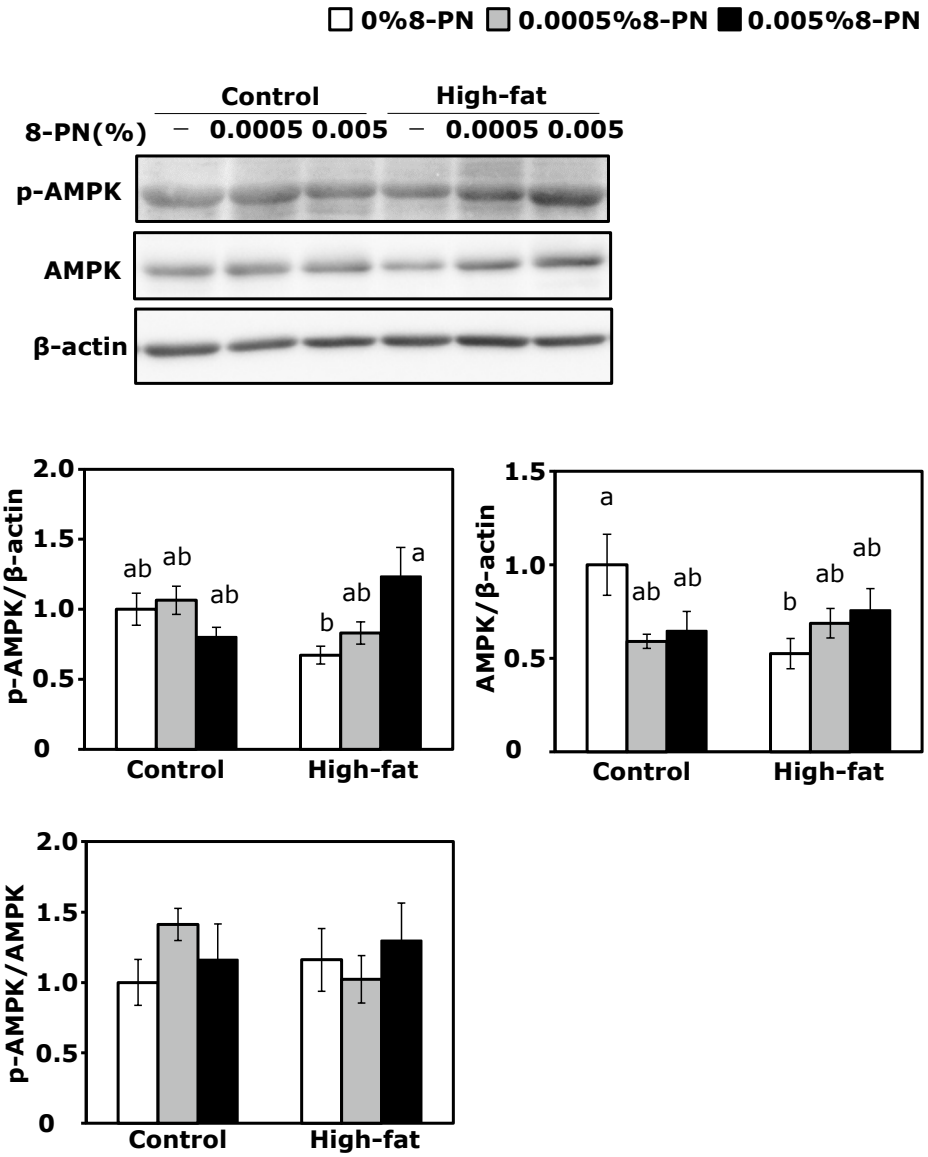

Fig. 3. Effects of 8-PN on protein levels and phosphorylation of AMPK. Tissue lysate was extracted from the epididymal white adipose tissue of mice fed a control or high-fat diet with or without 8-PN. Data are presented as mean ± SE (n = 5–7). Means were compared among the six groups using the Tukey–Kramer multiple comparison test; means with the same letter(s) are not significantly different from each other. The significance level was set as p<0.05. 8-PN, 8-prenylnaringenin; AMPK, adenosine monophosphate-activated protein kinase.

6A). The area under the curve results showed similar differences between groups (Fig. 6B). HOMA-IR, a index of insulin resistance at the end of feeding period, was calculated (Fig. 6C). The value of HF-0 mice was higher than C-0 mice, but 8-PN cancelled that increase. Also, high-fat diet intake decreased IRS-1 level in eWAT and 8-PN suppressed it (Fig. 6D). These results suggest that 8-PN intake suppresses high-fat diet-induced glucose intolerance and insulin resistance.

The effect of 8-PN on blood parameters in mice. Plasma cholesterol, triglycerides, FFAs, glucose, and insulin levels at the end of feeding are shown in Table 3. The plasma cholesterol, glucose, and insulin levels of HF-0 mice were significantly higher than those of C-0 mice. 8-PN suppressed these increases of high-fat diet. Plasma triglyceride and FFA levels were not affected by diet or 8-PN administration. These results indicate that 8-PN suppresses increases in plasma cholesterol, glucose, and insulin levels induced by a high-fat diet.

The effect of 8-PN on lipid accumulation and damage in the liver. Total lipid, triglyceride, and cholesterol levels in the liver are shown in Table 4. The total lipid and triglyceride levels in the livers of HF-0 mice were significantly higher than those of C-0 mice. 8-PN suppressed these due to high-fat diet. A high-fat diet did not affect liver cholesterol levels. We also measured AST and ALT activity (Supplemental Fig. 1*). ALT activity was increased by high-fat diet and 8-PN suppressed it, while AST activity was unchanged among all groups. These results suggest that 8-PN intake suppresses lipid accumulation and damage in the liver.

Discussion

Obesity causes a variety of diseases, including type 2 diabetes; therefore, obesity suppression using certain food factors could be an important approach by which to prevent disease. In the present study, we demonstrated that 8-PN intake suppressed high-fat

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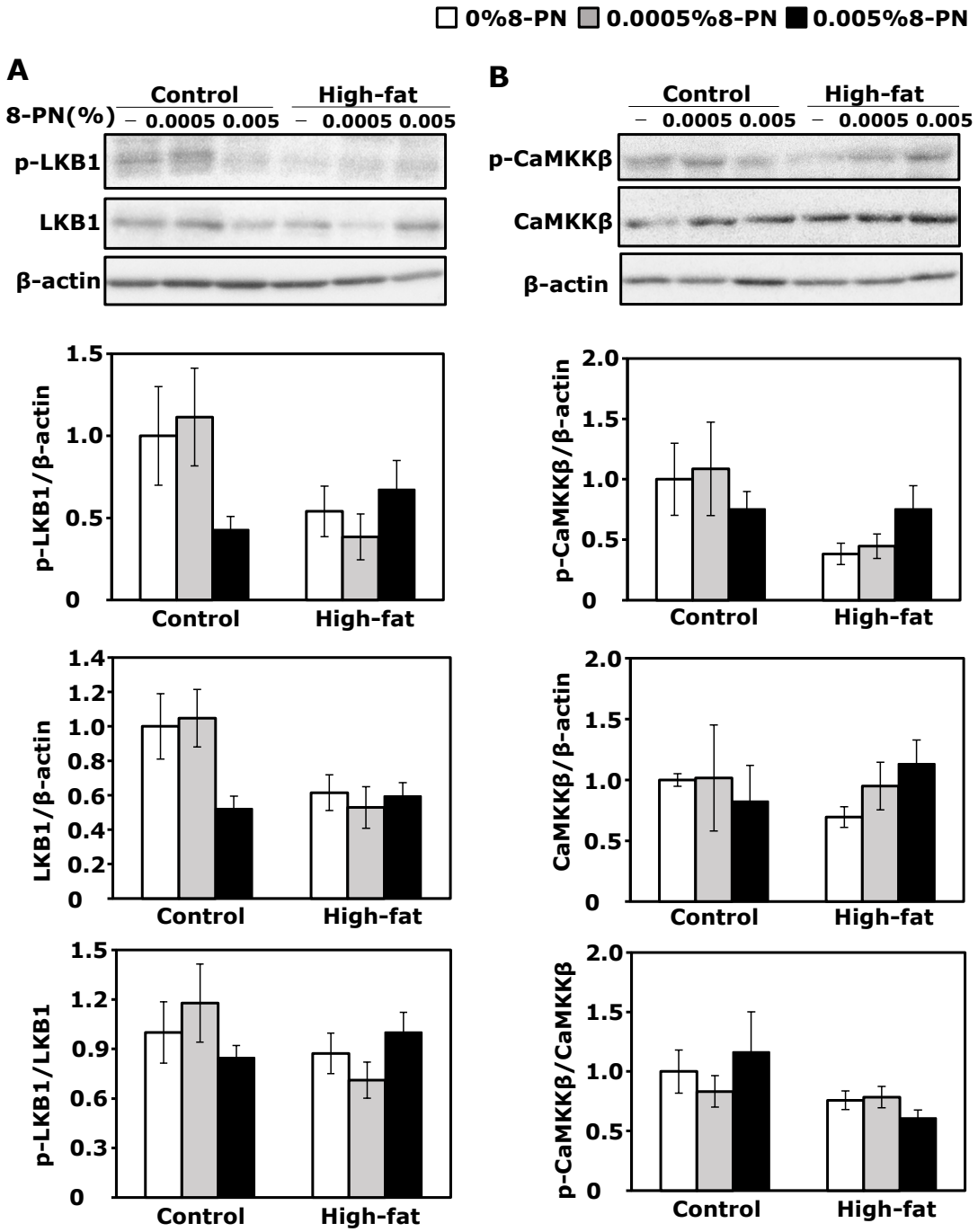

Fig. 4. Effects of 8-PN on protein levels and phosphorylation of LKB1 and CaMKKβ. (A, B) Protein levels and phosphorylation levels of (A) LKB1 and (B) CaMKKβ are shown. Tissue lysate was extracted from the epididymal white adipose tissue of mice fed a control or high-fat diet with or without 8-PN. Data are presented as mean ± SE (n = 5–7). Means were compared among the six groups using the Tukey–Kramer multiple comparison test; means with the same letter(s) are not significantly different from each other. The significance level was set as p<0.05. 8-PN, 8-prenylnaringenin; LKB1, liver kinase B1; CaMKKβ, calcium/calmodulin-activated kinase kinase β.

diet-induced obesity in mice via activation of the adiponectin/AMPK pathway in WAT. This is the first study to report the anti-obesity effect of 8-PN and its mechanism in adipose tissue. In the present study, we found that AMPK is important molecule in the anti-obesity effect of 8-PN. This suggested that intake of 8-PN also suppressed hyperglycemia, insulin resistance, fatty liver, and liver damage. LKB1 and CaMKKβ are generally known as AMPK activators; however, the expression and activity of these proteins were not affected by 8-PN (Fig. 4). Therefore, we investigated the involvement of adiponectin and found that 8-PN increased plasma adiponectin levels (Fig. 5A). We also calculated the adiponectin index, the plasma adiponectin level relative to total WAT: 8-PN administration resulted in an increase of this value in high-fat diet groups (Fig. 5B). This suggests that 8-PN promotes AMPK activity through adiponectin secretion from adipocytes.

There are few reports of LKB1- and CaMKKβ-independent pathways of AMPK activation by adiponectin. However, other

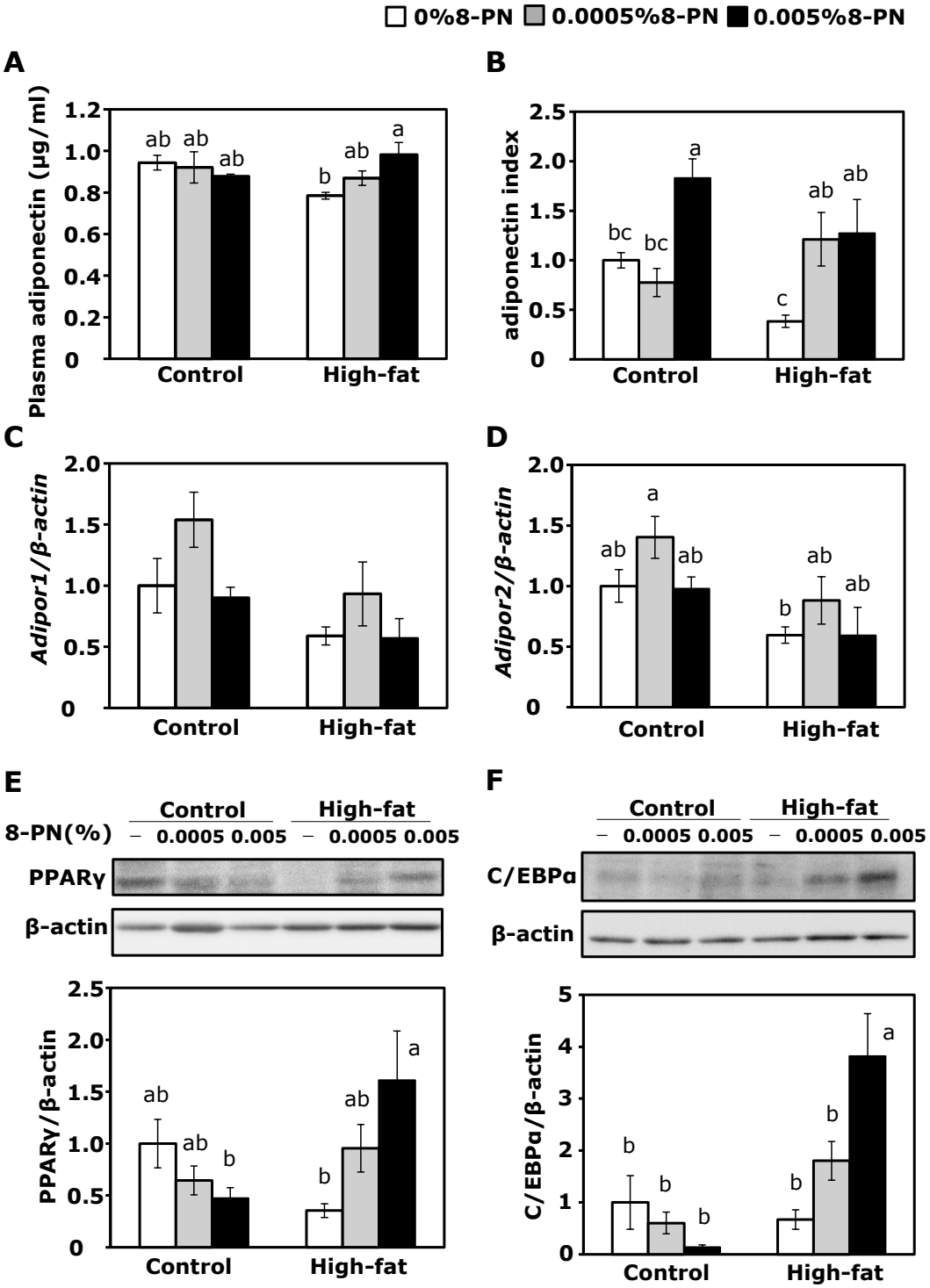

Fig. 5. Effects of 8-PN on plasma adiponectin and adipocyte differentiation. (A) Plasma adiponectin levels and (B) the adiponectin index of mice fed a control or high-fat diet with or without 8-PN are shown. (C–F) mRNA expression levels of (C) *AdipoR1* and (D) *AdipoR2* and protein expression levels of (E) PPARγ and (F) C/EBPα, adipocyte differentiation markers, are shown. Tissue lysate was extracted from the epididymal white adipose tissue of mice fed a control or high-fat diet with or without 8-PN. Data are presented as mean ± SE (n = 5–7). Means were compared among the six groups using the Tukey–Kramer multiple comparison test; means with the same letter(s) are not significantly different from each other. The significance level was set as p<0.05. 8-PN, 8-prenylnaringenin; PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT/enhancer-binding protein α.

factors, such as CaMKI and CaMKII, which are activated by the increase in intracellular calcium induced by adiponectin, may be involved. Indeed, several reports suggest that CaMKII acts upstream of AMPK.[19,20] As there have been few reports of compounds that activate AMPK via LKB1- and CaMKKβ-independent pathways, 8-PN may have a unique function in this regard. Furthermore, 8-PN has estrogen-like effects and binds to estrogen receptor-α. Several studies report an estrogen receptor-

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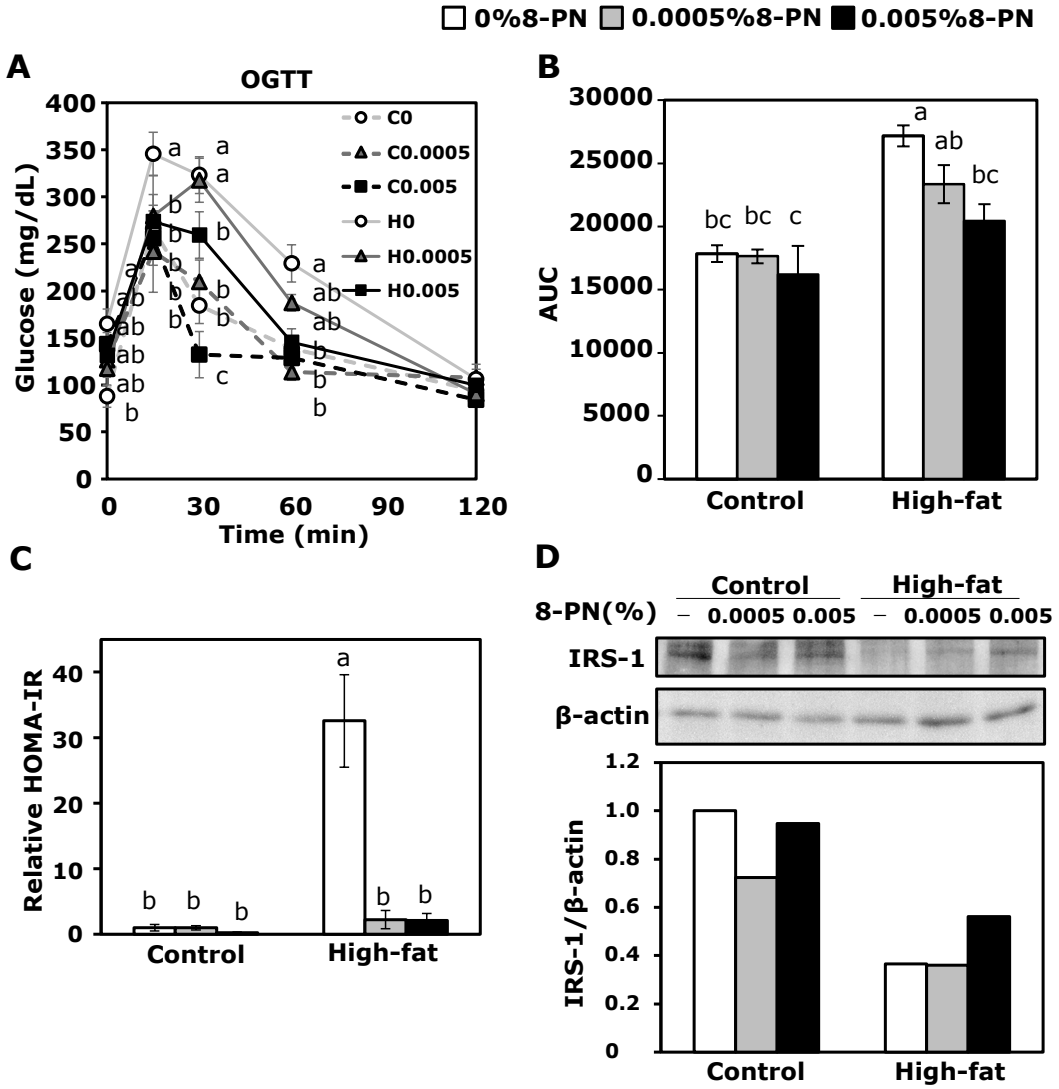

Fig. 6. Effects of 8-PN on glucose tolerance. (A) Results of an OGTT in mice fed a control or high-fat diet with or without 8-PN at week 7 of feeding are shown. (B) The AUCs from (A) are shown. (C) Relative HOMA-IR is shown. Plasma was collected from mice fed a control or high-fat diet with or without 8-PN. (D) The protein level of IRS-1 in mixture for each group is shown. Tissue lysate was extracted from the epididymal white adipose tissue of mice fed a control or high-fat diet with or without 8-PN. Data of (A)–(C) are presented as mean ± SE (n = 5–7). Means were compared among the six groups using the Tukey–Kramer multiple comparison test; means with the same letter(s) are not significantly different from each other. The significance level was set as p<0.05. 8-PN, 8-prenylnaringenin; OGTT, oral glucose tolerance test; AUC, area under the curve; HOMA-IR, homeostatic model assessment for insulin resistance; IRS-1, insulin receptor substrate-1.

Table 3. Plasma parameters of mice fed a control or high-fat diet with or without 8-PN

8-PN (%)	Control			High-fat		
	0	0.0005	0.005	0	0.0005	0.005
Triglyceride (mg/dl)	91.4 ± 15.5[a]	66.5 ± 9.6[a]	91.4 ± 9.6[a]	86.0 ± 15.1[a]	77.4 ± 12.7[a]	76.3 ± 10.9[a]
Cholesterol (mg/dl)	58.6 ± 4.17[a]	52.1 ± 3.48[a]	55.5 ± 2.65[a]	87.7 ± 7.18[b]	71.7 ± 5.76[a]	68.7 ± 3.15[a]
NEFA (mEq/dl)	0.57 ± 0.02[a]	0.69 ± 0.12[a]	0.56 ± 0.06[a]	0.78 ± 0.10[a]	0.89 ± 0.23[a]	0.73 ± 0.05[a]
Glucose (mg/dl)	138.4 ± 4.16[a]	145.1 ± 4.30[a]	133.9 ± 5.38[a]	193.4 ± 13.3[b]	155.5 ± 4.74[a]	147.5 ± 7.30[a]
Insulin (ng/ml)	1.74 ± 0.84[a]	1.65 ± 0.50[a]	0.47 ± 0.16[a]	41.49 ± 9.80[b]	3.55 ± 2.13[a]	3.41 ± 1.53[a]

Data are presented as mean ± SE (n = 6–7). Means with the same letter(s) are not significantly different from each other according to the Tukey–Kramer multiple comparison test (p<0.05). 8-PN, 8-prenylnaringenin; NEFA, non-esterified fatty acid.

α/AMPK pathway[21,22]; therefore, this could be another way in which 8-PN activates AMPK. However, whether either of these mechanisms caused AMPK activation after 8-PN supplementation requires further elucidation. Although it has been reported that 8-PN activates AMPK in the muscle and liver and contributes to suppressing hyperglycemia,[18] our research is the first

Table 4. Liver lipid levels of mice fed a control or high-fat diet with or without 8-PN

8-PN (%)	Control			High-fat		
	0	0.0005	0.005	0	0.0005	0.005
Total lipid (mg/g liver)	64.7 ± 2.45[a]	59.6 ± 3.15[a]	63.1 ± 4.15[a]	90.9 ± 7.17[b]	79.9 ± 6.61[a]	64.6 ± 4.74[a]
Triglyceride (mg/g liver)	29.1 ± 2.72[a]	30.1 ± 4.23[a]	32.0 ± 4.23[a]	43.2 ± 6.04[b]	41.3 ± 5.55[ab]	35.2 ± 3.83[a]
Cholesterol (mg/g liver)	2.48 ± 0.12[a]	2.54 ± 0.09[a]	2.64 ± 0.08[a]	3.36 ± 0.70[a]	3.32 ± 0.33[a]	3.39 ± 0.13[a]

Data are presented as mean ± SE (n = 6–7). Means with the same letter(s) are not significantly different from each other according to the Tukey–Kramer multiple comparison test (p<0.05). 8-PN, 8-prenylnaringenin.

to show that 8-PN activates AMPK through a non-canonical mechanism and contributes to obesity prevention.

We investigated molecules upstream of adiponectin secretion and found that PPARγ may be involved in 8-PN-induced adiponectin secretion. PPARγ is an important regulator of adiponectin expression[23,24] and is an adipocyte differentiation regulator; it also known to downsize adipocyte and promotes energy metabolism. In the present study, 8-PN intake increased expression of PGC-1α, a marker of mitochondrial biosynthesis and thermogenesis. Although these results suggest that 8-PN reduces adipocyte size and promotes energy metabolism, the detailed mechanism underlying this effect needs to be investigated in the future. One hypothesis is that 8-PN is a PPARγ agonist. 8-PN has a prenyl group, which makes it more hydrophobic than non-prenylflavonoids and gives it a higher affinity for biological membranes, which means it is easily taken up into cells. It also easily accumulates in tissues because of its slow rate of extracellular efflux.[13] Previously, other researchers reported that flavanones have PPARγ ligand activity and that a similar compound, apigenin, also binds to and activates PPARγ.[25,26] Therefore, it is possible that 8-PN is taken up into adipocytes and exerts its function by binding directly to PPARγ. Further studies are currently underway to verify this hypothesis.

8-PN exhibited the anti-obesity effects of a high-fat diet at very low concentrations in the present study, namely, 0.0005% and 0.005%. As mentioned above, 8-PN has high bioavailability because of its prenyl group. Therefore, when continuously ingested, its plasma level is higher than that of naringenin, which does not have a prenyl group.[27] The bioavailability of 8-PN is also 4–5 times higher than 6-prenylnaringenin, the structural isomer of 8-PN.[28] Thus, 8-PN could be highly functional at lower volumes than other similar compounds and may, therefore, play a more effective role against obesity than other compounds.

Conclusions

This study examined the effects of 8-PN on energy metabolism. When mice were fed a high-fat diet with 0.0005% and 0.005% 8-PN for 8 weeks, 8-PN activated AMPK, a master regulator of energy metabolism, and regulated lipogenesis and mitochondrial biosynthesis-related proteins in eWAT; PPARγ, C/EBPα, and adiponectin appeared to be involved in 8-PN-induced AMPK activation. Overall, our study suggests that 8-PN may be an effective compound for preventing high-fat diet-induced obesity and energy metabolism disorders.

Author Contributions

FO: writing – original draft, conceptualization, methodology, investigation, data curation, formal analysis, and visualization. AK and YU: design of study, writing – review, and funding. RM and HA: writing – review & editing, supervision. YY: writing – review & editing, supervision, project administration, and funding acquisition.

Abbreviations

ALT	alanine aminotransferase
AMPK	adenosine 5'-monophosphate-activated protein kinase
AST	aspartate aminotransferase
BSA	bovine serum albumin
CaMKKβ	calcium/calmodulin-dependent kinase kinase β
C/EBP	CCAAT/enhancer binding protein
CPT-1a	carnitine palmitoyltransferase 1a
eWAT	epididymal white adipose tissue
FFA	free fatty acid
HOMA-IR	homeostatic model assessment for insulin resistance
LDH	lactate dehydrogenase
LKB1	liver kinase B
NEFA	non-esterified fatty acid
OGTT	oral glucose tolerance test
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1α
PPARγ	peroxisome proliferator-activated receptor γ
8-PN	8-prenylnaringenin
SREBP-1	sterol regulatory element-binding protein 1
UCP-2	uncoupling protein 2

Conflict of Interest

This study was designed and funded by Daicel Corporation. Co-authors (AK and YU) are employees of Daicel Corporation. But the funding company has not conflict of interests. This company has approved the final version of the manuscript. This research was partly supprted by Grant-in-Aid for Scientific Research (C) Grant Number 20K11581 (RM).

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