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Identification of 6-octadecynoic acid from a methanol extract of *Marrubium vulgare* L. as a peroxisome proliferator-activated receptor γ agonist

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ABSTRACT

6-Octadecynoic acid (6-ODA), a fatty acid with a triple bond, was identified in the methanol extract of *Marrubium vulgare* L. as an agonist of peroxisome proliferator-activated receptor γ (PPARγ). Fibrogenesis caused by hepatic stellate cells is inhibited by PPARγ whose ligands are clinically used for the treatment of diabetes. Plant extracts of *Marrubium vulgare* L., were screened for activity to inhibit fibrosis in the hepatic stellate cell line HSC-T6 using Oil Red-O staining, which detects lipids that typically accumulate in quiescent hepatic stellate cells. A methanol extract with activity to stimulate accumulation of lipids was obtained. This extract was found to have PPARγ agonist activity using a luciferase reporter assay. After purification using several chromatographic methods, 6-ODA, a fatty acid with a triple bond, was identified as a candidate of PPARγ agonist. Synthesized 6-ODA and its derivative 9-octadecynoic acid (9-ODA), which both have a triple bond but in different positions, activated PPARγ in a luciferase reporter assay and increased lipid accumulation in 3T3-L1 adipocytes in a PPARγ-dependent manner. There is little information about the biological activity of fatty acids with a triple bond, and to our knowledge, this is the first report that 6-ODA and 9-ODA function as PPARγ agonists.

1. Introduction

Marrubium vulgare L. (White horehound), a perennial herb which is commonly distributed in the Mediterranean area including Northern Africa [1], is used as a traditional medicine to aid digestion, soothe a sore throat, relieve inflammation and treat diabetes [2]. Plant extracts were screened for anti-hepatic fibrosis activity, and the methanol extract of *Marrubium vulgare* L. was found to stimulate accumulation of lipids in hepatic stellate cell line, HSC-T6. In this process, Oil Red-O staining was employed to screen anti-fibrotic compounds, since lipid accumulation is a typical character of quiescent hepatic stellate cells [3]. Peroxisome proliferator-activated receptor γ, (PPARγ,) is a regulator of adipogenesis and is involved in the regulation of hepatic fibrosis [3]. The methanol extract of *Marrubium vulgare* L. was examined for PPARγ agonist activity. Thiazoloinediones, such as pioglitazone and rosiglitazone, are synthetic PPARγ agonists clinically used for treatment of type 2 diabetes by improving insulin resistance; 15d-PGJ_2 is known as an endogenous PPARγ agonist. Flavonoids such as naringenin also function as a PPARγ agonist from natural sources [4]. Fatty acids such as eicosapantaenoic acid (EPA), act on PPARγ as an agonist [5] but PPARγ agonist activity has not been reported in fatty acids with a triple bond.

In this study, 6-octadecynoic acid (6-ODA), a fatty acid with a triple bond, was identified from the methanol extract of *Marrubium vulgare* L. and its PPARγ agonist activity was confirmed by synthesized 6-ODA. Some fatty acids with a double bond have been found to have PPARγ agonists but agonist activity of fatty acids with a triple bond has not been reported. Little is known about the

Abbreviations: PPARγ, peroxisome proliferator-activated receptor; 6-ODA, 6-octadecynoic acid; 9-ODA, 9-octadecynoic acid; 15d-PDJ_2, 15-deoxy-Δ12,14-prostaglandin J_2.

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biological activity of 6-ODA except for anti-fungal activity [6]. In this study, both 6-ODA and 9-octadecynoic acid (9-ODA) with a triple bond in a position different from that of 6-ODA had similar PPARγ agonist activity in luciferase reporter assay and increased lipid accumulation in 3T3-L1 adipocytes in a PPARγ dependent manner despite these fatty acids were displayed different strength and spectrum in anti-fungal activity [6]. This study sheds light on the role of fatty acids with a triple bond as PPARγ agonists and their potential in fibrotic and diabetic therapy.

2. Materials and methods

2.1. Chemical reagents and instruments

Organic solvents for fractionation were purchased from Nacalai Tesque. 9-Octadecynoic acid was purchased from Wako. ^{1}H NMR spectra were measured and recorded on a Avance I 400 (reference TMS, Bruker, Germany). Flash column chromatography was performed using Wako gel C-200 (Wako) and Parallel FR-360 (YAMAZEN).

2.2. Cell culture

HSC-T6 and HepG2 cells were grown in Dulbecco's Modified Eagle Medium (Wako) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml streptomycin (Gibco) at 37 °C in humidified 5% CO_2 atmosphere. The conditions for culture of 3T3-L1 cells are described below.

2.3. Preparation of plant extracts

Dried aerial parts (170 g) of *Marrubium vulgare* L. cultivated at the Nippon Shinyaku Institute for Botanical Research were extracted with methanol (850 ml) for 1 week at room temperature. After filtration, the filtrate was evaporated to dryness in vacuo at 40 °C to afford the MeOH extract (14.8 g).

2.4. Fractionation of methanol extract of Marrubium vulgare

The MeOH extract (2.4 g) of *Marrubium vulgare* was partitioned between EtOAc (150 ml × 3), BuOH (150 ml × 3), and H_2O (150 ml). The EtOAc-soluble portion (0.7 g) was divided into 11 fractions (Fr. 1–11) using silica gel column chromatography (Wako gel C-200, Wako; Parallel FR-360, YAMAZEN) (φ10 × 300 mm; Hexane/EtOAc, 84:16 → 30:70 → 100% 2-propanol). Fr. 2 (40 mg) was subjected to a silica gel column chromatography (φ16 × 60 mm; Hexane/EtOAc, 91:9 → 50:50 → 100% 2-propanol) to afford 8 fractions (Fr. 2-1–2-8). Fr. 2–5 was separated into 5 fractions (Fr. 2-5-1–2-5-5) by preparative silica gel TLC (Merck Silica $60F_{254}$; 20 cm × 20 cm; $CHCl_3$/MeOH, 95:5). Fr. 2-5-4 was divided into 3 fractions (Fr. 2-5-4-1–2-5-4-3) using silica gel column chromatography (φ7 × 230 mm; Hexane/Et_2O, 85:15 → 45:55 → 100% EtOAc). Fr. 2-5-4-1 was subjected to gas chromatography/mass spectrometry (GC/MS) analysis after methyl-esterification.

2.5. Identification of 6-Octadecynoic acid using GC/MS analysis

To a solution of Fr. 2-5-4-1 (0.1 mg) in 20% MeOH/Hexane (1 ml) was added trimethylsillyldiazomethane, 2.0 M in Hexane (Sigma). The reaction mixture was stirred at room temperature for 1 h. The mixture was poured into H_2O (10 ml) and extracted with hexane (10 ml). The organic layer was subjected to GC/MS analysis. Electron impact mass spectra (MS) were obtained at 70 eV (ion source temperature, 200 °C) in the split-less mode on a Hewlett–Packard (HP) 5989B coupled with a gas chromatograph (GC) HP5890 Plus, using an HP-5 MS capillary column (0.25 mm id × 30 m, 0.25 μm in film thickness). The carrier gas was helium delivered at a constant flow of 1.2 ml/min, and the oven temperature was programmed from 60 °C (2 min hold) to 290 °C at a rate of 10 °C/min. The GC analysis was conducted with a Hewlett Packard 5890 Series II Plus equipped with a flame ionization detector (FID), using identical column and running conditions. The retention time and fragment pattern of fatty acids were identical to those of authentic compounds. 6-octadecynoic acid, methyl ester, GC t_R = 21.06 min; MS m/z (%): 294 (M^+, 9), 263 (9), 220 (11), 154 (58), 122 (28), 94 (71), 80 (100), 41 (35).

2.6. Synthesis of 6-Octadecynoic acid

Materials and methods of synthesis of 6-ODA are described in detail in the Supporting Information.

2.7. PPARγ reporter assay

A DNA fragment coding a ligand binding domain (204–505 amino acid residue) of mouse PPARγ2 (Genbank U09138) with *BamH*I and *Sal*I sites at the ends was amplified by PCR using genomic DNA of mouse 3T3-L1 cells as a template and was inserted to pSG424 [7] at *BamH*I and *Sal*I sites to construct pGal4-PPARγLBD. pGal4-PPARγLBD and pUAS-tk-luc [8] were transfected to HepG2 cells using Hily-max (Dojindo) and PPARγ agonist activity was determined by luciferase activity using the Luciferase Assay System according to the manufacturer's protocol (Promega). pact-βgal, carrying the β-galactosidase gene under control of the chicken β-actin promoter [9], was also co-transfected and normalized transfection efficiency by β-galactosidase activity using chlorophenol red β-D-galactopyranoside (Roche Diagnostics GmbH) as a substrate [10].

2.8. Measurement of triglyceride in 3T3-L1 cells

3T3-L1 predipocytes were maintained under an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (Wako) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Adipose differentiation was induced by treating confluent 3T3-L1 cells for 2 days with hormonal cocktail containing insulin (Sigma) (5 μg/ml), isobutylmethylxanthine (Sigma) (0.5 mM), and dexamethasone (Wako) (0.25 μM) and then for additional 2 days with insulin (5 μg/ml) alone. After incubation with these reagents, the basal medium with or without test samples was replenished every other day [11] for 8 days. Cells were sonicated and triglyceride contents were measured with a Triglyceride E-test kit (Wako) [12] normalized by protein contents measured with a Bio-Rad protein assay kit.

2.9. 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

3.1. Purification and identification of 6-octadecynoic acid from methanol extract of Marrubium vulgare L.

The methanol extract of *Marrubium vulgare* L. stimulated lipid accumulation in hepatic stellate cell lines HSC-T6 (Fig. 1). Lipid accumulation is a typical character of quiescent or non-fibrotic hepatic stellate cells and PPARγ is considered to be involved in

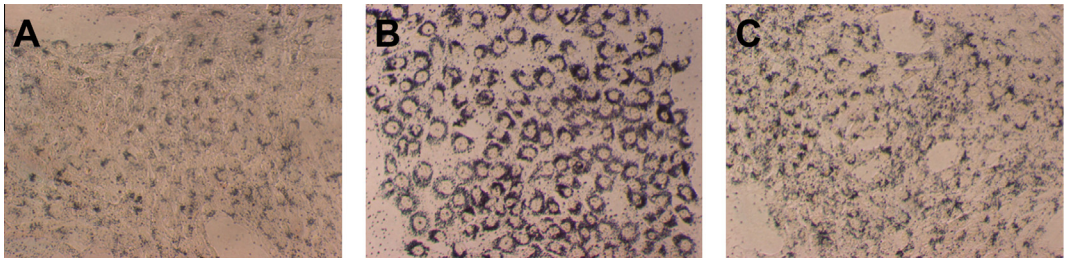

Fig. 1. Lipid accumulation in HSC-T6 cells. HSC-T6 cells were treated with (A) vehicle (DMSO), (B) Tetrandrine (2 μg/ml), an anti-fibrotic alkaloid used as a positive control, or (C) methanol extract of *Marrubium vulgare* L. (30 μg/ml) for three days, and fixed with 10% neutral formalin followed by staining with 0.3% Oil Red-O solution.

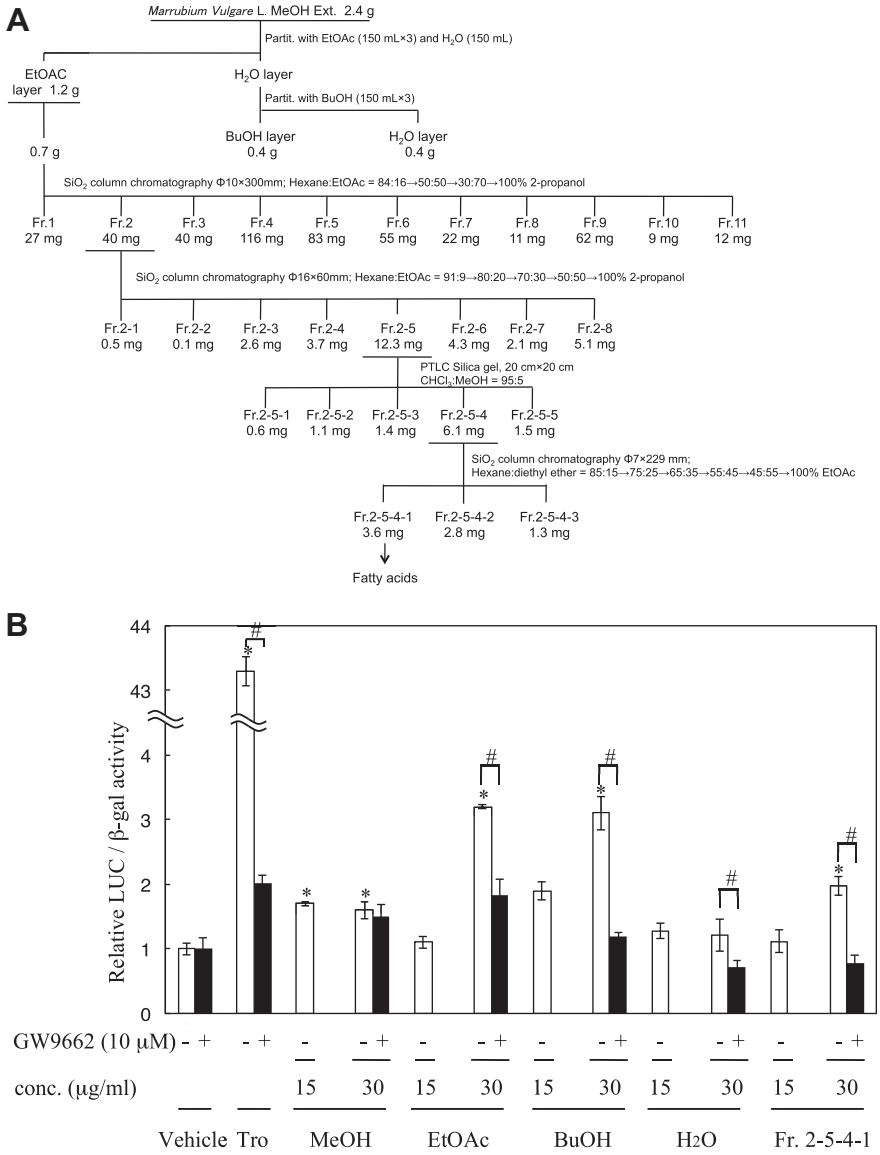

Fig. 2. Purification scheme of fractions containing transactivation potential of PPARγ from methanol extract of *Marrubium vulgare* L. (A) Purification scheme to identify 6-octacdecynoic acid. 6-ODA was identified in Fr. 2-5-4-1. The methanol extract was divided or gathered into sub-fractions according to the developing pattern analyzed by thin-layer chromatography of small fractions after separation by solvent extraction or separation using several kinds of chromatography. (B) Transcriptional activation potential of PPARγ by representative fractions. The luciferase assay was performed in HepG2 cells transiently co-transfected with pGal4-PPARγLBD, pUAS-tk-Luc reporter and pact-βGal plasmids. Relative luciferase activities were normalized by β-galactosidase activity. The data of vehicle control is denoted as 1. HepG2 cells were treated with the vehicle (0.1% DMSO), troglitazone (10 μM), a synthetic PPARγ agonist as a positive control, or methanol extract of *Marrubium vulgare* L. (15 or 30 μg/ml) and fractions including 2-5-4-1 fraction (15 or 30 μg/ml) for 4 h. PPARγ antagonist GW9662 at the dose of 10 μM was employed to examine the PPARγ-dependent activity. Results are presented as mean ± SD ($n = 3$). $^{*}P < 0.05$ compared with vehicle control. $^{\#}P < 0.05$ compared with cells treated without GW9662.

anti-fibrotic activity [3]. The PPARγ agonist activity of the extract was examined by GAL4 DNA-binding domain/PPARγ ligand binding domain chimera protein expression using plasmid pGAL4-PPARγLBD and a luciferase reporter plasmid pUAS-tk-Luc which contains the target sequence of GAL4. The methanol extract of *Marrubium vulgare* L. weakly but significantly activated PPARγ in

the luciferase reporter assay (Fig. 2B). The extract was subjected to bioassay-guided fractionation (Fig. 2A) to give several fractions that could activate PPARγ in the luciferase reporter assay (data not shown). ^{1}H NMR spectra indicated that Fr. 2-5-4-1, contains several free fatty acids. These fatty acids were identified as palmitic acid, oleic acid, linoleic acid and 6-octadecynoic acid (6-ODA) that possesses a triple bond, by GC/MS analysis after methyl-esterification using trimethylsillyldiazomethane.

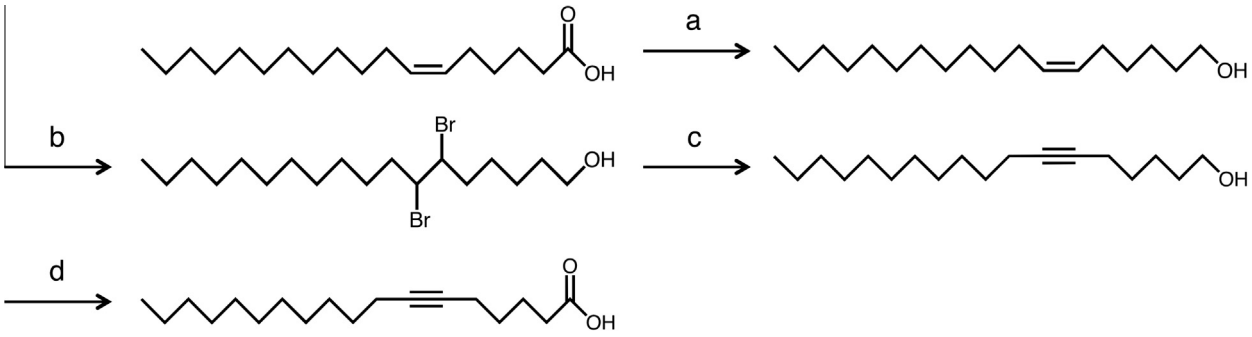

Fig. 3. Synthesis of 6-ODA. (a) $LiAlH_4$, diethyl ether, 69%; (b) bromine, diethyl ether, 93%; (c) DBU, neat, 82%; (d) PDC, DMF, 64%.

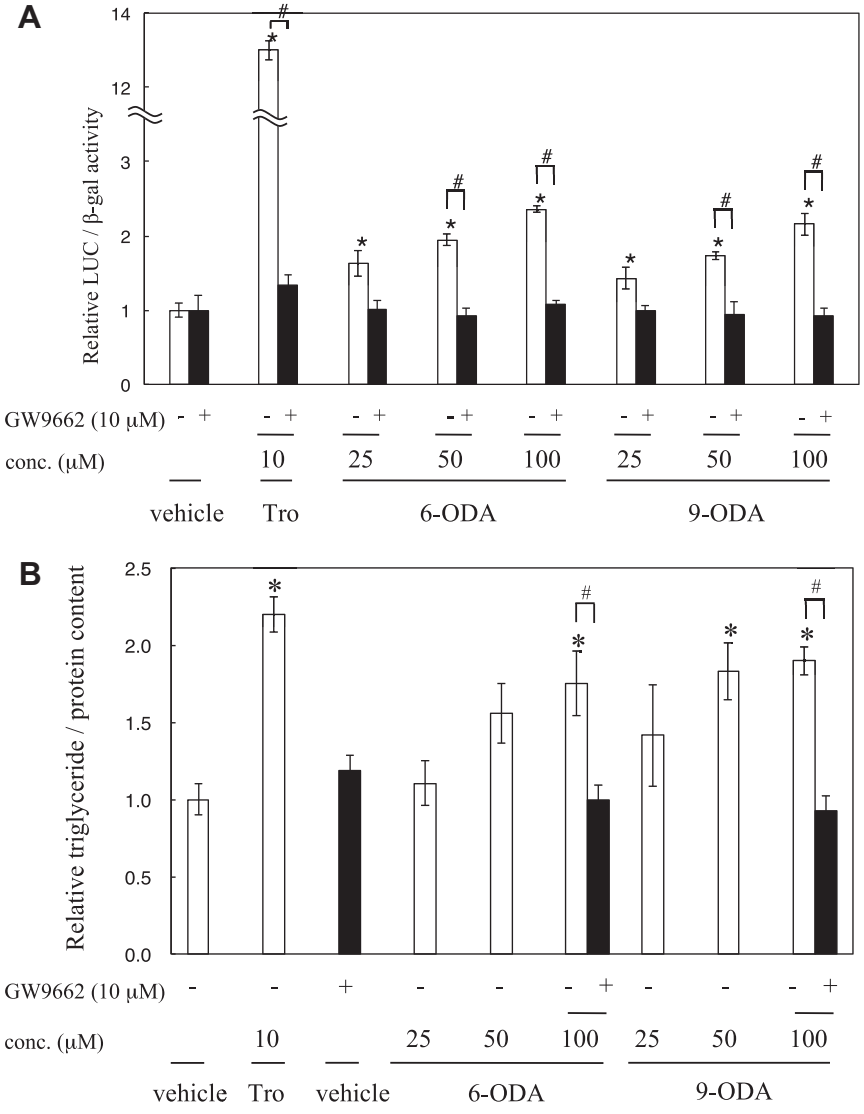

Fig. 4. Effect of 6-ODA or 9-ODA on PPARγ activation and accumulation of triglyceride. (A) Transactivation of PPARγ. Luciferase reporter assay was performed according to the method described in Fig. 2 legend. Dose-respond (25, 50, 100 μM) activation of PPARγ by 6- or 9-octadecynoic acid and its inhibition by GW9662 (10 μM). (B) Stimulation of triglyceride accumulation in 3T3-L1 cells by the treatment with 6- or -9-octadecynoic acid and its inhibition by GW9662 (10 μM). $^{*}P < 0.05$ compared with vehicle control. $^{\#}P < 0.05$ compared with cells treated without GW9662.

3.2. Synthesis of 6-ODA

Although 6-ODA was identified in the fraction of 2-5-4-1 as a candidate PPARγ agonist, its quantity was too small for further purification. Therefore, 6-ODA was also synthesized as described by Shak et al. [13] using petroselinic acid as a starting material (Fig. 3). The carboxyl group of petroselinic acid was reduced by $LiAlH_4$, to obtain (*Z*)-6-octadecen-1-ol in 69% yield. The C-6 alkene moiety of (*Z*)-6-octadecen-1-ol was brominated with bromine to afford dibromide in 93% yield. The bromine group of dibromide was subsequently eliminated using 1,8-diazabicyclo[5,4,0]undec-7-ene to give 6-octadecyn-1-ol in 82% yield. The primary alcohol of 6-octadecyn-1-ol was oxidized with piridinium dichrolomate provided 6-ODA in 64% yield. The final product was confirmed as 6-ODA by GC–MS, ^{1}H and ^{13}C NMR spectra data.

3.3. 6-ODA was a PPARγ agonist

6-ODA synthesized in this study and commercially available 9-ODA were examined for potent PPARγ agonist activity by a luciferase reporter assay. Both 6-ODA and 9-ODA similarly activated PPARγ in a dose-dependent manner which was completely inhibited by the PPARγ antagonist GW9662, suggesting that this activity was PPARγ-dependent (Fig. 4A).

3.4. Stimulation of lipid accumulation by 6-ODA

After induction of differentiation of 3T3-L1 preadipocytes to adipocyte by isobutylmethylxanthine, dexamathasone and insulin, addition of 6-ODA or 9-ODA to the culture medium of 3T3-L1 preadipocytes significantly increased the triglyceride contents of the cells, which was blocked by further simultaneous addition of PPARγ antagonist GW9662, suggesting that 6-ODA or 9-ODA stimulated lipid accumulation in 3T3-L1 cells in a PPARγ-dependent manner (Fig. 4B).

4. Discussion

In this study, 6-ODA, a rare fatty acid with a triple bond, was identified as a PPARγ agonist derived from *Marrubium vulgare* L. (Figs. 2A, 3). *Marrubium vulgare* L. (White horehound) is commonly distributed in the Mediterranean area [1] and is used as a traditional medicine to aid digestion, soothe sore throats and relieve inflammation. The leaf extract of *Marrubium vulgare* L. grown in Mexico was used in a clinical trial for type 2 diabetes [2]. PPARγ regulates glucose and lipid metabolism and its synthetic agonists, thiazolidinediones, such as pioglitazone, that improve insulin resistance are clinically utilized for diabetes therapy [14] and our results showed that crude methanol extracts of *Marrubium vulgare* L. plants grown in Japan exhibited PPARγ agonist activity comparable with that of the plants grown in Tunisia (data not shown). Many fractions derived from the methanol extract of *Marrubium vulgare* L. after separation by several kinds of chromatography had weak but significant PPARγ agonist activity in a luciferase reporter assay (Fig. 2, data not shown), suggesting that the extract contained several compounds that had potential for PPARγ agonists.

PPARγ, a member of the nuclear receptor family, has a quite unique large binding pocket with multiple sub-pockets [15], where several substances with quite different structures can bind; for example, an endogenous agonist 15d-PGJ_2, medium or long chain unsaturated fatty acids such as docosahexaenoic acid (DHA), and certain kinds of flavonoids such as naringenin that is a bitter taste substance found in grapefruit [4,16]. Fatty-acid metabolites are reported to activate PPARγ through conformational change of the Ω loop, while serotonin metabolites act as endogenous agonists for PPARγ by directly binding to helix 12 [15]. Mutational analyses suggested that the PPARγ ligand binding to each sub-pocket induces structural alterations at different sites on the outer ligand-binding domain surfaces, which interact with the co-regulators and heterodimer partner, indicating that the sub-pockets near the activation function 2 (AF-2) helix 12 (AF-2 pocket) and the Ω loop (Ω pocket) are specialized for the recognition of serotonin (5-HT) and fatty acid metabolites, respectively. The covalent modification of Cys285 by the antagonist, GW9662, inhibited the activation of PPARγ by a synthetic PPARγ agonist rosiglitazone [17] or a fatty-acid ligand. In this study, 6-ODA and 9-ODA dose-dependently increased accumulation of triglycerides in differentiated 3T3-L1 cells in a PPARγ-dependent manner since GW9662 completely abolished the increase of triglyceride (Fig. 4B). Among several kinds of fatty acids, linoleic acid increased triglyceride [18], while eicosapentaenoic acid or trans10, cis12-conjugated linoleic acid (t10,c12-CLA) decreased the triglyceride in differentiated 3T3-L1 cells [18,19]. However, t10, c12-CLA failed to activate PPARγ but selectively inhibited thiazolidine-induced activation of PPARγ in 3T3-L1 cells, suggesting that t10, c12-CLA was acting as a PPARγ modulator. As described above, PPARγ has a large ligand binding pocket with multiple sub-pockets and fatty acid metabolites are supposed to bind to Ω-pocket of ligand binding domain [15] which induced structural alternation with subtle differences depend on each fatty acid that regulate the increase or decrease of triglyceride in the cells. Octanoate and decanoate, 8-carbon and 10-carbon medium-chain fatty acids, decreased adipogenesis in 3T3-L1 preadipocytes when treated with a standard hormonal cocktail, but increased adipogenesis when treated with basal media [20], suggesting that the cellular condition or factors may regulate the increase or decrease of triglycerides in response to fatty acids.

6-ODA, the glyceride occupying 90% of seeds from *Picramnia sow* a widely found bush in Guatemala [21], was shown to be a acetylenic compound, tariric acid [22]. There are few detailed reports on the biological activity of 6-ODA except for anti-microbial or anti-fungal activity. Several acetylenic acids including 6-ODA have been evaluated for their *in vitro* anti-fungal activities against several pathogens, such as *Candida albicans* [6]. Against the examined pathogens, 6-ODA had stronger anti-fungal activity and wider anti-fungal spectrum than its derivative, 9-ODA that had a triple bond at a position in the carbon chain different from that of 6-ODA. Further studies are needed on other biological activities of acetylenic acids. The PPARγ agonist activity of other acetylenic acids is under investigation.

GPR40 and GPR120, G-protein coupled receptors whose ligands have recently been identified as medium- and long-chain free fatty acids, have been reported [23]. GPR40 showing exclusively high levels of expression in pancreatic β-cells [24] is involved in the glucose-dependent secretion of insulin and is also expressed in enteroendocrine cells [25]; its agonist TAK-875 is now in a phase 3 clinical trial for diabetic medicine after a phase 2 clinical trial where TAK-875 significantly improved glycemic control in patients with type 2 diabetes with minimum risk of hypoglycemia [26]. The possibility of 6-ODA transducing signals through these fatty acid receptors is also under investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.003.

References

[1] S. Sahpaz, N. Garbacki, M. Tits, F. Bailleul, Isolation and pharmacological activity of phenylpropanoid esters from *Marrubium vulgare*, J. Ethnopharmacol. 79 (2002) 389–392.

[2] A. Herrera-Arellano, L. Aguilar-Santamaria, B. Garcia-Hernandez, P. Nicasio-Torres, J. Tortoriello, Clinical trial of *Cecropia obtusifolia* and *Marrubium vulgare* leaf extracts on blood glucose and serum lipids in type 2 diabetics, Phytomedicine 11 (2004) 561–566.

[3] H. She, S. Xiong, S. Hazra, H. Tsukamoto, Adipogenic transcriptional regulation of hepatic stellate cells, J. Biol. Chem. 280 (2005) 4959–4967.

[4] H. Martin, Role of PPAR-gamma in inflammation. Prospects for therapeutic intervention by food components, Mutat. Res. 669 (2009) 1–7.

[5] C.D. Allred, D.R. Talbert, R.C. Southard, X. Wang, M.W. Kilgore, PPARgamma1 as a molecular target of eicosapentaenoic acid in human colon cancer (HT-29) cells, J. Nutr. 138 (2008) 250–256.

[6] X.C. Li, M.R. Jacob, S.I. Khan, M.K. Ashfaq, K.S. Babu, A.K. Agarwal, H.N. Elsohly, S.P. Manly, A.M. Clark, Potent in vitro antifungal activities of naturally occurring acetylenic acids, Antimicrob. Agents Chemother. 52 (2008) 2442–2448.

[7] I. Sadowski, M. Ptashne, A vector for expressing GAL4(1–147) fusions in mammalian cells, Nucleic Acids Res. 17 (1989) 7539.

[8] C.W. Pugh, J.F. O'Rourke, M. Nagao, J.M. Gleadle, P.J. Ratcliffe, Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit, J. Biol. Chem. 272 (1997) 11205–11214.

[9] A. Seiler-Tuyns, J.D. Eldridge, B.M. Paterson, Expression and regulation of chicken actin genes introduced into mouse myogenic and nonmyogenic cells, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 2980–2984.

[10] D.C. Eustice, P.A. Feldman, A.M. Colberg-Poley, R.M. Buckery, R.H. Neubauer, A sensitive method for the detection of beta-galactosidase in transfected mammalian cells, Biotechniques 11 (1991) 739–740. 742–733.

[11] H. Sakaue, W. Ogawa, M. Matsumoto, S. Kuroda, M. Takata, T. Sugimoto, B.M. Spiegelman, M. Kasuga, Posttranscriptional control of adipocyte differentiation through activation of phosphoinositide 3-kinase, J. Biol. Chem. 273 (1998) 28945–28952.

[12] Y. Gao, M.F. Yang, Y.P. Su, H.M. Jiang, X.J. You, Y.J. Yang, H.L. Zhang, Ginsenoside Re reduces insulin resistance through activation of PPAR-gamma pathway and inhibition of TNF-alpha production, J. Ethnopharmacol. 147 (2013) 509–516.

[13] S. Shak, N.O. Reich, I.M. Goldstein, P.R. Ortiz de Montellano, Leukotriene B4 omega-hydroxylase in human polymorphonuclear leukocytes. Suicidal inactivation by acetylenic fatty acids, J. Biol. Chem. 260 (1985) 13023–13028.

[14] P. Tontonoz, B.M. Spiegelman, Fat and beyond: the diverse biology of PPARgamma, Annu. Rev. Biochem. 77 (2008) 289–312.

[15] T. Waku, T. Shiraki, T. Oyama, K. Maebara, R. Nakamori, K. Morikawa, The nuclear receptor PPARgamma individually responds to serotonin- and fatty acid-metabolites, EMBO J. 29 (2010) 3395–3407.

[16] M. Penumetcha, N. Santanam, Nutraceuticals as Ligands of PPARgamma, PPAR Res. 2012 (2012) 858352.

[17] L.M. Leesnitzer, D.J. Parks, R.K. Bledsoe, J.E. Cobb, J.L. Collins, T.G. Consler, R.G. Davis, E.A. Hull-Ryde, J.M. Lenhard, L. Patel, K.D. Plunket, J.L. Shenk, J.B. Stimmel, C. Therapontos, T.M. Willson, S.G. Blanchard, Functional consequences of cysteine modification in the ligand binding sites of peroxisome proliferator activated receptors by GW9662, Biochemistry 41 (2002) 6640–6650.

[18] K. Kang, W. Liu, K.J. Albright, Y. Park, M.W. Pariza, Trans-10, cis-12 CLA inhibits differentiation of 3T3-L1 adipocytes and decreases PPAR gamma expression, Biochem. Biophys. Res. Commun. 303 (2003) 795–799.

[19] L. Granlund, L.K. Juvet, J.I. Pedersen, H.I. Nebb, Trans10, cis12-conjugated linoleic acid prevents triacylglycerol accumulation in adipocytes by acting as a PPARgamma modulator, J. Lipid Res. 44 (2003) 1441–1452.

[20] J.Y. Yang, M.A. Della-Fera, S. Rayalam, H.J. Park, S. Ambati, D.B. Hausman, D.L. Hartzell, C.A. Baile, Regulation of adipogenesis by medium-chain fatty acids in the absence of hormonal cocktail, J. Nutr. Biochem. 20 (2009) 537–543.

[21] A. Steger, J. van Loon, Fat of the seed of *Picramnia sow*, Recl. Trav. Chim. Pay. B 52 (1933) 593–600.

[22] P.B. Lumb, J.C. Smith, Higher aliphatic compounds X. Synthesis of tariric and petroselinic acids, J. Chem. Soc. (1952) 5032–5035.

[23] A. Hirasawa, T. Hara, S. Katsuma, T. Adachi, G. Tsujimoto, Free fatty acid receptors and drug discovery, Biol. Pharm. Bull. 31 (2008) 1847–1851.

[24] Y. Itoh, Y. Kawamata, M. Harada, M. Kobayashi, R. Fujii, S. Fukusumi, K. Ogi, M. Hosoya, Y. Tanaka, H. Uejima, H. Tanaka, M. Maruyama, R. Satoh, S. Okubo, H. Kizawa, H. Komatsu, F. Matsumura, Y. Noguchi, T. Shinohara, S. Hinuma, Y. Fujisawa, M. Fujino, Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40, Nature 422 (2003) 173–176.

[25] S. Edfalk, P. Steneberg, H. Edlund, Gpr40 is expressed in enteroendocrine cells and mediates free fatty acid stimulation of incretin secretion, Diabetes 57 (2008) 2280–2287.

[26] C.F. Burant, P. Viswanathan, J. Marcinak, C. Cao, M. Vakilynejad, B. Xie, E. Leifke, TAK-875 versus placebo or glimepiride in type 2 diabetes mellitus: a phase 2, randomised, double-blind, placebo-controlled trial, Lancet 379 (2012) 1403–1411.