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Dietary obacunone supplementation stimulates muscle hypertrophy, and suppresses hyperglycemia and obesity through the TGR5 and PPARγ pathway

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ABSTRACT

Obacunone is a limonoid that is predominantly found in *Citrus*. Although various biological activities of limonoids have been reported, little is known about the beneficial effects of obacunone on metabolic disorders. In the present study, we examined the effects of dietary obacunone supplementation on obese KKAy mice, to clarify the function of obacunone in metabolic regulation. Mice were pair-fed a normal diet either alone or supplemented with 0.1% w/w obacunone for 28 days. Compared with the control, obacunone-fed mice had lower glycosylated hemoglobin, blood glucose, and white adipose tissue weight, although there was no significant difference in body weight. Obacunone treatment also significantly increased the weight of the gastrocnemius and quadriceps muscles. Reporter gene assays revealed that obacunone stimulated the transcriptional activity of the bile acids-specific G protein-coupled receptor, TGR5, in a dose-dependent manner. In addition, obacunone inhibited adipocyte differentiation in 3T3-L1 cells and antagonized ligand-stimulated peroxisome proliferator-activated receptor γ (PPARγ) transcriptional activity. These results suggest that obacunone stimulates muscle hypertrophy and prevents obesity and hyperglycemia, and that these beneficial effects are likely to be mediated through the activation of TGR5 and inhibition of PPARγ transcriptional activity.

1. Introduction

The increasing prevalence of metabolic abnormalities including obesity and type 2 diabetes represents a global healthcare problem [1]. Although lifestyle modification involving regular physical activity is an effective strategy to treat metabolic disorders, adherence to an exercise regimen is challenging and requires strong motivation, which is the major obstacle for the success of lifestyle interventions. For this reason, huge efforts are made to develop more effective treatments that can cure these disorders and/or prevent their development.

TGR5, a member of the G protein-coupled receptor (GPCR) family, was identified as a lithocholic acid-activated receptor [2,3]. TGR5 is activated by binding of bile acids (BAs), and its activation leads to the elevation of intracellular cAMP levels [2,3]. TGR5 activation has been reported to prevent body weight gain and hepatic steatosis during high-fat feeding [4,5]. BA-dependent stimulation of TGR5 signaling improves glucose homeostasis by inducing intestinal glucagon-like peptide 1 (GLP-1) release [5,6]. These TGR5 properties indicate that its activation could beneficially affect several features of metabolic syndrome. To date, several natural compounds including oleanolic acid [7], betulinic acid [8,9], and nomilin [10] have been identified as TGR5 ligands.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the ligand-activated nuclear transcription factors and is enriched in adipose tissue, where it plays critical roles in adipocyte differentiation and maintenance of the characteristics of mature adipocytes [11,12]. PPARγ is a molecular target of thiazolidinedione anti-diabetic agents that ameliorate insulin resistance. It has been reported that several naturally occurring compounds, including flavonoids, can also act as PPARγ ligands [13,14].

Limonoids are a group of triterpene derivatives that exist in *Citrus*. It is known that the limonoid aglycones and glycosides accumulate in the seeds [15]. Although relative composition of limonoids in the seeds varies depending on the species, limonin, nomilin, deacetylnomilin and obacunone are the major limonoids

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[15]. The biological activities of limonoids have been investigated in several lines of research, including anti-cancer, antimalarial, and anti-microbial activities [16,17]. Obacunone inhibits the proliferation of tumor cells by inducing apoptosis [18] and caspase 3/7 activity [19]. It has also been reported that obacunone enhances vascular function in mice fed a high-cholesterol diet, indicating that it may contribute to the treatment of cardiovascular diseases caused by endothelial dysfunction [20]. However, the beneficial effects of obacunone on other metabolic disorders are largely unknown. Therefore, the aim of the present study was to investigate the physiological effects of obacunone on several features of metabolic syndrome when administered to obese diabetic KKAy mice.

2. Materials and methods

2.1. Materials

C. junos seeds were purchased from Marukyoseikatonya (Tokushima, Japan). Obacunone was purchased from ChromaDex (Irvine, CA, USA). Pioglitazone hydrochloride was purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals used were obtained from Sigma (St. Louis, MO, USA).

2.2. Cell culture

HEK293 cells were obtained from Dainippon Sumitomo Pharma Co Ltd. (Osaka, Japan). The cells were cultured in medium A [Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen Japan, Tokyo, Japan)].

3T3-L1 cells were obtained from the Health Science Research Resources Bank, (Osaka, Japan). 3T3-L1 preadipocytes were cultured in medium A. Two days after confluence, cells were differentiated into adipocytes by the addition of differentiation medium (medium A containing 0.5 mM 3-isobuthyl-1-methylxantine, 1 μM dexamethasone, 10 μg/mL insulin, and 5 μM pioglitazone hydrochloride) in the presence or absence of 100 μM obacunone (day 0). After 2 days, the 3T3-L1 cells were transferred to adipocyte-growing medium (medium A containing 5 μg/mL insulin and 5 μM pioglitazone hydrochloride) in the presence or absence of 100 μM obacunone, which was replenished every 2 days. Dimethyl sulfoxide (DMSO) was the vehicle control for the test compounds. On day 8, the differentiated adipocytes were photographed at × 40 magnification using an SP350 digital camera (Olympus, Tokyo, Japan) attached to an IMT-2 microscope (Olympus), and harvested for triglyceride (TG) quantification. The cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.3. Luciferase assays

Luciferase reporter assays were performed as described previously [10,21]. Briefly, HEK293 cells were plated in 12-well plates at a density of 1.0×10^5 cells/well and cultured with medium A for 20 h. Then, the cells were transfected with 100 ng of pCRE-Luc (a reporter plasmid), 100 ng of pcDNA-TGR5 (an expression plasmid for TGR5), and 100 ng of pEF-β-Gal (an expression plasmid for β-galactosidase) for determination of the TGR5 ligand activity. For the evaluation of PPARγ transcriptional activity, HEK293 cells were transfected with 50 ng of pG5Luc, 25 ng of pM-GAL4-PPARγ (an expression plasmid for a GAL4-DBD-PPARγ fusion protein), and 10 ng of pRL-CMV (a renilla luciferase control reporter plasmid) using lipofectamine 2000 (Life Technologies Japan, Tokyo, Japan). Four hours after transfection, the medium was replaced with DMEM supplemented with 10% dextran-charcoal-stripped FBS. After 20 h, the indicated test compounds or control vehicle (DMSO) was added to the medium. All test compounds were dissolved in DMSO. The final DMSO concentration in the culture medium was 0.1%. After incubation for another 5 h (for the determination of TGR5 ligand activity) or 24 h (for the evaluation of PPARγ transcriptional activity), the luciferase and β-galactosidase activities were measured as described previously [10,21]. Normalized luciferase values were determined by dividing the firefly luciferase activity by the β-galactosidase activity or renilla luciferase activity.

2.4. Triglyceride quantification in adipocytes

Triglycerides (TGs) accumulated in differentiated adipocytes were extracted by hexane-isopropyl alcohol (3:2, v/v). The extracted TGs were measured using the triglyceride E-test (Wako, Osaka, Japan), according to the manufacturer's instructions. TG values were normalized to protein contents and measured by the Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions.

2.5. Thin-layer chromatography

Thin-layer chromatography (TLC) was carried out with TLC Silica gel 60 F_{254} (EMD Millipore, St Charles, MO, USA), and the compounds were detected by spraying with 5% sulfuric acid ethanol.

2.6. Preparation of obacunone

Obacunone was prepared from *C. junos* seeds. After drying at 100 °C, the seeds were crushed in a food processor (Iwatani, Osaka, Japan). Extracts containing limonoids were isolated from the seed meal by incubation in ethanol at room temperature for 2 h. The extract was dried by evaporation using a rotary evaporator. The dry extract was suspended in double-distilled water and precipitated by centrifuge (5000 rpm, 20 min). The precipitate was washed twice with ice-cold ethanol. Subsequently, obacunone was isolated from the precipitate by silica gel column chromatography fractionation. The precipitate was chromatographed using a flash column on a silica gel eluted with dichloromethane-ethyl acetate (19:1, v/v). Eluted fractions were combined according to their TLC pattern to yield obacunone. The obacunone fraction was analyzed by high-performance liquid chromatography under the following conditions: system, Shimadzu SIL-10A autoinjector, SPD-10A UV spectrophotometric detector, CBM-20A communications bus module and LC-10AD pump; column, Shiseido CAPCELL PAK C18 MGIII (4.6 mm ID × 250 mm); eluent, acetonitrile-water (1:1, v/v); flow rate, 1 mL/min; detection wavelength, 210 nm. The concentration was determined by the absolute calibration method. The calibration curve was calculated using an acetonitrile solution of commercially available obacunone in the range of 10–1000 μg/mL. Obacunone was detected at a retention time of 11.1 min. The purity of obtained obacunone was above 90%.

2.7. Animal experimental procedures

The experimental protocols were approved by the Animal Research Control Committee of Kikkoman Corporation (permission number: 2013-Y24), and performed according to the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animals. All surgeries were performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Male KKAy mice were purchased from Clea Japan (Tokyo, Japan). At 4 weeks of age, the KKAy mice were divided into two groups

with similar average weight (control group, 22.5 g, and obacunone group, 22.1 g) and glycosylated hemoglobin (HbA_{1c}) levels (control group, 2.84%, and obacunone group, 2.83%; n = 8). Mice were maintained individually in an air-conditioned room (22 °C and 60% humidity under specific pathogen-free conditions) with a constant 12-h light/dark cycle. They were pair-fed a control diet (AIN-93G) or an obacunone-supplemented diet (AIN-93G supplemented with 0.1% obacunone). The mice were weighed every day. HbA_{1c} and blood glucose were measured every week. At 28 days after the initiation of obacunone supplementation, mice were fasted for 16 h and killed under isoflurane anesthesia. BAT, white adipose tissue (WAT), gastrocnemius muscles, and quadriceps muscles were rapidly excised and weighed. Blood samples were collected, dipeptidyl peptidase-4 (DPP-4) inhibitor (EMD Millipore) was immediately added and serums were separated and stored at −80 °C until further processing.

2.8. HbA_{1c} and blood glucose

HbA_{1c} and blood glucose were measured by ANTSENSE III (Horiba, Kyoto, Japan) and DCA2000 (Bayer Medical Japan, Tokyo, Japan), respectively.

2.9. Serum biochemistry

Serum GLP-1 level was determined using Lbis GLP-1 (active) ELISA Kit (Shibayagi, Gunma, Japan). Serum insulin level was measured using Lbis Insulin-mouse-T ELISA Kit (Shibayagi).

2.10. Statistical analysis

All values are represented as mean ± SD. In the animal experiments, unpaired Student's *t*-tests were used to analyze differences

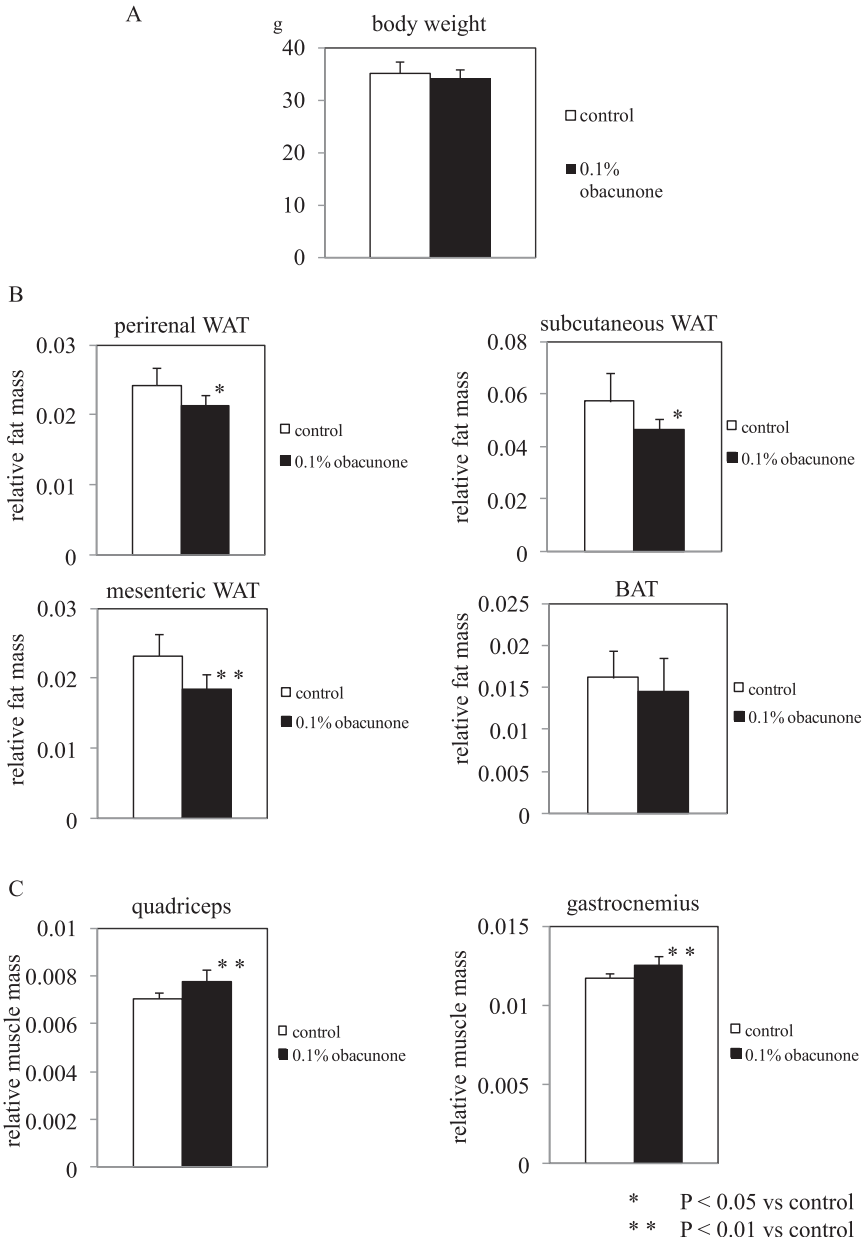

Fig. 1. The effects of dietary obacunone supplementation on body and tissue weight in KKAy mice. KKAy mice were pair-fed either a control diet (AIN-93G) or a diet containing 0.1% obacunone for 28 days. Body weight (A), relative adipose tissue mass (B), and relative muscle mass (C) are shown as the mean ± SD, n = 8.

between control and obacunone-treated animals. One-way analysis of variance followed by the Bonferroni/Dunn procedure was used to compare more than two groups. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Dietary obacunone supplementation reduces fat content and increases skeletal muscle mass in KKAy mice

To evaluate the effect of obacunone supplementation on metabolic parameters, obese diabetic KKAy mice were pair-fed either a control diet (AIN-93G) or a diet supplemented with 0.1% obacunone for 28 days. The body weight was not affected by dietary obacunone supplementation (Fig. 1A). On the contrary, obacunone treatment significantly decreased the accumulation of visceral and subcutaneous fat mass (Fig. 1B). There was no difference in BAT mass between control and obacunone-fed mice. Quadriceps and gastrocnemius muscle mass significantly increased in mice fed the obacunone diet (Fig. 1C). These results indicate that regular obacunone consumption would beneficially affect the features of metabolic syndrome. There were no overt adverse effects in each experimental group.

3.2. Obacunone treatment prevents hyperglycemia in KKAy mice

As shown in Fig. 2A, obacunone treatment significantly reduced fasting blood glucose and HbA_{1c} levels compared with those in the control group after 28 days of treatment. Although the changes were not statistically significant, mice fed with a 0.1% obacunone diet exhibited lower serum insulin levels and higher serum GLP-1 levels, compared with the control mice (Fig. 2B). GLP-1 is a gut peptide that is released to the circulation upon food ingestion, exerting glucose-lowering effects. These results suggest that dietary obacunone intake suppresses the onset of hyperglycemia, and it is possible that increased secretion of GLP-1 may contribute to the hypoglycemic effects of obacunone.

3.3. Obacunone is a TGR5 agonist

The above results suggest the possibility that obacunone inhibits the development of hyperglycemia by stimulating GLP-1 release. We therefore hypothesized that potentiation of the TGR5-GLP-1 pathway by obacunone treatment may contribute to the favorable effects on obesity and hyperglycemia. To further investigate this concept, we performed reporter assays using a TGR5 expression plasmid and a CRE-driven luciferase reporter plasmid. Obacunone and the positive control, taurolithocholic acid (TLCA), stimulated luciferase activity in the presence of the TGR5 expression plasmid (Fig. 3A). Moreover, this transcriptional activation by obacunone was dose dependent (Fig. 3B), indicating that obacunone exhibits TGR5 ligand activity.

3.4. Obacunone antagonizes PPARγ and inhibits lipid accumulation in differentiating adipocytes

We next examined the effects of obacunone on the transcriptional activity of PPARγ, a master regulator of adipogenesis. As shown in Fig. 4A, the pioglitazone-stimulated PPARγ transcriptional activity was significantly suppressed in the presence of obacunone, indicating that obacunone antagonizes ligand-stimulated PPARγ activity. Furthermore, obacunone significantly inhibited lipid accumulation during adipocyte differentiation under pioglitazone treatment (Fig. 4B and C). These findings suggest that obacunone is capable of inhibiting PPARγ activity, and the anti-obesity effects of obacunone are likely due to, at least in part, antagonism of PPARγ.

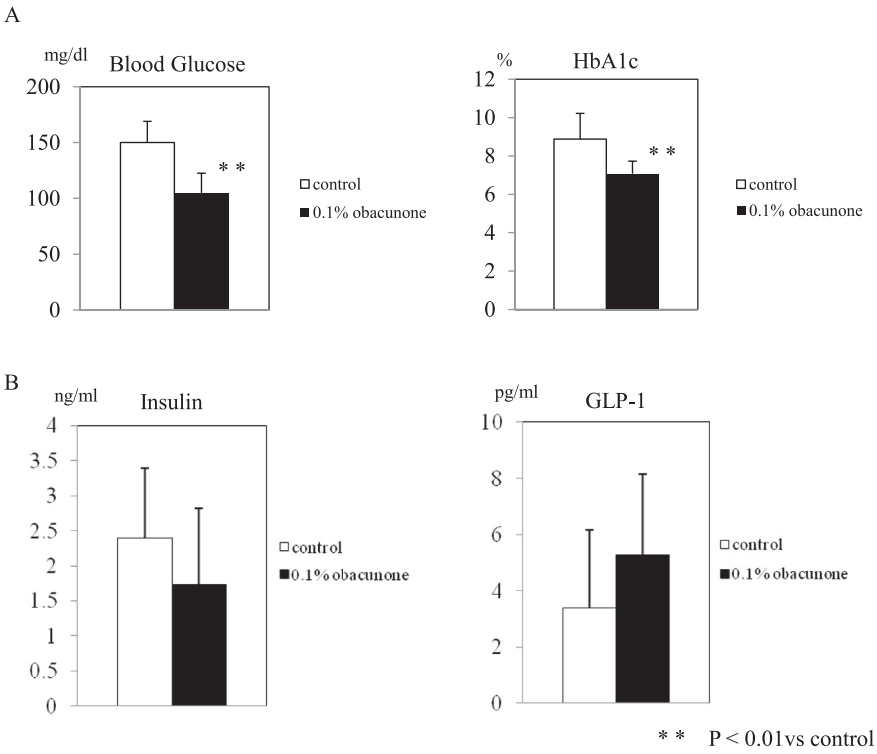

Fig. 2. Hypoglycemic effects of obacunone in KKAy mice. KKAy mice were pair-fed either a control diet (AIN-93G) or a diet containing 0.1% obacunone for 28 days. (A) Blood glucose and HbA_{1c} were measured after a 16-h fast. (B) GLP-1 and insulin were measured in serums collected after a 16-h fast. The data are represented as mean ± SD (n = 8).

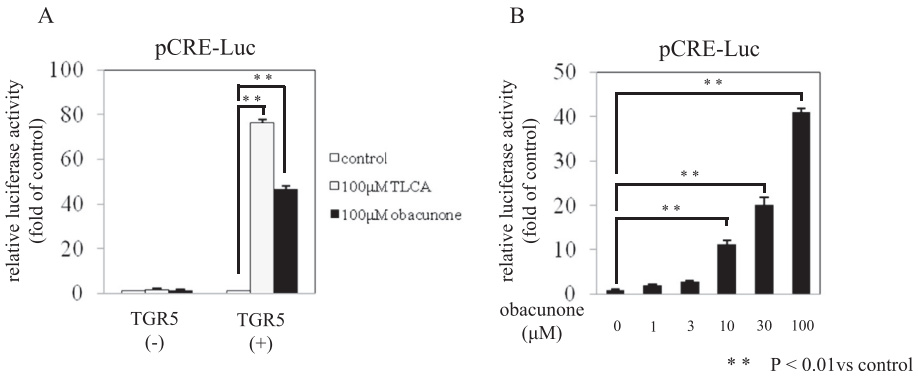

Fig. 3. Obacunone stimulates TGR5-mediated transcription. HEK293 cells were transfected with the CRE-driven reporter plasmid and the TGR5 expression plasmid. (A) Twenty-four hours after transfection, cells were treated with 100 μM TLCA (positive control) or 100 μM obacunone for another 5 h. (B) Twenty-four hours after transfection, cells were treated with obacunone at the indicated concentrations for 5 h. Luciferase reporter activities were then quantified. The data are shown as the mean ± SD of triplicate cultures.

4. Discussion

In this study, we examined the effects of obacunone on diabetic KKAy mice, and showed that obacunone exerts muscular hypertrophic, anti-hyperglycemia, and anti-obesity effects. The results of luciferase reporter assays suggest that modulation of the TGR5 and PPARγ pathway contributes to the favorable effects of obacunone. These results indicate that dietary obacunone intake could be a promising strategy for preventing hyperglycemia and obesity.

The incretin, GLP-1, is a gut peptide derived from a precursor that is synthesized in the enteroendocrine L-cells of the intestinal epithelium [22]. Increased intestinal secretion of GLP-1 induced by TGR5 activation leads to enhanced glucose tolerance in obese mice [5]. Although not statistically significant, the serum GLP-1 levels in obacunone-fed mice were higher than those in control mice, in this study (Fig. 2B). Considering obacunone stimulated TGR5 activity in a dose-dependent fashion (Fig. 3), it is conceivable that obacunone exerted glycemia-lowering effects, at least in part, through potentiation of the TGR5-GLP-1 pathway. The timing of the blood sample collection may be the reason for the weak trend in serum GLP-1 levels. In the present study, GLP-1 levels were measured in serum samples collected after an overnight fast. In general, the secretion of GLP-1 is induced shortly after food ingestion, and subsequently degraded by DPP-4. Therefore, the GLP-1 level in the blood samples used in this study may have returned to the pre-secretion basal level by the time of collection. It is possible that continuous

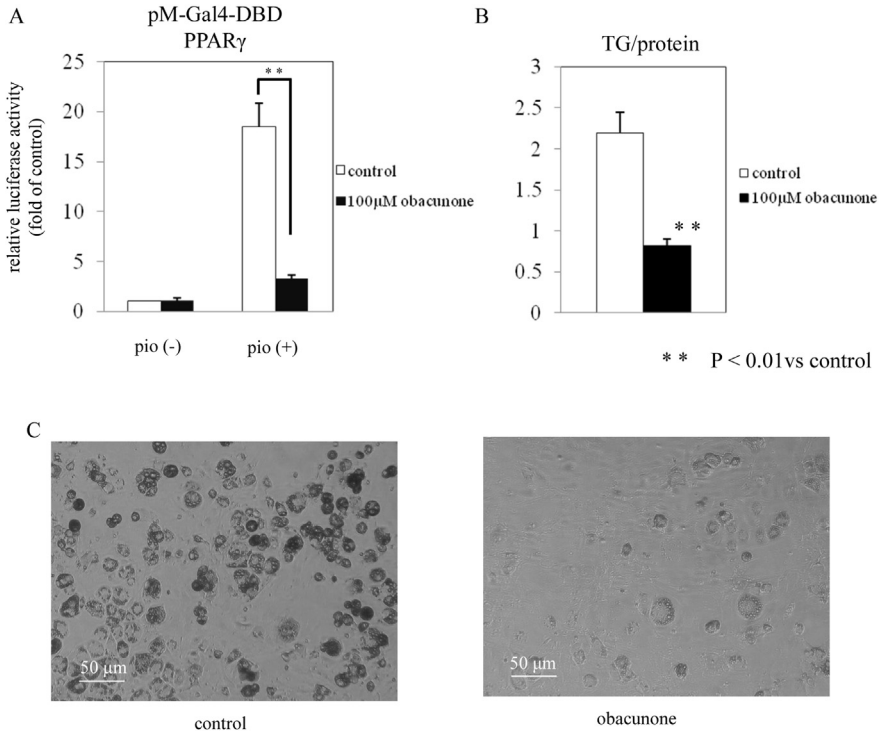

Fig. 4. Obacunone inhibits ligand-activated PPARγ transcription and lipid accumulation in differentiating adipocytes. (A) HEK293 cells were transfected with a reporter plasmid containing consensus Gal4-binding sites and the expression plasmid for the fusion protein containing the Gal4 DNA-binding domain and PPARγ ligand-binding domain. Twenty-four hours after transfection, cells were treated with 5 μM pioglitazone hydrochloride in the presence or absence of 100 μM obacunone for 24 h. Luciferase reporter activities were then quantified. (B) 3T3-L1 cells were differentiated for 8 days with 5 μM pioglitazone hydrochloride in the presence or absence of 100 μM obacunone. On day 8, TG accumulation was quantified, and normalized to protein content. (C) Photographs of the differentiated adipocytes on day 8. The data are represented as the mean ± SD of triplicate cultures.

stimulation of TGR5 activity by consumption of obacunone-containing diets contributed to the weak trend towards an increase in GLP-1 levels.

Interestingly, obacunone supplementation not only improved blood glucose metabolism, but also increased relative skeletal muscle mass (quadriceps and gastrocnemius) in KKAy mice (Fig. 1B). A recent study has reported the significant role of the TGR5-AKT-mTOR signaling pathway in glucose homeostasis through modulating functions of adipose tissue macrophages [23]. The AKT-mTOR pathway has been considered the main mediator of normal muscle development and muscle hypertrophy, as it coordinates multiple processes related to protein synthesis [24–27]. These observations indicate the possibility that obacunone consumption can augment muscle hypertrophy through modulating the TGR5-AKT-mTOR signaling pathway in skeletal muscle. However, some GPCRs have been shown to stimulate muscle hypertrophy [28]. These effects are probably mediated by selective activation of cAMP signaling in skeletal muscle, as inhibition of phosphodiesterase reduces atrophy [29,30]. Considering the fact that TGR5 couples $G\alpha_s$, leading to activation of adenylate cyclase and subsequent intracellular cAMP formation, TGR5 activation by obacunone may contribute to skeletal muscle hypertrophy. It is essential to determine whether orally administered obacunone also stimulates the TGR5-related hypertrophic pathway in skeletal muscle. This issue is now under investigation.

While obacunone stimulated the transcriptional activity of TGR5, it antagonized ligand-dependent PPARγ transactivation (Fig. 4A). PPARγ is a member of the nuclear hormone receptor superfamily, and plays a pivotal role in regulating the complex transcription network associated with glucose homeostasis and lipid metabolism. Thiazolidinediones, a class of compounds that act as PPARγ ligands, have been reported to improve insulin resistance, which in turn lowers blood glucose. However, their unique effectiveness is shadowed by the risk of body weight gain owing largely to the increase in fat pad mass [31–33]. On the contrary, several lines of studies have indicated that partial inhibition of PPARγ transcriptional activity may contribute to the prevention of the development of insulin resistance and obesity. It has been reported that heterozygous PPARγ-deficient mice are protected from the development of insulin resistance and adipocyte hypertrophy under high-fat diet conditions [34]. Other studies have reported that PPARγ antagonists inhibit adipocyte differentiation and adipogenesis [35,36]. Taking into account the fact that obacunone antagonized pioglitazone-induced PPARγ activity and inhibited lipid accumulation in differentiating adipocytes (Fig. 4), the observed effects on WAT in obacunone-fed mice (Fig. 1B) may have been due to moderate inhibition of PPARγ activity by obacunone. This inhibitory effect on PPARγ, as well as activation of TGR5 activity, may also contribute to the hypoglycemic effects of obacunone.

In summary, our study demonstrated that dietary obacunone supplementation suppressed hyperglycemia and increased muscle mass in diabetic KKAy mice, and this effect is likely to be mediated, at least in part, by the potentiation of multiple pathways associated with TGR5. Obacunone also exerted antagonistic activity on PPARγ, which contributes to inhibition of lipid accumulation in WAT. These results suggest that regular obacunone consumption could be beneficial in preventing the development of hyperglycemia, muscle atrophy, and obesity.

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