JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Diet Enriched with Korean Pine Nut Oil Improves Mitochondrial Oxidative Metabolism in Skeletal Muscle and Brown Adipose Tissue in Diet-Induced Obesity

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ABSTRACT: In this study, we investigated effects of pine nut oil (PNO) on high-fat-diet (HFD)-induced obesity and metabolic dysfunction in skeletal muscle and brown adipose tissue (BAT). Male C57BL/6 mice were fed a HFD with 15% energy from lard and 30% energy from either soybean oil (SBO-HFD) or PNO (PNO-HFD) for 12 weeks. The PNO-HFD resulted in less weight gain and intramuscular lipid accumulation than the SBO-HFD and was accompanied by upregulation of transcripts and proteins related to oxidative metabolism and phosphorylation of AMP-activated protein kinase (AMPK), as well as molecules selectively expressed in type I and type IIa muscle fibers. In addition, uncoupling protein-1 was upregulated in BAT. These beneficial metabolic effects were partly associated with the dual ligand activity of pinolenic acid, which is abundant in PNO, for peroxisome proliferator-activated receptors α and δ. Our findings suggest that PNO may have potential as a dietary supplement for counteracting obesity and metabolic dysregulation.

KEYWORDS: *Obesity, pine nut oil, oxidative metabolism, skeletal muscle, brown adipose tissue*

INTRODUCTION

Obesity-induced metabolic dysregulation in adipose tissue, liver, and skeletal muscle leads to metabolic complications, such as insulin resistance, type 2 diabetes, hepatic steatosis, and atherosclerosis.[1] Skeletal muscle, the largest metabolic tissue in the body, is a major site of lipid oxidation and insulin-stimulated glucose use and, thus, is important in maintaining energy homeostasis.[2] Impaired skeletal muscle metabolism and mitochondrial biogenesis and lowered oxidative metabolism are often observed in obese conditions.[3,4] Brown adipose tissue (BAT) also plays an important role in energy balance by upregulating the expression of uncoupling protein-1 (UCP1), which facilitates proton leakage across the mitochondrial membrane, leading to dissipation of chemical energy as heat.[5] In this context, food components that can enhance the mitochondrial function in skeletal muscle and/or in BAT may be beneficial for preventing obesity and its related metabolic disorders.

Pine nut oil (PNO) from Korean pine (*Pinus koraiensis*) is used in food preparation in several countries. It is composed of over 92% poly- and monounsaturated fatty acids,[6] which are considered healthy dietary fats.[7,8] Studies have shown that PNO has beneficial effects on lipoprotein metabolism, plasma triglyceride and cholesterol levels, and atherogenesis in hypertensive rats[9] and atherosclerotic mice.[10] However, it is not known whether PNO can improve the dysregulated lipid metabolism accompanied by impaired mitochondrial function frequently observed in obese individuals.[11] The aim of our study was to investigate the effect of a PNO-rich diet on high-fat-diet (HFD)-induced metabolic dysfunction in skeletal muscle and BAT.

MATERIALS AND METHODS

Animals and Diets. Five-week-old male C57BL/6 mice were individually housed in a specific pathogen-free animal facility, where a 12−12 h light−dark cycle was maintained. For 12 weeks, the mice were fed a control diet containing pine nut oil (10% energy from PNO, PNO-LFD) or soybean oil (10% energy from SBO, SBO-LFD) or a high-fat diet containing PNO (30% energy from PNO + 15% energy from lard, PNO-HFD) or SBO (30% energy from SBO + 15% energy from lard, SBO-HFD). The diets were custom-made by Dyets, Inc. (Bethlehem, PA), and the PNO was a gift from Dubio Co., Ltd. (GyeongGi-do, Korea). The composition of the experimental diets is described in Table 1, and the composition of fatty acids in the diets is shown in Table 2. The mice had free access to food and water and were weighed weekly to monitor changes in their body weight. All animal experiments were approved by the Animal Ethics Committee of Seoul National University and conformed to the guidelines of the National Institute of Health. After 12 weeks, the animals were killed by CO_2 asphyxiation. Skeletal muscle and BAT were dissected and immediately frozen in liquid nitrogen. For the polymerase chain reaction (PCR) analysis, tissues were stored at −20 °C in RNAlater buffer (Ambion, Austin, TX).

Cell Culture. The murine C2C12 myoblast cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). C2C12 cells (1×10^5 cells/mL) were grown at 37 °C in 5% CO_2 in

Received: August 17, 2012
Revised: November 10, 2012
Accepted: November 11, 2012
Published: November 11, 2012

Table 1. Composition of the Experimental Diets (g)[a]

	SBO-LFD (10% kcal of fat)	PNO-LFD (10% kcal of fat)	SBO-HFD (45% kcal of fat)	PNO-HFD (45% kcal of fat)
casein	200	200	200	200
L-cystine	3	3	3	3
sucrose	350	350	172.8	172.8
cornstarch	315	315	72.8	72.8
dyetrose	35	35	100	100
soybean oil	45	0	135	0
pine nut oil[b]	0	45	0	135
lard	0	0	67.5	67.5
t-butylhydroquinone	0.009	0.009	0.027	0.027
cellulose	50	50	50	50
mineral mix[c]	35	35	35	35
vitamin mix[d]	10	10	10	10
choline bitartrate	2	5	2	2
total weight	1045.009	1045.009	848.127	848.127
kcal/g diet	3.7	3.7	4.6	4.6

[a]Resource: Dyets, Inc, Bethlehem, PA. PNO, pine nut oil; SBO, soybean oil. [b]PNO was a gift from Dubio Co., Ltd. (GyeongGi-do, Korea). [c]A total of 33 g of mineral mix (Dyets, 210099) provides 4.9 g of calcium, 3.8 g of phosphorus, 3.4 g of potassium, 0.9 g of sodium, 1.5 g of chloride, 0.5 g of magnesium, 0.3 g of sulfur, 55 mg of manganese, 33 mg of iron, 28 mg of zinc, 6 mg of copper, 0.2 mg of selenium, 0.2 mg of iodine, and 3.9 g of sucrose. [d]A total of 10 g of vitamin mix (Dytes, 300050) provides 4000 IU of vitamin A, 1000 IU of vitamin D_3, 50 IU of vitamin E, 30 mg of niacin, 16 mg of pantothenic acid, 7 mg of vitamin B_6, 6 mg of vitamin B_1, 6 mg of vitamin B_2, 2 mg of folic acid, 0.8 mg of menadione, 0.2 mg of biotin, 10 μg of vitamin B_{12}, and 9.8 g of sucrose.

Table 2. Fatty Acid Composition of the Experimental Diets

	SBO-LFD (% of lipid)	PNO-LFD (% of lipid)	SBO-HFD (% of lipid)	PNO-HFD (% of lipid)
myristic acid (C14:0)			0.4	0.4
palmitic acid (C16:0)	11.9	7.0	14.0	10.5
stearic acid (C18:0)	4.8	3.6	6.9	6.2
total SFA	16.7	10.6	21.3	17.1
palmitoleic acid (C16:1 Δ9)			0.6	0.7
oleic acid (C18:1 Δ9)	21.1	27.4	27.7	31.7
total MUFA	21.1	27.4	28.3	32.4
linoleic acid (C18:2 Δ9,12)	54.9	47.2	44.9	39.7
α-linolenic acid (C18:3 Δ9,12,15)	7.4	0.8	5.5	1.0
pinolenic acid (C18:3 Δ5,9,12)		14.0		9.7
total PUFA	62.3	62	50.4	50.4

Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco), 100 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA), and 20 μg/mL gentamicin (Gibco). As cells reached confluence, the medium was replaced with differentiation medium containing DMEM and 2% horse serum (Gibco), which was changed daily. After 4 days of differentiation, the cells had fused into myotubes. Pinolenic acid (Cayman, Ann Arbor, MI) was dissolved in ethanol before being added to the culture medium. Four-day-old C2C12 myotubes were incubated with 25 and 50 μM pinolenic acid in DMEM plus 0.1% FBS. The treatment time was 24 h, and the cultured cells were washed 2 times with phosphate-buffered saline (PBS) before lysing with TRI Reagent (Invitrogen, Carlsbad, CA).

Luciferase Assay. Luciferase assays were performed using a GAL4/PPAR chimera system.[12] Briefly, p4xUASg-tk-luc (a reporter plasmid), pM-hPPARα, and pM-hPPARδ (expression vectors for the GAL4 DNA-binding domain and the human PPARα and PPARδ ligand-binding domains, respectively) were transfected into CV1 cells together with pRL-CMV (an internal control reporter plasmid) by the lipofectamine system (Invitrogen). After 24 h of transfection, the cells were treated with 20 and 50 μM pinolenic acid or α-linolenic acid. GW7647 (20 nM) and GW501516 (100 nM), which are PPARα- and PPARδ-specific agonists, respectively, were used for positive controls. Luciferase activity was measured using the Dual-Luciferase Reporter Gene Assay system (Promega, Madison, WI).

Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from 50 mg of tissue samples or lysed cells using TRI Reagent (Invitrogen). Samples (2 μg) of RNA were reverse-transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI). Real-time PCR amplification was conducted on a Thermal Cycler Dice Real Time system (TaKaRa Bio, Inc., Japan) using SYBR premix Ex Taq (TaKaRa Bio, Inc., Japan). All assays were performed in duplicate and in the same conditions. The samples were incubated at 95 °C for 10 s, followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s. The results were analyzed with Real Time System TP800 software, and all values were normalized to the levels of housekeeping genes: *β-actin* in skeletal muscle and *36B4* in BAT.

Western Blotting. Samples (50 μg) of total protein were analyzed by western blotting with the following polyclonal antibodies: anti-phospho-AMPK (Thr172) (2531, Cell Signaling, Beverly, MA), AMPK (2532, Cell Signaling), anti-phospho-p38 MAPK (Thr180/Tyr182) (4631, Cell Signaling), Troponin I-S (C-19) (sc-8119, Santa Cruz Biotechnology, Santa Cruz, CA), UCP1 (M-17) (sc-6529, Santa Cruz), COX IV (ab33985, Abcam, Cambridge, MA), β-actin (A5441, Sigma-Aldrich, St. Louis, MO), and α-tubulin (ab7291, Abcam). β-actin and α-tubulin served as protein-loading controls.

Muscle Triglyceride Content. Muscle tissue samples with a weight of about 500 mg were lysed overnight in 1 mL aliquots of 30% ethanolic KOH at 55 °C. The homogenates were dissolved in 50% ethanol and centrifuged at 3000 rpm for 5 min. The supernatants were collected and dissolved in 50% ethanol solution and neutralized with 1

M $MgCl_2$, and triglyceride contents were measured with a free-glycerol determination kit (FG0100, Sigma-Aldrich). The values were corrected for tissue weight.

Analysis of Fatty Acid Composition of the Experimental Diets. Lipids were extracted from a powdered diet according to Folch et al.[13] Extracted lipids were saponified with NaOH in methanol and then methylated in the presence of BF_3 in methanol at 100 °C. Fatty acid methyl esters were extracted with hexane, and 1 μL aliquots of the extracts were injected into a gas chromatograph (Agilant 7890A; Agilent, Santa Clara, CA) equipped with a flame ionization detector. The sample was injected in the split mode (1:10), and helium was used as a carrier gas at a flow rate of 1.5 mL/min. The capillary column used was a DB-carbowax (0.32 mm × 25 m, 0.2 μm, Agilent). The oven temperature was increased from 50 to 220 °C at a rate of 15 °C/min and held at maximum temperature for 20 min.

Statistical Analysis. The results are presented as means ± standard error of the mean (SEM). Variables were compared using Student's t test or analysis of variation (ANOVA) with Duncan's multiple-range test. Differences were considered significant at $p < 0.05$.

RESULTS

Effect of PNO on Body Weight and Intramuscular Fat Accumulation. As shown in Figure 1A, the mice consuming the PNO-HFD gained significantly less body weight than the SBO-HFD-fed mice, whereas no difference was observed between the LFD groups. To see whether PNO altered lipid metabolism, we measured the intramuscular triglyceride content, which was found to be significantly lower in the PNO-HFD than in the SBO-HFD-fed mice (Figure 1B).

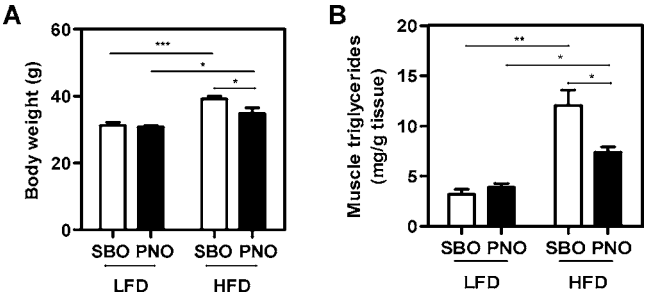

Figure 1. Body weight and intramuscular triglyceride content. Five-week-old male C57BL/6 mice were fed a low-fat diet (LFD; 10% energy from SBO or PNO) or a high-fat diet (HFD; 30% energy from SBO or PNO and 15% from lard) for 12 weeks. (A) Change in body weight. (B) Intramuscular triglyceride levels in quadriceps muscles. Data are presented as means ± SEM; $n = 5$ in each group. SBO, soybean oil; PNO, pine nut oil. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ compared between the PNO and SBO groups or between the LFD and HFD groups.

Effect of PNO on Mitochondrial Oxidative Metabolism in Skeletal Muscle. Following these findings, we examined whether PNO altered fatty acid oxidation in the skeletal muscle of the HFD-fed mice. A number of genes involved in oxidative metabolism were found to be upregulated in the PNO-HFD group relative to the SBO-HFD group. Among these were genes involved in the regulation of fatty acid oxidation, such as *Ppara* (2.32-fold), genes essential for transport of acyl-CoA into the mitochondria, such as *Cpt1b* (1.52-fold) and *Cpt2* (1.67-fold), and genes involved in mitochondrial β-oxidation, including *Acadm* (1.97-fold) and *Acadl* (1.66-fold) (Figure 2A). Also elevated was the expression of gene *Ndufa2* (1.62-fold) and protein COX IV, which are involved in mitochondrial oxidative phosphorylation (panels B and C of Figure 2). Although it did not reach biological significance (<1.50-fold),

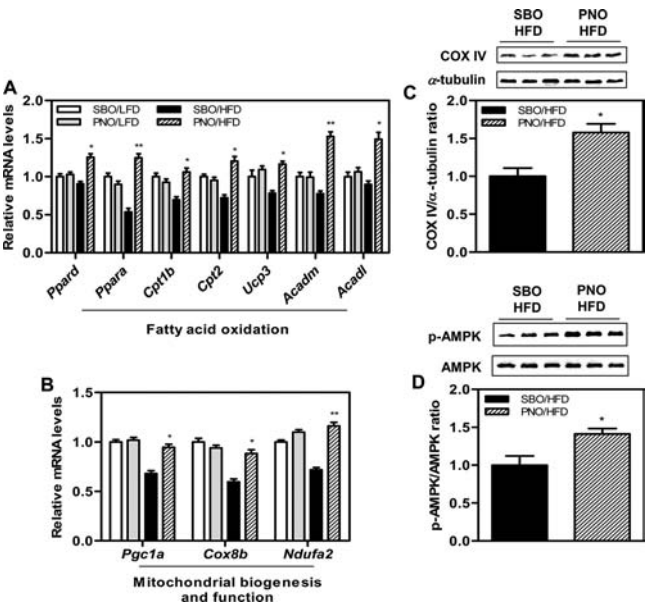

Figure 2. Effect of PNO on lipid oxidation and mitochondrial function in skeletal muscle. (A and B) qRT-PCR analysis for markers specific to (A) fatty acid oxidation (*Ppard*, *Ppara*, *Cpt1b*, *Ucp3*, *Cpt2*, *Acadm*, and *Acadl*) and (B) mitochondrial biogenesis and function (*Pgc1a*, *Cox8b*, and *Ndufa2*) in quadriceps muscles. (C and D) Western blot analysis for (C) COX IV/α-tubulin and (D) p-AMPK/total AMPK proteins in quadriceps muscles. Data are presented as means ± SEM; $n = 5$ in each group. (*) $p < 0.05$ and (**) $p < 0.01$ compared between the PNO-HFD- and SBO-HFD-fed mice.

there was a tendency of increase in the expression of genes involved in skeletal muscle oxidative metabolism, including *Ppard* (1.38-fold), *Ucp3* (1.49-fold), *Pgc1a* (1.39-fold), and *cox8b* (1.48-fold) in the PNO-HFD group (panels A and B of Figure 2). Furthermore, the PNO-HFD group had an elevated level of phosphorylated AMPK (Figure 2D), a major regulator of fatty acid oxidation.[14] Together, these findings suggest that the reduction in intramuscular fat accumulation because of PNO in HFD-induced obesity can be the result of enhanced fatty acid oxidation and increased mitochondrial biogenesis and function in skeletal muscle.

Effect of PNO on Fiber Types in Skeletal Muscle. Skeletal muscles are composed of slow-twitch (type I) and fast-twitch (types IIa, IIx, and IIb) fibers. Rich in mitochondria, type I and type IIa fibers are characterized by a high oxidative capacity. In contrast, type IIx and type IIb fibers have fewer mitochondria and a higher capacity for glycolysis.[15] Because the PNO-HFD appeared to increase mitochondrial biogenesis and oxidative metabolism, we investigated whether it altered the proportion of fiber types. As shown in Figure 3A, mRNA levels of genes specific to type I fibers, including *Myl2* (2.11-fold), *Myoglobin* (1.96-fold), *Myh7* (2.40-fold), *Tnni1* (1.75-fold), and *Tnnt1* (2.35-fold), were higher in the PNO-HFD-fed mice than in the SBO-HFD group. Also increased in this group was the expression of genes specific to type IIa fibers, such as *Myh2* (2.31-fold) (Figure 3A). Moreover, western blotting revealed that Troponin I (slow), a marker of type I fibers, was elevated in the PNO-HFD-fed mice compared to controls (Figure 3B). On the other hand, genes specific to fast-twitch fibers (e.g., *Tnni2*, *Tnnt3*, and *Myh4*) were not upregulated in the PNO-HFD-fed mice (Figure 3A).

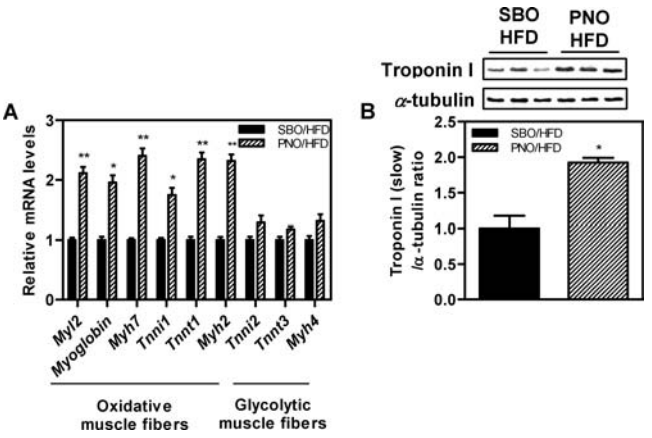

Figure 3. PNO-induced expression of genes specific to oxidative muscle fibers. (A) qRT-PCR analysis for markers specific to oxidative muscle fibers (*Myl2*, *Myoglobin*, *Myh7*, *Tnni1*, *Tnnt1*, and *Myh2*) and glycolytic muscle fibers (*Tnni2*, *Tnnt3*, and *Myh4*) in quadriceps muscles. (B) Western blot analysis for Troponin I (slow) protein expression. The density of the blots was normalized to α-tubulin. Data are presented as means ± SEM; $n = 5$ in each group. (*) $p < 0.05$ and (**) $p < 0.01$ compared to the SBO-HFD-fed mice.

Effect of PNO on Thermogenesis in BAT. To determine whether the reducing effect of PNO on weight gain was associated with changes in the thermogenic response in BAT, we compared the expression of genes involved in thermogenesis in BAT in the two HFD groups. The PNO-HFD appeared to increase the transcription (1.68-fold) (Figure 4A) and expression (Figure 4C) of UCP1, a key thermogenic protein in BAT. Likewise, the transcription of type II iodothyronine deiodinase (*Dio2*) and adrenergic receptor β3 (*Adrb3*), two major hormonal effectors that regulate the expression of UCP1,[16] was higher in this group, although their biological significances are not warranted (<1.5-fold) (Figure 4A). The PNO-HFD also appeared to upregulate the expression of phosphorylated p38 mitogen-activated protein kinase (MAPK) required for UCP1 expression[17,18] (Figure 4D) and the transcription of *Prdm16* (1.76-fold), which regulates brown adipocyte formation and UCP1 expression[19] (Figure 4A).

The thermogenic response is associated with mitochondrial biogenesis and function and fatty acid oxidation in BAT.[20] Thus, the next step was to investigate whether PNO altered fatty acid oxidation and mitochondrial biogenesis and function. As shown in Figure 4A, the levels of genes involved in mitochondrial biogenesis, such as *Tfam* (1.94-fold), and oxidative phosphorylation, such as *Cycs* (1.92-fold) and *Cox8b* (1.59-fold), were elevated in BAT from the PNO-HFD-fed mice compared to that from the SBO-HFD group. Key regulatory genes of fatty acid oxidation in BAT, *Cpt1a* (1.54-fold), *Acadm* (1.74-fold), and *Acadl* (1.61-fold), were also upregulated in this group, and relatively weak upregualtion was observed in *Ppara* (1.39-fold) and *Cd36* (1.44-fold) (Figure 4B). Consistent with these findings, the PNO-HFD-fed mice had increased levels of phosphorylated AMPK in BAT (Figure 4E).

Pinolenic Acid May Act as an Activator of Both PPARα and PPARδ. The nuclear receptors PPARα and PPARδ, which are expressed in skeletal muscle and BAT, play key regulatory

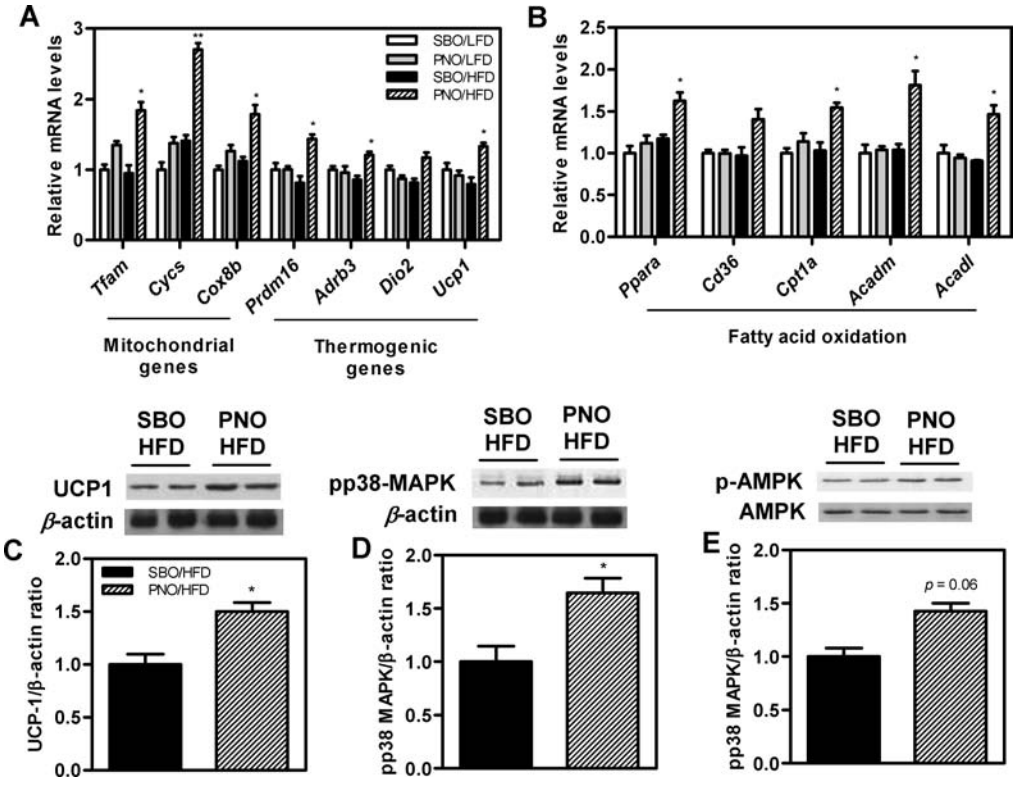

Figure 4. Effects of PNO on thermogenesis in BAT. (A and B) qRT-PCR analysis for markers specific to (A) thermogenesis (*Prdm16*, *Adrb3*, *Dio2*, and *Ucp1*), mitochondrial biogenesis and function (*Tfam*, *Cycs*, and *Cox8b*), and (B) fatty acid oxidation (*Ppara*, *Cd36*, *Cpta*, *Acadm*, and *Acadl*) in BAT. (C–E) Western blot analysis for UCP1, pp38-MAPK, p-AMPK, total AMPK, and β-actin proteins in BAT. Data are presented as means ± SEM; $n = 5$ in each group. (*) $p < 0.05$ and (**) $p < 0.01$ compared between the PNO-HFD- and SBO-HFD-fed mice.

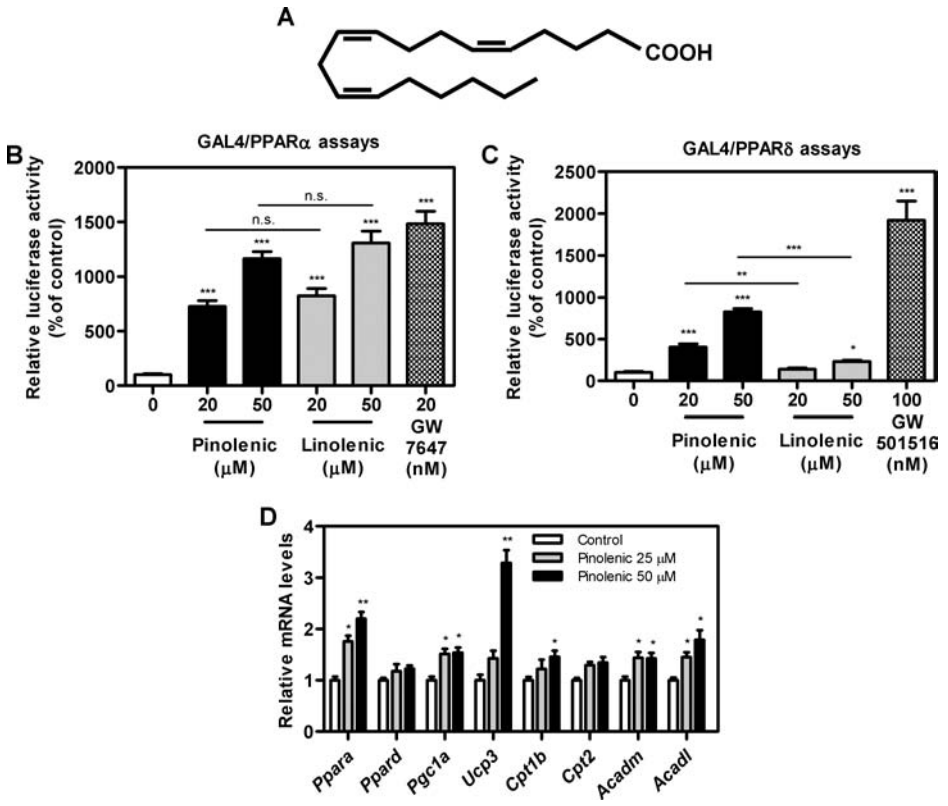

Figure 5. Pinolenic acid may act as an activator of PPARα and PPARδ. (A) Chemical structure of pinolenic acid. (B and C) Expression vectors for (B) PPARα and (C) PPARδ chimeric proteins (pM-hPPARα and pM-hPPARδ, respectively) were transfected into CV1 cells. After 24 h of transfection, the cells were incubated with 20 and 50 μM pinolenic acid or α-linolenic acid for 24 h. GW7674 (20 nM) and GW501516 (100 nM) are PPARα- and PPARδ-specific agonists, respectively, used as positive controls. Luciferase activity was measured using the dual-luciferase system. The activity of the vehicle control group was set at 100%, and relative luciferase activities are displayed as fold inductions with respect to the vehicle control. Data are means ± SEM of five tests. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ compared to the controls or between pinolenic acid and α-linolenic acid treatments. ns = not significant. (D) C2C12 myotubes were established for 4 days and then treated with 25 and 50 μM pinolenic acid for 24 h. qRT-PCR was performed to measure expression of *Ppara*, *Ppard*, *Pgc1a*, *ucp3*, *Cpt1b*, *Cpt2*, *Acadm*, and *Acadl*. mRNA levels are presented as fold inductions relative to the vehicle control. Data are means ± SEM of three independent duplicate experiments. (*) $p < 0.05$ and (**) $p < 0.01$ compared to the controls.

roles in oxidative lipid metabolism.[21] The PPARs are ligand-activated receptors that belong to the nuclear receptor superfamily. The structure of the PPAR protein comprises a transcriptional activation domain and the ligand-binding domain. Some fatty acids fit in the PPAR ligand-binding domain[22] and can regulate its transcriptional activity.[23] PNO contains approximately 14% pinolenic acid, whose chemical structure resembles γ-linolenic acid,[24] a natural ligand for PPARα and PPARδ.[25,26] Using the luciferase ligand assay system, we tested whether pinolenic acid (the chemical structure is shown in Figure 5A) elicits the ligand activities for PPARs. As shown in panels B and C of Figure 5, treatment with pinolenic acid increased the luciferase activities of both PPARα and PPARδ in a dose-dependent manner. We also found that both dosages of pinolenic acid enhanced the luciferase activities of PPARα, similar to α-linolenic acid (Figure 5B), but they elicited stronger activities for PPARδ than those of α-linolenic acid (Figure 5C). To investigate whether pinolenic acid activates PPARs in skeletal muscle cells, we examined the effects of pinolenic acid on the expression of PPAR target genes in C2C12 myotubes. Figure 5D shows that treatment with pinolenic acid significantly upregulated PPARα transcripts and downstream target genes of PPARα and PPARδ,[27,28] including *Pgc1a*, *Ucp3*, *Cpt1b*, *Acadm*, and *Acadl*. These findings indicate that PNO-derived pinolenic acid may act as a dual activator of PPARα and PPARδ.

DISCUSSION

Obesity-induced intramuscular lipid accumulation is associated with dysregulation of fatty acid oxidation and impaired mitochondrial biogenesis and/or function in skeletal muscle.[11] We observed that PNO markedly reduced triglyceride accumulation in the muscles of mice exposed to a high-fat diet, and we found that the expression of key genes involved in fatty acid oxidation (e.g., *Cpt1b*, *Cpt2*, *Ucp3*, and *Acadm*) and in mitochondrial biogenesis/function (e.g., *Pgc1a*, *Cox8b*, and *Ndufa2*) was significantly upregulated. Together with the observed increase in COX IV expression and AMPK phosphorylation, these findings suggest that PNO activates the mitochondrial oxidative pathway. Additionally, the observed upregulation of PGC-1α, a key regulator of mitochondrial biogenesis and function,[29] indicates that the enhancement of oxidative metabolism is associated with increased mitochondrial function. Interestingly, we also observed upregulation of genes and proteins specific to type I and IIa muscle fibers (e.g., *Myl2*, *Myoglobin*, *Myh2*, and Troponin I1), which possess high mitochondrial densities and oxidative capacities.[15] Thus, it appears that the increase in oxidative metabolism because of PNO is accompanied by fiber-type switching in skeletal muscle

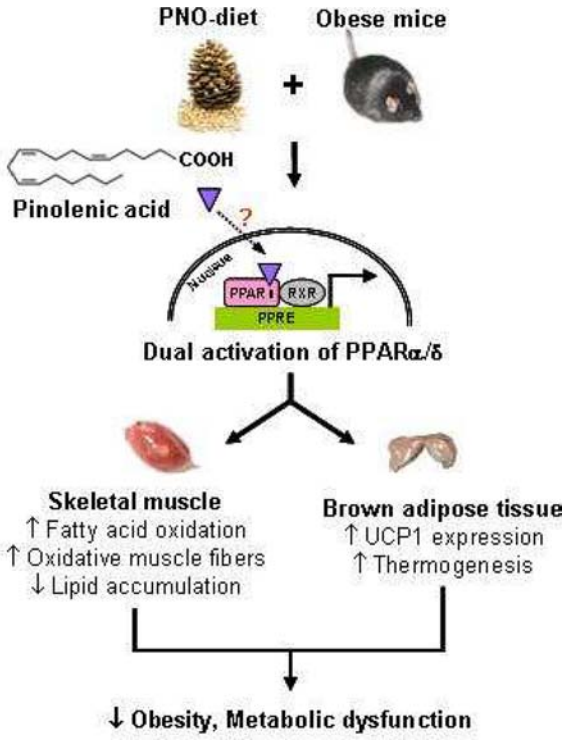

Figure 6. Schematic presentation for the effect of PNO on oxidative metabolism in skeletal muscle and BAT in diet-induced obesity. PNO contributes to the reduction of obesity and metabolic dysfunction, and this may be associated with dual ligand activity of pinolenic acid, which is abundant in PNO, for PPARα/δ.

toward higher oxidative capacity, leading to reduced intramuscular lipid accumulation and body weight gain.

Because of its ability to dissipate chemical energy as heat, BAT is considered an important defense mechanism against obesity,[5,30] and UCP1 is a key protein responsible for this process.[31] Upregulation of UCP1 expression in mice is thought to protect against obesity induced by a HFD.[19,32] We found that PNO upregulated UCP1 transcription and expression in BAT, which was accompanied by upregulation of genes involved in the regulation of thermogenesis (e.g., *Adrb3* and *Prdm16*), mitochondrial biogenesis and oxidative metabolism (e.g., *Tfam*, *Ppara*, and *Cpt1a*), and AMPK activation. These findings help to explain the mechanism behind the observed increase in oxidative metabolism and energy expenditure that led to lower body weight gain in the PNO-HFD-fed mice.

The nuclear receptors PPARα and PPARδ are expressed predominantly in skeletal muscle and BAT, where they play key regulatory roles in lipid metabolism.[21] Their transcriptional activity requires the binding of specific ligands, heterodimerization with the retinoid X receptors, and interaction with transcription coactivators, such as PGC-1α.[33−35] Upon activation, PPARα/δ receptors induce the expression of genes involved in fatty acid metabolism,[27,28] and PPARδ also upregulates the transcription of PGC-1α.[36] In our experiments, PNO upregulated the transcription of both PPARα/δ and PGC-1α, as well as their target genes and proteins involved in oxidative metabolim in skeletal muscle and BAT. These indicate that PNO may have potential to activate PPARs. It has been shown that some polyunsaturated fatty acids can bind to PPARs[23] and, thus, enhance their transcriptional activity to modulate gene expressions involved in fatty acid oxidation.[22,23] Interestingly, PNO contains approximately 14% pinolenic acid, whose chemical structure resembles γ-linolenic acid,[24] a natural ligand for PPARα and PPARδ.[25,26] We found that pinolenic acid elicited the ligand activity for PPARα similar to α-linolenic acid, but its activity for PPARδ was stronger than that of α-linolenic acid. Furthermore, treatment of skeletal muscle cells with pinolenic acid markedly enhanced levels of downstream target genes of PPARα and PPARδ (e.g., *Ucp3*, *Cpt1b*, and *Acadm*). These findings indicate that a PNO-enriched diet containing pinolenic acid may induce PPARα and/or PPARδ activation in skeletal muscle and BAT and enhance oxidative fatty acid metabolism and thermogenesis, leading to attenuation of the HFD-induced obesity and metabolic dysfunction (Figure 6). Further studies are needed to assess some other potential components in PNO, which may have beneficial effects on the prevention of obesity and metabolic dysfunction.

In conclusion, PNO enhances mitochondrial oxidative metabolism in skeletal muscle and thermogenesis in BAT, at least in part by activating PPARα and/or PPARδ. This leads to a reduction in muscle lipid content and body weight gain. PNO may have potential as a dietary supplement for preventing obesity and metabolic dysregulation in skeletal muscle and BAT.

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Funding

This work was supported by the grant from National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST) (grant number 2010-0024878), and by a program of the Science Research Center (Center for Food & Nutritional Genomics Research, grant number 2011-0000919) of the NRF of Korea funded by the MEST.

Notes

The authors declare no competing financial interest.

ABBRIVIATIONS USED

AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferator activated receptor γ, coactivator 1α; Tfam, transcription factor A, mitochondrial; Cycs, cytochrome *c*; Cox8b, cytochrome *c* oxidase, subunit VIIIb; COX IV, cytochrome *c* oxidase, subunit IV; Ndufa2, NADH dehydrogenase ubiquinone 1α subcomplex 2; Ppard, peroxisome proliferator-activated receptor δ; Ppara, peroxisome proliferator-activated receptor α; Cpt1a, carnitine palmitoyltransferase a; Cpt1b, carnitine palmitoyltransferase 1b; Cpt2, carnitine palmitoyltransferase 2; Acadm, acyl-coenzyme A dehydrogenase, medium chain; Acadl, acyl-coenzyme A dehydrogenase, long chain; Cd36, fatty acid translocase; Prdm16, PR domain containing 16; Adrb3, adrenergic receptor, β3; Dio2, iodothyronine type II deiodinase; Ucp1, uncoupling protein 1; Myl2, myosin light chain 2; Myh7, myosin heavy chain 7; Myh2, myosin heavy chain 2; Myh4, myosin heavy chain 4; Tnni1, troponin I1, slow; Tnni2, troponin I2, fast; Tnnt1, troponin T1, slow; Tnnt3, troponin T3, fast

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