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PPARγ Ligand-binding Activity of Fragrin A Isolated from Mace (the Aril of *Myristica fragrans* Houtt.)

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Abstract Peroxisome proliferator-activated receptor-gamma (PPARγ), a member of the nuclear receptor of ligand-activated transcription factors, plays a key role in lipid and glucose metabolism or adipocytes differentiation. A lignan compound was isolated from mace (the aril of *Myristica fragrans* Houtt.) as a PPARγ ligand, which was identified as fragrin A or 2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)-propane. To ascertain whether fragrin A has PPARγ ligand-binding activity, it was performed that GAL-4/PPARγ transactivation assay. PPARγ ligand-binding activity of fragrin A increased 4.7, 6.6, and 7.3-fold at 3, 5, and 10 μM, respectively, when compared with a vehicle control. Fragrin A also enhanced adipocytes differentiation and increased the expression of PPARγ target genes such as adipocytes fatty acid-binding protein (aP2), lipoprotein lipase (LPL), and phosphoenol pyruvate carboxykinase (PEPCK). Furthermore, it significantly increased the expression level of glucose transporter 4 (GLUT4). These results indicate that fragrin A can be developed as a PPARγ agonist for the improvement of insulin resistance associated with type 2 diabetes.

Keywords: peroxisome proliferator-activated receptor-gamma (PPARγ), mace, *Myristica fragrans* Houtt. (nutmeg), fragrin A, type 2 diabetes

Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of a nuclear receptor superfamily. PPARs have 3 members of subtype α, β, and γ. PPARα is highly expressed in the liver, skeletal muscle, and kidney and regulates the expression of target genes related in lipid catabolism (1). PPARβ is ubiquitously expressed and thought to be involved in thermogenesis, cell proliferation, and carcinogenesis (2). PPARγ, expressed predominantly in adipose tissue, plays a central role in the control of adipocyte gene expression and differentiation (3). It has been reported that PPARγ is a regulator of the insulin resistance and glucose metabolism in adipose tissue and it is the predominant molecular target for the insulin-sensitizing thiazolidenedione (TZD) drugs of type-2 diabetes such as troglitazone, pioglitazone, and rosiglitazone (4).

However, the TZD class of compounds, which includes rosiglitazone, pioglitazone, and troglitazone, are associated with a number of adverse effects. These side effects are body weight gain, hemodilution, edema, and increasing the risk of heart failure (5). Particulary, troglitazone was ultimately withdrawn due to cases of irreversible liver toxicity (6). Thus, the safety concerns have recently led to late stage development failures of synthetic PPARγ agonists (7). For reducing this side effect, PPARγ activators of edible plants origin can confer advantages to provide health benefits without toxicity concerns.

Plants have played a significant role in maintaining human health and improving the quality of life for thousands of years. It has been reported that some herbal medicines improve diabetes mellitus, hyperlipidemia, and cardiovascular diseases associated with an abnormality in lipid metabolism (8,9). During our search for effective PPARγ activators from medicinal plants, it was found that mace or the aril of *Myristica fragrans* Houtt. (nutmeg) has potent PPARγ ligand-binding activity.

M. fragrans is a perennial herb native to Indonesia and cultivated in the South Africa, the Molucca Islands, India, and other tropical areas. Mace has been traditionally used for spice and also possesses carminivative, astringent, hypolipidaemic, antithrombotic, anti-platetlet aggregation, antifungal, aphrodisiac, anxiogenic, anti-diarrheal, and anti-inflammatory activities (1). However, its activity as a PPARγ agonist has not as yet been reported to date. This research focused on the PPARγ ligand-binding activity of an active compound isolated from mace and its effect on the expression of PPARγ target genes in 3T3-L1 adipocytes.

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Materials and Methods

Plant material and chemicals Mace was collected in Jakarta, Indonesia, and identified by Dr. Nam-In Baek, Department of Oriental Medicinal Materials and Processing, Kyunghee University, Yongin, Korea. Mace was dried and deposited at 4°C in the Department of Biotechnology, Yonsei University (Seoul, Korea). Troglitazone and bisphenol A diglycidyl ether (BADGE) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of fragrin A The powdered mace (100 g) was extracted twice with 75%(v/v) methanol for 5 days at room temperature, and the extract (13.26 g) was fractionated with ethyl acetate, butanol, and water in turn (ethyl acetate fraction 10.29 g, butanol fraction 0.17 g, water fraction 2.80 g). The ethyl acetate fraction was applied to silica gel column (70-230 mesh, Merck & Co., Whitehouse Station,

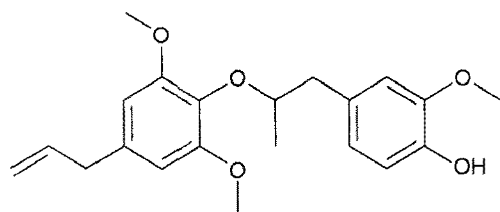


Fig. 1. Chemical structure of fragrin A.

NJ, USA) and eluted with chloroform and ethyl acetate (9:1, v/v) to give 10 fractions (Fraction I to X). Fraction V (0.52 g) was further separated using a silica gel column with chloroform, ethyl acetate, and acetone (24:0.3:1, v/v/v). Third fraction (Fraction V-C) of the second silica column was eluted with 100% methanol using recycling preparative high performance liquid chromatography (HPLC, column: GS-310, 21.5 mm i.d.×500 mm l, Japan Analytical Industry Co., Ltd., Tokyo, Japan) and compound V-C (0.03 g) was finally obtained as a single compound. Careful comparison of several spectral data of compound V-C including ^{13}C-nuclear magnetic resonance (NMR), ^{1}H-NMR, ^{13}C-DEPT, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, and fast atom bombardment-mass spectra (FAB-MS) with those in the literature (10) suggested the chemical structure to be fragrin A (Fig. 1) or 2-(4-allyl-2,6-dimethoxyphenoxy)-1-(4-hydroxy-3-methoxyphenyl)-propane which belongs to a kind of lignan compounds.

Instrumentation NMR spectra were recorded on a Bruker Avance-600 spectrometer (Rheinstein, Germany) at 600 MHz for ^{1}H- and ^{13}C- in $CDCl_3$ with tetramethylsilane (TMS) as an international standard. Complete proton and carbon assignments were based on 1D (^{1}H-, ^{13}C-, ^{13}C-DEPT) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC) NMR experiments. FAB-MS were measured using JMS-700 (Jeol Ltd., Tokyo, Japan). All instrumental data are available upon request.

Cell culture and cell viability Mouse 3T3-L1 pre-adipocytes and monkey COS-7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with antibiotics (100 U/mL of penicillin A and 100 U/mL of streptomycin) and 10% heat-inactivated fetal bovine serum (FBS, Gibco, Rockville, MD, USA). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

GAL-4/PPARγ transactivation assay PPARγ ligand-binding activity was measured using a GAL-4/PPARγ transactivation assay (11). COS-7 cells were inoculated into 100 mm^2 culture plate at 2×10^6 cells/plate, and incubated in 5% CO_2/air at 37°C for 24 hr. DMEM containing 10% FBS and 10 mL/L penicillin-streptomycin (5,000 IU/mL and 5,000 mg/mL) (Gibco) was used as the medium. Cells were transfected with pFA-hPPARγ, pFR-Gal4 (UAS-Gal4-luciferase), and pFR-β-galactosidase (Stratagene, La Jolla, CA, USA) using Lipofectamin and Plus reagent (Invitrogen, Madison, WI, USA). In an internal control, pFA and pFR-Gal4 (UAS-Gal4-luciferase) were transfected into COS-7 cells. After 4 hr from transfection, the medium was changed with DMEM containing 10% FBS and the cells were further cultured for 16 hr. Then, the cells were cultured with samples for 24 hr. Subsequently, cell were washed with Dulbecco's phosphate buffered saline (D-PBS) and luciferase assay substrate (Promega, Madison, WI, USA) was added. The intensity of emitted luminescence was determined using a luminescence plate reader (PerSeptive Biosystems Inc., Wiesbaden-Nordenstadt, Germany). PPARγ ligand-binding activity of the samples were detected as the relative light unit (RLU) to that of control.

Adipogenesis 3T3-L1 cells were plated into 96-well culture plate at 1×10^4 cells/well and maintained for 2 days after reaching confluence. Then, medium was exchanged with differentiation medium (DMEM containing 10% FBS, 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 10 μg/mL insulin) and cells were incubated for 2 days. Finally, differentiation medium was replaced with adipocyte growth medium (DMEM supplemented with 10% FBS and 10 μg/mL insulin), which was refreshed every 2 days. At day 1, 3, 5, 7, and 9 after differentiation medium was added, cells were retrieved and neutral lipid accumulation

Table 1. Primer sequences for PPARγ target gene with annealing temperatures, product sizes, and accession number of NCBI GenBank

Gene[1)]	Primers (from 5' to 3')	Tm (°C)	Size (bp)	NCBI GenBank (Accession No.)
aP2	forward: ACAGCTCCTCCTCGAAGG reverse: GCGTAAATGGGGATTTGGTCA	54	151	NM_024406
PEPCK	forward: AGGCATCGATGAGCCGCT reverse: GCTGCAGAACACAAGGGCC	48	241	NM_011044
LPL	forward: ATCCATGGATGGACGGTAACG reverse: CTGGATCCCAATACTTCGACCA	52	368	NM_008509
GLUT4	forward: ATCCATGGATGGACGGTAACG reverse: CTGGATCCCAATACTTCGACCA	51	367	NM_009204
β-Actin	forward: TGGAATCCTGTGGCATCCATGAAC reverse: TAAAACGCAGCTCAGTACAGTCCG	52	369	NM_007393

[1)]aP2, Adipocyte fatty acid-binding protein; PEPCK, phosphoenol pyruvate carboxykinase; LPL, lipoprotein lipase; GLUT4, glucose transporter 4.

was measured using as described previously (12). Briefly, cells were washed with D-PBS, fixed with 10% buffered formalin, and stained with Oil Red O solution (0.5 g in 100 mL isopropanol) for 10 min. After removing the staining solution, the stained lipid in the cells was dissolved into isopropanol and quantity of lipid accumulation was determined at $OD_{540\,nm}$ with a microplate reader (Molecular Devices Corp., San Francisco, CA, USA).

RNA preparation and reverse transcriptase (RT)-polymerase chain reaction (PCR) Total RNA was isolated with Trizol reagent (Invitrogen) at 3 days after differentiation. The oligonucleotide primers of PPARγ target genes were designed using a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database (Table 1). The reaction solution (25 μL final volume) contained 0.5 μL of avian myeloblastosis virus (AMV) reverse transcriptase (5 Unit), 12.5 μL of AccessQuick™ RT-PCR System Mater Mix (2X) (Promega) and 100 pM of each primer. The conditions of RT-PCR were as follows: RT reaction at 48°C for 45 min, 25 cycles of denaturation at 95°C for 60 sec, annealing at each appropriate temperature as described for 60 sec, and extension at 72°C for 60 sec.

Statistical analysis Each experiment was performed at least independently in triplicate. All data are presented as the mean±standard error of the mean (SEM). The data analysis was performed using one-way analysis of variance (ANOVA). The difference between treated and control groups were also analyzed by the Duncan test (SPSS 12.0). Values of $p<0.05$ and $p<0.01$ were considered statistically significant.

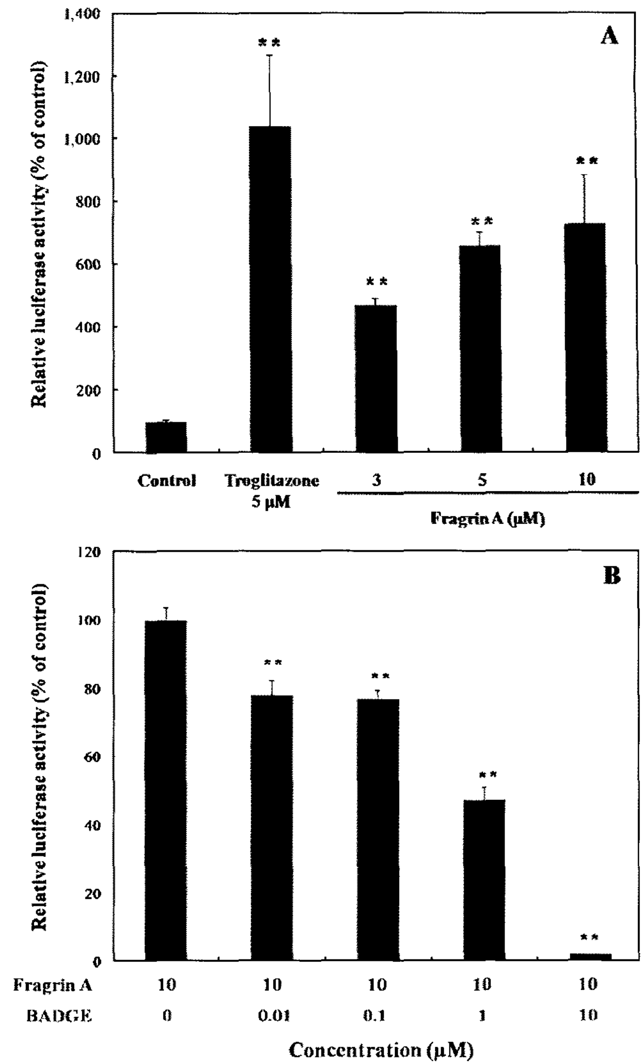


Fig. 2. PPARγ ligand-binding activity of fragrin A in GAL-4/PPARγ transactivation assay. (A) The values are means±SEM of 3 independent experiments. *$p<0.05$ compared with the vehicle control. Positive control was troglitazone (5 μM). (B) The activity of 10 μM fragrin A without BADGE was set at 100%. The values are means±SEM of 3 independent experiments. Significance was determined using the Duncan test vs. vehicle control (**$p<0.01$).

Results and Discussion

PPARγ ligand-binding activity of fragrin A GAL-4/PPARγ transactivation assay was carried out to investigate the effect of fragrin A on the PPARγ ligand-binding activity. Fragrin A exhibited PPARγ ligand-binding activity and its relative luminescence activity was increased 4.7, 6.6, and 7.3-fold at concentrations of 3, 5, and 10 μM, respectively, when compared with a vehicle control (Fig. 2A). The concentrations of fragrin A used in these assays had no influence on cell viability (data not shown). Furthermore, PPARγ ligand-binding activity was confirmed by BADGE, which binds to the PPARγ ligand binding domain and strongly inhibits PPARγ ligand-mediated transactivation (13). BADGE suppressed fragrin A-induced transactivation of PPARγ in a dose-dependent manner (Fig. 2B), indicating that fragrin A plays a role as a competitive ligand in PPARγ-mediated transactivation. These results support that fragrin A potentially activate PPARγ as an agonist.

Effect of fragrin A on adipogenesis Adipocyte differentiation, which is also referred to as adipogenesis, is the process by which preadipocytes are converted into mature adipocyte (14). PPARγ is a central regulator of adipocyte differentiation and is a key transcription factor for induction of adipogenic marker genes (15). As shown in Fig. 3, incubation of 3T3-L1 adipocytes with fragrin A resulted in increasing lipid accumulation when compared to a vehicle control. However, the lipid accumulation was less than troglitazone treatment, which was consistent with the result of the PPARγ ligand-binding activity. This result indicates that fragrin A can induce adipogenesis via relatively lower PPARγ activation in 3T3-L1 adipocytes than troglitazone. It is conceivable that fragrin A can modulate serious increase of fat mass which is the side effect of synthetic agonists like troglitazone.

PPARγ target genes expression In adipocytes, PPARγ regulates the expression of numerous target genes involved in lipid metabolism, glucose homeostasis, and adipocyte differentiation, including adipocytes fatty acid-binding protein (aP2), lipoprotein lipase (LPL), and phosphoenol pyruvate carboxykinase (PEPCK) (16). aP2 is a carrier protein for fatty acids that is primarily expressed in adipocytes (17). LPL is an enzyme that hydrolyzes lipids in lipoproteins, like those found in chylomicrons and very

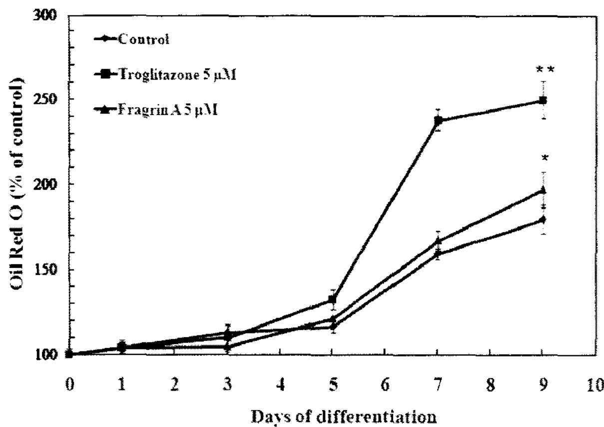
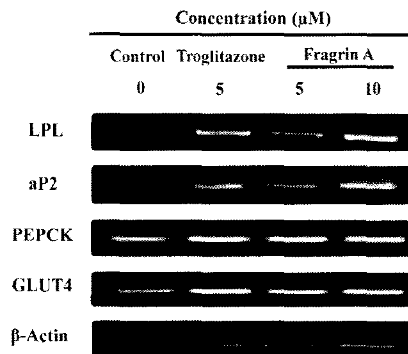


Fig. 3. Effect of fragrin A on 3T3-L1 adipocyte differentiation. Each value is expressed as the relative % compared to a vehicle control at 0 day after differentiation. The values are the means± SEM of 3 independent experiments. Significance was determined using the Duncan's test vs. a vehicle control at 9 day after differentiation (*p<0.05; **p<0.01).



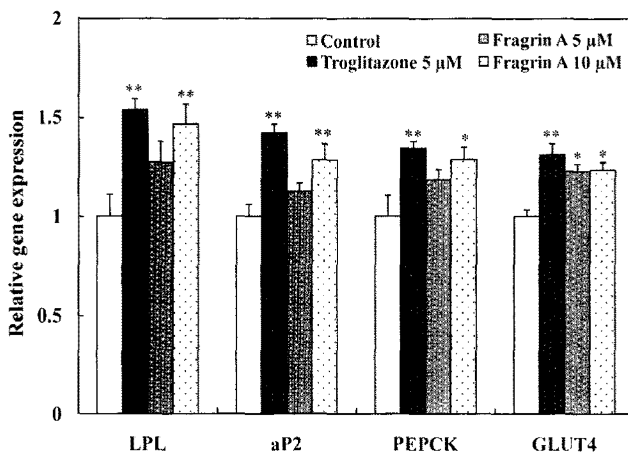


Fig. 4. Effect of fragrin A on PPARγ target gene expression. The relative expression levels are presented as fold induction to that of the vehicle control. All values are means±SEM of 3 independent experiments. Significance was determined using the Duncan's test vs. vehicle control (**p<0.01).

low density lipoproteins (VLDL), into 3 free fatty acids and 1 glycerol molecule (18). PEPCK is an enzyme that catalyzes the conversion of oxaloacetate and ATP to phosphoenolpyruvate, carbon dioxide, and ADP. The blood glucose level is maintained within well-defined limits in part due to precise regulation of PEPCK gene expression (19).

In order to provide biological evidence of fragrin A as a PPARγ ligand, it was investigated that the expressions of adipocyte marker gene related to adipocyte differentiation in fragrin A-treated 3T3-L1 cells. Fragrin A induced expression of aP2, LPL, and PEPCK in adipocytes in a dose dependent manner (Fig. 4). These results demonstrate that fragrin A can induce adipocyte differentiation by increasing expressions of aP2, LPL, and PEPCK via PPARγ activation. We also studied the effect of fragrin A on gene expression of GLUT4 in 3T3-L1 adipocytes. GLUT4, which is related to glucose homeostasis, is an insulin-responsive glucose transporter associated with insulin sensitivity in adipocytes (20). In 3T3-L1 adipocytes treated with 10 μM of fragrin A, GLUT4 mRNA expression was significantly increased when compared with a vehicle control (Fig. 4). These results indicate that fragrin A acts as an insulin sensitizer through up-regulation of GLUT4.

In the present study, fragrin A isolated from mace was found as a PPARγ agonist. Fragrin A, showing PPARγ ligand-binding activity, up-regulated adipogenesis, and increased the expressions of PPARγ target genes and GLUT4 related with insulin sensitivity in 3T3-L1 adipocyte. Fragrin A may contribute to insulin-sensitizing effects of PPARγ through regulating lipid and glucose metabolism in 3T3-L1 adipocyte. It is anticipated that fragrin A regulates type 2 diabetes and metabolic pathways as a natural PPARγ agonist.

Acknowledgments

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