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Ramalin inhibits differentiation of 3T3-L1 preadipocytes and suppresses adiposity and body weight in a high-fat diet-fed C57BL/6J mice

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ABSTRACT

Obesity is a serious global health problem. Natural substances that could be effective remedies for treatment of obesity, and which are relatively safe, are desired. The aim of this study was to examine the anti-obesity effect and the mechanism of ramalin in 3T3-L1 preadipocytes and high fat diet (HFD)-induced obese mice. In this study, 3T3-L1 cells were treated with various concentrations of ramalin (1, 5, and 10 μg/ml). Ramalin reduced the accumulation of intracellular lipid droplets in 3T3-L1 cells. In addition, ramalin inhibited 3T3-L1 adipocyte differentiation by blocking adipogenic gene expression including CCAAT enhancer binding proteins (C/EBPs), peroxisome proliferator-activated receptors γ (PPARγ), adipocyte fatty acid-binding protein (aP2), and leptin. The suppression of adipogenesis by ramalin was mediated through the inhibition of MAPK pathways. Ramalin also reduced the secretion of TNF-α and IL-6 in 3T3-L1 adipocytes. Oral administration of ramalin (50 and 100 mg/kg) to HFD-fed mice reduced body weight gain and abdominal fat accumulation without changes in food intake. Ramalin also attenuated organ weight and basal serum level by inhibiting liver X receptors (LXRs), sterol regulatory element-binding protein-1c (SREBP-1c), and lipoprotein lipase (LPL) mRNA expression in HFD-fed mice. Taken together, these results indicate that ramalin inhibits adipogenesis in 3T3-L1 preadipocytes and prevents HFD-induced obesity. The present study also provides insight into the mechanisms underlying the anti-obesity activity of ramalin and suggests that ramalin has the potential to prevent obesity.

1. Introduction

Obesity is a major risk factor for several metabolic disorders including type 2 diabetes, hypertension, cardiovascular diseases and osteoarthritis [1]. Obesity is increasing in prevalence globally and obesity has become a significant global health threat. Cellular and animal studies on the development of obesity have revealed that dietary factors can change the number of adipocytes and adipocyte size [2]. Adipose tissue stores excess energy and is an endocrine organ [3].

In response to an adipogenic cocktail (MDI) consisting of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin, postconfluent 3T3-L1 preadipocytes undergo a series of transcriptional activations [4]. The process begins with CCAAT/enhancer binding protein beta (C/EBPβ) expression, followed by C/EBP δ, peroxisome proliferator-activated receptor gamma (PPARγ), and C/EBPα, which play an important role in adipocyte phenotype [5,6]. The process of adipocyte differentiation is divided into the early, intermediate, and late stages [7]. The early stage of adipogenesis is mainly controlled by C/EBPδ and C/EBPβ, which are rapidly increased by inducers of cell division and hormonal stimulation [8]. Increased levels of C/EBPδ and C/EBPβ trigger changes in the expressions of PPARγ and C/EBPα, which are involved in the

Abbreviations: CCAAT/enhancer-binding protein alpha, C/EBPα; CCAAT/enhancer-binding protein beta, C/EBPβ; Peroxisome proliferator-activated receptors gamma, PPARγ; Adipocyte fatty acid-binding protein, aP2; Liver X receptor alpha, LXRα; Liver X receptor beta, LXRβ; Lipoprotein lipase, LPL; Sterol regulatory element-binding protein-1c, SREBP-1c; Mitogen-activated protein kinase, MAPK; Interleukin-6, IL-6; Tumor necrosis factor-alpha, TNF-α; Normal fat diet, NFD; High fat diet, HFD; 3-isobutyl-1-methylxanthine, IBMX.

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regulation of the late stage of adipogenesis [9]. The activation of PPARγ and C/EBPα regulates the expression of multiple adipogenic genes necessary for fat accumulation.

Intracellular mitogen-activated protein kinase (MAPK) signaling pathways play a critical role in the regulation of cell proliferation and differentiation [10]. MAPK pathways are involved in the early stage of adipocyte differentiation and regulate the expression of C/EBPα and PPARγ, contributing to the expression and phosphorylation of downstream proteins related to adipocyte differentiation in 3T3-L1 cells [8,10]. Therefore, it is plausible that MAPK pathways are a potential target for the treatment of obesity. Understanding the mechanism by which preadipocytes differentiate into adipocytes would aid in developing therapeutic strategies to prevent obesity.

Various food materials or natural compounds isolated from edible plants that show an anti-obesity effect have been screened for use as functional foods or dietary supplements. Metabolites extracted from Antarctic lichen reportedly have a variety of bioactivities that include antibiotic, anti-mycobacterial, anti-viral, anti-inflammatory, analgesic, anti-pyretic, anti-proliferative, and cytotoxic effects [11]. In addition, they have been applied in natural cosmetics and medicines, and have demonstrated fewer side effects compared to industrial products. However, there is still no adequate information pertaining to the health-promoting properties of the bioactive compounds in lichen and their pharmaceutical potential.

Ramalin, isolated from the Antarctic lichen, *Ramalina terebrata* (Ramalinaceae) has anti-oxidant and anti-inflammatory activities [12,13]. There is increasing evidence that inflammation plays a central role in the metabolic consequence of obesity [14]. Recent reports also showed that the plasma levels of inflammatory mediators, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), are increased in the insulin resistant states of obesity and type 2 diabetes [15]. In preliminary experiments, we have shown that the exposure of 3T3-L1 preadipocytes to ramalin induces significant changes in the expression pattern of adipogenesis-related genes [16]. These findings lead us to explore the effect of ramalin on the regulation of lipid accumulation *in vitro* and *in vivo*. In the present study, we examined the effects of ramalin on adipogenesis in 3T3-L1 cells *in vitro* and whether ramalin can decrease obesity in high fat diet (HFD)-fed C57BL/6J mice *in vivo*. The results demonstrate that ramalin inhibits adipogenesis and reduces obesity in HFD-fed mice.

2. Materials and methods

2.1. Reagents

Unless otherwise indicated, all chemicals used in this study were purchased from Sigma-Aldrich Co. (St Louis, MO). Orlistat was were purchased from Cayman Chemical (Ann Arbor, MI). Bio-Rad DC protein assay was obtained from Bio-Rad (Hercules, CA). Antibodies against extracellular signal-regulated kinase (ERK), phospho-ERK, p38, phospho-p38, c-Jun N-terminal kinase (JNK), phospho-JNK, phospho-C/EBPβ, and PPARγ were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against C/EBPβ, cyclin A, CDK2, p27, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) kit was obtained from Amersham (Arlington Hights, IL). Trizol Reagent and SuperScript II cDNA synthesis kit were purchased from Invitrogen (Carlsbad, CA). Mouse TNF-α and IL-6 Enzyme-linked immunoassay (ELISA) MAX™ Standards were obtained from Biolegend, Inc. (San Diego, CA).

2.2. Synthesis of ramalin

Ramalin was synthesized as described previously and the synthesis of ramalin was already patented (Fig. 1) [17]. Briefly, 1.2-protected L-glutamic acid and 1-benzyl 2-aminophonol were the starting materials. Total synthesis of ramalin required four steps. The first step was 1′ carboxyl acid and 2′ amino site protected glutamic acid activation step for coupling reaction. Another coupling agent, phenyl hydrazine, was synthesized from 1-benzyl 2-aminophonol using $SnCl_2$ reagent. Coupling reaction with GPH-09 and GPH-04 produced GPH-11. Final deprotection reaction with palladium (on carbon) and H_2 gas produced ramalin.

2.3. Measurement of cell viability

3T3-L1 preadipocytes were seeded at a concentration of 8×10^3 cells/well in 96-well tissue culture plates and treated with the indicated concentrations of ramalin for 48 h. Cell viability was measured by a quantitative colorimetric assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which indicates the mitochondrial activity of living cells. The extent of reduction of MTT to formazan within cells was quantified by measuring the optical density at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

2.4. Determination of lipid accumulation by Oil Red O staining

Oil Red O staining was performed 8 days following ramalin exposure to stain the accumulated lipid droplets in the differentiated adipocytes. The cells were washed with phosphate buffered saline (PBS) and fixed with 10% formalin for 1 h at room temperature. The cells were rinsed with 60% isopropanol and stained with filtered Oil Red O solution for 20 min at room temperature. After removing the staining solution, the stained cells were washed with distilled water and dried. The stained lipid droplets were microscopically examined and photographed. To quantify intracellular lipid accumulation, the stained lipid droplets were dissolved with 100% isopropanol for 10 min. The optical density was measured at 490 nm using the aforementioned microplate reader.

2.5. Western blot analysis

After cell lysis, lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4°C. Protein concentration was determined using the Bio-Rad DC protein assay with bovine serum albumin (BSA) as the standard. The whole cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fractionated proteins were electrophoretically transferred to PVDF membranes (Amersham, Arlington Hights, IL) and probed with antibodies to extracellular signal-regulated kinase (ERK), phospho-ERK, p38, phospho-p38, c-Jun N-terminal kinase (JNK), phospho-JNK, phospho-C/EBPβ, PPARγ, C/EBPβ, cyclin A, CDK2, p27, and β-actin. The blots were developed using an enhanced chemiluminescence (ECL) kit. In all immunoblotting experiments, the blots were reprobed with an anti-β-actin antibody as a control for protein loading.

2.6. Cell cycle progression analysis

Cell cycle progression was measured by flow cytometric analysis after propidium iodide (PI) staining. Post-confluence preadipocytes were treated with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin in the presence or absence of 1, 5, and 10 μg/ml ramalin and 100 μg/ml orlistat for 24 h. A suspension of the cells was fixed overnight with 70% ethanol at 4°C, and then incubated with 10 μg/ml of RNase A and 50 μg/ml of propidium iodide staining buffer for 30 min at room temperature in the dark. Then, 10,000 cells per experiment were analyzed using a