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Myricanol mitigates lipid accumulation in 3T3-L1 adipocytes and high fat diet-fed zebrafish via activating AMP-activated protein kinase

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ABSTRACT

Myricanol is a diarylheptanoid isolated from Chinese bayberry. Through virtual docking strategy, myricanol was discovered as an AMP-activated protein kinase (AMPK) activator among a series of structural analogs, with high affinity for the γ subunit of AMPK. Myricanol was also evaluated for regulatory effects on lipid accumulation and insulin sensitivity in 3T3-L1 adipocytes and adiposity in high-fat diet-fed zebrafish. Myricanol suppressed lipid accumulation in 3T3-L1 cells in the initial stage (days 0–2) by suppressing adipogenesis and in the terminal stage (days 4–7) by inducing lipolysis and lipid combustion through activating AMPK. Moreover, myricanol enhanced insulin-stimulated glucose uptake by activating the insulin signaling pathway. In high-fat diet-fed zebrafish, myricanol inhibited lipid accumulation by suppressing adipogenic factors including peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα). In summary, the results indicate that myricanol could be a potential therapeutic agent against obesity by activating the AMPK signaling pathway.

1. Introduction

Obesity has reached epidemic proportions worldwide, and obesity-associated insulin resistance, diabetes, atherosclerosis, and cardiovascular diseases are increasing at an alarming rate (WHO, 2016 http://www.who.int/mediacentre/factsheets/fs311/en/). Adipocyte dysfunction is a key factor in the onset of obesity. Excess energy is stored in adipocytes in the form of triglycerides, via adipocyte hyperplasia (increased number of adipocytes) and hypertrophy (increased size of adipocytes) (Rosen & MacDougald, 2006). Therefore, modulating the function of adipocytes is crucial for treating obesity and its related diseases.

The differentiation of preadipocytes to adipocytes is important in regulating fat mass. 3T3-L1 cells represent a well-characterized model of adipocyte differentiation (Gregoire, Smas, & Sul, 1998; Rosen & Spiegelman, 2000). The differentiation of preadipocytes into adipocytes, also called adipogenesis, is categorized into initial, middle, and terminal stages (Rosen & Spiegelman, 2000). Adipogenesis is tightly regulated by numerous complex biomolecules, including adipogenic transcriptional factors, lipogenic enzymes, and triglyceride synthesis enzymes (Lowe, O'Rahilly, & Rochford, 2011). Peroxisome proliferator-activated receptor γ (PPARγ) is expressed at the early stage of adipocyte differentiation and is associated with lipogenesis because this nuclear receptor regulates the expression of fatty acid synthase (FAS). Lipid accumulation is initiated at the middle stage and accelerates in the late stage of differentiation (Rosen & Spiegelman, 2000). Hormone-sensitive lipase (HSL) is a rate-limiting enzyme for lipolysis. Carnitine palmitoyltransferase 1 (CPT1) is an enzyme that is important for lipid metabolism and promotes the transport of long-chain fatty acids into the mitochondria. Uncoupling protein 1 (UCP1) plays a key role in regulating the thermogenic capacity of adipose tissues. This protein enables the separation of lipid oxidation from ATP production, thus inducing higher metabolic rate and the conversion of nutritional energy to heat (Cypess et al., 2013; Harms & Seale, 2013). PPARγ coactivator-1α (PGC-1α) is a cold-inducible transcriptional coactivator (Finck & Kelly, 2006; Lin, Handschin, & Spiegelman, 2005). PGC-1α stimulates mitochondrial biogenesis and electron transport activity and potently induces the expression of genes implicated in energy homeostasis in almost every cell type through interacting directly with and co-activating the mitochondrial regulators, such as PPARs (Wang et al., 2003).

AMP-activated protein kinase (AMPK) is an important energy sensor that regulates adipocyte metabolism (Zhang, Zhou, & Li, 2009). AMPK

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activation could insulin-independently promote cellular glucose uptake, enhance fatty acid oxidation, and inhibit lipogenesis through short-term regulation of protein phosphorylation or long-term effects on gene expression (Zhang et al., 2009). Previous studies have demonstrated that AMPK activators, including 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and A-769662, inhibit adipogenesis (Lee, Kang, Bae, & Yoon, 2011). Moreover, AMPK activation suppresses the expression of PPARγ in adipocytes (Yang et al., 2001). Chronic AMPK activation in hyperleptinemic rats was associated with increased expression of PGC-1α, higher mitochondrial content, upregulation of UCPs, elevated expression of enzymes involved in β-oxidation, and decreased expression of lipogenic enzymes in white adipose tissue (Orci et al., 2004). Thus, AMPK is one of the most promising drug targets for obesity treatment.

Chinese bayberry, *Myrica rubra* (Lour.) Sieb. et Zucc (Myricaceae), is an economically important plant that has been cultivated in southern China for more than 2000 years. Chinese bayberry is well recognized for its nutritional and medicinal values as it is rich in polyphenols. Previous studies have disclosed that different extracts from Chinese bayberry exhibited potential effects for treating diabetes. In streptozotocin-induced diabetic mice, the bayberry fruit extract rich in cyaniding-3-*O*-glucoside significantly reduced blood glucose, improved glucose tolerance, and protected the insulin secretion ability of pancreatic *β* cells (Sun et al., 2012). In another study, a bayberry extract protected pancreatic *β* cells against H_2O_2-induced necrosis and apoptosis via extracellular signal-regulated kinases 1/2- and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)-mediated heme oxygenase-1 upregulation (Zhang et al., 2010). Recently, the flavonoids-rich extract from Chinese bayberry was found to stimulate glucose uptake and consumption in hepatocytes and diabetic KK-A(y) mice (Zhang et al., 2016). However, until now, the role of Chinese bayberry in lipid metabolism and insulin sensitivity in adipocytes remains unclear.

We previously isolated several diarylheptanoids from the barks of Chinese bayberry (Shen, Xia, Li, Liu, & Pan, 2015). In the current study, virtual docking for AMPK activators was performed using AutoDock 4 (version 4.2). Myricanol (MY) was identified as an AMPK activator among a series of diarylheptanoids. The effect of MY in regulating adipogenesis and insulin sensitivity in adipocytes, as well as lipid accumulation in high-fat diet-fed zebrafish, are described.

2. Materials and methods

2.1. Molecular docking study

Crystal structure of AMPK used in the current study was obtained from the Brookhaven Protein Data Bank (PDB, http://www.rcsb.org/pdb). The PDB entry is 4rew (Li et al., 2015). Python Molecular Viewer (PMV version 1.5.6) (Sanner, 1999) was employed to deal with both the ligand and receptor. Whole structure of AMPK was edited including deleting water molecule, α and β subunits, since the known activators like metformin and AICAR interact with the γ subunit AMPK (Day et al., 2007; Zhang, Wang, et al., 2012), γ subunit was retained for receptor preparation. Hydrogens were added using AutoDockTool (Morris et al., 2009; Sanner, 1999) integrated in PMV. The structures of compounds downloaded from ChemSpider database (http://www.chemspider.com/) were treated as ligand. For the ligand, Gasteiger charges were assigned with nonpolar hydrogens merged. The atom types and bond types were assigned, which integrated in PMV. The docking area was defined by a 60 × 100 × 100 3D grid centered around the ligand binding site with a 0.375 Å grid space. The grid maps were generated using the auxiliary program autogrid4 package. All bond rotations for the ligand was ignored and the Lamarckian genetic algorithm (LGA) was employed for docking process. Compound C (CC), an AMPK inhibitor, and MY were performed the docking analysis.

2.2. Reagents

The isolation procedure of MY was described previously (Shen et al., 2015). MY, AICAR, resveratrol (RSV) and CC were dissolved in DMSO. In the cell-based assays, the final DMSO concentration was less than 0.1%. Phosphate-buffered saline (PBS) powder, Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin (P/S), calf serum (CS), fetal bovine serum (FBS) and Nile red were purchased from Life Technologies (Grand Island, NY, USA). All other chemicals (analytical grade) unless specified were obtained from Sigma-Aldrich (St. Louis, MO, USA). All antibodies were purchased from Cell Signaling Technology (Boston, MA, USA).

2.3. Cell culture

Murine preadipocyte 3T3-L1 cells were obtained from ATCC (Manassas, VA, USA). 3T3-L1 preadipocytes were maintained in high-glucose (4.5 g/l) DMEM with 10% CS and 1% P/S. To induce differentiation, 2 days post confluence (day 0), cells were treated with DMEM containing 10% FBS and DMI [1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl xanthine (IBMX) and 5 μg/ml insulin] for 2 days. Cells were then maintained in DMEM with 10% FBS and 5 μg/ml insulin, and medium was changed every other day. Cells were harvested on day 8. Cells were treated with MY, AICAR, RSV or CC as indicated.

2.4. Cell viability assay

Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously (Feng et al., 2017). Cells were seeded in 96-well plates at a density of 1×10^5 cells/well. When at approximately 70–80% confluence, cells were treated with or without MY at different concentrations for 24, 48 and 72 h, respectively. Doxorubicin (60 μM) was added as a positive control. Then cell viability was determined by incubation with DMEM containing MTT (1 mg/ml) for 4 h, followed by dissolving the formazan crystals with DMSO. The absorbance at 570 nm was measured by a SpectraMax M5 microplate reader (Molecular Devices, CA, USA). The calculation equation for relative cell viability was as following: cell viability (%) = (As − A0)/(Ac − A0) × 100%, where As, A0 and Ac were the absorptions of test sample, blank control and negative control (DMSO), respectively.

2.5. Nile red staining

To detect intracellular lipid accumulation, Nile red staining was performed on fully differentiated adipocytes. Cells were washed twice with PBS and then incubated in PBS containing 1 μg/ml Nile red for 15 min at 37 °C. After washed with PBS twice, the fluorescence was monitored at an excitation wavelength of 488 nm and an emission wavelength of 550 nm, using a SpectraMax M5 microplate reader.

2.6. Western blot analysis

Cells were washed twice with cold PBS and then lysed with cold RIPA buffer containing freshly added phosphatase inhibitor cocktails and phenylmethylsulfonyl fluoride (PMSF) on ice for 30 min. Whole cell lysates were centrifuged at 17,400 *g* for 20 min and the supernatants were transferred into new tubes. Protein concentration of each sample was quantified using the BCA protein assay kit (Life Technologies, Grand Island, NY, USA). The same amount of proteins (30 μg) were separated by 8% SDS-PAGE, transferred to PVDF membranes (Bio-Rad Laboratories, Inc.), blocked with 5% nonfat milk in TBST buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 0.1% Tween-20) for 1 h at room temperature, and incubated with specific primary antibodies overnight at 4 °C. After washing with TBST three times, a horseradish