Fitoterapia 101 (2015) 57–63

Contents lists available at ScienceDirect

Fitoterapia

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

Identification of evodiamine as the bioactive compound in evodia (*Evodia rutaecarpa* Benth.) fruit extract that activates human peroxisome proliferator-activated receptor gamma (PPARγ)

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ARTICLE INFO

Article history:
Received 14 November 2014
Accepted in revised form 10 December 2014
Accepted 16 December 2014
Available online 24 December 2014

Keywords:
Evodia fruit extract
Evodiamine
Evodia rutaecarpa Benth
Peroxisome proliferator-activated receptor gamma
Rutaecarpine

ABSTRACT

The dried unripe fruit from *Evodia rutaecarpa* Benth., known as Wu zhu yu in China, has long been used in traditional Chinese medicine. In this research, we provide evidence that evodia fruit extract activates peroxisome proliferator-activated receptor gamma (PPARγ) and, as identified through HPLC fractionation and mass spectroscopy, the activating phytochemical is evodiamine. Evodiamine was shown to bind to and activate PPARγ. It was also shown to activate PPARγ-regulated gene expression in human hepatoma cells similar to known PPARγ ligands and that the expression was blocked by a PPARγ specific antagonist.

1. Introduction

The dried unripe fruit from *Evodia rutaecarpa* Benth., known as Wu zhu yu in China, has long been used in traditional Chinese medicine for the treatment of gastrointestinal disorders (abdominal pain, dysentery), headache, amenorrhea, and postpartum hemorrhage [1–3]. It also has a history of use as a cardiotonic, a central stimulant with transient hypertensive and positive inotropic and chronotropic effects [4,5]. In phytochemical studies, the indoloquinazoline alkaloids from the Evodia fruit (*Evodiae fructus*), evodiamine and rutaecarpine, have been linked to these cardiotonic effects via activation of the vanilloid receptor [6] and subsequent production of calcitonin gene-related peptide [7]. These compounds also act on other cardiovascular-related targets, rutaecarpine inhibits platelet aggregation and evodiamine is a vasorelaxant [8,9].

Rutaecarpine and evodiamine have also been shown to act at additional biological targets. In inflammation, rutaecarpine directly inhibits COX-2 enzymatic activity [10], while evodiamine inhibits COX-2 gene expression via an NF-κB/Akt dependent pathway [11,12]. In aberrant cell growth, evodiamine has been shown to inhibit cellular growth, invasion, and metastasis of a wide variety of tumor cells, while having little toxicity on normal human peripheral blood cells [13]. In energy metabolism, evodiamine affects biological targets involved in obesity and adipocyte maturation while reducing diabetic complications in vivo [14,15]. It has been shown to block adipogenesis by activating ERK thru EGFR activation and PKCα [15,16].

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand activated nuclear receptor that directly binds to and regulates gene expression (for review see [17]). Its activation

Abbreviations: COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; FABP1, human fatty acid binding protein 1; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; HPRT1, hypoxanthine phosphoribosyltransferase; LBD, ligand-binding domain; PCK1, phosphoenolpyruvate carboxykinase 1; PPAR, peroxisome proliferator-activated receptor; PKCα, protein kinase C alpha; TR-FRET, time-resolved fluorescence resonance energy transfer; UAS, upstream activating sequence.

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http://dx.doi.org/10.1016/j.fitote.2014.12.009

affects energy metabolism as well as inflammation and tumor growth. In adipocytes, PPARγ activation stimulates lipids and glucose uptake, leading to lipid storage. However, evodiamine blocks PPARγ induced adipogenesis [18]. Therefore, evodiamine is not an obvious PPARγ agonist. PPARγ ligands have a positive effect on insulin action and improve glucose tolerance in diabetic animals and humans, as well as improvements in inflammation and cognitive function [17]. These observations are consistent with the effects seen for evodiamine in obesity, diabetic, and Alzheimer's disease animal models [14,15,19].

In this research, we showed that evodia fruit extracts demonstrate a robust response in a PPARγ luciferase reporter assay. We further identified through HPLC fractionation and mass spectroscopy that the activating phytochemical is evodiamine.

2. Materials and methods

2.1. Chemicals and reagents

Evodiamine, rutaecarpine, and PPARγ agonists, rosiglitazone, and troglitazone were obtained from Sigma Corporation (St. Louis, MO). Evodiamine is a synthetic product (Sigma Corp.) and as such is a mixture of chiral forms [20,21]. The PPARγ antagonist, T0070907, and PPARα agonist, GW-7647, were from Tocris Biologicals (Minneapolis, MN).

Vectors pGL4.35 9XUAS luc2P and pFN26A BIND, and Fugene 6 were purchased from Promega Inc. (Madison, WI). Restriction enzymes were from New England Biolabs (Ipswich, MA), D-luciferin was from Biotium (Hayward, CA), and LanthaScreen TR-FRET PPARγ competitive binding assay was from Life Technologies (Grand Island, NY).

Dulbecco's minimum essential media (DMEM), Ham's F-12K (Kaighn's, F12K) media, Dulbecco's phosphate buffered saline (DPBS), fetal bovine serum (FBS), pennicillin/streptomycin, and amphotericin B were purchased from Fisher Scientific (Pittsburgh, PA); hygromycin from EMD Millipore (Temecula, CA); and bovine serum albumin (BSA), G-418, and β-mercaptoethanol (BME) were purchased from Sigma Corporation (St. Louis, MO).

RNeasy Plus Mini kit, and quantitative polymerase chain reaction (qPCR) primer sets for human fatty acid binding protein 1 (FABP1), phosphoenolpyruvate carboxykinase 1 (PCK1), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), and the housekeeping gene, hypoxanthine phosphoribosyltransferase 1 (HPRT1) were purchased from Qiagen (Toronto, ON). First strand cDNA synthesis kit, iScript, and the real-time qPCR kit, SsoFast EvaGreen, were from Bio-Rad Laboratories (Hercules, CA).

2.2. Evodia rutaecarpa fruit extracts

E. rutaecarpa fruit was harvested from plants cultivated in China. The fruit was dried, milled into a homogenous feedstock, and extracted at room temperature for 24 h in methanol or chloroform using a ratio of 1:10 (fruit:solvent). The extract was filtered, and solvent was removed by drying under nitrogen. The resulting solids were quantified by mass, suspended in the original extraction solvent, aliquoted, re-dried, sealed, and stored at −80 °C. Before biological testing, the dried solids were suspended in 70:30 DMSO:water, shaken for 2 h, and diluted in cell culture media prior to assay.

Powdered *E. rutaecarpa* fruit extract was suspended in methanol:water (50:50) at 50 mg/mL, sonicated for 10 min, then filtered through a 0.45 μm PVDF membrane syringe filter into an autosampler vial for UPLC fractionation and LC-MS analysis.

2.3. UPLC fractionation and LC-MS analysis

Chromatographic separation was performed on an Acquity UPLC-H chromatograph with photodiode array detector (PDA) and XBridge Shield RP18 (5 μm, 4.6 × 250 mm) column. The mobile phase solutions used for gradient separation were: A, 0.1% acetic acid in water and B, 0.1% acetic acid in acetonitrile. The mobile phase gradient (A:B), at ambient temperature and a flow rate of 0.8 mL/min, was initially set at 95:5 and linearly changed to 0:100 from 0 to 30 min, held for 2 min, and then, returned to initial conditions at 32.1 min and held till 35 min. The injection volume was 40 μL for both samples and standards and the chromatogram recorded between 210 and 800 nm at a 1.2 nm resolution.

The effluent was split after the PDA, with the majority going to a fraction collector configured for 96 well plates with 2 mL/well volume. Fractions were collected at 20 s intervals for 32 min. The plates were frozen at −80 °C overnight, and the solvent was removed by freeze drying. The sample plates were sealed and stored dry at −20 °C until assayed for activity.

The remaining effluent was directed to a Waters Synapt G2 mass spectrometer equipped with an electrospray ion source and operated at 25 V cone voltage. Accurate mass spectra were collected in positive ion mode from *m/z* 100 to 1200 at 0.5 s/scan, with alternating spectra collected at 0 V and 20 V collision energy in the transfer cell (MS^e mode). The mass spectra, UV spectra, and fractions were time aligned for identification of active compounds.

2.4. Plasmid constructs

To study the activation of PPARγ and PPARα in a luciferase reporter assay, Gal4-PPARγ-ligand-binding domain (LBD) and Gal4-PPARα-LBD vectors were created in a modified pFN26A (BIND) vector (Promega, Inc.). The barnase gene was excised and a short cloning region (5′-cgcagagctcaaaagcg-3′) was inserted at the PvuI/EcoRI site to create pBIND3. The PPARγ-LBD (residues 203–477) was qPCR amplified from human MGC: 5041 (pSPORT6; Open Biosystems, Inc.) using forward and reverse primers 5′-acgatcgaacagctgaatccagagtccgctga-3′ and 5′-tctagactagtacaag tccttgtagatctcctgcaggagc-3′, incorporating pvuI and xbaI sites at the 5′ and 3′ ends, respectively. The PPARγ-LBD fragment was then ligated into the pBIND3 PvuI/XbaI site forming a Gal4-PPARγ-LBD fusion protein. Similarly, the PPARα-LBD (residues 201–468) vector was created using forward and reverse primers, 5′-cgatcgcagacctcaaatctctgggc-3′ and 5′-gaattcagtacatgtctctgtagatctcttgc-3′, to amplify the PPARα-LBD from mouse MGC:18607 (Invitrogen). The PPARα-LBD fragment was then ligated into the pBIND3 PvuI/EcoRI site forming a Gal4-PPARα-LBD fusion protein.