Bioorganic & Medicinal Chemistry Letters 24 (2014) 3168–3174

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

trans-Caryophyllene is a natural agonistic ligand for peroxisome proliferator-activated receptor-α

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ARTICLE INFO

Article history:
Received 28 September 2013
Revised 13 April 2014
Accepted 28 April 2014
Available online 6 May 2014

Keywords:
trans-Caryophyllene
PPAR-α
Hepatocyte
Lipid metabolism

ABSTRACT

Intake of dietary aroma compounds may regulate cellular lipid metabolism. We demonstrated that *trans*-caryophyllene, a flavor compound in plant foods and teas, activates peroxisome proliferator-activated receptor (PPAR)-α through direct interaction with the ligand-binding domain of PPAR-α. The agonistic activity of *trans*-caryophyllene was investigated by the luciferase reporter assay, surface plasmon resonance, and time-resolved fluorescence resonance energy transfer assay. Following the stimulation of cells with *trans*-caryophyllene, intracellular triglyceride concentrations were significantly reduced by 17%, and hepatic fatty acid uptake was significantly increased by 31%. The rate of fatty acid oxidation was also significantly increased. The expressions of PPAR-α and its target genes and proteins in fatty acid uptake and oxidation were significantly up-regulated as well. In HepG2 cells transfected with small interfering RNA of PPAR-α, the effects of *trans*-caryophyllene on PPAR-α responsive gene expressions, intracellular triglyceride, fatty acid uptake and oxidation were disappeared. These results indicate that the aroma compound, *trans*-caryophyllene, is PPAR-α agonist thus regulates cellular lipid metabolism in PPAR-α dependent manners.

Hypertriglyceridemia (HTG) is an independent risk factor for atherosclerosis and coronary heart disease, the leading causes of death worldwide.[1,2] HTG can be caused by various interactions between genetic and environmental factors, and is usually associated with diabetes, obesity, chronic renal failure, infection or stress.[3,4] Metabolic turnover studies indicated that HTG mainly arises from the overproduction of or the defective clearance of plasma triglyceride (TG)-rich lipoproteins.[5,6]

Peroxisome proliferator-activated receptors (PPARs) are members of the superfamily of nuclear transcription factors, which regulate lipid metabolism and glucose homeostasis and play a role in cellular proliferation, differentiation and apoptosis.[7–9] Activated PPARs can heterodimerize with the retinoid X receptor (RXR) and alter the transcription of target genes through binding to PPAR response elements located in the promoter of target genes.[10,11] PPARs are composed of three different PPAR subtypes termed PPAR-α, PPAR-δ/β and PPAR-γ. PPAR-α is widely expressed in the liver, kidney, muscle and heart and regulates diverse aspects of lipid metabolism by regulating the expression of genes involved in hepatic fatty acid uptake and fatty acid oxidation, thereby modulating plasma TG and cholesterol concentrations.[12,13] PPAR-α is a ligand-activated transcription factor and can be activated by a structurally diverse group of compounds including fibrates, fatty acids and eicosanoids.[14] PPAR-α agonists have been shown to be effective in lowering plasma TG levels by decreasing hepatic TG synthesis and increasing circulating TG clearance.[15] As a consequence, PPAR-α agonists, such as fibrates, are widely used in the treatment of HTG in humans.[16]

We hypothesized that aroma compounds with pleasant scents are bioactive and regulate cellular metabolism when ingested.

Abbreviations: PPAR, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; SPR, surface plasmon resonance; TR-FRET, time-resolved fluorescent resonance energy transfer; TG, triglyceride; HTG, hypertriglyceridemia; RXR, retinoid X receptor; EO, essential oils; LDL, low-density lipoprotein; SREBP-1c, sterol regulatory element binding protein; HDL, high-density lipoprotein; EC_{50}, the half maximal effective concentration; K_D, equilibrium dissociation constant; FATP4, fatty acid transport protein 4; ACS, acyl-CoA synthetase; CPT1, carnitine palmitoyl transferase; ACOX, acyl-CoA oxidase; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase; LPL, lipoprotein lipase; siRNA, small interfering RNA.

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http://dx.doi.org/10.1016/j.bmcl.2014.04.112
0960-894X/

A line of evidence suggested that the intake of herbal essential oils may regulate cellular lipid and glucose metabolism; however, identification of active compound(s) and the exact molecular targets of action have not been clearly elucidated. *trans*-Caryophyllene (Fig. 1A) is a widely distributed natural bicyclic sesquiterpene, which has been found in the essential oils (EO) of various plants. Traditionally, it has been used in perfumes and cosmetics for its woody and spicy aroma.[17] It has recently been reported to have various biological activities, including anti-inflammatory, antioxidant, anticarcinogenic, antibiotic and local anesthetic activities.[17–19] Therefore, some researchers have considered this compound as a lead for drug development.[17] Several studies have demonstrated that *trans*-caryophyllene is an agonist of the cannabinoid receptor type 2, which makes *trans*-caryophyllene an excellent candidate for the treatment of inflammation and atherosclerosis.[20,21] It is believed that cannabinoid receptor type 2 interacts closely with the PPAR-γ pathway.[19] However, *trans*-caryophyllene shows no binding affinity or transactivation for PPAR-γ.[22] Several studies have suggested plant extracts containing *trans*-caryophyllene regulate cellular lipid metabolism. Chung et al.[23] reported that wormwood EO (16.3% *trans*-caryophyllene) showed hypocholesterolemic activities. Lin et al.[24] suggested that *Alpinia zerumbet* seed EO (2.95% *trans*-caryophyllene) is a potent high-density-lipoprotein (HDL) cholesterol elevating effects. Another in vivo study suggested a strong hypolipidemic effect of the EO extracted from Chios mastic gum (2.04% *trans*-caryophyllene), which significantly reduced the plasma TG concentrations and cholesterol levels in hyperlipidemic rats.[25] These studies suggested that EOs containing *trans*-caryophyllene may have beneficial effects on lipid metabolism. However, the direct molecular target of *trans*-caryophyllene has not been identified. In this study, we investigated the PPAR-α agonistic activity of *trans*-caryophyllene and its molecular mechanism of action in hepatic lipid metabolism.

The PPAR-α transactivation of *trans*-caryophyllene was assessed by the luciferase reporter assay in HEK293 cells.[26] The transcriptional activity of PPAR-α increased in a sigmoidal manner with the increasing concentrations of *trans*-caryophyllene, and the half-maximal effective concentration (EC_{50}) value was 9.58 μM (Fig. 1B and Table 1). The transactivation of PPAR-β and PPAR-γ

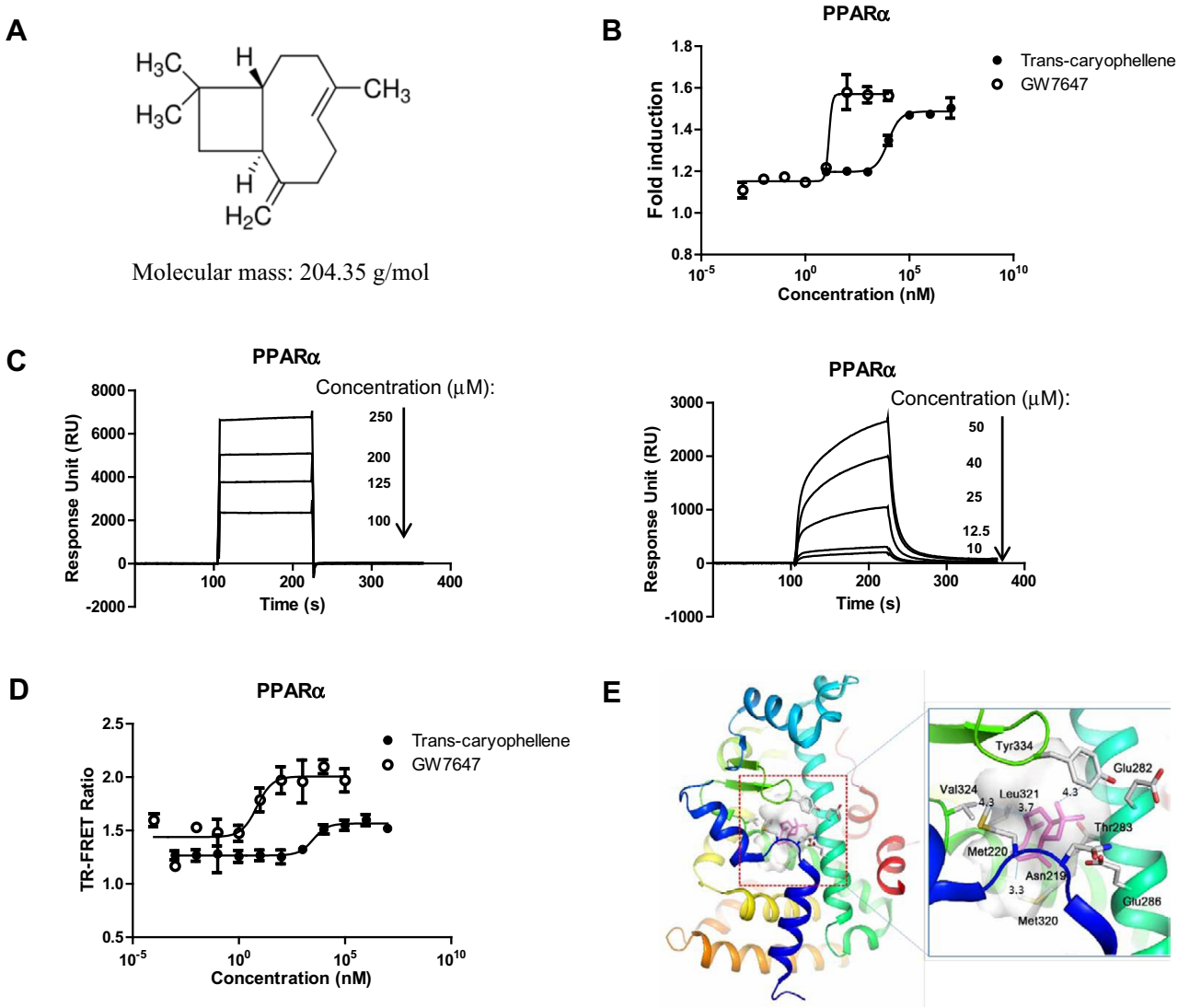

Figure 1. *trans*-Caryophyllene induces PPAR-α activation. (A) Chemical structure of *trans*-caryophyllene. (B) Effects of *trans*-caryophyllene and GW7647 on the transactivation of PPAR-α using a reporter gene assay. (C) *trans*-Caryophyllene binds directly to PPAR-α LBD. SPR sensorgrams were obtained from the Biacore after injection of a series of concentrations of *trans*-caryophyllene (left panel of C), GW7647 (right panel of C) over the immobilized PPAR-α LBD response. (D) TR-FRET assay with *trans*-caryophyllene and GW7647. (E) In silico modeling of *trans*-caryophyllene docking into PPAR-α LBD. Values indicate distance between *trans*-caryophyllene and an amino acid residue in LBD. Data represent the mean ± SEM.

Table 1
Half-maximal effective concentrations (EC_{50} values) of *trans*-caryophyllene and PPAR-α agonist GW7647, as measured by the luciferase reporter assay (LC-EC_{50}) and the TR-FRET assay (TR-EC_{50}) and the equilibrium dissociation constants (K_D values) for the binding of hPPAR-α LBD, as measured by SPR

Compound	Values		
	LC-EC_{50}	TR-EC_{50}	K_D
trans-Caryophyllene	9.58 μM	3.2 μM	1.93 μM
GW7647	13.6 nM	7.1 nM	32.4 nM

were unaltered (data not shown). We next investigated the direct interactions between the PPAR-α ligand binding domain (LBD) proteins and *trans*-caryophyllene using the surface plasmon resonance (SPR)-BIAcore system.[27] The data indicated that *trans*-caryophyllene was directly associated with PPAR-α (Fig. 1C), and the equilibrium dissociation constants (K_D) value of *trans*-caryophyllene to PPAR-α was 1.93 μM (Table 1). The direct interaction of *trans*-caryophyllene to PPAR-α LBD proteins was further confirmed by a time-resolved fluorescence resonance energy transfer (TR-FRET) assay as described previously.[28] The results (Fig. 1D) showed that *trans*-caryophyllene induced the recruitment of fluorescein-labeled coactivator peptide to PPAR-α LBD in a dose-dependent manner, with an EC_{50} value of 3.2 μM (Table 1).

In structure, PPARs contain 13 helices and a small four beta-sheets with a large Y-shaped hydrophobic binding pocket (1300–1400 $Å^3$). Molecular weights of PPAR ligands are approximately 350–500 g/mol and generally have a polar head group, which usually allows four hydrogen bonds in the amino acids in the LBD. The polar head group has been suggested as critical for PPAR activation and coactivator binding by interacting with AF2 helix (helix 12). PPARα and γ ligands, in many cases, form hydrogen bonds with S289 of helix3, H323 of helix4, H499 of helix11, Y473 of helix12 in the LBD, however, the various ligands may interact with LBD differently. The structural aspect of ligand and LBD interactions have reviewed in several excellent papers.[29,30] Among three PPAR subtypes, PPAR-α has the largest and the most hydrophobic pocket[31] and hydrophobic interaction between a ligand and the hydrophobic pocket of LBD plays a role to modify the biological potency.[32]

trans-Caryophyllene is a hydrophobic molecule that cannot form an H-bond with amino acid residues in the LBD and has a relatively small molecular weight of 204 g/mol compared with conventional PPAR ligands. However, results from the TR-FRET and SPR analyses demonstrated direct binding and activation of PPARα by *trans*-caryophyllene. In silico modeling analysis,[33] *trans*-caryophyllene docked the hydrophobic pocket of LBD successfully. The compound interacts with L321 (~3.7 Å), V324 (~4.3 Å), Y334 (~4.3 Å) and M320 (~3.3 Å) (Fig. 1E) possible by van der Waals interactions. Hydrophilic pocket of LBD is possibly occupied with water molecules as shown in several co-crystal structures of PPARs. Understanding of correct interaction between *trans*-caryophyllene and LBD requires X-ray crystallographic studies.

trans-Caryophyllene is a ligand for CB_2 receptor as well. Molecular docking study showed that *trans*-Caryophyllene bound to the hydrophobic regions of the water accessible cavity of CB_2 receptor interacting with F117, W258, I198, V113, M265 with K_i value of 780 ± 12 nM. Most of CB_2 receptor ligands form multiple H-bonds at ligand binding site but *trans*-caryophyllene potently activated CB_2 receptor by hydrophobic interaction only.[20]

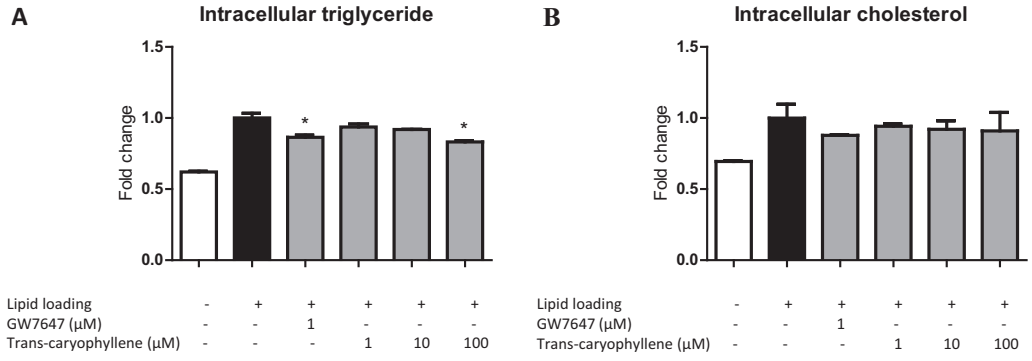

Figure 2. *trans*-Caryophyllene reduces the intracellular triglyceride and cholesterol concentrations in lipid-loaded hepatocytes. (A) Cellular triglyceride contents. (B) Cellular cholesterol concentrations. Lipid concentrations are relative to those of lipid-loaded cells. $^{*}p < 0.05$, $^{**}p < 0.01$. Data represent the mean ± SEM.

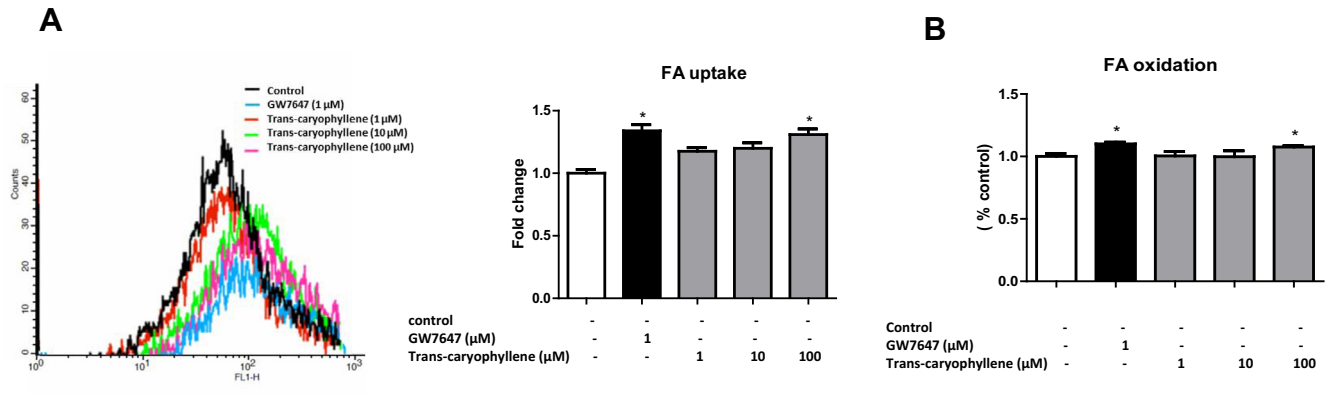

Figure 3. *trans*-Caryophyllene induces fatty acid uptake and fatty acid oxidation in HepG2 cells. (A) FACS-based cellular fatty acid uptake using BODIPY-labeled fatty acids. (B) Fatty acid oxidation measured using [1−^{14}C] palmitate. Data represent the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

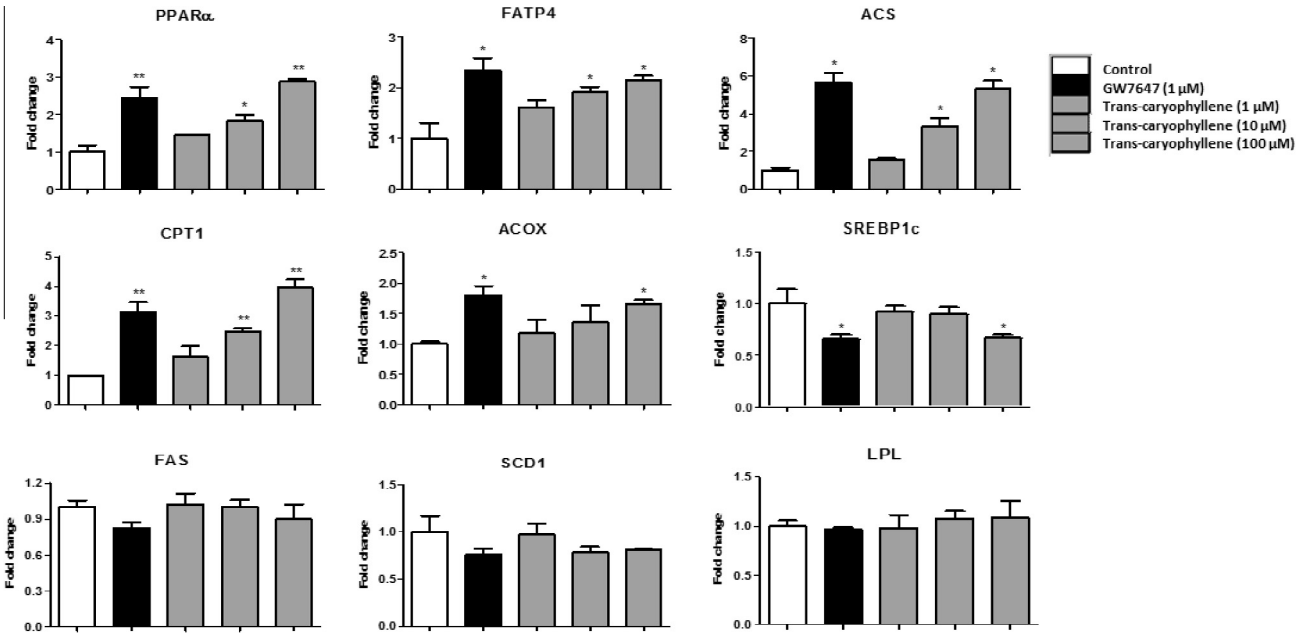

Figure 4. The expression of PPAR-α and its target genes in hepatocytes. Expression levels of the lipid metabolism genes were assessed with qPCR in cells stimulated with *trans*-caryophyllene or GW7647. The results are normalized to the GAPDH mRNA level. Data represent the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

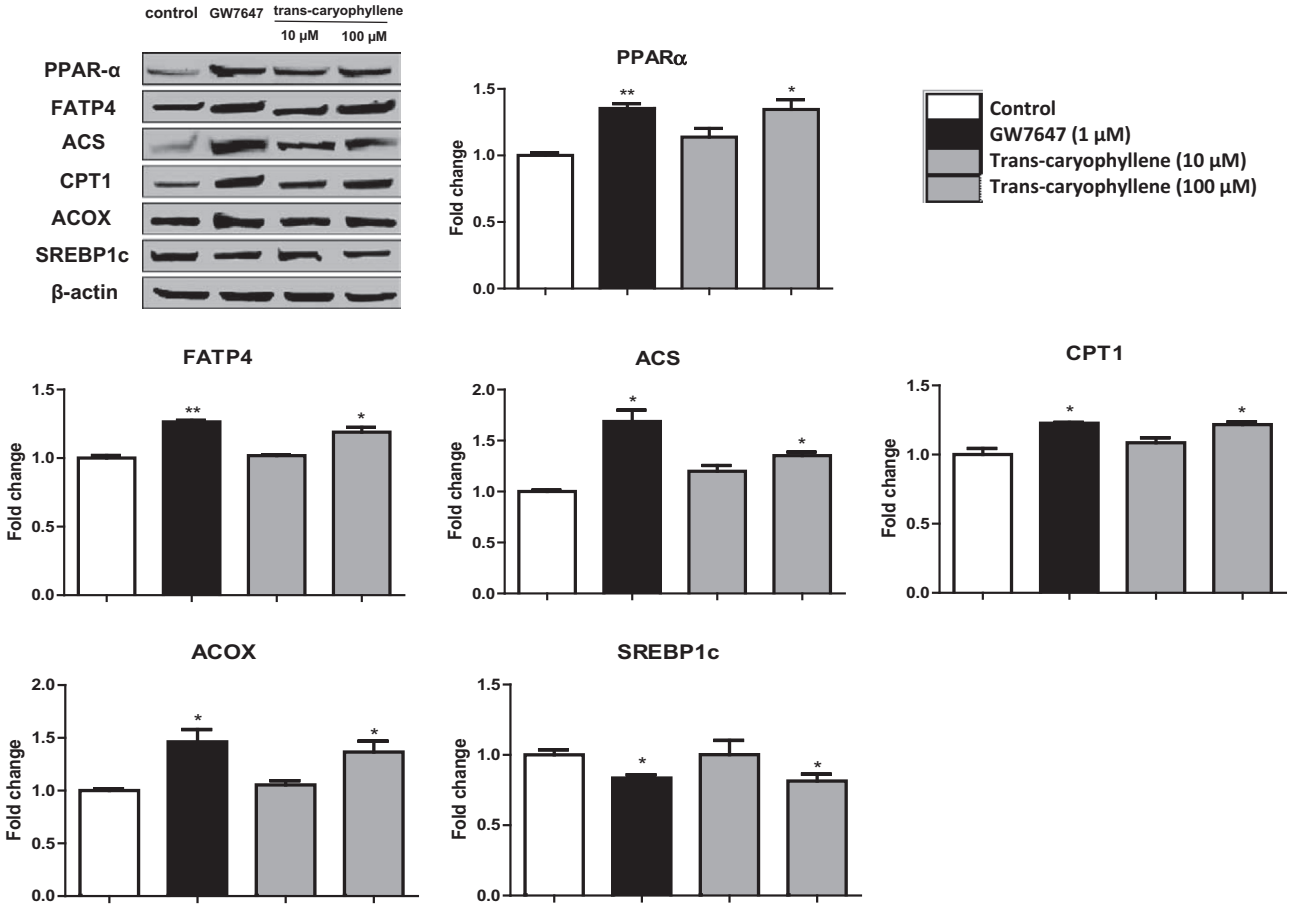

Figure 5. The expression levels of protein were determined by immunoblotting. Data represent the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

PPAR-α plays a crucial role in intracellular lipid metabolism. Therefore, the effect of *trans*-caryophyllene treatment on the cellular lipid concentration in lipid-loaded HepG2 cells was investigated.[34] Intracellular TG concentrations were significantly reduced by *trans*-caryophyllene in HepG2 cells by 16.8% at 100 μM compared to the levels in the lipid loading group

(Fig. 2A). Cholesterol concentrations were marginally altered (Fig. 2B).

PPAR-α activation is also involved in the stimulation of cellular fatty acid uptake and the rate of fatty acid oxidation. We quantified the BODIPY-labeled fatty acid uptake by the FACS assay,[35,36] and measured the rate of fatty acid oxidation using [1−^{14}C] palmitate.[37] Stimulation of cells with *trans*-caryophyllene induced fatty acid uptake in a dose-dependent manner; 100 μM *trans*-caryophyllene treatment increased fatty acid uptake significantly by 31% (Fig. 3A). The rate of fatty acid oxidation was increased significantly treated with 100 μM *trans*-caryophyllene (Fig. 3B). Compared with the EC_{50} on PPARα (9.58 μM), *trans*-caryophyllene induced hepatic fatty acid uptake and oxidation at high concentration in HepG2 cells. This may be due to a low cellular bioavailability of *trans*-caryophyllene or the compound may undergo structural modification after uptake. Further research is required to clarify the issue.

trans-Caryophyllene induces the expression of PPAR-α and its target genes in hepatocytes. Real-time qPCR and immunoblotting analysis were performed as previously described, respectively.[38,39] PPAR-α expression was increased in HepG2 cells stimulated with *trans*-caryophyllene (Fig. 4). Treatment with 10 or 100 μM *trans*-caryophyllene significantly increased the levels of PPAR-α mRNA by 82% and 187%, respectively, suggesting that *trans*-caryophyllene mediated the expression of PPAR-α in hepatocytes. PPAR-α activation also induces the catabolism of fatty acids by increasing the gene expression of the rate-limiting enzymes involved in fatty acid uptake and fatty acid oxidation, including fatty acid transport protein 4 (FATP4), acyl-CoA synthetase (ACS), carnitine palmitoyl transferase (CPT1) and acyl-CoA oxidase (ACOX).[40,41] FATP4 is the main protein involved in fatty acid uptake, which can facilitate the uptake of long chain fatty acids in the liver.[40] Treatment with 10 or 100 μM *trans*-caryophyllene increased the levels of FATP4 mRNA significantly by 91% and 115%, respectively (Fig. 4). PPAR-α activation up-regulates the gene expression of ACS, which prevents an efflux of fatty acids from the cell by esterifying fatty acids to acyl-CoA derivatives.[42] Treatment with 10 or 100 μM *trans*-caryophyllene increased ACS gene expression by 232% and 432%, respectively (Fig. 4). CPT1 and ACOX are two rate-limiting enzymes involved in fatty acid oxidation. CPT1 is responsible for the transport of fatty acids into the mitochondria;[41] and ACOX is responsible for the oxidation of acyl-CoA esters, to reduce fatty acid levels.[40] In our study, the gene expressions of CPT1 and ACOX were significantly increased by *trans*-caryophyllene. Treatment with 10 or 100 μM *trans*-caryophyllene increased CPT1 gene expression by 149% and 295%, respectively (Fig. 4). The results also showed that treatment with 100 μM *trans*-caryophyllene significantly increased ACOX gene expression by 65% (Fig. 4). Immunoblotting analysis showed results similar to those of the qPCR analysis (Fig. 5).

The activation of PPAR-α can suppress the LXR-SREBP-1c pathway through reducing the binding of LXR/RXR to LXRE[43] and can regulate two target genes of SREBP-1c, fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD1). The results showed that SREBP-1c gene expression was significantly reduced by 33%

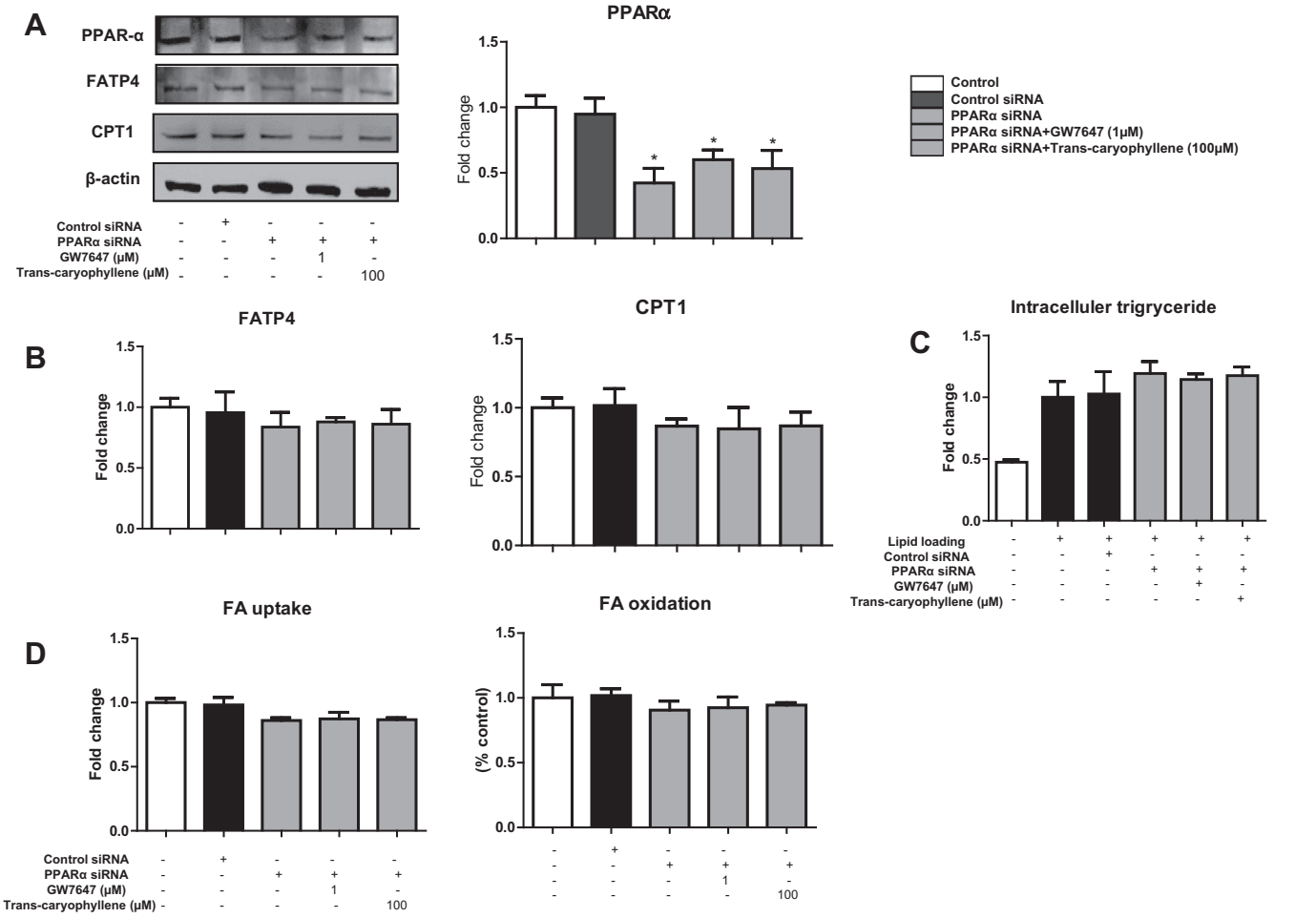

Figure 6. The effects of *trans*-caryophyllene on cells with PPAR-α knockdown. (A) The protein expression of PPAR-α in HepG2 cells transfected with small interfering RNA of PPAR-α. (B) Expression of PPAR-α responsive genes. (C) Intracellular TG concentrations. (D) FACS-based cellular fatty acid uptake and fatty acid oxidation. Data represent the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

stimulation with 100 μM *trans*-caryophyllene. It is associated with similar immunoblotting analysis (Fig. 5). The gene expressions of FAS and SCD1 were also reduced at the 100 μM *trans*-caryophyllene treatment. However, we found that treatment with *trans*-caryophyllene had no significantly effect on the lipoprotein lipase (LPL) gene expression, which is the major enzyme for the hydrolysis of TG.[44] Therefore, we propose that the reduction in TG contents observed in our study might be obtained through reducing the gene expression of SREBP1c and SCD1, which is required for the synthesis of TG, cholesterol esters and phospholipids.[45]

To determine whether the effects of *trans*-caryophyllene on lipid metabolism were PPAR-α specific, we performed experiments in HepG2 cells transfected with PPAR-α small interfering RNA (siRNA).[46] The protein level of PPAR-α in the transfected cells was reduced by 58%, as assessed by immunoblotting analysis (Fig. 6A). The induction of FATP4 and CPT1 expression, which were induced by *trans*-caryophyllene in HepG2 cells (Fig. 5), disappeared in cells with PPAR-α knockdown (Fig. 6A). Most importantly, the effects of *trans*-caryophyllene on intracellular TG concentrations, fatty acid uptake, and oxidation were abolished when HepG2 cells were transfected with PPAR-α siRNA (Fig. 6). These results indicate that the effects of *trans*-caryophyllene on hepatic lipid metabolism including TG accumulation, fatty acid uptake, and oxidation are PPAR-α-dependent processes.

PPAR-α activation is involved in the regulation of various pathways improving lipid profiles, associated with reducing plasma levels of LDL and TG, and elevating HDL levels.[47] Generally, PPAR-α activation stimulates LPL activity and inhibits the transcription of apolipoprotein C-III, thereby increasing TG hydrolysis and LDL uptake; it increases the expression of apolipoprotein AI and AII, thus leading to increased HDL production.[47,48] It also inhibits TG synthesis by enhancing fatty acid uptake and reducing fatty acid synthesis through decreasing the expression of SREBP-1c and its responsive genes.

In conclusion, it is suggested that *trans*-caryophyllene served as the PPAR-α ligand and activated the receptor. We investigated how *trans*-caryophyllene directly binds to PPAR-α LBD proteins using SPR, TR-FRET assays, and molecular docking. Treatment with *trans*-caryophyllene increased the expression of the genes involved in fatty acid uptake and β-oxidation and suppressed the LXR-SREBP-1c pathway, which led to an increase in fatty acid uptake, fatty acid oxidation and a reduction in intracellular TG concentrations in hepatic lipid metabolism. In addition, PPAR-α siRNA results indicated that these effects of *trans*-caryophyllene are dependent on PPAR-α.

Acknowledgments

This work was supported by Basic Science Research Program of the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (No. 2013R1A2A2A01016176), and a grant (14162MFDS973) from Ministry of Food and Drug Safety in 2014. The SPR instrument was provided by the Korea Basic Science Institute. We thank Professor Hyun-Gyu Song for the advice on FRET analysis.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.112.

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26. HEK293 cells were seeded in 24-well plates at a density of 1.6×10^5/well. pSG5-PPAR-α was co-transfected with pCMV-3xPPRE-Luc. Transfection was performed with Hilymax (Dojindo, MD, USA) according to the manufacturer's protocol. At 24 h post-transfection, cells were stimulated with different concentrations of *trans*-caryophyllene (ranging from 1 nM to 10 mM) in 1% DMSO, and the luciferase activity was assayed with a firefly luciferase assay kit (Biotium, Hayward, CA, USA).
27. The binding affinities of the immobilized hPPAR-α ligand binding domain (LBD) to ligands were evaluated using a Biacore 2000 instrument (GE Healthcare, Uppsala, Sweden). All Biacore data were obtained at 25 °C using phosphate-buffered saline (PBS) with 1% DMSO as the running buffer at a constant flow of 25 μL/min. The BIA evaluation software version 3.1 (GE Healthcare, Uppsala, Sweden) and a 1:1 Langmuir binding fitting model were used to analyze the equilibrium dissociation constants (K_D values) of *trans*-caryophyllene and GW7647 binding.
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33. To gain insight into the mode of binding, *trans*-caryophyllene was docked into the PPAR-α receptor LDB (PDB ID: 2P54). Total 10 binding sites were predicted assuming the protein structure to be rigid. Coordinates for *trans*-caryophyllene were prepared by GRADE server (Global Phasing Ltd) with Mogul assisted modeling. In the left panel *trans*-caryophyllene in stick model (cyan) was shown in the putative binding site as predicted by AutoDock 4. The side chains of residues lining *trans*-caryophyllene were also depicted in stick model. The binding pocket was rendered as a semitransparent surface in white color. The ribbon model of human PPAR-α was colored in rainbow scheme from blue (N-terminus) to red (C-terminus). Right panel was a blow up of the dashed box in which residues involved in binding *trans*-caryophyllene were labelled.
34. HepG2 cells were seeded in 6-well plates at a density of 10^6/well for 24 h. The medium was removed, and the cells were washed with PBS and loaded with free fatty acids (400 μM palmitic acid and 400 μM oleic acid) with 0.5% bovine serum albumin (BSA, Bovogen Biologicals, Melbourne, Australia) for 24 h. The following day, cells were treated with *trans*-caryophyllene (1, 10 or 100 μM) or 1 μM GW7647 for a further 24 h. HepG2 cells were washed twice with PBS and treated with 1 mL hexane/isopropanol (2:1) for 30 min at room temperature. The cellular extracts were evaporated using a Speed Vac (n-Biotech, Korea) and then dissolved in 95% ethanol. The cellular contents of TG and cholesterol were analyzed using an enzymatic method with a Cobas C111 automatic analyzer (Roche Diagnostic Systems Inc., Indianapolis, IN, USA).

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46. HepG2 cells were seeded in 12-well plates at a density of 2×10^5/well. When the cells were 50–60% confluent, they were transfected with a PPAR-α siRNA duplex (4 μL/well; Sant Cruz, USA) using Fugene HD reagent (Promega, USA) for 24 h. The cells were retransfected with the siRNA duplex again after the initial transfection for 4 h. After the second transfection, the cells were treated with vehicle, 1 μM GW7647 or 100 μM *trans*-caryophyllene for 24 h. Then cell lysates were prepared, and protein levels of PPAR-α and target genes were determined.
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