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Catalposide is a natural agonistic ligand of peroxisome proliferator-activated receptor-α

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ABSTRACT

Peroxisome proliferator-activated receptor-alpha (PPARα) is a nuclear receptor that regulates the expression of genes related to cellular lipid uptake and oxidation. Thus, PPARα agonists may be important in the treatment of hypertriglyceridemia and hepatic steatosis. In this study, we demonstrated that catalposide is a novel natural PPARα agonist, identified from reporter gene assay-based activity screening with approximately 900 natural plant and seaweed extracts. Results of time-resolved fluorescence resonance energy transfer analyses suggested that the compound interacted directly with the ligand-binding domain of PPARα. Cultured hepatocytes stimulated with catalposide exhibited significantly reduced cellular triglyceride concentrations, by 21%, while cellular uptake of fatty acids was increased, by 70% ($P < 0.05$). Quantitative PCR analysis revealed that the increase in cellular fatty acid uptake was due to upregulation of fatty acid transporter protein-4 (+19% *vs.* the control) in cells stimulated with catalposide. Additionally, expression of genes related to fatty acid oxidation and high-density lipoprotein metabolism were upregulated, while that of genes related to fatty acid synthesis were suppressed. In conclusion, catalposide is hypolipidemic by activation of PPARα via a ligand-mediated mechanism that modulates the expression of in lipid metabolism genes in hepatocytes.

1. Introduction

Hypertriglyceridemia is a clinical condition characterized by elevated plasma triglyceride (TG) concentrations with concomitant accumulation of plasma TG-rich lipoproteins, and has been found to be a risk factor for the pathogenesis of atherosclerosis, coronary heart disease (CHD), and hepatic steatosis, and a component of metabolic syndrome [1,2]. Hypertriglyceridemia can be caused by multiple factors, including dietary, genetic, and life-style issues [3,4]. The prevalence of hypertriglyceridemia is also associated with the development of obesity and diabetes [5,6]. Peroxisome proliferator-activated receptor-alpha (PPARα) is a subtype of PPAR nuclear receptors that functions as a transcription factor. PPARα is activated by ligand binding and the active form can regulate the transcription of a series of target genes involved in fatty acid and glucose metabolism [7]. Hepatocytes, cardiomyocytes, enterocytes, and proximal tubule cells of the kidney are the major cells that express PPARα at high levels [8]. Pharmacological activation of PPARα has been shown to reduce the risk of cardiovascular disease by reducing plasma and cellular TG accumulation [9,10]. Fatty acid derivatives and metabolic substrates for acyl-coenzyme A oxidase are known to be natural PPARα ligands, and several synthetic PPARα agonists, the fibrate drugs, have been used in the treatment of dyslipidemia and other metabolic diseases [11,12]. Evidence suggests that fibrates may reduce the risk of cardiovascular disease by lowering plasma TG concentrations and reducing the number of LDL particles, although the effect is less potent than that of the statins [13,14]. Activation of PPARα increases lipolysis and the elimination of TG-rich particles from plasma by activating lipoprotein lipase [15] and may induce HDL particle production, by stimulating apolipoprotein A1 (ApoA1) gene expression leading to more HDL particles [16]. This, in turn, mediates reverse cholesterol transport and removal of redundant cholesterol from subendothelial macrophages [17].

We previously screened approximately 900 Korean natural plant and marine organism extracts for PPARα agonistic activity using a reporter gene assay. We found that a methanol extract of

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Catalpa ovata potently activated PPARα. Subsequent purification of the single active compound and structural determination by liquid chromatography–mass spectrometry-based dereplication with the *C. ovata* extract resulted in the identification of catalposide (Fig. 1A, MW = 482.45), which has been reported to have anti-inflammatory and anti-oxidant effects [18,19]. In this study, we investigated the ligand binding activity of PPARα and the mechanism of catalposide in hepatic lipid metabolism.

2. Materials and methods

2.1. Purification and identification of catalposide from Catalpa ovata

The dried stem of *C. ovata* (3.0 kg) was extracted with MeOH at room temperature. After removal of the solvent under vacuum, the resulting extract was suspended in H_2O and then partitioned with *n*-hexane and CH_2Cl_2 consecutively. The CH_2Cl_2-soluble fraction, which showed the most potent PPAR activation, was subjected to silica gel column chromatography and eluted with a CH_2Cl_2–MeOH step gradient system to give six fractions (COS-A to COS-F). Fraction COS-E was further subjected to Sephadex LH-20 column chromatography and eluted with a H_2O–MeOH step gradient system to give five fractions (COSE-1 to COSE-5). Fraction COSE-3 was further purified by semi-preparative HPLC (Waters, YMC ODS H-80, 150 × 20 mm i.d., H_2O–MeCN = 62:38) to obtain catalposide (25.5 mg). Optical rotation and ESI-MS were obtained using a JASCO DIP-1000 polarimeter and LCQ Fleet mass spectrometer, respectively. ^1H and ^{13}C NMR spectra were recorded using a Bruker DRX 500 NMR spectrometer.

2.2. Cell culture

Human HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air/5% CO_2 [20].

2.3. Reporter gene assays

Experiments were performed according to method a reported previously [21]. HepG2 cells were seeded in 24-well plates at 1×10^5/well. The following day, the cells were co-transfected with the pGL3-PPRE3-TK-luc reporter vector, which contains the firefly luciferase gene under control of a PPRE, with an expression vector encoding full-length human PPARα and an expression vector encoding β-galactosidase using Hilymax (Dojindo Laboratories, Rockville, MD, USA). The medium was removed after a 4-h incubation and replaced with high-glucose DMEM. After 18 h, cells were treated with catalposide (4 or 0.8 μM) or fenofibrate (10 μM) for 24 h and lysed in Firefly Luciferase Lysis Buffer (Biotium, Inc., Hayward, CA, USA). The luciferase activity in the cell lysate was measured using a Firefly Luciferase Assay Kit (Biotium), according to the manufacturer's protocol. The β-galactosidase activity was determined using the β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA). Luciferase activity was normalized to that of β-galactosidase in the same lysate.

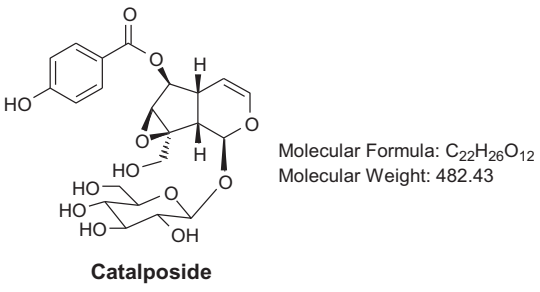

Fig. 1. Chemical structure of catalposide isolated from *Catalpa ovata*.

2.4. Time-resolved fluorescence resonance energy transfer (TR-FRET) assay for PPAR-α ligand-binding activity

TR-FRET assays for PPAR-α were performed with a commercial kit from Invitrogen (Madison, WI, USA). Various concentrations of catalposide and fenofibrate were incubated with a solution containing the ligand binding domain (LBD) of PPARα and a co-activator peptide, fluorescein-PGC1. After incubation for 2 h at room temperature, the samples were analyzed on a Spectra Max instrument with time-resolved fluorescence laser excitation at 340 nm, emission at 495 nm, and PPARα binding detection at 520 nm. The ratio of the emission signals at 520 and 495 nm was plotted against the log of the ligand concentration to generate a binding curve. To determine the concentration required to produce a 50% effect (EC_{50}), the data were fitted to a sigmoidal dose–response curve (varying slope) using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

2.5. Fatty acid uptake assay

HepG2 cells were plated in six-well plates at a density of 10^5/well and grown for 48 h. A fluorescence-labeled fatty acid (C1-BODIPY 500/510 C12; 4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid; 2 μM in Hank's buffered salt solution containing 0.1% bovine serum albumin) was added to each well together with a catalposide or fenofibrate solution and the culture plates were incubated for 1 min at 37 °C. The assay was terminated by the addition of ice-cold Hank's buffered salt solution containing 0.2% bovine serum albumin to each sample. The cells were resuspended in FACS buffer and analyzed by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA) by determining FL-1 fluorescence. Data were analyzed using the Cell Quest Pro software (BD Biosciences) [21].

2.6. Cellular TG and cholesterol measurement

HepG2 cells were seeded in six-well plates at density of 10^5/well. After washing with PBS, cells were treated with mixture of hexane and isopropanol (2:1) for 30 min. The cellular extracts were evaporated using a Speed Vac (*n*-Biotech, Korea), then dissolved in 95% ethanol. Intracellular TG and cholesterol concentrations were quantified using an enzymatic method and a Cobas C111 automatic analyzer (Roche Diagnostic Systems Inc., Indianapolis, IN, USA). Lipid levels were normalized to the total cellular protein concentration, as determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA) [22].

2.7. Lipid staining

HepG2 cells were fixed in 10% formalin for 10 min at room temperature. The formalin was then discarded and replaced with fresh formalin for at least 1 h. Cells were washed with PBS twice and incubated with 60% isopropanol for 5 min. The solvents were aspirated and cells were dried under a hood. Oil red O working solution (Sigma) was added and incubated for 12 h. Images were acquired using an inverted microscope (Eclipse T*i*-s, Nikon). For fluorescence images of intracellular lipids, 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) dye was added without a fixation step. Fluorescence-labeled dye was incubated for 20 min and washed for 30 min. Images were acquired using a confocal laser scanning microscope (LSM 5 Exciter, Carl-Zeiss).

2.8. Total RNA extraction

Total RNA was isolated using a total RNA extraction reagent (RNAiso Plus; Takara Bio Inc., Shiga, Japan). After the addition of 1 mL of RNA extraction reagent, the samples were incubated for 5 min at room temperature (RT). The samples were extracted with 200 μL chloroform; the hydrophilic layer was collected and the RNA was precipitated with isopropanol. The RNA pellets were washed with 75% ethanol and dried at RT. The RNA concentration was measured spectrophotometrically (Smart Spec Plus; Bio-Rad).

2.9. Reverse transcription and quantitative PCR (qPCR) analysis

For cDNA synthesis, total RNA (2 μg) was incubated with 1 μL of oligo(dT) primer (50 pmol) and 4 μL of dNTP mixture (2.5 nM) in Rnase-free water at 65 °C for 15 min, and then mixed with 1 μL of reverse transcriptase (Mbiotech, Seoul, Korea), 5 × buffer, and 5 μL of Rnase-free water, followed by incubation at 42 °C for 60 min and 70 °C for 15 min. The synthesized cDNA was amplified by real-time PCR (iCycler iQ5; Bio-Rad) using qPCR premix solution containing SYBR Green (SYBR Premix Ex Taq II; Takara) and appropriate primers. Amplification was performed with an initial denaturation step at 95 °C for 30 s, followed by 60 cycles of denaturation at 95 °C for 10 s, annealing at 55–61 °C for 15 s, and extension at 68 °C for 20 s. The fluorescence signal was detected automatically at the end of each PCR cycle [23].

2.10. Statistics

Data are expressed as means ± standard error of the mean (SEM). Differences were considered statistically significant at $P < 0.05$, assessed using Student's *t*-test.

3. Results and discussion

3.1. Structural analysis of catalposide

The active principle was obtained as a white amorphous powder with physico-chemical properties of $[\alpha]_D^{20} = -156°$ (*c* 0.5, MeOH); ESI-MS *m/z*: 483 $[M+H]^+$; ^{1}H NMR (500 MHz, CD_3OD) δ: 7.97 (2H, d, *J* = 8.5 Hz, H-2″, 6″), 6.89 (2H, d, *J* = 8.5 Hz, H-3″, 5″), 6.41 (1H, dd, *J* = 1.5, 5.9 Hz, H-3), 5.24 (1H, d, *J* = 9.1 Hz, H-1), 5.16 (1H, dd, *J* = 1.0, 7.0 Hz, H-6), 5.04 (1H, dd, *J* = 4.1, 6.0 Hz, H-4), 4.84 (1H, d, *J* = 7.9 Hz, H-1′), 4.22 (1H, d, *J* = 13.2 Hz, H-10a), 3.97 (1H, dd, *J* = 1.9, 12.0 Hz, H-6′a), 3.89 (1H, d, *J* = 13.2 Hz, H-10b) 3.69 (1H, dd, *J* = 6.7, 12.0 Hz, H-6′b), 3.79 (1H, d, *J* = 1.0 Hz, H-7), 3.25–3.48 (4H, m, H-2′, 3′, 4′, 5′), 2.71 (1H, m, H-5), 2.67 (1H, m, H-9); ^{13}C NMR (125 MHz, CD_3OD) δ: 167.9 (C═O), 163.8 (C-4″), 142.4 (C-3), 133.0 (C-2″, 6″), 121.8 (C-1″), 116.3 (C-3″, 5″), 103.0 (C-4), 99.7 (C-1′), 95.1 (C-1), 81.6 (C-6), 78.7 (C-3′), 77.7 (C-5′), 74.9 (C-2′), 71.8 (C-4′), 66.8 (C-8), 62.9 (C-6′), 61.3 (C-10), 60.3 (C-7), 43.2 (C-9), 36.8 (C-5). The structure of catalposide (Fig. 1) was confirmed by comparison of its physico-chemical and spectroscopic data with those reported previously [18,19,24].

3.2. Catalposide is an agonistic ligand for PPARα

The PPARα agonistic activity of catalposide was examined using a reporter gene assay in HepG2 cells. In the assay, catalposide activated PPARα activity by 57% (0.8 μM) and 80% (4 μM), compared with the controls ($P < 0.01$ in both; Fig. 2A). Gene expression levels of PPARα were also induced by catalposide stimulation in hepatocytes (Fig. 2B).

PPARα transactivation could be achieved by either direct ligand binding or indirect induction of endogenous ligand synthesis. Thus, we further investigated direct ligand binding of catalposide to PPARα with a cell-free FRET assay. TR-FRET quantifies ligand-dependent coactivator recruitment on the PPARα-ligand binding domain (LBD). Both fenofibrate and catalposide increased the TR-FRET signal by enhancing the recruitment of fluorescein-labeled coactivator peptide to PPAR-LBD. The EC_{50} were 1 nM and 5.2 μM for fenofibrate and catalposide, respectively. These results demonstrated that catalposide is a ligand for PPARα, interacting with PPARα-LBD directly although the binding was considerably weaker than that of fenofibrate (Fig. 2C).

3.3. Catalposide increased fatty acid uptake in hepatocytes

PPARα regulates cellular lipid metabolism through the induction of fatty acid uptake and fatty acid β-oxidation in hepatocytes, resulting in reduced cellular and plasma TG concentrations. Accordingly, synthetic and natural PPARα agonists could ameliorate hypertriglyceridemia. Lipid reduction could be achieved by the regulation of PPARα target gene expression in hepatic lipid metabolism.

First, we investigated whether catalposide could induce hepatic fatty acid uptake and reduce lipid concentrations. We quantified cellular fatty acid uptake in HepG2 cells with a FACS-based assay using fluorescently labeled fatty acid (Fig. 3A). Stimulation of cells with fenofibrate or catalposide induced fatty acid uptake significantly ($P < 0.01$), by 43 and 70%, respectively. Additionally, FATP4 expression was upregulated at 48 h in cells stimulated with catalposide, by 19% compared with controls (Fig. 3B). FATP4 is a PPARα target gene and the FATP4 protein is known as a major fatty acid transporter in hepatocytes [25]. These data suggest that catalposide induces hepatic fatty acid uptake by upregulation of FATP4 in hepatocytes.

3.4. Catalposide reduced lipid concentration in hepatocytes

The effects of catalposide on cellular lipid levels were investigated. Intracellular cholesterol concentrations tended to decline in cells stimulated with catalposide, and cells incubated with fenofibrate showed a significant reduction (Fig. 3C). Catalposide stimulation in HepG2 cells reduced intracellular TG concentrations significantly, by 14% ($P < 0.05$) at 0.8 μM and 21% at 4 μM ($P < 0.01$). Indeed, the effect was comparable to that of fenofibrate (−15%, $P < 0.05$, Fig. 3C). Two types of lipid staining results showed similar trends (Fig. 3D).

Fatty acids removed from circulation are subsequently synthesized into hepatic TG for storage. Stearoyl-CoA desaturase 1 (SCD1) is the lipogenic enzyme that converts palmitoyl- and stearoyl-CoA into palmitoleoyl- and oleoyl-CoA. These monounsaturated fatty acid metabolites are major substrates for the biosynthesis of phospholipids, TG, and cholesteryl esters [26]. Catalposide reduced the expression of SCD1, by 22%, suggesting inhibition of the fatty acid synthetic pathway (Fig. 4). TG is lipolysed to fatty acids in the fasting state to sustain gluconeogenesis, and LPL is the lipase responsible for hepatic TG hydrolysis [27]. The resulting free fatty acids are subsequently converted into acyl CoA, the initial substrate for β-oxidation by carnitine palmitoyltransferase 1 (CPT1) in the mitochondrial membrane. Acyl-CoA oxidase (ACOX) transforms the fatty acyl-CoA to acetyl-CoA, which enters the TCA cycle [28]. PPARα activated by catalposide markedly increased the expression of LPL, CPT1, and ACOX, by 78%, 66%, and 59%, respectively (Fig. 4). Additionally, uncoupling proteins 2 (UCP2), a target gene of PPARα expressed in the mitochondrial membrane that mediates thermogenesis, was up-regulated in cells stimulated significantly with catalposide treatment (39%); this could contribute to the reduction in hepatic TGs (Fig. 4) [29]. Collectively, these gene expression results suggest the reduction in

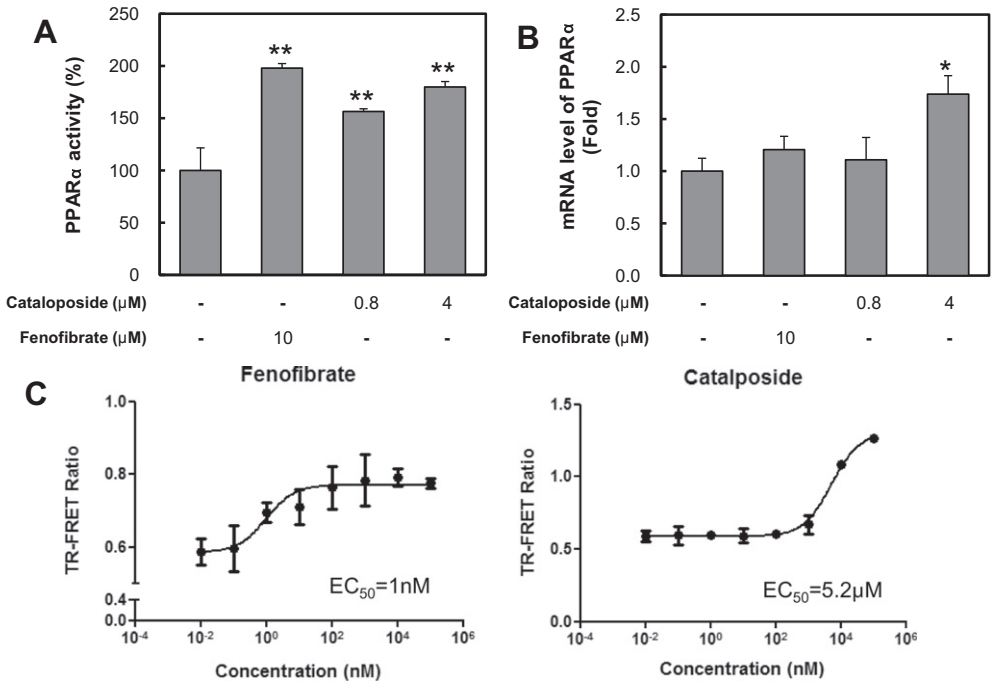

Fig. 2. Catalposide is an agonistic ligand of PPARα. (A) PPARα activation assessed using a reporter gene assay in HepG2 cells stimulated with catalposide. (B) mRNA expression of PPARα in cells stimulated with catalposide for 48 h. Levels of mRNAs were assessed by qRT-PCR and normalized to cyclophilin mRNA level, as described in the Methods. (C) Cell-free TR-FRET assay with fenofibrate and catalposide. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. controls (no treatment). Data are means ± SEM.

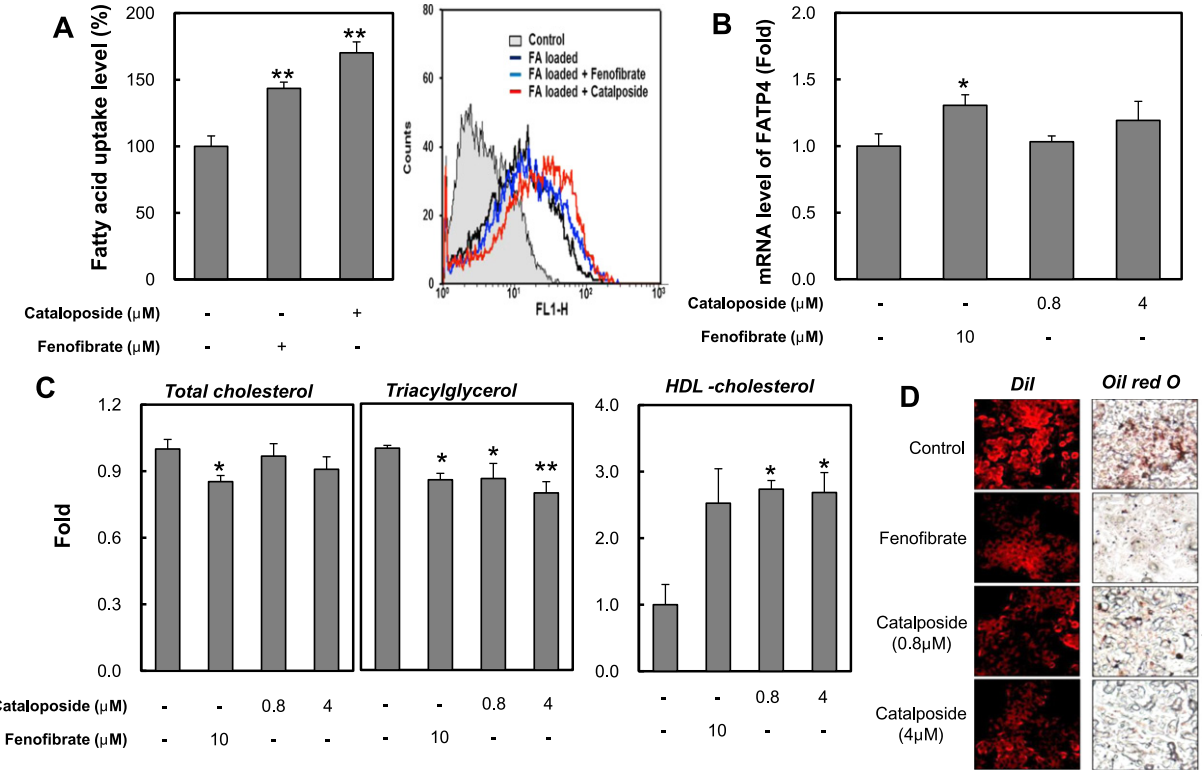

Fig. 3. Effects of catalposide on hepatic lipid accumulation. (A) FACS-based cellular fatty acid uptake using BODIPY-labeled fatty acids. (B) mRNA levels of FATP4. HepG2 cells were stimulated with catalposide for 48 h before RNA extraction. Levels of mRNAs were assessed by qRT-PCR and normalized to the cyclophilin mRNA level. (C) Cellular TG, total cholesterol, and HDL cholesterol concentrations in HepG2 cells were measured enzymatically. (D) Lipid staining of HepG2 incubated with fenofibrate and catalposide shows reduction of cellular lipid stained with DiI or Oil red O. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. control (no treatment). Data are means ± SEM.

hepatic TG concentrations is the result of induction of the hepatic fatty acid oxidation and thermogenesis pathways.

Additionally, HDL cholesterol concentrations in the media of the cells stimulated with catalposide and fenofibrate were increased significantly. Results showed that cholesterol and Apo A1, the main protein of high-density lipoprotein (HDL) and secreted by the liver, was expressed significantly higher in the catalposide group, by 36%. Apo A1 forms discoidal pre-β-HDL and starts the first step

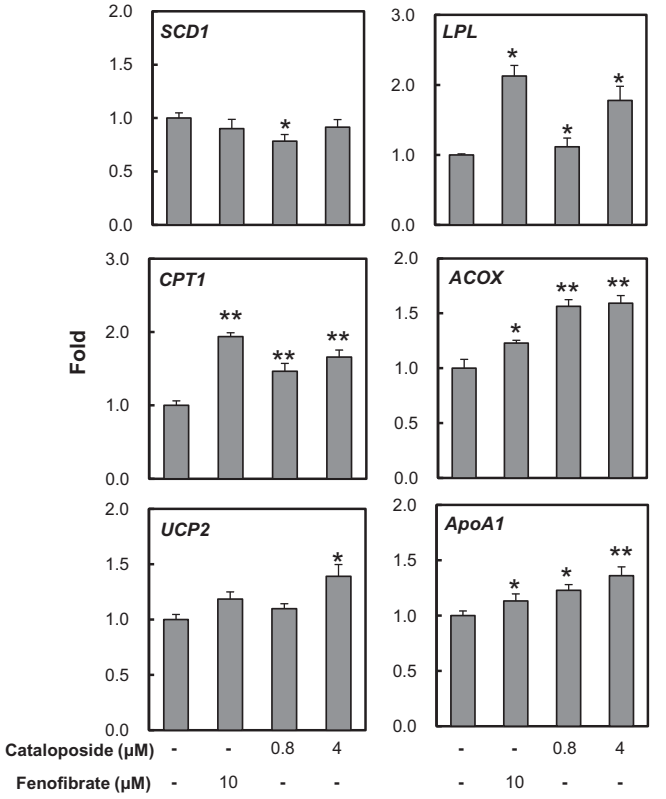

Fig. 4. The expression of PPARα target genes in hepatocytes. Expression levels of lipid metabolism genes were assessed with qRT-PCR in cells stimulated with fenofibrate or catalposide. Levels of mRNAs were assessed by qRT-PCR and normalized to the cyclophilin mRNA level. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. the control (no treatment). Data are means ± SEM.

of the HDL particle formation cycle [30]. Additionally, catalposide increased cholesterol concentrations in the media and Apo A1 expression was upregulated in cells stimulated with catalposide.

In conclusion, catalposide is a novel natural ligand of PPARα that regulates hepatic lipid metabolism. Treatment with catalposide led to an increase in lipid uptake in hepatocytes and increased gene expression in β-oxidation, thermogenesis, and lipid hydrolysis, which may explain the reduction in hepatic TG concentrations.

Acknowledgments

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