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Anti-diabetic effect of amorphastilbol through PPARα/γ dual activation in *db*/*db* mice

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) have been considered as desirable targets for metabolic syndrome treatments, even though their specific agonists have several side effects, including body weight gain, edema, and tissue failure. The effects of amorphastilbol (APH) on glucose- and lipid metabolism were investigated with *in vitro* 3T3-L1 adipocyte systems and *in vivo db/db* mice model. APH selectively stimulates the transcriptional activities of both PPARα and PPARγ, which are able to enhance fatty acid oxidation and glucose utilization. Furthermore, APH improves glucose and lipid impairment in *db/db* mice. More importantly, there are no significant side effects, such as weight gain or hepatomegaly, in APH-treated animals, implying that APH do not adversely affect liver or lipid metabolism. All our data suggest that APH can be used as potential therapeutic agents against type 2 diabetes and related metabolic disorders, including obesity, by enhancing glucose and lipid metabolism.

1. Introduction

Type 2 diabetes mellitus (T2DM), which accounts for more than 90% of diabetes cases, is a common metabolic disease that is characterized by the resistance of target tissues to insulin stimulation [1]. T2DM is usually associated with hyperglycemia, dyslipidemia, obesity, hypertension, fatty liver disease, atherosclerosis, certain cancers, and cardiovascular diseases [2]. Multiple defects in intracellular events, including an impairment of the insulin signaling axis, diminished glucose metabolism and reduced glycogen synthesis, contribute to insulin resistance [3,4]. T2DM patients also manifest adipocyte resistance to the antilipolytic effects of insulin [5], and the resulting increase in concentration of the plasma free fatty acid impairs insulin secretion [6,7] and aggravates insulin resistance in liver and muscle tissues [8,9].

Currently, relieving the insulin resistance has been considered to be an approach to treating T2DM [10]. Thiazolidinediones and fibrate drugs are some of the most commonly used medications in the treatment of T2DM, hyperlipidemia and insulin resistance. These drugs bind to and activate peroxisome proliferator-activated receptors (PPARs), which results in the upregulation of several genes involved in glucose and lipid metabolism [11]. The PPAR family consists of three isoforms, PPARα, PPARβ/δ, and PPARγ [12]. PPARγ, mainly expressed in adipose tissue and vascular tissue/macrophages [13] and present in muscle and β cells in low abundance [14], affects genes involved in lipid synthesis and storage and glucose homeostasis. PPARγ agonists, such as thiazolidinediones including rosiglitazone and pioglitazone, control mobilization of lipid into adipocytes by promoting adipogenesis and inducing the expression of such lipid transport genes as adipocyte fatty acid-binding protein (*aP2*), thereby reducing lipotoxicity [15,16]. However, several concerns, such as the weight gain associated with increased excess fat, arise in T2DM patients [17]. Accumulating evidence indicates that the activation of PPARα, predominantly expressed in the liver [18], would stimulate lipid consumption by enhancing the expression of fatty acid oxidation genes, resulting in the amelioration of hyperlipidemia. PPARα agonists, such as fenofibrate (used to treat hyperlipidemia and cardiovascular disease), have potent effects on the reduction of plasma triglycerides [19]. Due to the distinct metabolic effects of PPARα and PPARγ agonists on insulin sensitivity and lipid metabolism, development of novel drugs has focused on dual PPARs that possess PPARγ and PPARα activities. It has been proposed that the simultaneous activation of PPARα and PPARγ would guarantee more desirable effects with alleviated adverse effects [20,21]. Many PPARα/γ dual agonists have been identified and tested in obese and insulin-resistant individuals; however, most of these drugs have shown unexpected side effects, including weight gain, heart

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failure, renal failure, urinary cancer and anemia [22,23]. Therefore, the development of novel PPARα/γ dual agonists with few adverse effects is in urgent need.

Recent our report shows that amorphastilbol (APH) from *Amorpha fruticosa* stimulates transcriptional activities of PPARα/γ [24], however, its effect is not evaluated *in vivo*. In the present study, we explored the pharmacological properties of APH on glucose and lipid metabolisms *in vivo*. APH is able to activate PPARα and PPARγ directly, leading to the amelioration of glucose and lipid abnormalities. Furthermore, in obese and diabetic *db/db* mouse models, APH improved the abnormalities in glucose and lipid metabolism without exhibiting the previously reported side effects of other PPARγ agonists, such as weight gain, hepatomegaly, and hepatotoxicity. Collectively, these data suggest that APH can be potential therapeutic agents for T2DM, obesity, and lipid dysregulation through PPARα/γ dual activation.

2. Materials and methods

2.1. Cell culture and reagents

CV-1 and 3T3-L1 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). HepG2 cells were purchased from ATCC and cultured in Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS), supplemented with 10% FBS, 1% penicillin/streptomycin, 1X non-essential amino acid (WelGENE, Daegu, Korea) and 1 mM sodium pyruvate (WelGENE) at 37 °C with 5% CO_2 in air. Amorphastilbol (APH) was synthesized by Dr. J. Ham (Korea Institute of Science and Technology [KIST] Gangneung Institute) for *in vivo* study [24]. Rosiglitazone, troglitazone, tesaglitazar, and WY14643 were purchased from Sigma–Aldrich Co. (St Louis, MO), and GW501516 was purchased from Santa Cruz Inc. (Santa Cruz, CA).

2.2. Cell-based transactivation assay and adipocyte differentiation

Transactivation assay for PPARs was measured by reporter gene (PPRE-luciferase plasmid) analysis, and adipocyte differentiation was measured by oil-red O staining as described previously [25].

2.3. Ligand binding assay

The LanthaScreen™ TR-FRET PPAR competitive binding assay (Invitrogen) was performed according to the manufacturer's instructions [24].

2.4. Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity

After treatment, differentiated 3T3-L1 adipocytes were rinsed twice with PBS, scraped into 200 μl enzyme extraction buffer (provided by a kit, Takara MK426) and sonicated. The GPDH activity was determined according to the decreases in the NADH activity by measuring the decrease in the absorbance at 340 nm, according to the manufacturer's protocol.

2.5. Gene expression analysis

Total RNA was isolated from 3T3-L1 adipocytes or tissue using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA concentration of each sample was determined by spectrophotometry at 260 nm; the integrity of each RNA sample was evaluated using the Agilent 2100 BioAnalyzer (Agilent Technologies, SantaClara, CA). cDNA synthesis was performed using 1 μg of total RNA in 20 μl with random primers and Superscript II reverse transcriptase. Quantitative real-time PCR (Q-PCR) analyses were performed with SYBR green fluorescent dye using the 7500 Real-Time PCR System (Applied Biosystem, Foster City, CA). Data analyses were performed using 7500 System SDS software version 1.3.1 (Applied Biosystem). The sequences of the primers used in this study are described in Supplementary Table 1.

2.6. Animal experiments

All of the experiments were performed according to the procedures approved by the KIST's Institutional Animal Care and Use Committee. Seven week-old male C57BLKS/J lar-*Lep*$^{db/db}$ mice were purchased from the Shizuoka Laboratory Animal Center (Japan). The mice were housed under conditions of 23 ± 2 °C and 55 ± 5% humidity with standard light cycles (12 h light/dark). The mice were orally administered 4 mg/kg rosiglitazone, 1 mg/kg tesaglitazar or 20 mg/kg APH once a day for 8 weeks prior to the gene expression or blood biomarker analyses. For glucose-tolerance tests, 7 week mice were orally administered 4 mg/kg rosiglitazone, 1 mg/kg tesaglitazar or 20 mg/kg APH once a day for 8 weeks and fasted overnight before the oral administration of 2 g/kg D-glucose. Glucose was measured by tail vein bleeds at the indicated time intervals using an Accu-Chek glucometer (Roche), and the serum insulin concentrations were determined by ELISA (Shibayagi, Japan). At the end of the experimental period, the liver/body weight ratios were measured, and blood samples were obtained from the abdominal aorta to determine plasma biomarker concentrations.

2.7. Analysis of plasma biomarkers

After the experiment, blood was collected in tubes containing 0.18 M EDTA and centrifuged at 5000 rpm for 5 min at 4 °C. After centrifugation, the plasma was separated for the estimation of the total cholesterol, LDL-cholesterol, triglycerides, and free fatty acids. The total cholesterol levels were measured by enzymatic methods using SICDIA L T-CHO reagents (Eiken Chemical, Tokyo, Japan), and the LDL-cholesterol levels were determined by enzymatic methods using L-Type LDL-C reagents (Wako Pure Chemical, Osaka, Japan). The triglyceride levels were measured by GPO-HMMPS using the SICDIA L TG reagent (Eiken Chemical), and free fatty acids were measured by enzymatic methods using NEFA-ZYME-S (Eiken Chemical).

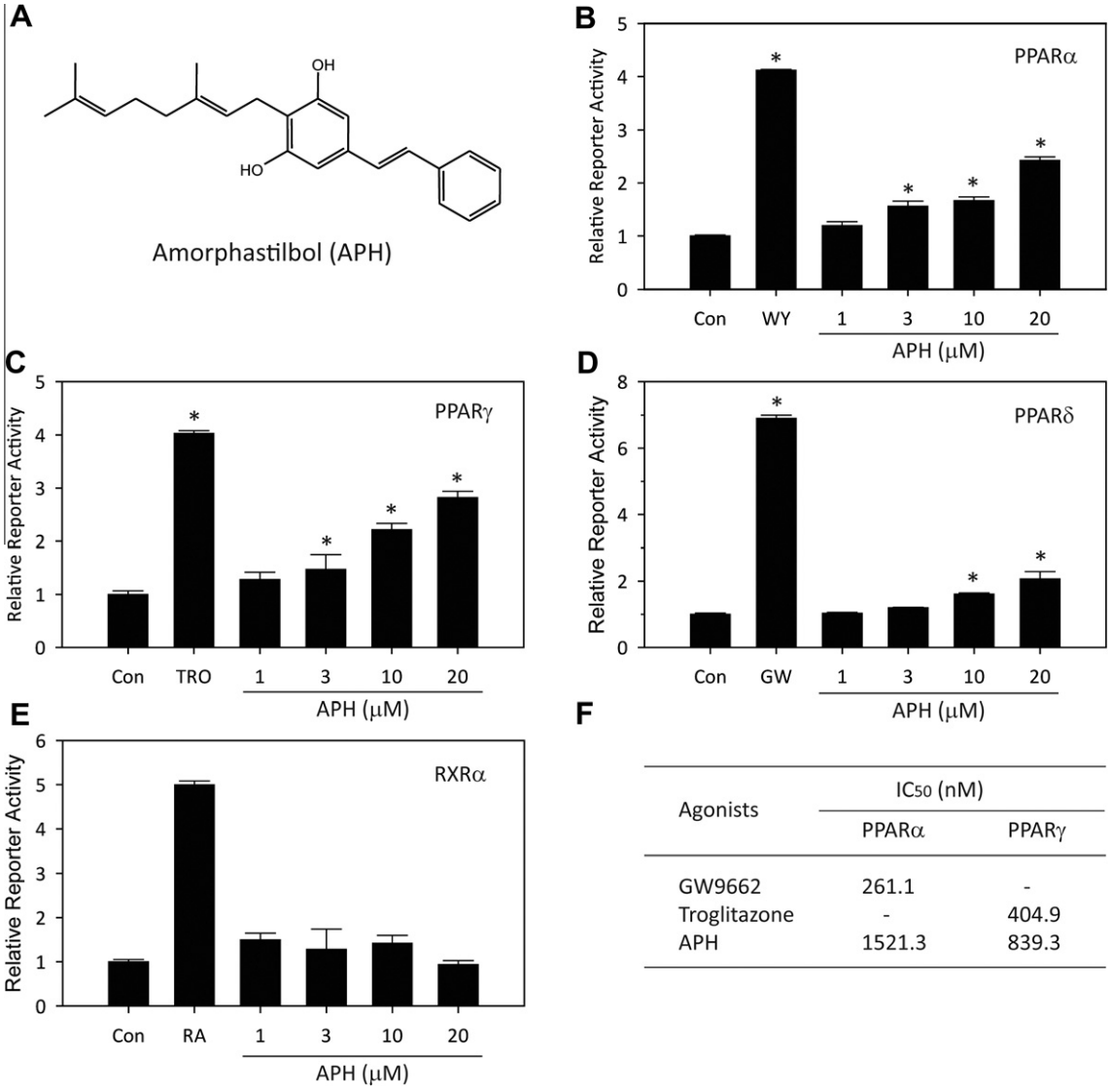
2.8. Statistics

The data are expressed as the mean ± SD. Differences between the mean values in the two groups were analyzed using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Amorphastilbol is a PPARα/γ dual agonist

To explore the pharmacological properties of APH (Fig. 1A), we reexamined the effects of APH on transcriptional activation of PPARs. As shown in Fig. 1B and C, APH treatment led to an increase in both PPARα- and PPARγ-reporter gene activities in a dose-dependent manner, and the EC_{50} values to induce PPARα and PPARγ activation were estimated to be 7.4 μM and 5.4 μM, respectively. However, APH exhibited a minimal effect on PPARδ transcription (Fig. 1D) and no detectable effect on RXRα (Fig. 1E). We next examined the binding affinities of APH to PPARα and PPARγ



Agonists	IC50 (nM)	
	PPARα	PPARγ
GW9662	261.1	-
Troglitazone	-	404.9
APH	1521.3	839.3

Fig. 1. Amorphastilbol selectively activates PPARα and PPARγ *in vitro*. (A) Chemical structure of amorphastilbol (APH). Human PPARα (B), PPARγ (C), or PPARδ (D) and the RXRα (E) expression vector, PPRE-luciferase reporter construct, and pRL-SV40 vector were transiently cotransfected in CV-1 cells. The cells were then treated with 10 μM WY14643 (WY), 10 μM troglitazone (TRO), 1 μM GW501516 (GW), or various concentrations of APH (1, 3, 10, or 20 μM) for 24 h. A reporter assay was performed as described in Materials and methods. Each bar represents the mean ± SD of duplicates. $^{*}P < 0.05$ vs. control. (F) Binding affinities of APH to PPARα and PPARγ were analyzed using the LanthaScreen™ TR-FRET PPAR competitive binding assay, as described in Materials and methods.

using a LanthaScreen competitive binding assay and found that APH could bind to both PPARα and PPARγ, as shown in Fig. 1F. However, the APH binding affinities to PPARα and PPARγ are weaker than those of the positive controls, GW9662 and troglitazone, which is in accordance with the weak ability of APH to activate the transcription of PPARα/γ in comparison to that of the positive control (Fig. 1B and C). These findings suggest that APH acts as a PPARα/γ dual agonist through the directly binding to the PPARα/γ ligand-binding domain.

3.2. APH up-regulates specific genes involved in both adipocyte differentiation and fatty acid oxidation

We next examined the potential effect of APH on adipogenesis of 3T3-L1 preadipocytes. As shown in Fig. 2A, the APH treatment caused a dramatic increase in lipid accumulation in the differentiated adipocytes, which was comparable to the effect of troglitazone. To characterize the adipogenic potential of APH further, the cellular triglyceride content and glycerol-3-phosphate dehydrogenase (GPDH) enzyme activity were measured. The triglyceride content in the differentiated adipocytes was also increased by the APH treatment up to 2.0- and 3.2-fold in the presence of 1 μM and 10 μM APH, respectively (Fig. 2B); this observation was in accordance with the result that APH causes lipid droplet accumulation in adipocytes, as shown in Fig. 2A. In addition, the GPDH activity was significantly enhanced by the APH treatment (Fig. 2C). Altogether, these results strongly support the notion that APH stimulates adipocyte differentiation in 3T3-L1 cells. Because adipogenesis is governed by the increased expression of various transcription factors and adipocyte-specific genes, we examined the effect APH on the expression of adipogenic transcription factors and marker genes in differentiated adipocytes. After the induction of adipocyte differentiation in the presence of APH, the mRNA levels of *C/EBPα, PPARγ, aP2, adiponectin,* and *resistin* were measured using Q-PCR. In the APH-treated adipocytes, the mRNA levels of these genes were dramatically increased compared to their levels in the absence of APH (Fig. 2D). In addition, the APH treatment led to a drastic increase in the mRNA level of *GLUT4*, which is

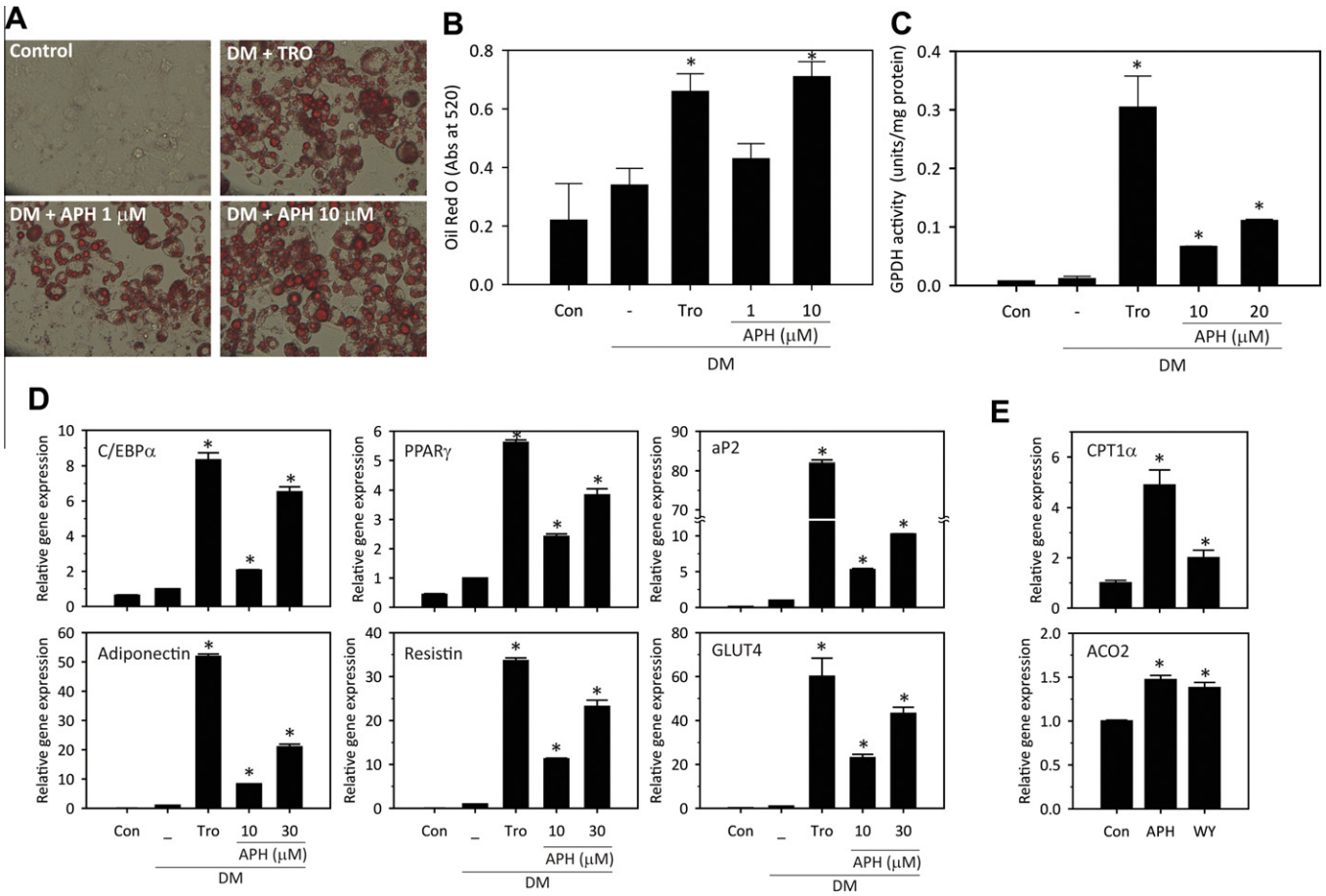


Fig. 2. *In vitro* functional analysis of APH. (A) Lipid accumulation in differentiated 3T3-L1 cells treated with troglitazone (TRO) or APH (1 or 10 μM) following Oil-red O staining. DM: differentiation media. (B) The quantification of the lipid accumulation was based on the OD values measured at 520 nm of destained Oil Red O from the adipocytes. (C) The GPDH activity was measured at day 8 after differentiation (C). $^{*}P < 0.05$ vs. DM control. (D) The expression of adipocyte-enriched genes in these cells was analyzed by Q-PCR ($n = 3$) and normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each bar represents the mean ± SD. $^{*}P < 0.05$ vs. DM control. (E) HepG2 cells transfected with human PPARα were incubated with WY or APH for 48 h. The relative gene expression was analyzed by Q-PCR and normalized to the GAPDH expression. Each bar represents the mean ± SD of triplicate experiments. $^{*}P < 0.05$ vs. control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

responsible for insulin-mediated glucose uptake, indicating that the insulin sensitivity of adipose tissue could be enhanced by APH treatment. Next, we examined the effect of APH on fatty acid oxidation to confirm its PPARα agonistic effect. In PPARα-transfected HepG2 cells, two different PPARα target genes involved in β-oxidation, carnitine-palmitoyl transferase 1α (CPT1α) and acyl-CoA oxidase 2 (ACO2), were induced by the APH treatment (Fig. 2E), supporting the hypothesis that APH promotes fatty acid oxidation through the activation of PPARα.

3.3. APH alleviates glucose impairment and glucose tolerance in db/db mice

To evaluate the effects of APH *in vivo*, we administered APH to obese and diabetic *db/db* mice and monitored the plasma glucose levels in each animal. As shown in Fig. 3A, treatments with APH led to a significant decrease in glucose levels, which is comparable to the effect of rosiglitazone, a PPARγ agonist, or tesaglitazar, a PPARα/γ dual agonist, treatment. However, unlike rosiglitazone that causes significant weight gain, APH treatments did not promote significant gains in body weight (Fig. 3B) without changes in food intake (Supplementary Fig. 1A and B). Interestingly, although liver weights in rosiglitazone- or tesaglitazar-treated animals were dramatically increased, we did not observe increased liver weight in APH-treated groups (Fig. 3C). Furthermore, glucose tolerance was significantly attenuated by APH treatments (Fig. 3D). Taken together, these results strongly suggest that APH can efficiently alleviate glucose impairment without the side effects of significant weight gain and hepatomegaly.

3.4. APH improves metabolic markers in the liver, blood, and white adipose tissue

Because hepatic gluconeogenesis gene expression is markedly increased in diabetic animals and contributes to hyperglycemia, the expression of glucose-6-phosphatase (G6Pase), one of the key enzymes in gluconeogenesis, were measured in the liver tissue of *db/db* mice. The expression level of G6Pase significantly reduced in APH-treated mice (Fig. 4A), supporting the anti-diabetic effects of APH. In addition, the APH treatment increased the expression of uncoupling protein 2 (UCP2), which is involved in the regulation of energy expenditure in the liver; however, the effects of APH are weaker than that of rosiglitazone or tesaglitazar (Fig. 4B). Next, we examined whether APH is capable of alleviating lipid abnormalities. As shown in Fig. 4C, the plasma levels of triglycerides and free fatty acids significantly decreased in APH-treated mice, comparable to the effects observed following rosiglitazone or tesaglitazar treatment. Furthermore, unlike rosiglitazone or tesaglitazar, AF

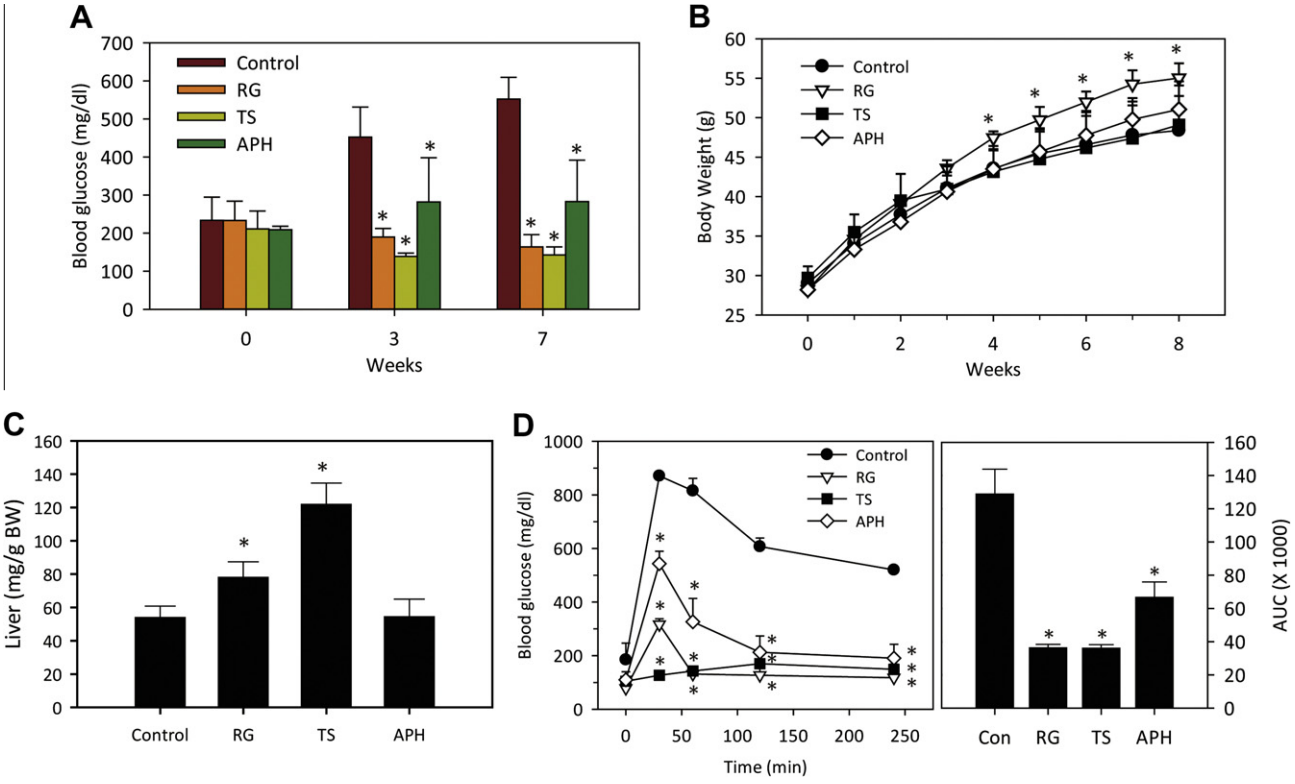


Fig. 3. Antidiabetic activity of APH in *db/db* mice. (A) Non-fasting blood glucose changes in rosiglitazone (RG; 4 mg/kg)-, TS (1 mg/kg)-, or APH (20 mg/kg)-treated obese and diabetic *db/db* mice ($n = 7$ for each group). $^{*}P < 0.05$ vs. control. (B) Whole-body weight changes over 8 weeks following drug administration. $^{*}P < 0.05$ vs. control. (C) Average liver weights of RG-, TS-, or APH-treated *db/db* mice. Each bar represents the mean ± SD. $^{*}P < 0.05$ vs. control. (D) Oral glucose tolerance test in *db/db* mice. Mice were fasted and injected with glucose (2 g/kg), and the blood glucose levels were measured at the baseline ($t = 0$ min) and blood samples drawn at the indicated time points ($n = 7$). The area under the curve (AUC) of the glucose tolerance test was calculated. $^{*}P < 0.05$ vs. control.

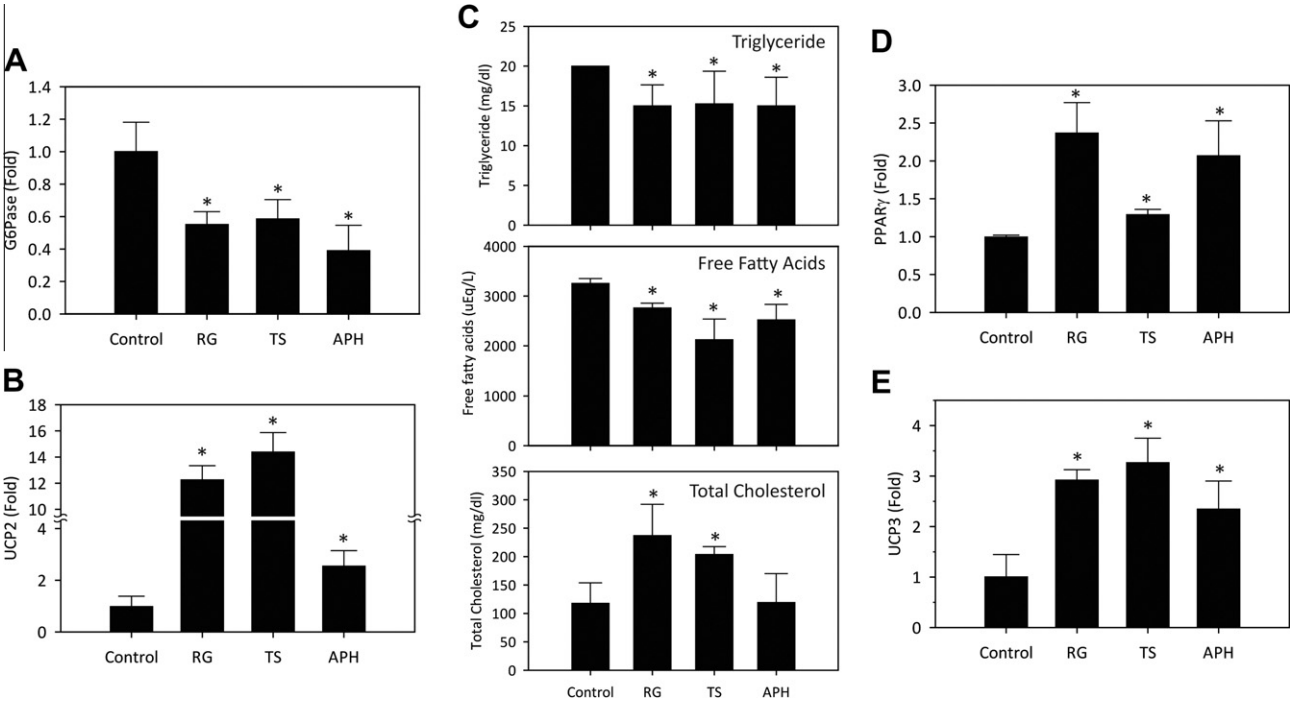


Fig. 4. APH improves metabolic markers in the liver, blood, and white adipose tissue (WAT) of *db/db* mice. Relative gene expression of glucose-6-phosphatase (G6Pase) (A) and uncoupling protein 2 (UCP2) (B) from the livers of the indicated drug-treated obese *db/db* mice ($n = 7$) were determined by Q-PCR. Data represent the means ± SD. $^{*}P < 0.05$ vs. control. (C) The blood triglyceride, free fatty acids, and total cholesterol levels were measured as described in Materials and methods. Each bar represents the means ± SD. $^{*}P < 0.05$ vs. control. Relative gene expression of PPARγ (D) and UCP3 (E) from the WAT were determined by Q-PCR. Data represent the means ± SD. $^{*}P < 0.05$ vs. control.

and APH treatment did not affect the total plasma cholesterol levels (Fig. 4C), indicating that APH can alleviate lipid abnormalities and prevent hypertriglyceridemia and hypercholesterolemia. We next analyzed the expression levels of PPARγ and UCP3 in white adipose tissue because UCP3 expression is directly up-regulated by PPARγ agonists [26,27]. The expression of PPARγ was dramatically increased in the white adipose tissues (Fig. 4D) after APH treatments, and this induction of PPARγ was accompanied by increased UCP3 expression in white adipose tissues (Fig. 4E), which might be related to the increased rate of lipid metabolism.

4. Discussion

T2DM and its related complications arise as a serious health problem in modern societies. In this study, we demonstrated the anti-diabetic and hypolipidemic potentials of APH, as evidenced by the observations that APH improve glucose impairment in *db/db* mice through the dual activation of PPARαγ transcription. Unlike other PPAR agonists, APH exhibited favorable effects on obesity and hyperlipidemia without the severe side effects like weight gain and hepatomegaly.

Although insulin sensitizers are considered as a better agent for the treatment of T2DM patients with insulin resistance, the clinical use of thiazolidinediones, especially rosiglitazone, is currently challenged by their severe adverse effects, including hepatotoxicity, weight gain, dyslipidemia, and the possible worsening of cardiovascular risk [28]. Thus, enormous efforts are concentrated on the development of novel insulin sensitizers having less toxicity. In this study, we showed that APH has anti-diabetic and hypolipidemic effects *via* the dual agonistic action on PPARαγ. APH shows dual agonistic activities for both PPARα and PPARγ; however, their binding affinities to PPARα are weaker than to PPARγ. Although APH acts as a PPARγ agonist, its adipogenic potential and PPARγ-binding affinity are milder than those of thiazolidinedione, and our observation that APH efficiently stimulates PPARα activity indicates that APH may primarily govern lipid metabolism by fatty acid oxidation. Indeed, APH increases the expression of PPARα and its target genes, suggesting the enhancement of lipid catabolism.

Several reports have demonstrated that rosiglitazone is associated with an increased risk of heart attacks, which potentially limits its appeal and further clinical use, in spite of its potent improvement of glucose metabolism [28]. However, pioglitazone, another thiazolidinedione PPARγ agonist, is less frequently associated with increased cardiovascular risk [29], implying that PPARγ activity itself may not necessarily be associated with this cardiovascular risk and that the chemical structure of the individual agonists may be responsible. Therefore, the development of novel PPAR agonists that are not structurally related to thiazolidinediones, especially rosiglitazone, remains appealing. From this point of view, it is noteworthy that APH, which are derived from natural products, is a novel and weak PPARα/γ dual agonists and that the chemical structure of APH is unique from those of thiazolidinediones. Taken together, these facts support, in part, the relative safety of APH with regard to cardiovascular risk. In addition, PPAR agonists such as rosiglitazone and tesaglitazar show hepatomegaly as a side effect, which is caused by fatty liver, aberrant PPARα activation, and peroxisome proliferation. However, our data show that APH do not increase liver weight in *db/db* mice (Fig. 4C), indicating that APH would improve fatty liver without severe hepatomegaly, a fatal side effect of potent PPARα activators [16,30]. However, their exact molecular mechanisms remain to be unraveled.

In summary, we demonstrate here that APH has beneficial effects on glucose and lipid metabolism in the improvement metabolic disorders by selectively activating both PPARα and PPARγ without the severe adverse reactions that have been observed for other PPAR agonists, such as weight gain and hepatomegaly. Therefore, APH have strong reasons to be further developed as anti-metabolic agents to correct glucose and lipid abnormalities, insulin resistance and obesity. APH has a potential to be a lead compound to combat insulin resistance and obesity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.083.

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