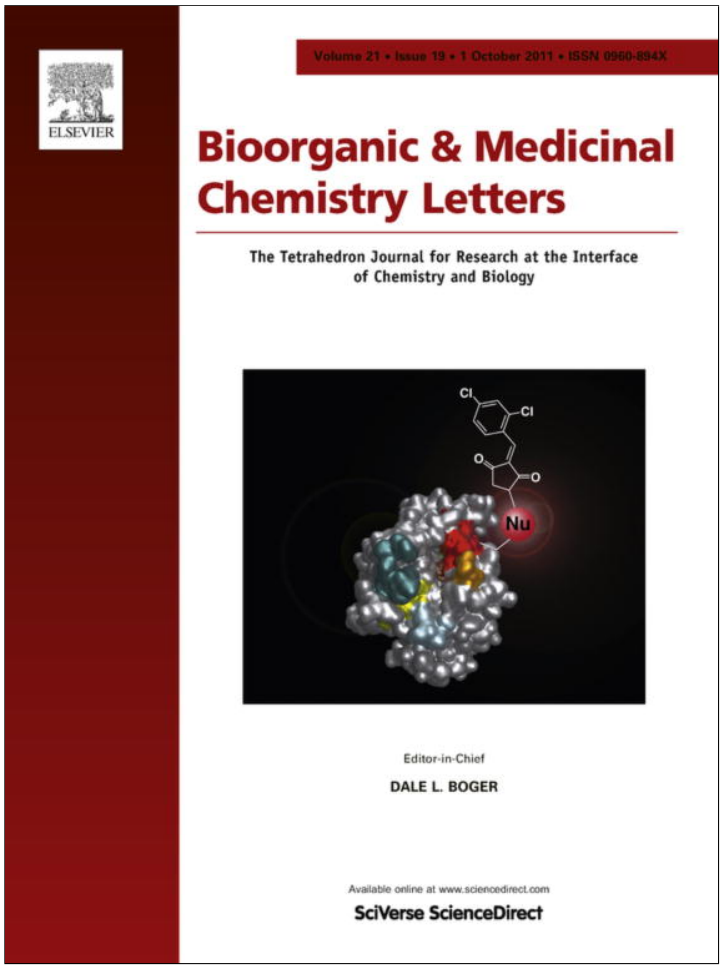

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Ursolic acid is a PPAR-α agonist that regulates hepatic lipid metabolism

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ABSTRACT

In this study, we confirmed that ursolic acid, a plant triterpenoid, activates peroxisome proliferator-activated receptor (PPAR)-α in vitro. Surface plasmon resonance and time-resolved fluorescence resonance energy transfer analyses do not show direct binding of ursolic acid to the ligand-binding domain of PPAR-α; however, ursolic acid enhances the binding of PPAR-α to the peroxisome proliferator response element in PPAR-α-responsive genes, alters the expression of key genes in lipid metabolism, significantly reducing intracellular triglyceride and cholesterol concentrations in hepatocytes. Thus, ursolic acid is a PPAR-α agonist that regulates the expression of lipid metabolism genes, but it is not a direct ligand of PPAR-α.

Hypertriglyceridemia is associated with type II diabetes, obesity, and metabolic syndrome; is an independent risk factor for cardiovascular disease,[1] which is a leading cause of mortality worldwide. Because the peroxisome proliferator-activated receptor (PPAR)-α protein, which is highly expressed in liver, kidney, heart, and muscle, plays a central role in the regulation of hepatic lipid metabolism, synthetic or natural PPAR-α agonists are attractive as potential therapeutic agents for treating hypertriglyceridemia.[2] PPAR-α is a transcription factor that regulates diverse aspects of lipid metabolism, including the induction of cellular fatty acid uptake and β-oxidation of fatty acids, thereby modulating hepatic fatty acid synthesis and plasma triglyceride and cholesterol concentrations.[3] It enhances the transcription of PPAR-α-responsive hepatic lipid-regulating genes[4] by binding to the peroxisome proliferator response element (PPRE) sequence in their promoter regions.[5,6] PPAR-α binds to the PPRE as part of a heterodimeric complex with retinoid X receptor (RXR)-α; this complex forms when PPAR-α is activated by the binding of natural or synthetic agonists.

In a reporter gene assay-based screen of approximately 800 Korean natural plant extracts for PPAR-α-agonistic activity, we identified that the ethyl acetate extract of *Actinidia arguta* (hardy kiwi vine) had potent PPAR-α agonist activity. Subsequent analysis of liquid chromatography–mass spectrometry-based dereplication with the *Actinidia arguta* extract, resulted in the identification of ursolic acid (Fig. 1A, MW = 456.7 g/mol), a pentacyclic triterpenoid found in many medicinal herbs and plants, as a possible source of the agonistic activity. Some research showed that there are several terpenoids having PPAR-α agonistic activity.[7] Several pharmacological effects, such as anti-tumor, anti-inflammatory, and antimicrobial activities, had already been attributed to ursolic acid.[3] Additionally, the potential PPAR-α-agonistic activity of ursolic acid was previously suggested by its ability to improve the epidermal permeability barrier function through PPAR-α activation.[8] However, in another study, PPAR-α-stimulated epidermal keratinocyte differentiation was not activated by ursolic acid.[9] In the current study, we investigated the PPAR-α agonistic activity of ursolic acid and examined its effect on hepatic lipid metabolism. Our findings confirm that ursolic acid is a PPAR-α agonist that has hypolipidemic effects in hepatocytes but does not bind directly to PPAR-α. It appears that ursolic acid induces the cellular synthesis of endogenous physiologic ligand for PPAR-α activation by promoting uptake of cellular fatty acids and lipoprotein lipase (LPL) upregulation as reported with some agonist.[10]

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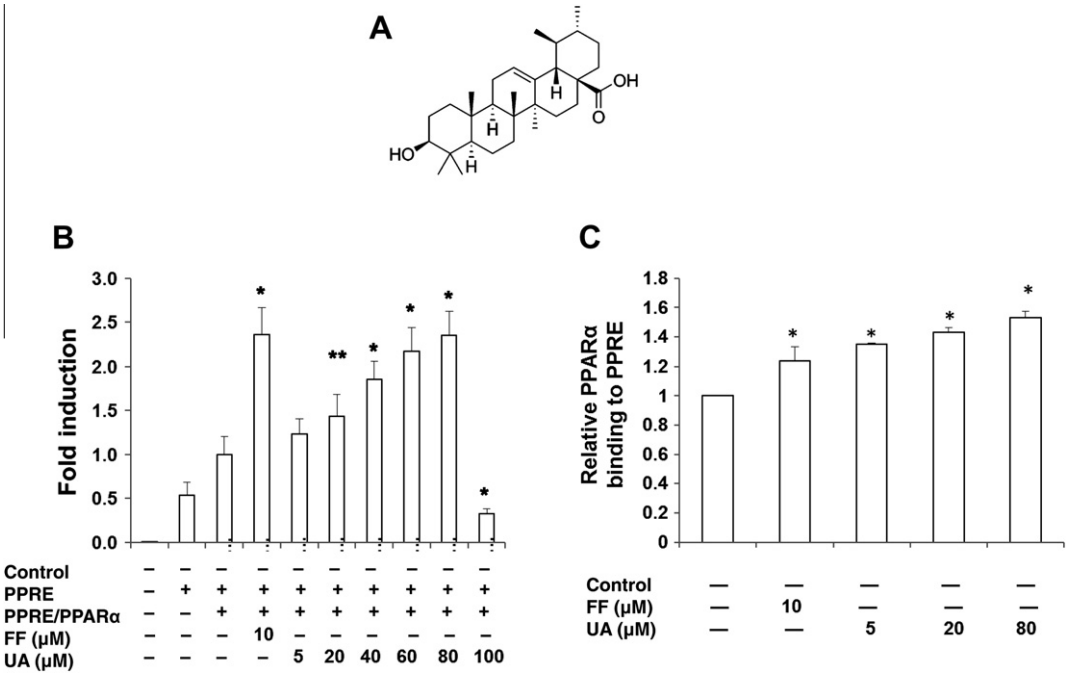

Figure 1. Ursolic acid induces PPAR-α activation and binding to the PPRE. (A) Structure of ursolic acid. (B) Ursolic acid activates PPAR-α transactivation activity. HepG2 cells were co-transfected with the pGL3-PPRE3-TK-luc reporter vector, a human PPAR-α expression vector, and a β-galactosidase expression vector. Then, luciferase activity was assayed and normalized to β-galactosidase activity.[12] (C) Ursolic acid enhances PPAR-α binding to a PPRE-containing DNA probe. The binding of activated PPAR-α protein to a PPRE probe was quantified using an ELISA-based assay.[14]

Reporter gene assays were performed in cultured HepG2 cells[11] to measure the activation of PPAR-α transcriptional activity were performed in HepG2 cells co-transfected with a PPAR-α expression vector and a PPRE-driven luciferase reporter-gene construct.[12] In a previous study using PPAR-α-expressing CV1 and HaCAT cells, ursolic acid at 1 or 10 μg/mL did not significantly increase PPRE-driven luciferase reporter activity, although PPAR-α transactivation activity did increase.[9] In our study, ursolic acid treatment of the cells increased the luciferase reporter activity in a significant, dose-dependent manner (+214% at 80 μM; P <0.01 vs. control; Fig. 1B), and the EC_{50} of ursolic acid was 41.31 μM, as would be expected for a PPAR-α agonist.[13]

The binding of activated PPAR-α to the PPRE was quantified using an enzyme-linked immunosorbent assay (ELISA)-based technique.[14] In a cell-free binding assay using nuclear extracts prepared from treated HepG2 cells, treatment with 20 μM ursolic acid increased PPAR-α binding to the PPRE DNA sequence by 46%, demonstrating that ursolic acid is a PPAR-α agonist (Fig. 1C). We next performed time-resolved fluorescence resonance energy transfer (TR-FRET) and surface plasmon resonance (SPR) experiments to examine whether ursolic acid directly interacts with the PPAR-α LBD as described in Supplementary data. First, we measured the ability of ursolic acid and fenofibric acid (FF, 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid, molecular mass 318.75 g/mol) to enhance the recruitment of recombinant LBD to a fluorescein-labeled coactivator peptide by quantifying the increase in the Lanthascreen TR-FRET signal. As expected, FF substantially increased recruitment of the fluorescein-labeled PGC1α co-activator peptide to the LBD in a dose-dependent manner (EC_{50} = 4.5 μM; Fig. 2A). In contrast, ursolic acid did not significantly affect recruitment of the co-activator peptide (Fig. 2B), demonstrating that ursolic acid does not directly activate PPAR-α. We also performed Biacore SPR analysis to investigate whether ursolic acid directly interacts with the PPAR-α LBD. This analysis also demonstrated significant binding of FF to the LBD but failed to show a significant association between ursolic acid to the LBD, strongly suggesting that ursolic acid is not a direct ligand for PPAR-α; rather, it activates PPAR-α via an indirect mechanism (Fig. 2C).

Activation of PPAR-α was previously reported to reduce intracellular triglyceride levels.[15] Moreover, ligand-mediated PPAR-α activation was reported to reduce cholesterol levels by down-regulating nuclear translocation of sterol regulatory element-binding protein-2 (SREBP-2), probably via upregulation of the Insig protein.[16] Therefore, we next investigated the effect of ursolic acid treatment on the intracellular triglyceride and cholesterol content of HepG2 cells.[17] Ursolic acid treatment at 5, 20, and 80 μM significantly reduced the triglyceride content (P <0.01) by 23.5%, 38.8%, and 44.7%, respectively, relative to the control treatment. It also significantly reduced the intracellular cholesterol content by 11.0%, 18.9%, and 26.5%, respectively, relative to the control. These results suggest that the PPAR-α-agonistic property of ursolic acid has a hypolipidemic effect in hepatocytes (Fig. 3A and B).

Enhanced hepatic uptake of circulating fatty acids is protective against atherosclerosis, coronary heart disease, and insulin resistance.[18] Stimulation of cells with a PPAR-α agonist has been shown to enhance hepatic fatty acid uptake by inducing the expression of genes involved in this process.[19] Here, we quantified the uptake of a BODIPY-labeled fatty acid in HepG2 cells using FACS analysis.[20] The average cellular fatty acid uptake increased in a dose-dependent manner as the concentration of ursolic acid increased (Fig. 3C). Based on the average fluorescence intensity of the cells, treatment with 80 μM ursolic acid increased fatty acid uptake significantly (P <0.01), by 108.7% (Fig. 3D). This increase in uptake was associated with significant upregulation of the fatty acid transport protein 4 (FATP4) gene which is a known PPAR-α target gene and a major fatty acid transporter in hepatocytes[21]; treatment with 20 or 80 μM ursolic acid increased the level of FATP4 mRNA by 185% and 259%, respectively (Fig. 3C and D).

Next, we performed quantitative PCR analysis.[22] Ursolic acid treatment also significantly activated PPAR-α gene expression in HepG2 cells (Fig. 4; P <0.05 or P <0.01). At ursolic acid concentrations of 5, 20, and 80 μM, the PPAR-α mRNA level increased by 134%, 156%,

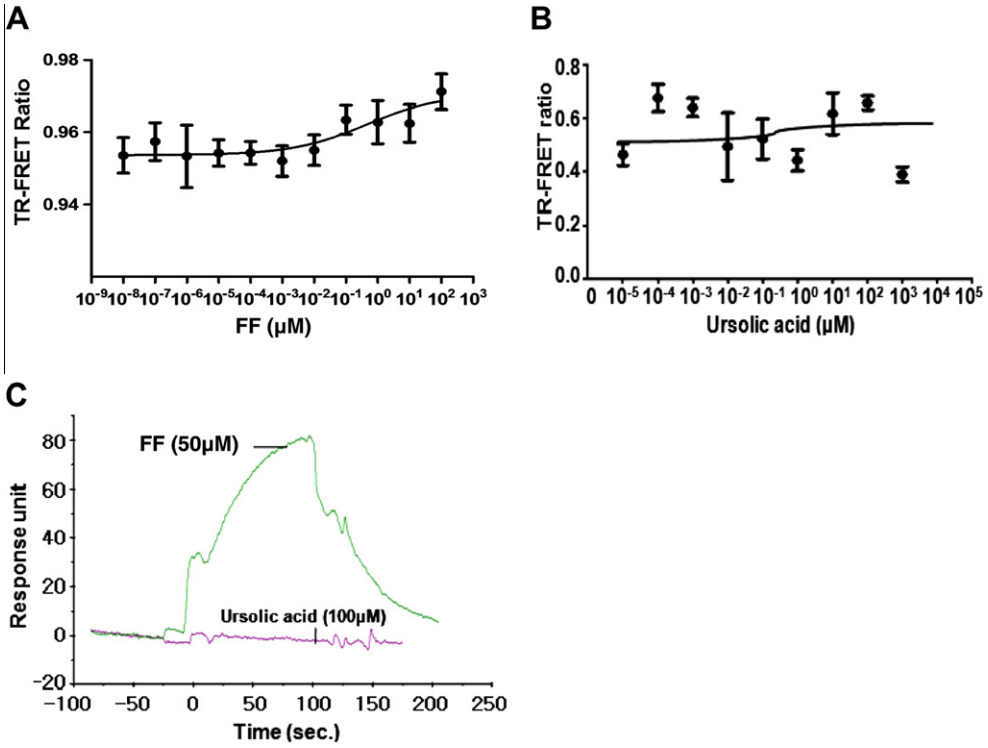

Figure 2. Ursolic acid did not directly interact with the ligand-binding domain of PPAR-α. (A) LanthaScreen TR-FRET assay with FF as a putative PPAR-α ligand. This assay was the positive control for the experiment shown in (B). (B) LanthaScreen TR-FRET assay with ursolic acid as a putative PPAR-α ligand. (C) The direct interactions between ursolic acid (100 μM) or FF (100 μM) and the immobilized PPAR-α-LBD were examined by SPR on a Biacore instrument. Data shown represent means ± SE; $^{*}P$ <0.01 and $^{**}P$ <0.05 versus control (n = 3).

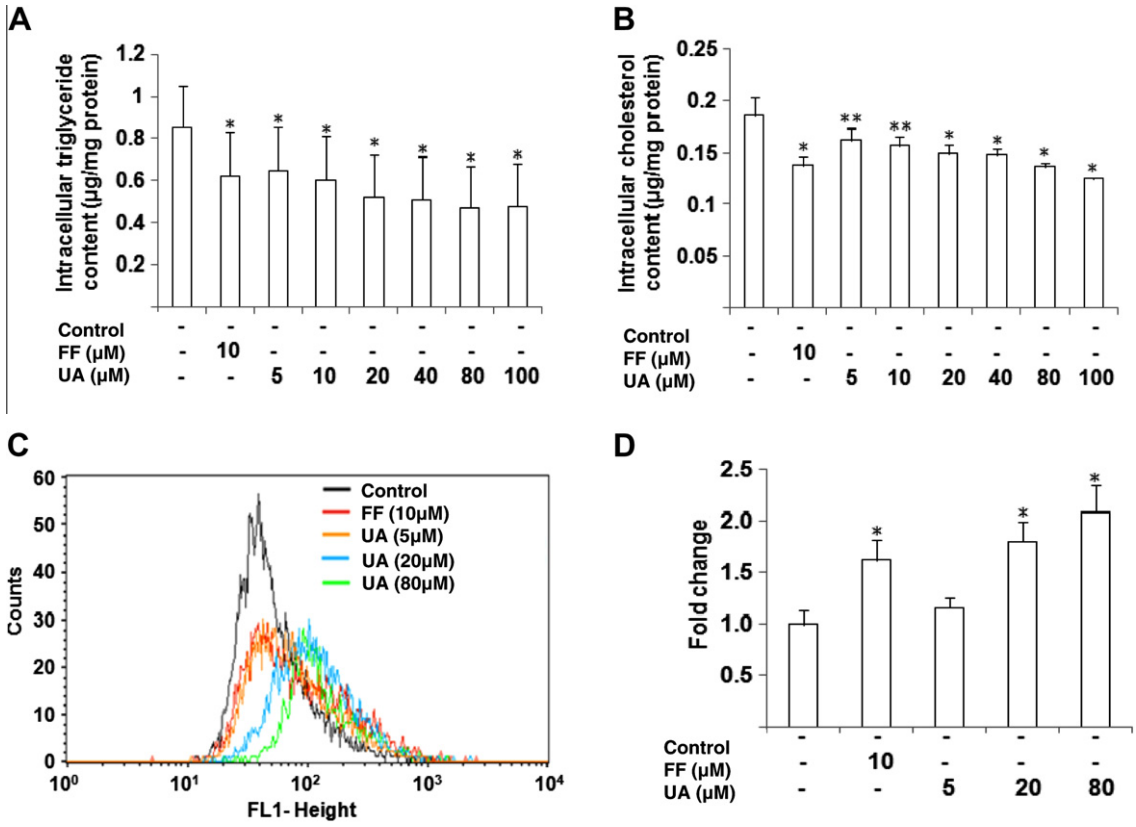

Figure 3. Ursolic acid reduces intracellular lipid levels and induces fatty acid uptake in HepG2 cells. (A) Intracellular triglyceride concentrations. (B) Intracellular cholesterol concentrations. (C) and (D) Fatty acid uptake was quantified using a BODIPY-labeled fatty acid and FACS analysis.[20] (C) FACS profile of fatty acid uptake. (D) Relative fatty acid uptake. Data shown represent means ± SE. $^{*}P$ <0.01 and $^{**}P$ <0.05 compared with control (n = 3).

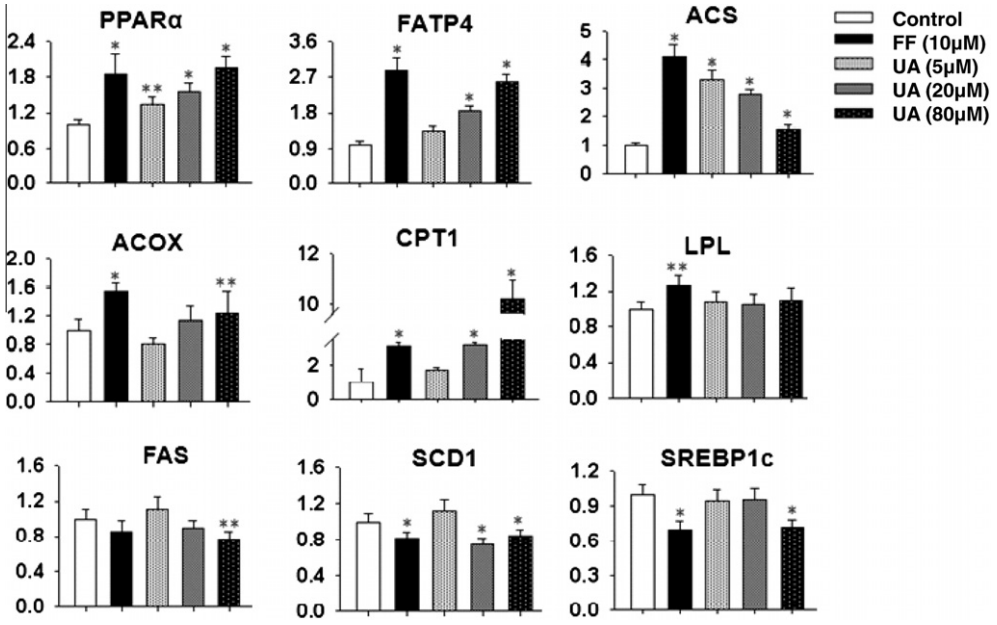

Figure 4. Ursolic acid-induced changes in hepatocyte gene expression. Expression of lipid metabolism-related genes in ursolic acid-stimulated hepatocytes. Levels of mRNA for genes involved in lipid metabolism were assessed using qPCR and iQ5 optical system software from Bio-Rad Laboratories, Inc. Results are normalized to the GAPDH mRNA level. $^{*}P$ <0.01 and $^{**}P$ <0.05 compared with control (n = 3).

and 195%, respectively, suggesting that ursolic acid mediates expression of PPAR-α in hepatocytes. The PPAR-α-agonistic activity and hypolipidemic effects of ursolic acid led us to investigate whether it also regulates the transcription of PPAR-α target genes involved in lipid metabolism. Using qPCR, we quantified the effect of ursolic acid treatment of HepG2 cells on the expression of the genes encoding two key proteins in hepatic fatty acid synthesis, acyl-CoA synthetase (ACS), which mediates the esterification of fatty acids to keep them from escaping the cell,[21] and carnitine palmitoyl transferase (CPT1), which is responsible for mitochondrial transport of activated acyl-CoA esters. Ursolic acid significantly (P <0.01) increased the expression of the ACS and CPT1 genes (Fig. 4). At 80 μM, ursolic acid increased CPT1 gene expression by 1021%, consistent with a report that PPAR-α up-regulates CPT1 gene expression through a functional PPRE in the promoter region of the gene.[23]

PPAR-α promotes the peroxisomal β-oxidation of acyl-CoA esters by acyl-CoA oxidase (ACOX)[24] and reduces de novo fatty acid synthesis by blocking fatty acid synthase (FAS).[24] We found that treatment with 80 μM ursolic acid increased ACOX gene expression by 24% (P <0.05) and decreased FAS gene expression by 23% (P <0.05); both of these effects would tend to reduce fatty acid levels. On the other hand, it had no significant effect on the expression of the gene for LPL, which hydrolyzes triglycerides. However, the ursolic acid-induced reduction in intracellular triglyceride content observed in our study might be achieved through an alternative mechanism in which a reduction in stearoyl-CoA desaturase (SCD1) expression reduces adiposity and liver steatosis.[25] Indeed, 80 μM ursolic acid treatment decreased SCD1 gene expression by 15% (P <0.01).

PPAR-α activation can regulate lipogenesis by suppressing the LXR-SREBP1c pathway via a reduction in the formation of the LXR/RXR complex.[26] In our experiments, 80 μM ursolic acid treatment reduced SREBP1c expression by 28% (P <0.01; Fig. 4), indicating that the suppression of major lipogenic, SREBP1c target genes, including FAS and SCD1, may account for the reduction in intracellular cholesterol and triglyceride levels. PPAR-α agonists, fibrates, are potent hypolipidemic drugs and have been used increasingly to treat cardiovascular disease. PPAR-α agonists lower plasma triglyceride levels markedly and also increase high-density-lipoprotein (HDL) level. The former effect occurs by stimulating hepatic fatty acid oxidation and reducing apoCIII expression, whereas the latter effect is due to induction of apolipoprotein-AI and apolipoprotein A-II expression, both mediated by PPAR-α. PPAR-α agonists may also have a hypoglycemic and thus anti-diabetic effect, as a consequence of their hypolipidemic action, thus future studies will have to establish whether PPAR-α agonist may be applicable in the treatment of type II diabetes.

In this work, ursolic acid has been confirmed to be a novel PPAR-α agonist. We conclude that ursolic acid increases the binding of activated PPAR-α to PPRE, thereby regulating the transcription of PPAR-α target genes involved in cellular lipid metabolism. It also reduces cellular triglyceride and cholesterol levels in hepatocytes, probably by increasing fatty acid uptake and oxidation and by inhibiting fatty acid synthesis. This is first report to show ursolic acid effect on lipid metabolism. However, our SPR and co-activator recruitment analyses demonstrate that ursolic acid is not a direct ligand for PPAR-α. It is possible that ursolic acid induces the synthesis of endogenous ligands for PPAR-α thus activates PPAR-α. It has been shown that the stimulation of LPL in hepatocytes, as shown by ursolic acid treatment, activates PPAR-α due to the increased synthesis of endogenous ligands for PPAR-α.[10] Finally, although the PPAR-α agonistic activity of ursolic acid is relatively weak compared with synthetic ligands, one can synthesize a more potent hit compound based on the ursolic acid chemical structure. Alternatively, ursolic acid is a natural phytochemical that could be consumed as food supplements or functional foods. For the use of supplement or functional food, relatively weak activity may not be a problem if its safety is insured.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.07.095.

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11. HepG2 cells were cultured as previously described **21**, A. s.-c., the monolayer was washed with phosphate-buffered saline and then incubated for 24 h in serum-free, high-glucose Dulbecco's minimal Eagle medium (DMEM) containing ursolic acid (5, 20, or 80 μM; Sigma-Aldrich, St. Louis, MO, USA), the PPAR-α ligand FF (10 μM; Sigma-Aldrich; positive control), or dimethyl sulfoxide (1%; DMSO; vehicle control).
12. HepG2 cells were seeded in 24-well plates at 1×10^5 cells per well. The following day, the cells were co-transfected with the reporter vector pGL3-PPRE3-TK-luc (which contains the firefly luciferase gene under control of a PPRE), with an expression vector encoding full-length human PPAR-α and an expression vector encoding β-galactosidase using Hilymax (Dojindo Laboratories, Rockville, MD USA). The medium was removed after 4 h of incubation and replaced with high-glucose DMEM. After 18 h, the cells were treated with ursolic acid (0, 5, 20, 40, 60, 80, or 100 μM) or FF (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. β-Galactosidase activity was determined using the β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA). To normalize the results for transfection efficiency, luciferase activity was expressed relative to the β-galactosidase activity in the same lysate.
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14. Nuclear extracts from ursolic acid- or FF-treated HepG2 cells were isolated with a Nuclear Extraction Kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions. After protein concentrations in the nuclear extracts were determined using a BCA protein assay kit (Pierce Biotechnology, Cramlinton, UK), PPAR-α binding assays were performed using a PPAR-α transcription factor assay kit (Cayman). Briefly, 40-μg aliquots of extract were placed into the wells of an assay plate coated with a PPRE-containing double-stranded DNA probe. The plate was incubated overnight at 4 °C and then washed. An anti-PPAR-α primary antibody was added to the wells, and the plate was incubated at room temperature for 1 h. After washing, a horseradish peroxidase-conjugated goat anti-rabbit secondary was added to the wells. The plate was incubated at room temperature for 1 h and then washed. After addition of developing solution, the absorbance of the solution at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amount of PPAR-α protein bound to the PPRE-containing probe was quantified using a standard curve.
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20. HepG2 cells were plated in 6-well plates at a density of approximately 10^5 cells/well and grown for 24 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 ursolic acid or FF solution or 1% DMSO 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 25. The data were analyzed using Cell Quest Pro software (BD Biosciences).
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