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Basic nutritional investigation

Magnolol promotes thermogenesis and attenuates oxidative stress in 3T3-L1 adipocytes

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ABSTRACT

Objective: The aim of this study was to explore the browning and antioxidative effects of magnolol in 3T3-L1 adipocytes, as recruitment of beige-like adipocytes (browning) by natural compounds is being considered as a promising strategy to fight against obesity.
Methods: Magnolol-induced browning effect was evaluated by determining the expression levels of specific marker genes and proteins using real-time polymerase chain reaction and immunoblotting, respectively. Induction of thermogenesis and suppression of oxidative stress in 3T3-L1 adipocytes were further validated by immunofluorescence.
Results: Magnolol significantly enhanced expression of a core set of brown fat–specific marker genes (Ucp1, *Cd137, Prdm16, Cidea*, and *Tbx1)* and proteins (UCP1, PRDM16, and PGC-1α). Increased expression of UCP1 and other brown fat–specific markers contributed to the browning of 3T3-L1 adipocytes possibly via activation of the AMPK, PPARγ, and protein kinase A (PKA) pathways. In addition, magnolol up-regulated key fatty acid oxidation and lipolytic markers (CPT1, ACSL1, SIRT1, and PLIN) and down-regulated lipogenic markers (FAS and SREBP1). Magnolol also reduced the production and release of reactive oxygen species.
Conclusion: The current data suggest possible roles for magnolol in browning of white adipocytes, augmentation of lipolysis, and thermogenesis, as well as repression of oxidative stress and lipogenesis. Thus, magnolol may be explored as a potentially promising therapeutic agent for the prevention of obesity and other metabolic disorders.

Introduction

Obesity incidence is increasing worldwide at an alarming pace and has become a major threat to public health [1]. Indeed, obesity facilitates the development of hypertension, diabetes, stroke, osteoarthritis, and cancer [2,3]. It is well accepted that obesity results from an imbalance between energy expenditure and energy consumption, which causes excess energy storage in the form of lipids in white adipose tissue (WAT). Methods that either increase energy expenditure or reduce energy consumption are potential antiobesity strategies [4]. WAT enlargement is associated with elevation of body weight, whereas brown adipose tissue (BAT) prevents storage of excess energy by dissipation in the form of heat. Hence, BAT plays a fundamental role in the protection against cold and obesity [5,6].

Earlier reports have proposed that WAT and BAT in mammals have opposite functions because of their structural differences, as WAT has a unilocular structure and BAT has a multilocular structure [7,8]. Of importance, brown fat–like cells ("beige" fat) have been reported to have potential to combat obesity and other obesity-related complications [9]. White and brown adipocytes originate from different lineage precursor cells having the property of interconversion [10]. In response to various external stimuli mediated by different factors, multilocular structure cells expressing UCP1 in white fat depots become characterized as beige or brown in white adipocytes [11]. Cold exposure and activation of β3-adrenergic receptor by various external stimuli are fundamental factors responsible for the appearance of beige adipocytes in WAT via remodeling of progenitor WAT fat cells [12,13].

The authors have no conflicts of interest to declare.

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Recent advances in the obesity treatment have indicated that WAT depots countered with certain external factors could lead to brown fat–like phenotype. Different pharmacologic and dietary compounds have been proposed as remedies for increasing energy expenditure and preventing lipid accumulation in mammals [14,15]. In response to pharmacologic treatment such as capsaicin, beriberine, irisin, or PPARγ agonist, a brown fat–like gene expression program is induced in certain adipocytes of WAT [16,17].

Magnolol is an active component isolated from *Magnolia officinalis* (magnolia bark) and is distributed in many Asian countries. It is a traditional medicine widely used as a remedy to facilitate bowel movement and ameliorate cough, pain, anxiety, and cardiovascular diseases [18–20]. Magnolol was found to act as an antiinflammatory molecule to inhibit progression of diabetes and body fat accumulation in an obese mice model [21,22]. Magnolol has also been reported to have various biological effects, including antioxidant activity, smooth muscle relaxant activity, and an antithrombotic effect [23,24]. Moreover, magnolol administration was reported to significantly reduce weight gain caused by white adipocytes as well as adipocyte size and protect against insulin resistance induced in obese mice [22]. Therefore, the present study was undertaken to elucidate the effects of magnolol on induction of the brown fat–like phenotype in 3T3-L1 adipocytes as well as its possible molecular mechanism for the regulation of thermogenesis.

Methods

Chemicals

Magnolol (99% purity; Fig. 1A) was purchased from Cayman Chemical and Arbor Assays (Ann Arbor, MI, USA). GW9622 and dorsomorphin were purchased from Tocris Bioscience (Bristol, UK) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. All other chemicals used in this study were of analytical grade.

Cell culture and differentiation

3T3-L1 and HIB1 B preadipocytes were cultured in complete media, which consists of high-glucose Dulbecco's Modified Eagle's Medium (DMEM, Thermo, Waltam, MA, USA), 10% fetal bovine serum (FBS, PAA laboratories, Pashing, Austria) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) according to previously described protocol [25]. For magnolol treatment, 2-d confluent preadipocytes were incubated with different doses of magnolol (1, 5, 10, and 20 μM) during differentiation until mature adipocyte formation. Cells treated with DMSO (1:1000 dilutions) during differentiation and maturation were used as a control. Sufficiently confluent cells were maintained in differentiation induction medium consisting of 10 μg/mL of insulin (Sigma), 0.25 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) in complete media, followed by maturation medium containing complete media and 10 μg/mL of insulin. For induction of browning of 3T3-L1 adipocytes, differentiation induction medium was supplemented with 50 to 100 nM triiodothyronine (T3, Sigma), and maturation medium was supplemented with 50 nM triiodothyronine with 1 μM rosiglitazone (Rosi, Abcam, Cambridge, UK) (browning cocktail). To elucidate the molecular mechanism, 3T3-L1 cells were treated with antagonists of each target protein, including dorsomorphin, 5 μM, GW9622, 5 μM, and H-89 dihydrochloride, 10 μM [25,26] during the differentiation and maturation period until cells were harvested.

Cell viability assay

3T3-L1 preadipocytes were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated at 37°C and 5% CO_2 with complete media for 24 h. After 24 h, cells were treated with different concentrations of magnolol (1, 5, 10, and 20 μM) for 48 to 72 h. Preadipocytes treated with DMSO (1:1000) were used as a control. MTT solution was then added to the plate according to the manufacturer's instructions. Absorbance was measured at 570 nm with background subtraction at 690 nm using a microplate reader (Tecan Infinite M200 pro, Mannedorf, Switzerland). Six replicate wells were used for each data point throughout the experiments.

Oil red O staining

3T3-L1 preadipocytes were seeded in a six-well plate and allowed to reach 70% confluency. Cells were treated with cocktail and magnolol in the differentiation and maturation medium. After 72 h of treatment, cells were washed with phosphate-buffered saline (PBS), fixed with 10% formalin for 1 h at room temperature, and washed again three times with deionized water. A mixture of oil red O (ORO) solution (0.6% ORO dye in isopropanol) and water at a ratio of 6:4 was layered onto cells for 20 min, followed by washing four times with deionized water. Intracellular lipid accumulation was quantified using ORO staining. The stained lipid droplets were visualized using an inverted microscope. Intracellular lipid content was quantified after extracting ORO bound to cells with 100% isopropanol, and absorbance at 500 nm was determined in triplicate wells using a microplate reader.

Immunofluorescence

3T3-L1 cells were cultured on sterilized coverslips placed on six-well plate and allow the cells to recover for 24 h. Cells were treated with differentiation media, followed by maintenance media along with doses of magnolol as mentioned in the cell culture section. Cells grown on coverslips were fixed with 4% *p*-formaldehyde, followed by washing with PBS three times for 5 min. Unreacted groups were blocked by incubating with 1% bovine serum albumin (BSA) for 1 h at room temperature, followed by washing with 1 X PBS three times for 5 min and then permeabilization with 0.1% triton X-100 (Sigma). Cells were washed again with 1 X PBS three times. Primary polyclonal anti-UCP1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in primary antibody dilution buffer (1:200) was then added, followed by incubation overnight at 4°C and three washes with 1 X PBS. Cells were then incubated with FITC-conjugated antigoat secondary antibody (1:500 dilutions). Fluorescence images were captured using an Olympus 1 X51 inverted microscope (Olympus Co., Tokyo, Japan). For staining of mitochondria, Mito Tracker Red (1 mM, Cell Signaling Technology, Beverly, MA, USA) was directly added to the growth media at a concentration of 20 to 25 nM, and cells were kept for 30 to 40 min at 37°C. After incubation, cells were washed with 1 X PBS and fixed with 4% *p*-formaldehyde, followed by a single wash with PBS and immunostaining.

Total RNA extraction

Preadipocytes were cultured in six-well cell culture plates and incubated until 70% confluency. Differentiation was initiated by addition of differentiation media, which was replaced after 2 d with maintenance media. Cells were also treated with either magnolol alone or combined with browning cocktail. After maturation, cells were harvested and then used to extract total RNA according to an earlier RNA isolation method [25].

Real-time reverse transcriptase polymerase chain reaction

Complementary DNA was synthesized from RNA (1 μg) using Maxime RT premix (iNtRON Biotechnology, Sungnam, Korea). Power SYBR green (Roche Diagnostics Gmbh, Mannheim, Germany) was employed to quantitatively determine transcription levels of genes with reverse transcriptase polymerase chain reaction (Stratagene 246 mix 3000 p QPCR System, Agilent Technologies, Santa Clara, CA, USA). Polymerase chain reactions were run in duplicate for each sample, and transcription level of each gene was normalized to the level of *β-actin*. Sequences of primer sets used in this study are listed in Table 1.

Immunoblot analysis

Cell lysates were prepared using RIPA buffer (Sigma) by homogenization and centrifugation at 14 000 × *g* for 20 min. Cell extract was diluted in 5 X sample buffer (50 mM Tris at pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue) and heated for 5 min at 95°C before 8, 10, or 12% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, samples were transferred to a polyvinylidene difluoride membrane (PVDF, Santa Cruz Biotechnology) and then blocked for 1 h with TBS-T (10 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk/BSA. The membrane was rinsed three times consecutively with TBS-T buffer, followed by overnight incubation with 1:1000 dilutions of primary polyclonal antibodies antiβ-actin, anti-PPARγ, anti-pAMPK, anti-PRDM16, anti-UCP1, anti-PGC-1α, anti-CPT1, anti-SIRT1, anti-ACSL, anti-ACC, anti-SREBP1 (Santa Cruz Biotechnology), anti-pHSL, anti-pACC (Cell Signaling Technology), and anti-PLIN, (Abcam) in TBS-T buffer containing