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Fisetin regulates obesity by targeting mTORC1 signaling

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Abstract

Fisetin, a flavonol present in vegetables and fruits, possesses antioxidative and anti-inflammatory properties. In this study, we have demonstrated that fisetin prevents diet-induced obesity through regulation of the signaling of mammalian target of rapamycin complex 1 (mTORC1), a central mediator of cellular growth, cellular proliferation and lipid biosynthesis. To evaluate whether fisetin regulates mTORC1 signaling, we investigated the phosphorylation and kinase activity of the 70-kDa ribosomal protein S6 kinase 1 (S6K1) and mTORC1 in 3T3-L1 preadipocytes. Fisetin treatment of preadipocytes reduced the phosphorylation of S6K1 and mTORC1 in a time- and concentration-dependent manner. To further our understanding of how fisetin negatively regulates mTORC1 signaling, we analyzed the phosphorylation of S6K1, mTOR and Akt in fisetin-treated TSC2-knockdown cells. The results suggested that fisetin treatment inhibits mTORC1 activity in an Akt-dependent manner. Recent studies have shown that adipocyte differentiation is dependent on mTORC1 activity. Fisetin treatment inhibited adipocyte differentiation, consistent with the negative effect of fisetin on mTOR. The inhibitory effect of fisetin on adipogenesis is dependent of mTOR activity, suggesting that fisetin inhibits adipogenesis and the accumulation of intracellular triglycerides during adipocyte differentiation by targeting mTORC1 signaling. Fisetin supplementation in mice fed a high-fat diet (HFD) significantly attenuated HFD-induced increases in body weight and white adipose tissue. We also observed that fisetin efficiently suppressed the phosphorylation of Akt, S6K1 and mTORC1 in adipose tissue. Collectively, these results suggest that inhibition of mTORC1 signaling by fisetin prevents adipocyte differentiation of 3T3-L1 preadipocytes and obesity in HFD-fed mice. Therefore, fisetin may be a useful phytochemical agent for attenuating diet-induced obesity.

Keywords: Fisetin; mTORC1; 3T3-L1; Akt; Obesity

1. Introduction

Fisetin (3,7,3′,4′-tetrahydroxyflavone) is a naturally occurring flavonoid found in fruits and vegetables, especially onions, strawberries, blueberries, mangoes and the skin of cucumbers [1]. Fisetin has been widely investigated for its strong antioxidant activity and other cellular regulatory properties [2,3]. Recent studies have shown that fisetin not only possesses antioxidative activity but may also possess strong anticancer activity against a wide variety of cancer cells through regulation of various signaling pathways, including the AMP-activated kinase, extracellular-signal-regulated protein kinases 1 and 2, p53 and nuclear factor kappa B pathways [4–9]. A recent study has also suggested that fisetin is a novel regulator of mammalian target of rapamycin complex 1 (mTORC1) signaling in prostate cancer cells, leading to the induction of autophagic cell death [10]. As a master growth regulator, mTORC1 is stimulated by nutrients and growth factors, and substantial evidence suggests that mTORC1 functions in the regulation of obesity [11,12].

Recent findings have indicated that the tuberous sclerosis protein 1–2 complex (TSC1–TSC2) regulates adipocyte differentiation through the control of mTORC1 activity and peroxisome proliferator-activated receptor γ (PPAR-γ) expression [13], suggesting that mTORC1 may, at least in part, regulate adipogenesis. Many independent groups have reported that rapamycin inhibits the adipogenic process both *in vivo* and *in vitro* [14–17]. Genetic knockdown of raptor, a binding partner of mTORC1, in 3T3-L1 preadipocytes is reported to reduce adipogenesis [18], suggesting that mTORC1 plays a key role in adipogenesis in mammalian cells. Although the precise mechanism underlying mTORC1 signaling in adipogenesis is unknown, observations from previous studies suggest that mTORC1 controls adipogenesis/lipogenesis by inducing the translation of RNA encoding key constituents of the adipogenic process, namely, PPAR-γ and CCAT/enhancer binding protein-α (C/EBP-α) [19,20]. Interestingly, mTORC1 also promotes the expression of genes involved in fatty acid and cholesterol biosynthesis by activating the transcription factor sterol regulatory element-binding protein 1 (SREBP-1) [21]. Activation of SREBP-1 triggers the production of endogenous ligands for PPAR-γ, thereby promoting the transactivational activity of the

Abbreviations: mTORC1, mammalian target of rapamycin complex 1; S6K1, 70-kDa ribosomal protein S6 kinase 1; TSC2, tuberous sclerosis protein 2; HFD, high-fat diet; PPAR-γ, peroxisome proliferator-activated receptor γ; SREBP-1, sterol regulatory element-binding protein 1; C/EBP-α, CCAT/enhancer binding protein-α; Rheb, a Ras family GTPase.

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nuclear receptor [22]. Peterson and colleagues have suggested that mTORC1 regulates SREBP-1 by controlling the nuclear entry of lipin 1, a phosphatidic acid phosphatase that promotes triglyceride synthesis by catalyzing the conversion of phosphatidic acid into diacylglycerol [23]. These observations suggest that mTORC1 signaling plays a fundamental role in adipogenesis and lipogenesis. Through similar such studies, investigators should be able to determine the extent to which an mTORC1 inhibitor may be useful in the potential treatment of metabolic syndrome.

Recent findings show that fisetin could improve hyperglycemia and glycation in diabetic complications by regulating the key enzymes of carbohydrate metabolism [24–26] and might act as a potent inhibitor of the intestinal sugar transporter GLUT2 [27]. It is possible that fisetin can ameliorate or prevent obesity since many of these activities of fisetin have the potential to reduce the metabolic disorders associated with diabetes mellitus. In this study, we investigated whether fisetin inhibits mTORC1 activity and assessed mTORC1 as a putative target for reducing obesity.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine calf serum (CS), sodium pyruvate and penicillin–streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). Antibodies against PPARγ (sc-7273), CD36 (sc-13572), TSC2 (sc-893) and mTOR (sc-1549) and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against FAS (#3180), β-actin (#4967), C/EBPα (#2295s), p-mTOR (#5536s), mTOR (#9272), p-70-kDa ribosomal protein S6 kinase 1 (S6K1) (#9205), S6K1 (#9202), p-TSC2 (#3615s), p-Akt (#9271) and Akt (#9272) were purchased from Cell Signaling Technology (Danvers, MA, USA). Isobutylmethylxanthine (IBMX, I7018), dexamethasone (D4902) and Oil Red O (O0625) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triglyceride (TG, #21001) and total cholesterol (TC, # 1Y004) kits were purchased from Shinyang Diagnostics (Seoul, South Korea). Insulin (#80-INSMSU-E01) and leptin (#22-LEPMS-E01) enzyme-linked immunosorbent assay (ELISA) kits were obtained from Alpco Diagnostics (Windham, NH, USA). The mTOR kinase assay kit (#CBA055) was obtained from Calbiochem (San Diego, CA, USA).

2.2. Cell culture

3T3-L1 preadipocytes cells were cultured in DMEM with 1% penicillin–streptomycin and 10% CS at 37°C in 5% CO_2. Cells were regularly subcultured before reaching 70% confluence, and the passage number was kept at less than 8.

2.3. Knockdown of TSC2 by using siRNA

siTSC2 (sc-36763) and control siRNA (sc-36869) were purchased from Santa Cruz Biotechnology. Cells were seeded (2×10^5 cells) in six-well plates and transfected with siRNA using siRNA transfection reagent (sc-29528) according to the manufacturer's instructions.

2.4. Lentiviral preparation, viral infection and stable cell line generation

Lentiviral shRNA transduction was performed as described previously [28]. The pLKO.1 shRNA vectors encoding shTSC2 or scrambled shRNA were provided by Dr. Do-Hyung Kim (University of Minnesota, MN, USA), and the target sequences of the mouse TSC2-specific shRNA and scrambled shRNA were 5′-GCCTCGTATGAAGATGGCTAT-3′ and 5′-AACGTACGCGGAATACTTCGA-3′, respectively. The constructs were transfected into HEK293T cells with lentiviral packaging vectors pHR′8.2ΔR and pCMV-VSV-G (provided by S. Stewart, Washington University, MO, USA) using FuGENE 6 transfection reagent (Promega, Fitchburg, WI, USA). The 3T3-L1 cells were infected with the collected viruses in the presence of Polybrene and were selected by puromycin treatment (2 mg/ml).

2.5. Measurement of cell viability

The 3T3-L1 preadipocytes were seeded into a 96-well plate at a concentration of 5000 cells in 100 μl media per well. After 24 h of preconditioning, the cells were exposed to a variety of concentrations of fisetin for 24 h. Subsequently, 10 μl of solution from cell counting kit-8 (#CK04, Dojindo Molecular Technology, Kumamoto, Japan) was added into each well and further incubated at 37°C for 3 h to detect cell survival. The cell viability was calculated by measuring the absorbance using a microplate reader (Infinite M200 PRO; Tecan Group Ltd., Männedorf, Switzerland) at an excitation wavelength of 450 nm.

2.6. Cell differentiation

The cells were seeded into a six-well plate at a density of 4×10^5 cells/well. At 2 days postconfluence (day 0), the cells were treated for 2 days with an MDI solution containing 0.5 mM of 3-IBMX, 1 μM of dexamethasone and 1 μg/ml of insulin in DMEM with 10% FBS. After induction, the medium was replaced with DMEM containing 10% FBS and 100 nM insulin for a 2-day incubation period. The cells were maintained in DMEM with 10% FBS until maturity. To examine the effects of fisetin on the differentiation of preadipocytes into adipocytes, the cells were treated with various concentrations of fisetin at 2 days postconfluence (day 0).

2.7. Oil Red O staining

Cells were fixed with 10% neutral formalin for 1 h at room temperature for quantification, washed with phosphate-buffered saline and then stained for 1 h with 0.5% Oil Red O in 60% isopropanol. After the stained cells were washed with distilled water, they were observed under a fluorescence microscope. Oil Red O was extracted from cells with 100% isopropanol. Optical density was determined at an excitation wavelength of 490 nm.

2.8. Animal models

Male 4-week-old C57BL/6J mice were obtained from Orient Bio Inc. (Seoul, South Korea) and acclimatized to laboratory conditions comprising a 12:12-h light–dark cycle, temperature of 24°C and humidity of 55% for 1 week. The mice were then randomly divided into four groups: mice fed with the American Institute of Nutrition (AIN)-76A diet (normal or N group), test mice fed a high-fat diet (HFD group) and test mice fed an HFD supplemented with 0.2% or 0.5% (w/w) fisetin (HFD+LF or HFD+HF group, respectively) for 10 weeks. The experimental diets were prepared by supplementation of the basic AIN-76 diet with 20% fat and 0.5% cholesterol (w/w). Body weight and average daily food intake were measured weekly. The care and use of the animals followed our institutional and national guidelines, and all experimental procedures were approved by the Korea Food Research Institute Animal Care and Use Committee (KFRI-IACUC, #2011-0019).

2.9. Sample preparation and procedures

Ten weeks after supplementation with the experimental diets, the mice were subjected to fasting for 12 h and then sacrificed. Blood samples were collected from the abdominal aorta, centrifuged at 1000*g* for 15 min and stored at −80°C. The serum TG, TC and high-density lipoprotein (HDL) cholesterol levels were measured with the appropriate kits. The serum insulin and leptin concentrations were measured according to the protocol provided by the manufacturers of the mouse insulin and leptin ELISA kits, respectively. Epididymal and retroperitoneal tissues were excised, weighed and stored at −80°C for future use. Epididymal fat pads were either fixed in a 4% formalin solution and processed for histological analysis or were snap frozen in liquid nitrogen and stored at −80°C until protein extraction.

2.10. Histological analysis

The epididymal fat pads were fixed in 4% neutral-buffered formalin, embedded in paraffin, sliced into 5-μm–thick sections and stained with hematoxylin and eosin (H&E) stain. The pathological changes were assessed and photographed using an Olympus microscope (BX-51; Olympus, Tokyo, Japan).

2.11. Western blotting

The white adipose tissues of the HFD mice and the 3T3-L1 preadipocytes were lysed with cell lysis buffer containing 40 mM HEPES, pH 7.4; 120 mM NaCl; 1 mM EDTA; 50 mM NaF; 1.5 mM Na_3VO_4; 10 mM β-glycerophosphate and 1% Triton X-100 supplemented with EDTA-free phosphatase and protease inhibitor cocktail (#78441; Thermo Scientific, Waltham, MA, USA). After centrifugation at 14,000*g* for 20 min at 4°C, the supernatants were boiled in sodium dodecyl sulfate loading buffer, loaded onto Tris-glycine gels, transferred to polyvinylidene fluoride membranes and visualized with a chemiluminescence reagent (Amersham Bioscience, Piscataway, NJ, USA).

For the mTOR kinase assay, endogenous mTOR was isolated by immunoprecipitation using anti-mTOR antibody (sc-1549) from 3T3-L1 cells treated in the presence or absence of fisetin. The kinase assay was performed using an mTOR kinase assay kit (#CBA055) according to the manufacturer's protocol.

2.12. Statistical analysis

The results have been displayed as mean±S.D. values. The differences among the four groups were calculated using an analysis of multiple ranges and a one-way variance test using the GraphPad Prism 4 software (San Diego, CA, USA).