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Research paper

Trans-anethole ameliorates obesity via induction of browning in white adipocytes and activation of brown adipocytes

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ABSTRACT

To treat obesity, suppression of white adipose tissue (WAT) expansion and activation of brown adipose tissue (BAT) are considered as potential therapeutic targets. Recent advances have been made in the induction of brown fat-like adipocytes (beige) in WAT, which represents an attractive potential strategy for the management and treatment of obesity. Use of natural compounds for browning of white adipocytes can be considered as a safe and novel strategy against obesity. Here, we report that trans-anethole (TA), a flavoring substance present in the essential oils of various plants, alleviated high fat diet (HFD)-induced obesity in mice models via elevation of the expression of beige-specific genes such as *Ppargc1α, Prdm16, Ucp1, Cd137, Cited1, Tbx1,* and *Tmem26*. TA also regulated lipid metabolism in white adipocytes via reduction of adipogenesis and lipogenesis as well as elevation of lipolysis and fat oxidation. Moreover, TA exhibited thermogenic activity by increasing mitochondrial biogenesis in white adipocytes and activating brown adipocytes. In addition, molecular docking analysis enabled us to successfully predict core proteins for fat browning such as β3-adrenergic receptor (β3-AR) and sirtuin1 (SIRT1) based on their low binding energy interactions with TA for promotion of regulatory mechanisms. Indeed, agonistic and antagonistic studies demonstrated that TA induced browning of 3T3-L1 adipocytes through activation of β3-AR as well as the AMPK-mediated SIRT1 pathway regulating PPARα and PGC-1α. In conclusion, TA possesses potential therapeutic implications for treatment of obesity by playing multiple modulatory roles in the induction of white fat browning, activation of brown adipocytes, and promotion of lipid catabolism.

1. Introduction

Obesity is a serious health problem that has become one of the most common health concerns of modern times. Adipose tissue has increasingly become an area of focus for researchers since it plays an important role in the human body not only in fat accumulation but also in endocrine roles [1,2]. To treat obesity, suppression of white adipose tissue (WAT) expansion and activation of brown adipose tissue (BAT) are considered as potential therapeutic targets [3]. In particular, recent advancements have been made in utilizing

Abbreviations: ACC, acyl-CoA carboxylase; ACO, acyl-coenzyme A oxidase 1; AMPK, AMP-activated protein kinase; AR, adrenergic receptor; ATGL, adipose triglyceride lipase; BAT, brown adipose tissue; Beige, brown in white; *Cidea*, gene encoding cell death-inducing DFFA-like effector a; *Cited1*, gene encoding Cbp/p300-interacting transactivator 1; C/EBP/*Cebp*, CCAAT/enhancer-binding protein/encoding gene; *Cox4*, gene encoding cytochrome *c* oxidase subunit 4; CPT1, carnitine palmitoyltransferase 1; ERK1/2, mitogen-activated protein kinase 3/1; *Eva1*, gene encoding myelin protein zero-like 2; FAS, fatty acid synthase; HFD, high fat diet; HSL, hormone-sensitive lipase; *Lhx8*, gene encoding LIM/homeobox protein Lhx8; *MtDNA*, gene encoding mitochondiral DNA gene *Eve*; PGC-1α/*Ppargc1α*, peroxisome proliferator-activated receptor gamma co-activator 1-alpha/encoding gene; *Nrf1*, nuclear respiratory factor 1-encoding gene; PKA, protein kinase A; PPAR, peroxisome proliferator-activated receptor; PRDM16/*Prdm16*, PR domain-containing 16/encoding gene; p38-MAPK, mitogen-activated protein kinase 14; SIRT1, Sirtuin1; TA, trans-anethole; *Tbx1*, gene encoding T-box protein 1; *Tfam*, gene encoding mitochondrial transcription factor; *Tmem26*, gene encoding transmembrane protein 26; UCP1/*Ucp1*, uncoupling protein 1/encoding gene; iWAT, inguinal white adipose tissue; *Zic1*, gene encoding zinc finger protein ZIC1.

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BAT function, which increases oxidative metabolism at the expense of fat storage in order to dissipate energy as heat [4]. Recently, induction of the brown fat-like phenotype in WAT (formation of *brite* or beige adipocytes) represents another attractive potential strategy for the management and treatment of obesity [4–6]. Apart from classical BAT, recruitment of beige adipocytes has been observed in WAT depots in response to specific stimuli such as chronic cold exposure, endogenous signals, as well as dietary factors and pharmacological agents [2,7].

Due to the serious side effects of synthetic anti-obesity medicines in the commercial market, many natural products with anti-obesity effects have recently become more appealing to consumers [8]. Of note, a wide variety of natural compounds are known to favor the acquisition of brown adipocyte-like features in white adipocytes [2,7,9–13]. In the screening of natural anti-obesity compounds, we observed that trans-anethole possessed the capacity to recruit beige adipocytes both in cultured 3T3-L1 adipocytes and obese mice fed a high fat diet.

Trans-anethole (*trans*-1-methyoxy-4-propenyl-benzene, Fig. 1A) is a flavoring substance present in the essential oils of various plants of more than 20 species, including fennel, anise, and star anise, and has been used for culinary purposes for centuries [14]. Two isomers of anethole occur in nature: E– or trans-anethole and Z- or cis-anethole. About 90% of natural anethole is trans-anethole (TA), and only TA is considered as food grade due to the higher toxicity of cis-anethole [15]. Although TA was accorded Generally Recognized as Safe status by the FDA, several toxicity studies have reported carcinogenicity in high dose animal groups [16,17]. A long-term feeding study reported that TA is unlikely to be a rodent carcinogen and can thus be considered as non-genotoxic and non-carcinogenic [18,19]. TA has been widely used as a flavor agent in foods, cosmetics, and perfumes and attempts to explore its pharmaceutical potential in human chronic diseases have recently been made [15]. Animal and cell line data suggest that TA may have beneficial effects in several chronic diseases, including cancer [20,21], diabetes [22,23], inflammation [24,25], wound-healing [26], immunomodulation [27], as well as neurological [28] and skin diseases [29], where several molecular targets of TA have been partly identified [15].

Recent advances in the field of bioinformatics have provided detailed perspectives and an intense understanding of interacting proteins at the molecular level, highlighting modulation of protein behavior due to binding of small drug-like compounds forming complexes [30]. Molecular docking data contributes to determination of the mechanisms of action driving protein interactions by imparting insights into structural stability, energy levels of binding modes, and selectivity of unique residues of amino acids, which play major roles during intermolecular interactions [31]. Thus, *in silico* analytical tools were considered and utilized to characterize the molecular relationships between lipid metabolic proteins and our compound of interest.

To date, the anti-obesity effect of TA has not been reported. The objective of the present study was to investigate the anti-obesity effect of TA by stimulating thermogenic activity through induction of the *beige* phenotype in cultured 3T3-L1 white adipocytes as well as on diet-induced obese mice and activation of brown adipocytes. Further validation by computational techniques was performed to improve our understanding about the mechanism of action of TA on browning pathways.

2. Materials and methods

2.1. Chemicals

Trans-anethole (99% purity) and resveratrol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BRL 37344 and L-748.337 were purchased from Tocris Bioscience (Bristol, UK). EX527 was purchased from Selleckchem (Houston, TX, USA). All other chemicals used in this study were of analytical grade.

2.2. Cell culture and differentiation

Dulbecco's Modified Eagle's Medium (DMEM, Thermo, Waltam, MA, USA) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories, Pasching, Austria) and 100 μg/ml of penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) was used to culture 3T3-L1 (ATCC, Manassas, VA, USA) at 37 °C in a 5% CO_2 incubator. Sufficiently confluent cells were maintained in differentiation induction medium consisting of 10 μg/ml of insulin (Sigma, St. Louis, MO, USA), 0.25 μM dexamethasone (Dex, Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma) in DMEM, followed by maturation medium containing 10% FBS and 10 μg/ml of insulin. During treatments, unless otherwise stated, cells were maintained in complete medium containing 100 μM TA (dissolved in 99% ethanol) for 6–8 days before further analysis, and maturation medium was changed every 2 days. Cytotoxicity of TA was evaluated by MTT assay as described previously [32].

2.3. Animal experiments

Five-week-old C57BL/6 mice were acclimatized with normal chow for 1 week and then divided into two groups *viz*. HFD (60% fat)-fed control mice (CON group) and HFD-fed mice treated with TA by oral administration (TA group). TA was administered daily by oral gavage to mice at a dose of 100 mg/kg body weight for 8 weeks. In order to minimize volatilization during TA feeding, stock solution of TA was prepared as a small aliquot dissolved in 30% ethanol. All animal experiments were approved by the Committee for Laboratory Animal Care and Use of Daegu University.

2.4. Quantitative real-time RT-PCR

Total RNA was isolated from mature cells (4–8 days) using a total RNA isolation kit (RNA-spin, iNtRON Biotechnology, Seongnam, Korea). RNA (1 μg) was converted to cDNA using Maxime RT premix (iNtRON Biotechnology). Power SYBR green (Roche Diagnostics Gmbh, Mannheim, Germany) was employed to quantitatively determine transcription levels of genes with RT-PCR (Stratagene 246 mix 3000p QPCR System, Agilent Technologies, Santa Clara, CA, USA). PCR reactions were run in duplicate for each sample, and transcription levels of all genes were normalized to the level of *β-actin*. Sequences of primer sets used in this study are listed in Table 1.

2.5. Oil Red O staining

Cells were matured for 4–8 days, followed by washing with phosphate-buffered saline (PBS), fixation with 10% formalin for 1 h at room temperature, and washing again three times with deionized water. A mixture of Oil Red O solution (0.6% Oil Red O dye in isopropanol) and water at a 6:4 ratio was layered onto cells for 20 min, followed by washing four times with deionized water, and images were captured under a microscope.

2.6. Immunoblot analysis

Cell lysates were prepared using RIPA buffer (Sigma) by homogenization and centrifugation at 14000 ×*g* for 20 min. Cell extract was diluted in 5X sample buffer (50 mM Tris at pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol