Research Article

4′,6-dimethoxyisoflavone-7-*O*-β-D-glucopyranoside (wistin) is a peroxisome proliferator-activated receptor α (PPARα) agonist in mouse hepatocytes

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors that regulate lipid and glucose metabolism. PPARα mainly affects fatty acid metabolism, and its activation lowers lipid levels. PPARγ is involved in the regulation of adipogenesis, insulin sensitivity, energy balance, and lipid biosynthesis. We have previously reported that 4′,6-dimethoxyisoflavone-7-*O*-β-D-glucopyranoside (wistin) can activate PPARγ. The purpose of the present study is to investigate the PPARα agonist activity of wistin. Using a luciferase reporter assay system of PPARα in monkey COS7 kidney cells, we showed that wistin could activate PPARα ($P < 0.01$ at 10 μg/mL) in a dose-dependent manner. Moreover, the addition of wistin upregulated the expression of PPARα ($P < 0.01$ at 10 μg/mL) and PPARα target genes including carnitine palmitoyltransferase 1a ($P < 0.05$ at 10 μg/mL), acyl-CoA oxidase ($P < 0.01$ at 10 μg/mL), acyl-CoA synthase ($P < 0.05$ at 10 μg/mL), PPARγ coactivator 1α ($P < 0.05$ at 10 μg/mL), uncoupling protein 2 ($P < 0.05$ at 1 μg/mL), and uncoupling protein 3 ($P < 0.05$ at 10 μg/mL), which are genes involved in lipid efflux and energy expenditure, in mouse primary hepatocytes. Furthermore, wistin inhibited cellular triglyceride accumulation in hepatocytes ($P < 0.05$ at 10 μg/mL) in a dose-dependent manner. These results indicate that wistin could suppress lipid accumulation through PPARα activation. The ~~dual~~ action of wistin on PPARα could be of interest for the amelioration of lipid metabolic disorders. To the best of our knowledge, wistin is the first reported isoflavonoid *O*-glycoside with PPARα agonist activity.

Key words: *Fatty acid oxidation, PPARα, wistin, PPARα agonist*

INTRODUCTION

Metabolic syndrome, which is characterized by dyslipidemia and hyperglycemia, is becoming increasingly prevalent in developed countries [1]. The main risk factors of metabolic syndrome are closely linked to obesity and a lack of physical activity, which occur when energy intake exceeds energy expenditure [2, 3]. Insulin resistance may also increase the risk of metabolic syndrome. In the last decade, the pharmacological activation of peroxisome proliferator-activated receptors (PPARs) has been implicated as an effective therapeutic approach to address certain aspects of metabolic syndrome mainly hyperlipidemia and type 2 diabetes [4].

PPARs are transcription factors belonging to the superfamily of nuclear receptors. PPARs have been implicated in the regulation of lipid and lipoprotein metabolism, glucose homeostasis, insulin sensitivity, and cellular differentiation [5]. Upon activation by a ligand, PPARs heterodimerize with 9-cis-retinoic acid receptors (RXRs) and promote the transcription of their target genes by binding to the peroxisome proliferator response element (PPRE) [6]. There are three subtypes of PPARs: PPARγ, PPARα, and PPARδ. PPARγ is predominantly expressed in adipocytes, and is responsible for adipogenesis. Its agonists, such as thiazolidinedione derivatives, are potent insulin sensitizers that can be used for treating type 2 diabetes [6, 7]. PPARα is involved in lipid metabolism regulation in the liver [8]. A PPARα agonist such as bezafibrate can reduce triglyceride (TG) levels and increase plasma high density lipoprotein cholesterol levels [9, 10]. PPARδ is ubiquitously expressed and is thought to affect the lipid profile by reducing adiposity, which in turn prevents the development of obesity [11]. Among the different PPARs, PPARα is a master regulator of lipid metabolism that controls the transcription of its target genes such as acyl-CoA oxidase (*ACO*), acyl-CoA synthase (*ACS*), carnitine palmitoyltransferase 1a (*CPT1a*), PPARγ coactivator 1α (*PGC1α*), uncoupling protein 2 (*UCP2*), and *UCP3* [12, 13]. The activation of PPARα has been reported to enhance fatty acid β-oxidation in the liver and decrease the level of cellular lipids in obese patients with diabetes [10, 14]. Therefore, dedicated efforts have been made to develop new therapeutic agents for hyperlipidemia and insulin resistance according to their

activity toward PPARs.

In a previous study, we reported that 4′,6-dimethoxyisoflavone-7-*O*-β-D-glucopyranoside (wistin), isolated from *Wisteria floribunda* seeds, is a PPARγ agonist that can induce the mRNA expression of the PPARγ target gene, adiponectin, in mouse 3T3-L1 cells [15]. An increased level of adiponectin enhances insulin sensitivity by increasing fat oxidation, resulting in reduced levels of circulating fatty acids and intracellular triacylglycerol content in the liver and skeletal muscle [16]. Therefore, it is important to evaluate the effect of wistin as an activator of PPARα. The purpose of this study was to examine the activity of wistin in the transactivation of PPARα and induction of PPARα target gene expression in mouse primary hepatocytes.

MATERIALS AND METHODS

Luciferase assay

Monkey COS7 kidney cells from the American Type Culture Collection (ATCC, Manassas, VA, USA) were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Trace, Melbourne, Australia) in a humidified atmosphere of 5% CO_2. Luciferase assays were performed using a GAL4/PPARα chimera system [17]. We transfected COS7 cells with pM-hPPARα (a chimeric protein expression plasmid incorporating the GAL4 DNA-binding domain and human PPARα ligand-binding domain), p4xUASg-tk-luc (a reporter plasmid), and pSV-β-Gal (an internal control plasmid to normalize transfection efficiency) using Lipofectamine (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Transfected cells were seeded in 96-well plates and incubated with medium containing wistin (INDOFINE, Hillsborough, NJ, USA) or dimethyl sulfoxide (DMSO, 0.1%) vehicle for 24 h. Luciferase activity was assayed using the Steady-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol in a Centro XS^3 LB 960 fluorescence microplate reader (Berthold, Bad Wildbad, Germany). The transiently transfected COS7 cells were assayed for β-galactosidase (β-gal) activity using 180 μL of β-gal assay buffer (0.1 M PBS [pH 7.2], 2.8 mM $MgCl_2$, 133 mM 2-mercaptoethanol, 2.8 mg/mL 2-nitrophenyl β-D-galactopyranoside) was added to 20 μL of cell lysates, and the plates were incubated for 2 h at 37 °C prior to measuring the absorbance at 415 nm using a MTP-310 microplate reader (CORONA, Ibaragi, Japan). Data were quantified relative to β-gal activity and expressed as percentage of DMSO vehicle-treated cells.

Preparation of mouse primary hepatocytes

Mouse hepatocytes were prepared according to a previous protocol [18]. C57/BL/6j male mice

were intraperitoneally anesthetized with pentobarbital, and the liver was perfused with Liver Perfusion Medium (Invitrogen), followed by Liver Digestion Medium (Invitrogen). Hepatocytes were dispersed in Hepatocyte Wash Medium (Invitrogen) by dissection and gentle shaking. After filtration through a 100 μm nylon mesh filter, the hepatocytes were isolated by repeated centrifugation (three times) at 50 *g* for 3 min. The isolated hepatocytes were cultured in type 1 collagen-coated 12-well plates (Iwaki, Tokyo, Japan) at a cell density of 2×10^5 cells/well. After incubation for 5 h at 37 °C in a 5% CO_2 atmosphere, the hepatocytes were treated with 1 μg/mL (2.17 μM) or 10 μg/mL (21.7 μM) wistin for 48 h.

The experiments were conducted in accordance with the Guideline for Animal Experimentation of Nagahama Institute of Bio-Science and Technology. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Nagahama Institute of Bio-Science and Technology (Permit No. 035).

RNA isolation

Two C57/BL/6j male mice were used for RNA quantification assay. Total RNA was extracted from mouse primary hepatocytes using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. The extracted RNA was dissolved in diethyl pyrocarbonate-treated water. Concentration (absorbance 260 nm, A260) and purity (A260/280 ratio) of the total RNA samples were measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Evaluation of mRNA expression

Isolated RNA was used as a template for reverse transcription with random primers (ABI High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA). Real-time qPCR was performed using the SYBR Premix Ex Taq II Kit (TaKaRa, Otsu, Japan) in a Roter-Gene 6000 thermocycler (Corbett Research, Mortlake, Australia). The reaction was performed using an initial

denaturation step for 10 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Subsequently, the melting curve of qPCR products was examined by changing the ramp temperature from 60 °C to 95 °C. The oligonucleotide primer sets of PPARα and PPARα target genes are shown in Table 1. The Ct value was determined, and the abundance of gene transcripts was calculated from the Ct value using *Hprt1* for normalization according to the comparative Ct method (User Bulletin No.2; Applied Biosystems).

Measurement of TG accumulation

Two C57/BL/6j male mice were used for the TG accumulation assay. After 48 h of incubation, mouse primary hepatocytes were washed with PBS and placed immediately in RIPA buffer (Wako, Osaka, Japan). The lysate was centrifuged at 10,000 rpm for 5 min, and supernatants was collected. The TG level in the supernatants was measured using the TG E-test Wako kit (Wako). Protein was quantified using the Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data are expressed as the mean ± S.E. The control and treatment groups were compared using Student's t-test. Differences were considered significant at $P < 0.05$.

Conflict of interest

The authors declare that they have no conflict of interest.

RESULTS AND DISCUSSION

Activation of PPARα in luciferase assays by wistin

First, we investigated whether wistin itself activates PPARα by performing luciferase assays using the GAL4/PPARα chimera system. As shown in Fig. 1, wistin increased PPARα reporter activity in a dose-dependent manner. The PPARα activation level with 10 μg/mL wistin was approximately 2-fold higher than that with the vehicle control.

Induction of the expression of PPARα target genes in hepatocytes by wistin

The liver plays a central role in glucose and lipid homeostasis, and the hepatic regulation of genes involved in lipid metabolism is dependent on PPARα [19]. Initially, to determine whether wistin affects PPARα in mouse primary hepatocytes, the mRNA expression of PPARα was examined. Wistin significantly increased PPARα mRNA levels in a dose-dependent manner (Fig. 2a).

The activation of PPARα is important for lipid metabolism in peripheral tissues including the liver and muscles. To determine whether wistin induces PPARα activation, we examined the mRNA expression level of PPARα target genes in mouse primary hepatocytes in the presence of wistin. PPARα activation induces the gene expression of proteins required for fatty acid oxidation. CPT1a is involved in the first limiting step of mitochondrial β-oxidation, which is the entry flux of fatty acyl carnitine for translocation across the inner mitochondrial membrane. CPT1a is regulated by PPARα and is strongly induced by fibrates [20]. ACO catalyzes the rate-limiting step of peroxisomal β-oxidation and represents a direct target of PPARα. Induction of ACO is generally considered as a marker of peroxisome proliferation [21]. On the other hand, ACS is a vital enzyme for lipid metabolism. ACS catalyzes the first step of fatty acid metabolism, and plays a key role in both the synthesis of cellular lipids and the degradation of fatty acids via β-oxidation [22]. The addition of 10 μg/mL wistin resulted in a 2.5-fold, 7.2-fold, and 14.8-fold increase in the expression levels of *CPT1a, ACO,* and *ACS,* respectively, compared with that of the vehicle control (Fig. 2b, c, d).

PPARα activation also induces the gene expression of proteins required for energy dissipation.

PGC1α is a transcriptional coactivator involved in the regulation of energy homeostasis [23]. It is preferentially expressed in tissues with high oxidative capacity, such as the liver, skeletal muscle and brown fat, where it participates in the regulation of biological pathways related to energy metabolism. PGC1α is required for the activation of gluconeogenesis and fatty acid oxidation in the liver during fasting [24]. On the other hand, UCP2 and UCP3 are mitochondrial proteins similar to the thermogenic UCP1 protein present in brown adipose tissues [25]. Although their precise biological functions are a matter of debate, they appear to be involved in the regulation of biological processes associated with mitochondrial energy metabolism and fat metabolism [25, 26]. The addition of wistin significantly induced *PGC1α*, *UCP2*, and *UCP3* expression (Fig. 2e, f, and g). These data indicate that wistin could regulate the expression of the promoters of PPARα target genes via PPARα activation in mouse hepatocytes.

Effect of wistin on the amount of cellular TG

To confirm the observed induction of PPARα activation by wistin, we examined the effect of wistin on the amount of cellular TG. Treatment with wistin for 48 h significantly decreased the amount of cellular TG in mouse primary hepatocytes in a dose-dependent manner (Fig. 3). These findings suggest that wistin deceases the amount of cellular TG via PPARα activation in mouse primary hepatocytes.

Several isoflavones, such as daidzein, biochanin A, and genistein, have been reported to act as activators of PPARα and PPARγ [27]. Specifically, structure-activity relationship studies have found that the 7-hydroxybenzopyran-4-one scaffold is an important determinant of the PPARα/γ dual agonist activity [28]. To date, isoflavonoid *O*-glycosides have not been reported to have PPAR agonist activity. In particular, the 7-*O*-β-D-glucopyranoside, ononin, does not exert PPARγ agonist activity [29]. However, we have reported that its 6-methoxy derivative, wistin, is a PPARγ agonist that can induce the mRNA expression of the PPARγ target gene adiponectin in mouse 3T3-L1 cells [15]. Moreover, wistin was also

found to exhibit PPARα agonist activity in this study. To the best of our knowledge, wistin is the first reported isoflavonoid *O*-glycoside with both PPARα and γ agonist activity.

Prevention of lifestyle-related diseases is a global concern. There have been many studies on the functionality of natural chemicals in plants, food and drinks. We showed that wistin, isolated from *Wisteria floribunda* seeds, activates both PPARγ and PPARα. The dual activation of both PPARγ in adipocytes and PPARα in the liver would be important for treating hyperlipidemia and diabetes associated with obesity. PPARα/γ dual agonists have recently been developed as therapeutic agents for the treatment of type 2 diabetes with dyslipidemia [30]. These dual agonists have anti-diabetic activity. Furthermore, they reduce arteriosclerosis development and improve endothelial functions, thus decreasing plasma free fatty acids and lowering blood pressure [31].

Despite the various benefits of PPARα/γ dual agonists, recent studies have shown that some PPARγ agonists also result in undesirable side effects, including weight gain, bone fractures, edema, congestive heart failure and increased risk of myocardial infarction [32-34]. PPAR agonists have different properties and specificities for individual PPARs, different absorption and distribution profiles, and distinctive gene expression profiles, which can lead to different clinical outcomes [35-37]. The potential health benefits of wistin in PPARγ- and PPARα-regulated mechanisms warrant further *in vivo* characterization.

In summary, luciferase reporter assays revealed that wistin induced PPARα activation in a dose-dependent manner. Furthermore, wistin upregulated the target genes of PPARα and inhibited cellular triglyceride accumulation in mouse primary hepatocytes, suggesting that fatty acid oxidation and energy expenditure were enhanced by the addition of wistin in hepatocytes through PPARα activation. The PPAR-associated health benefits of wistin warrant further investigation *in vivo*.

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Table 1. Primer sets used for qPCR and GenBank accession numbers

Gene	Forward (F), Reverse (R)	GenBank accession number
PPARα	F: TGAACAAAGACGGGATG	BC016892
	R: TCAAACTTGGGTTCCATGAT	
CPT1a	F: CTCAGTGGGAGCGACTCTTCA	NM_013495
	R: GGCCTCTGTGGTACACGACAA	
ACO	F: ATATTTACGTCACGTTTACCCCGG	NM_015729
	R: GGCAGGTCATTCAAGTACGACAC	
ACS	F: ACATCCACGTGTATGAGTTCTACGC	AF033031
	R: AGTAGACGAAGTTCTCACGGTCGAT	
PGC1α	F: GCCCGGTACAGTGAGTGTTC	XM_006503774
	R: CTGGGCCGTTTAGTCTTCCT	
UCP2	F: GCTGGTGGTGGTCGGAGATA	NM_011671
	R: ACAGTTGACAATGGCATTACGG	
UCP3	F: CCGATACATGAACGCTCCC	NM_009464
	R: AAGCTCCCAGACGCAGAAAG	
Hprt1	F: CCTGGTTCATCATCGCTAATC	AH003453
	R: TTTTCGCCAGACTCCTCCT	

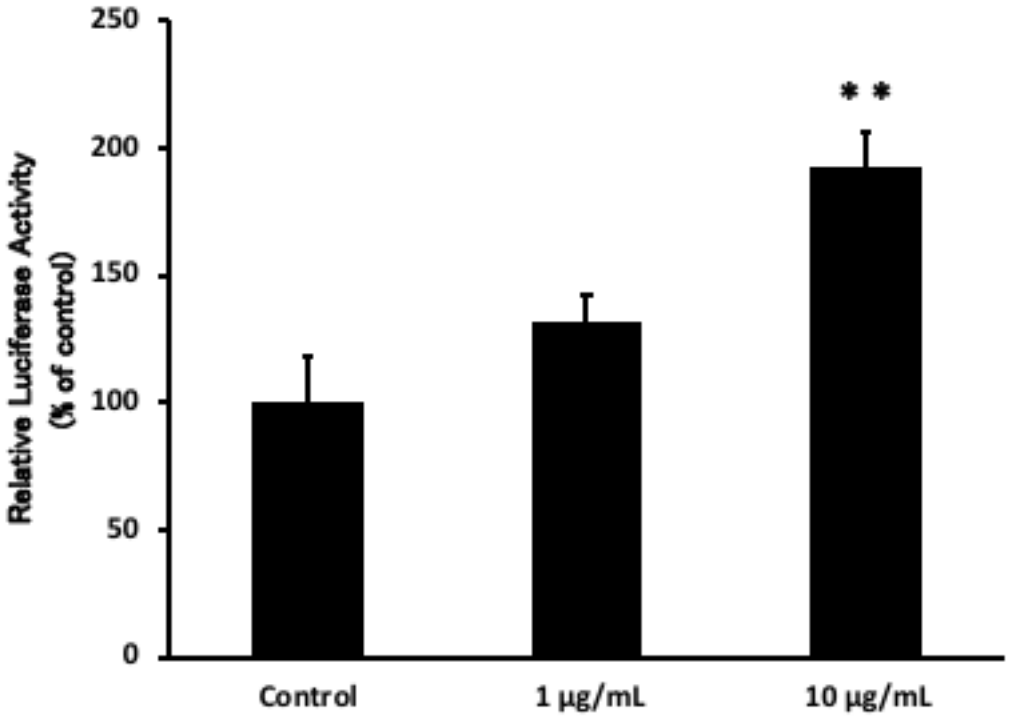

Fig. 1 Effect of wistin on PPARα activation in the luciferase assay. Values are the mean ± S.E. of four replicated cultures. DMSO (0.1%) was used as the vehicle control. The activity of the vehicle control is set at 100% and the relative luciferase activities are presented as fold-differences relative to the vehicle control. $^{**}P < 0.01$ compared with the vehicle control

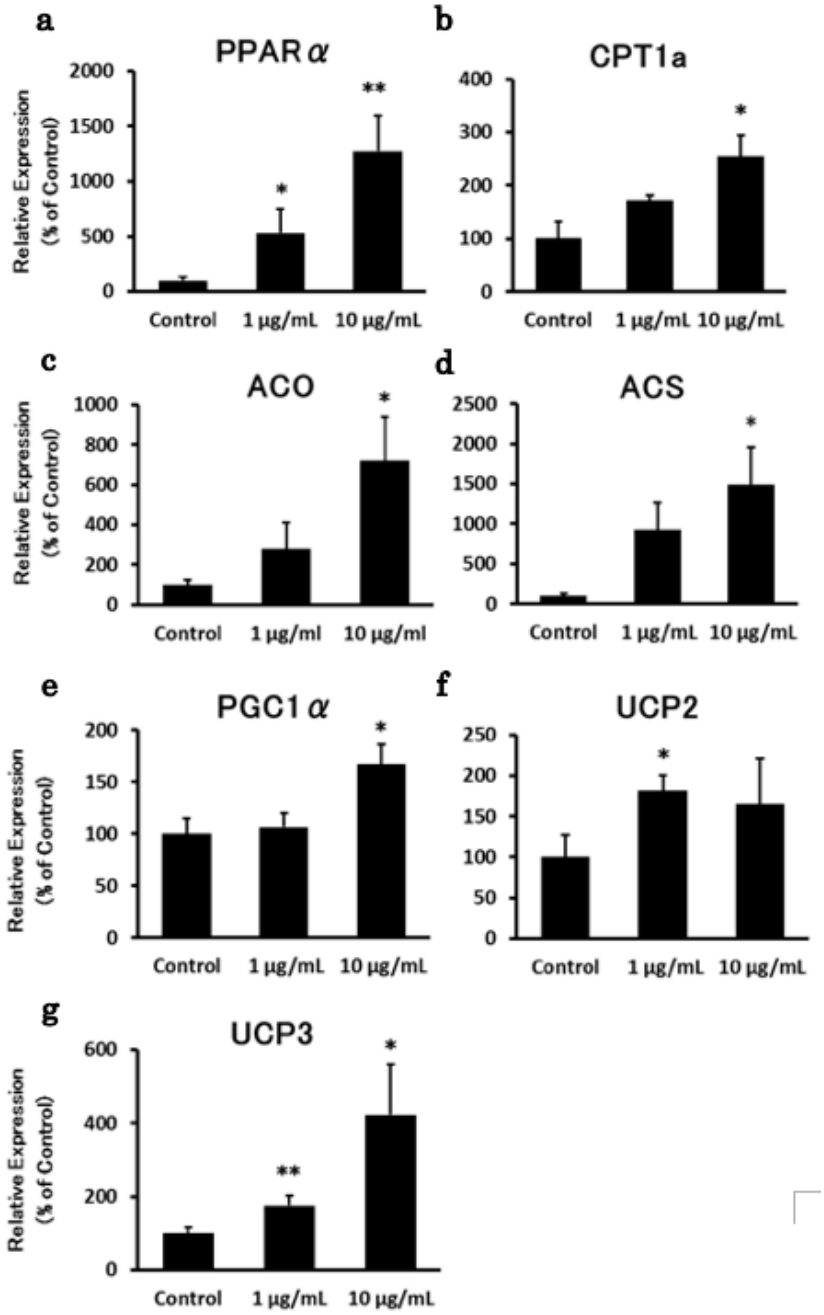

Fig. 2 Effect of wistin on *PPARα* (a), *CPT1a* (b), *ACO* (c), *ACS* (d), *PGC1α* (e), *UCP2* (f) and *UCP3* (g) expression in mouse primary hepatocytes. Values are the mean ± S.E. of four replicated cultures. DMSO (0.1%) was used as a vehicle control. The expression level of the vehicle control is set at 100% and the relative expression levels are presented as fold-differences relative to the vehicle control. *$P <$ 0.05 and **$P < 0.01$ compared with the vehicle control

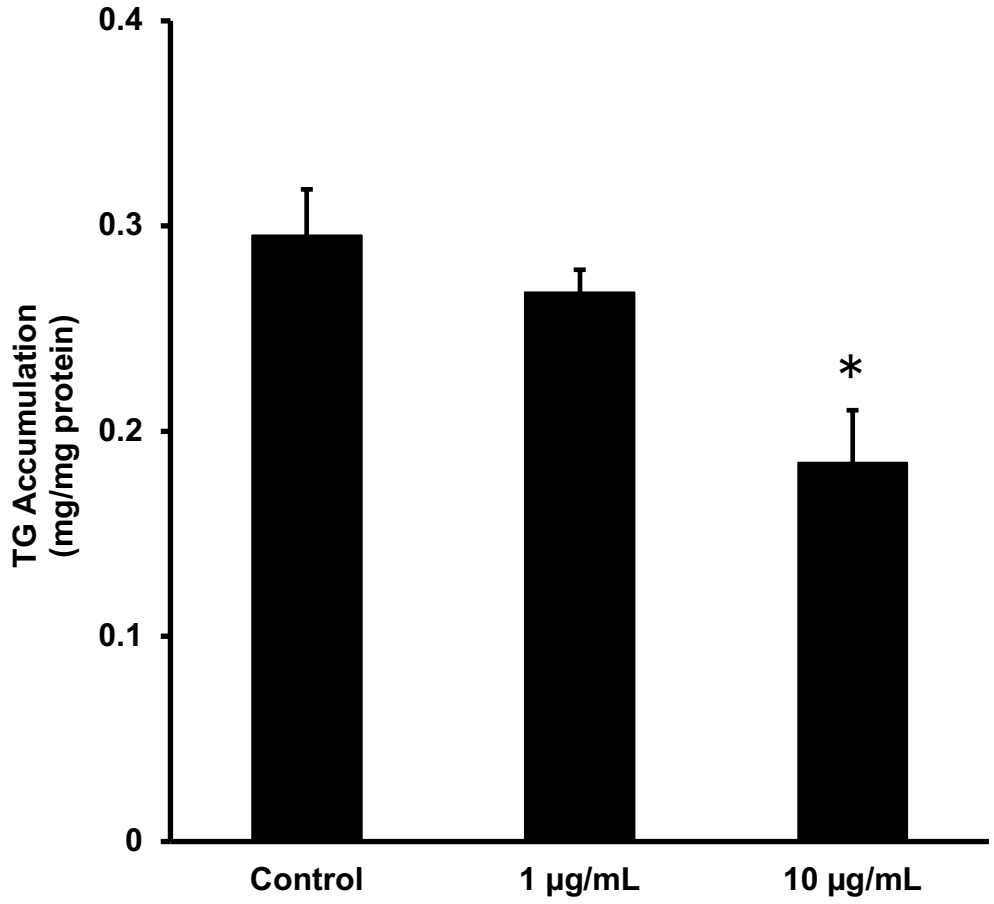

Fig. 3 Effect of wistin on cellular TG accumulation in mouse primary hepatocytes. TG amount in the hepatocytes was measured by an enzymatic method. Values are the mean ± S.E. of four replicated cultures. DMSO (0.1%) was used as a vehicle control. **P* < 0.05 compared with the vehicle control