Aralia cordata Inhibits Triacylglycerol Biosynthesis in HepG2 Cells

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ABSTRACT Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first committed step in triacylglycerol (TAG) and phospholipid biosynthesis, and has been considered as one of the drug targets for treating hepatic steatosis, insulin resistance, and other metabolic disorders. The aim of this study was to investigate the GPAT inhibitors from natural products and to evaluate their effects. The methanol extract of *Aralia cordata* roots showed a strong inhibitory effect on the human GPAT1 activity. A further bioactivity-guided approach led to the isolation of *ent*-pimara-8(14),15-dien-19-oic acid, (PA), one of the major compounds of *A. cordata*, which suppressed the GPAT1 activity with IC₅₀ value of 60.5 μ M. PA markedly reduced *de novo* lysophosphatidic acid synthesis through inhibition of GPAT activity and therefore significantly decreased synthesis of TAG in the HepG2 cells. These results suggest that PA as well as *A. cordata* root extract could be beneficial in controlling lipid metabolism.

KEY WORDS: • *Aralia cordata* • *ent*-pimara-8(14),15-dien-19-oic acid • glycerol-3-phosphate acyltransferase • lysophosphatidic acid • triacylglycerol

INTRODUCTION

BIOSYNTHESIS OF TRIACYLGLYCEROL (TAG) is fundamentally important for the maintenance of cellular energy homeostasis. Almost all excess calories, whether from fats, proteins, or carbohydrates, are converted to TAGs and stored primarily in adipose cells, which constitute the adipose tissues. However, excessive accumulation of TAG in adipose tissue leads to obesity. In nonadipose tissue, such as the pancreas, skeletal muscle, and liver excessive TAG induces adverse effects, including lipotoxicity, leading to insulin resistance and type 2 diabetes.^{1,2} Elevated TAG in the blood stream is a risk factor for cardiovascular disease and coronary artery disease.³ Therefore, pharmacological inhibition of TAG biosynthetic enzymes has been recently suggested to be one of the potential strategies to treat these disorders.

There are two major pathways for TAG synthesis; the glycerol phosphate pathway involved in *de novo* lipogenesis and the monoacylglycerol pathway, which plays a major role in lipid absorption. The first committed rate-limiting

step in the glycerol phosphate pathway is catalyzed by glycerol-3-phosphate acyltransferase (GPAT). Currently, four mammalian GPAT isoforms have been identified—two mitochondrial GPAT (GPAT1 and GPAT2)^{4,5} and two microsomal GPAT (GPAT3 and GPAT4), each encoded by separate genes.^{6–8} The activity of GPAT1 can be differentiated from other isoforms by their resistance to sulfhydryl group reactive reagents such as *N*-ethylmaleimide (NEM).⁹ GPAT1 is most highly expressed in the liver and adipose, and accounts for 30–50% of the total GPAT activity in the liver.¹⁰ GPAT1 is regulated at both the transcriptional and post-transcriptional levels. GPAT1 mRNA is upregulated by a high carbohydrate, insulin, and sterol regulatory element binding protein-1 (SREBP-1)¹¹ and downregulated by AMP-activated protein kinase (AMPK).¹² The activation of GPAT1 is closely related with the requirements for *de novo* TAG synthesis, suggesting a role for this enzyme in regulating TAG biosynthesis.

TAG synthesis is catalyzed sequentially by enzymes that have multiple isoforms, including GPAT, 1-acylglycerol-3-phosphate acyltransferase (AGPAT), and diacylglycerol acyltransferase (DGAT). The inhibition of these enzymes using genetic alterations results in decreased TAG in various tissues.^{13–17} Noteworthy, the previous studies have reported that mice with genetically modified GPAT in the liver^{14,18,19} are closely developing hepatic steatosis, suggesting that the

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GPAT enzyme can be the therapeutic target. Recently, a couple of GPAT inhibitors have been reported.^{20,21} However, the pharmacological validation of their use in cells and animal models remains to be examined.

Aralia cordata Thunb. belongs to the family Araliaceae, which has long been recognized in Korea, China, and Japan as therapeutic herbs with antinociceptive,²² antidementia,²³ antioxidant,²⁴ anticancer,^{25,26} and anti-inflammatory²⁷ activities. The root of this plant has been used to treat rheumatism, lumbago, common cold, migraines, and lameness clinically. A previous study showed that the dichloromethane fraction of the root contains essential oil, saponins, sesquiterpenes and diterpene acids, diterpenes, polyacetylenes, and sterols.²⁸ During the screening of human GPAT1 inhibitors from natural sources, we found that the MeOH extract from the root of *A. cordata* strongly inhibited the GPAT1 enzymatic activity. The further bioactivity-guided approach led to the isolation of the diterpene compound, *ent*-pimara-8(14),15-dien-19-oic acid (PA). In this study, we isolated and identified a bioactive molecule from *A. cordata*, which affected the GPAT1 activity and TAG metabolism.

MATERIALS AND METHODS

Materials

Optical rotations were determined on a JASCO P-1020 Polarimeter using a 100-mm glass microcell. ¹H-NMR (300 MHz) and ¹³C-NMR (100 MHz) spectra were obtained on a Varian Unity Inova 400 spectrometer using CDCl₃ as the solvent. Electrospray ionization (ESI) mass spectrometry (MS) was measured on a Finnigan navigator spectrometer. Stationary phases for column chromatography (Kieselgel 60, 70–230, and 230–400 mesh, and Lichroprep RP-18 gel, 40–63 μm; Merck) and TLC plates (Kieselgel 60 F₂₅₄ and RP-18 F₂₅₄; Merck) were purchased from Merck. Bovine serum albumin and palmitoyl-CoA were obtained from Sigma Chemical Co. [1-¹⁴C] acetic acid (250 μCi) was purchased from Amersham Biosciences. [1-¹⁴C] glycerol (50 μCi) and [1-¹⁴C] oleate (50 μCi) were purchased from PerkinElmer.

Extraction and isolation

The dried roots of *A. cordata* (3 kg) were purchased from a herbalist in Daejeon (Korea) and extracted with MeOH by maceration for 15 days at room temperature to give 373 g of dried MeOH extract, which was suspended in 1 L of water and then extracted with an equal volume of CHCl₃. A part of the CHCl₃-soluble fraction (50 g) was separated by silica gel column chromatography (9.5 cm diameter × 35.0 cm, 2 kg, 70–230 mesh; Merck) using a stepwise gradient of hexane: EtOAc (10:1–1:1), which afforded 11 subfractions (C1–C11). The fraction C3 (3 g) was rechromatographed over a silica column chromatography (5 cm diameter × 120 cm; 230–400 mesh; eluting solvent: 100% hexane) to yield crude PA, which was recrystallized from MeOH yielding a diterpene compound, PA (1.3 g). The compound was sealed and stored in a dark place at –20°C.

ent-pimara-8(14),15-diene-19-oic acid: C₂₀H₃₀O₂, colorless needle (MeOH), ESI-MS: *m/z* 301 [M-H]⁺, 357 [M+Na]⁺; UV (MeOH); λ_{max} 203 nm, mp: 165–166 °C; [α]_D²⁵ = –120° (*c* 0.75, CHCl₃); ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 5.71 (1H, dd, *J* = 10.2, 17.1 Hz, H-15), 5.15 (1H, br s, H-14), 4.95 (1H, dd, *J* = 1.8, 10.2 Hz, H-16b), 4.91 (1H, dd, *J* = 1.8, 17.1 Hz, H-16a), 1.26 (3H, s, H-18), 1.00 (3H, s, H-17), 0.66 (3H, s, H-20); ¹³C-NMR (100 MHz, CDCl₃): δ (ppm) 39.2 (C-1), 19.6 (C-2), 38.0 (C-3), 44.0 (C-4), 56.1 (C-5), 24.1 (C-6), 35.8 (C-7), 137.9 (C-8), 50.5 (C-9), 39.2 (C-10), 19.2 (C-11), 36.4 (C-12), 39.2 (C-13), 128.0 (C-14), 147.2 (C-15), 112.8 (C-16), 29.3 (C-17), 29.1 (C-18), 183.8 (C-19), 13.8 (C-20).

Human GPAT1 expression in Sf-9 cells and membrane preparation

Human GPAT1 genes were overexpressed in Sf-9 insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer's instructions. hGPAT1 (gene accession number: BC030783) cDNA clone is a gift from 21C Human Gene Bank, Genome Research Center, KRIBB, Korea. The cDNA with an in-frame C-terminal FLAG tag was cloned into the pFastBac1 donor vector (Invitrogen) to obtain the recombinant bacmid. Sf-9 cells infected with the resultant baculovirus at 10 MOI for 56 h were harvested in ice-cold PBS and homogenized using a sonicator (Fisher Scientific) in the sucrose solution (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM DTT). Nuclei were removed by centrifuging at 600 *g* for 15 min at 4°C. The resulting supernatants were further centrifuged at 8000 *g* for 15 min at 4°C to collect crude mitochondria. The pellets were resuspended and the protein concentration was quantified using the Bradford protein assay method.

The GPAT activity was measured according to the method of Hammond *et al.*⁴ The assay buffer contained 75 mM Tris, pH 7.5, 4 mM MgCl₂, 8 mM NaF, 100 μM palmitoyl-CoA, 1.8 μM [¹⁴C] glycerol-3-phosphate, and 2 mg/mL BSA. To measure the NEM-resistant GPAT activity, protein samples were mixed with NEM (2 mM final concentration) for 15 min at 4°C before starting the reaction. The reaction was started by mixing 2 μg of mitochondrial membrane fraction with the assay buffer in a final volume of 200 μL and shaking the samples for 20 min at 26°C. The reaction was stopped by adding 1 mL of water-saturated butanol and then adding 0.5 mL of butanol-saturated water to extract lysophosphatidic acid (LPA). After vortexing, 0.8 mL of the organic phase was transferred to a tube and washed once with 0.8 mL of butanol-saturated water. The radioactivity of the top phase was determined by liquid scintillation counting (Tri-Carb 2900TR Liquid Scintillation Analyzer; PerkinElmer).

Cell culture and viability test

Human hepatocyte HepG2 cells were obtained from the American Type Culture Collection. Cells were maintained in the DMEM (Invitrogen) supplemented with 10%

heat-inactivated FBS and 1% penicillin–streptomycin (Sigma) in 5% CO₂ at 37°C. To test whether the PA has cytotoxic effects on HepG2 cells, we determined viability by the MTT assay. Cells were seeded in 96-well plates (2 × 10⁴ cells/well) and treated with compounds for 24 h. The absorbance of the formazan dye was read using the ELISA plate reader at 560 nm.

Cellular TAG and LPA formation assay using HepG2 cells

The cell-based assay for confirmation of GPAT inhibition was performed in HepG2 cells. To determine the *de novo* synthesis of LPA and TAG, the cells were co-treated with PA or vehicle at the indicated concentrations and [¹⁴C] glycerol (0.5 μCi) or [¹⁴C] acetate (0.5 μCi) or [¹⁴C] acetate (0.5 μCi). At the end of the incubation, in-

tracellular lipids were extracted with a mixture of hexane/isopropanol (3:2, v/v). Cellular lipids were resolved on silica plates by thin layer chromatography (Kieselgel 60 F₂₅₄ plates; Merck) using either a solvent system consisting of hexane/diethyl ether/acetic acid (80:20:1, v/v) for TAG or chloroform/ethanol/water/triethylamine (30:35:6:35, v/v/v/v) for LPA. The isotope-labeled lipids were detected and quantified with a bioimage analyzer (FLA-7000; Fuji).

Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed using the Student's *t*-test (unpaired) with GraphPad Prism 4 software. A *P* value of < .05 was considered to be significant.

FIG. 1. Effect of MeOH extract from *Aralia cordata* and its CHCl₃ and H₂O phases on GPAT1 activity and *de novo* triacylglycerol (TAG) synthesis. (A) The MeOH extract and its partitions were tested against *in vitro* GPAT1 activity. (B) MTT assay was performed with increasing concentrations up to 30 μM for 24 h. (C, D) Lipid profile analyzed by TLC using either [¹⁴C] acetate or [¹⁴C] glycerol as radiolabeled substrates. Relative percentage of vehicle control represents was shown as a graph. Similar results were obtained in more than three independent experiments. **P* < .05, ***P* < .01, ****P* < .001 vs. vehicle control. AC-M, crude MeOH extract; AC-C, CHCl₃-soluble part separated out of AC-M; DMSO, dimethyl sulfoxide; BBR, berberine (positive control).

FIG. 2. Activity-guided isolation of *ent*-pimara-8(14),15-dien-19-oic acid (PA) from the roots of *A. cordata* and its hGPAT1-inhibitory activity. **(A)** CHCl₃ partitions were sequentially fractionated into 11 sub-fractions using a stepwise gradient of hexane: EtOAc (10:1–1:1) and individual fractions analyzed for their effects on GPAT1 activity. Pure active compound (PA) was isolated using a silica column chromatography and chemical structure was shown below of schematic representation. **(B)** *In vitro* GPAT1 inhibitory activity of PA. Mitochondrial fractions of Sf-9 cells expressing hGPAT1 were prepared as described in **Materials and Methods** and the effects of PA on hGPAT1 activity with increasing concentrations were performed. Values are means ($\pm$ standard deviation [SD]) for three separate preparations.

RESULTS

MeOH extract and its fractions of A. cordata inhibit human GPAT1 activity and intracellular TAG synthesis

The crude MeOH extract (AC-M) showed considerable inhibition of the human GPAT1 activity with an IC₅₀ value of 19.7 μg/mL by confirming the *in vitro* enzymatic activity assay. The extract was separated into two portions with a CHCl₃-soluble part (AC-C) and remaining water residue (AC-H). The AC-C exhibited more potent GPAT1 inhibitory effects with an IC₅₀ value of 19.4 μg/mL, suggesting that putative bioactive molecules were transferred into the CHCl₃ phase (Fig. 1A). To examine whether AC-M and AC-C can inhibit newly synthesized TAG through the inhibition of GPAT1, we analyzed isotope-labeled TAG contents after treatment of [¹⁴C] acetate or [¹⁴C] glycerol as a substrate in the presence or absence of compounds in HepG2 cells. First, cell viability assay was performed to avoid any toxic effects on cells using the MTT assay. The treatment of both fractions (AC-M and AC-C) up to 20 μg/mL for 24 h had no effect on the viability of HepG2 cells (Fig. 1B). TLC analyses revealed that the AC-M and AC-C efficiently inhibit incorporation of TAG from both acetates and glycerol in a dose-dependent manner (Fig. 1C, D). Moreover, these data showed that AC-M and AC-C have similar or better efficacy compared with berberine (BBR, a positive control, 15 μg/mL) at the same concentration. A previous study showed that BBR inhibits TAG synthesis through AMPK activation.²⁹

Isolation of PA as an hGPAT1 inhibitory compound

Further bioactivity-guided fractionation of the AC-C led to the isolation of an active substance with appreciable GPAT1 inhibitory activity. The structure of the isolate was identified as one of the pimarane-type diterpenes, PA by direct comparison of its physical and spectral properties (ESI-MS, ¹H-NMR, and ¹³C-NMR) with reference data (Fig. 2A). PA was one of the major compounds of the MeOH extract (AC-M) of *A. cordata* roots with a yield of 0.62% w/w and occupied 2.6% w/w of chloroform fraction (AC-C). To evaluate the activity of PA against GPAT1, we performed *in vitro* GPAT1 enzymatic assay using the mitochondrial membrane fraction prepared from human GPAT1-overexpressed Sf-9 cells. PA showed considerable inhibitory efficacy against GPAT1 with an IC₅₀ value of 60.5 μM (Fig. 2B).

To determine whether PA exhibits multifunctional effects on other acyltransferases, we examined its inhibitory effects on human DGAT1 (acyl CoA:diacylglycerol acyltransferase-1), which is well known to catalyze the esterification of *sn*-1,2-diacylglycerol with long-chain fatty acyl CoA in the final step in TAG synthesis. As a result, PA exhibited weak DGAT1 inhibition with an IC₅₀ value of 193 μM (data not shown), suggesting it was more specific against hGPAT1 than hDGAT1.

PA inhibits endogenous GPAT

Because PA is the main isolate from the AC-C, we next assessed the ability of PA to inhibit the endogenous GPAT

FIG. 3. Effect of PA on lysophosphatidic acid (LPA) formation in HepG2 cells. **(A)** Lipid profile analyzed by TLC using [¹⁴C] glycerol as radiolabeled substrates. **(B)** The radioactivity of LPA bands were quantified by densitometry using the Multi-Gauge V3 program (Fuji Photo Film Co.) and relative percentage of vehicle control represents was shown as a graph. Data represent mean ($\pm$ SD) obtained from three independent experiments. ***P* < .01, ****P* < .001 versus vehicle control. STD, standard (¹⁴C-labeled LPA).

activity by measuring its product LPA in HepG2 cells. In the intracellular condition, the glycerol could be metabolized and sequentially converted into glycerol-3-phosphate, LPA, phosphatidic acid, and TAG through the glycerol phosphate pathway. When incubated with ^{14}C -labeled glycerol to estimate *de novo* LPA synthesis, cells are expected to show increased generation of ^{14}C -labeled LPA, which was attenuated by treatment of PA in a dose-dependent manner (Fig. 3A, B).

PA blocks intracellular TAG synthesis in HepG2 cells

Finally, we examined whether PA inhibits *de novo* intracellular TAG synthesis by suppressing the GPAT1 activity. TAG synthesis was determined using ^{14}C acetate or ^{14}C glycerol incorporation, whereas TAG assembly was assessed by the incorporation of ^{14}C oleate in HepG2 cells. Cells were incubated with $0.5\ \mu\text{Ci}$ ^{14}C acetate or ^{14}C glycerol for 6 h and ^{14}C oleate for 2 h in the presence of PA ($10\sim 30\ \mu\text{M}$) or BBR ($15\ \mu\text{g/mL}$ [$40.3\ \mu\text{M}$], a positive control). As observed in Figure 4A, 20 and $30\ \mu\text{M}$ PA in-

hibited ^{14}C acetate incorporation into TAG by 21.5% and 72.1%, respectively. At the same time, PA suppressed ^{14}C glycerol incorporation into TAG by 24.1% and 79% at 20 and $30\ \mu\text{M}$, respectively (Fig. 4B). Moreover, PA abolished incorporation of ^{14}C oleate to TAG by 57.1% and 39.4% at 20 and $30\ \mu\text{M}$ (Fig. 4C), suggesting that TAG assembly was also affected during the treatment. These findings concluded that PA could inhibit *de novo* TAG synthesis through the regulation of GPAT1 positioning on the first step in the glycerol phosphate pathway.

DISCUSSION

Previous studies revealed that overexpression of GPAT1 in hepatocytes increased TAG synthesis rather than phospholipids.³⁰ In addition, liver-directed overexpression of GPAT1 in lean mice and rats resulted in hepatic steatosis and hepatic insulin resistance through the increase of TAG secretion.³¹ Conversely, selective hepatic GPAT1 knockout in *ob/ob* mice resulted in decreased hepatic steatosis, and improved plasma glucose and cholesterol levels.^{13,32} Thus,

FIG. 4. Effect of PA on *de novo* intracellular TAG synthesis in HepG2 cells. Lipid profile analyzed by TLC using either ^{14}C acetate (A), ^{14}C glycerol (B), or ^{14}C oleate (C) as radiolabeled substrates. Relative percentage of vehicle control represents was shown as a graph. Similar results were obtained in more than three independent experiments. Data represent mean ($\pm$ SD) obtained from three independent experiments. * $P < .05$, ** $P < .01$, versus vehicle control.

inhibiting GPAT1 may be an attractive way to interfere with hepatic TAG accumulation and related diseases.¹⁹

Recently, the inhibition of lipogenesis was shown to increase fatty acid oxidation.^{20,33} Carnitine palmitoyl-transferase-1 (CPT-1), the rate-limiting enzyme of the mitochondrial fatty acid β -oxidation, and GPAT1 are both located on the outer mitochondrial membrane and compete for long-chain acyl-CoAs as their substrate. Genetic or pharmacological GPAT inhibition resulted in an increase in oxidation because the acyl-CoA substrates were partitioned toward CPT-1 for β -oxidation rather than the TAG synthetic pathway.²⁰ Conversely, GPAT1 overexpression in rat hepatocytes resulted in a dramatic decrease in fatty acid oxidation and a significant increase in hepatic diacylglycerol and phospholipid biosynthesis.³⁰ Therefore, further studies are needed to investigate the effect of PA on fatty acid β -oxidation and alteration of related gene profile.

Previous studies reported that PA has anti-inflammatory³⁴ and vasorelaxant effects,³⁵ however, there has been no comprehensive research on the function of PA in TAG metabolism. Therefore, this is the first time report of PA on downregulation of TAG synthesis in human HepG2 cells.

In this study, we showed that the MeOH extract and its fractions of *A. cordata* roots have strong inhibitory effects on human GPAT1 activity. Also, PA, which was isolated from the MeOH extract of *A. cordata* roots, markedly decreased new syntheses of LPA and TAG by inhibiting GPAT1 in the HepG2 cells. Therefore, these results suggest that PA as well as the extract of *A. cordata* roots could be beneficial in regulating lipid metabolism and useful for preventing or treating lipid metabolism disorders and their complications.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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