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Invited Perspective

Sulfuretin has therapeutic activity against acquired lymphedema by reducing adipogenesis

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ABSTRACT

Acquired lymphedema is a pathological condition associated with lymphatic dysfunction caused by surgical treatments for cancer. Although global estimates of the prevalence of acquired lymphedema have been rising, there are currently no effective therapeutics available. Since adipose tissue accumulation is a clinical hallmark of lymphedema, we hypothesized that regulation of adipogenesis in lymphedematous tissue could be used as a therapeutic intervention against lymphedema. Toward this, we investigated the possibility of anti-adipogenic 30% ethanol *Rhus verniciflua* Stokes (RVS) extract as a potential lymphedema treatment. Oral administration of RVS extract ameliorated volumetric symptoms of lymphedema in a mouse model. RVS administration also reduced adipose tissue accumulation in lymphedematous tissue and downregulated expression of adipocyte markers, including Pparγ and Fabp4. Sulfuretin was identified as a major bioactive compound in the 30% ethanol RVS extract in liquid chromatography-mass spectrometry analysis. Similar to the activities of RVS, sulfuretin inhibited adipocyte differentiation in 3T3-L1 preadipocytes. Moreover, treatment with sulfuretin on lymphedema-induced mice reduced lymphedema volume, decreased the expression of adipogenic markers, but induced the expression of markers associated with lymphangiogenesis. Taken together, our data raise the possibility that sulfuretin might be used in therapeutic interventions against acquired lymphedema.

1. Introduction

Lymphedema is a pathological condition associated with lymphatic dysfunction and is classified into two types, congenital and acquired [1,2]. While congenital cases make up only a small percentage of all lymphedema cases, the majority of patients with lymphedema suffer from acquired lymphedema caused by surgical treatments for cancer [3]. The reported incidences of acquired lymphedema by cancer types are as follows: 49% breast, 21.8% gynecologic, 16% melanoma, 10% genitourinary, and 6% head and neck following surgical lymph node dissection and/or radiation therapies [4–11].

Currently, however, no effective therapeutics against lymphedema is available. Although physical therapies such as complete decongestive therapy can be administered to alleviate the symptoms of lymphedema [12], these therapies produce only transient results and are not effective. Therefore, further investigation into the etiology of lymphedema and the development of new strategies for treating lymphedema are urgently required.

The histopathologic symptoms of lymphedema include the excessive accumulation of interstitial protein-rich fluid and tissue swelling, adipogenesis, and tissue fibrosis [13,14]. One hypothesis is that the regulation of lymphangiogenesis recovery or inhibiting fibrosis as lymphedema progresses could constitute a putative therapeutic strategy. Alternatively, adipogenesis may also play a significant role in lymphedema pathology [15–17]. Thus, proper control of adipocyte differentiation and lipid accumulation in lymphedematous tissue could also constitute a promising therapeutic intervention against lymphedema.

Recent technological advances have renewed interest in exploring natural products in the context of drug development [15,16]. Therapeutic effects of natural products for diseases have been intensively studied and their safety is an important advantage in drug development. Especially, *Rhus verniciflua* Stokes (RVS) has been shown to exhibit various biological effects including

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anti-cancer, antioxidant, anti-platelet, anti-inflammatory, anti-fibrotic, anti-apoptotic, and anti-adipogenic activities [18–22]. Polyphenolic compounds from RVS are believed to mediate these biological effects. Most of the identified bioactive compounds are anti-adipogenic, including fisetin, fustin, kaempferol, gallic acid, quercetin, protocatechuic acid, butein, and sulfuretin [23,24]. Based on these findings, we hypothesized that the suppression of adipogenesis by RVS extract and its bioactive compounds would ameliorate the symptoms associated with lymphedema.

In this study, we investigated the effects of orally administrated RVS extract in a mouse model of lymphedema and identified bioactive compounds responsible for the beneficial effects of RVS. Our findings highlight the potential use of RVS and its major component sulfuretin in therapeutic interventions against lymphedema and further elucidate the critical roles of adipogenesis in lymphedema pathology.

2. Materials and methods

2.1. Preparation of plant materials

A 30% ethanol extract of RVS was provided by the Department of Oriental Medical Science at Kyunghee University. To generate the extract, the RVS was detoxified, filtered, and extracted with ethanol. The filtrate was freeze-dried to obtain the extract following evaporation in a rotary vacuum evaporator. The resultant RVS powder was dissolved in 30% ethanol immediately before treatment and stored at 4 °C until use. Sulfuretin (PubChem CID: 5281295) and butein (PubChem CID: 5281222) were prepared from the Gyeonggi Institute of Science & Technology.

2.2. Preparation and identification of chemical compounds

Sulfuretin, (*Z*)-2-(3,4-dihydroxybenzylidene)-6-hydroxybenzofuran-3(2H)-one was synthesized at the Gyeonggi Institute of Science & Technology Promotion (Supplemental Fig. 1). To a mixture of 150 mg (1 mM) of 6-hydroxybenzofuran-3(2H)-one and 138 mg (1 mM) of 3,4-dihydroxybenzaldehyde in ethanol (5 ml), 12 N HCl (1.5 ml) was added dropwise at 0 °C. Then the reaction mixture was allowed to stir at 60 °C until the substrates were disappeared on TLC. The mixture was poured into water and the resulting precipitate was filtered and dried in vacuum. The crude product was recrystallized by MeOH/H_2O to yield 216 mg (79%) of sulfuretin as an orange powder. The synthesized sulfuretin was identified by LC/MS. LC was performed on WATERS ACQUITY UPLC BEH C18 column (2.1 × 100 mm; Milford, MA, USA). The mobile phase A was 0.1% formic acid in water and phase B was 0.1% formic acid in acetonitrile. Mass spectrum was performed with Micromass Quattro Micro API mass spectrometer (Milford, MA, USA). The monoisotopic mass of deprotonated sulfuretin $[M-H]^-$ was detected at a retention time of 3.3 min on low resolution mass spectrometry (LRMS). In addition, protonated sulfuretin $[M+H]^+$ was confirmed by high resolution mass spectrometry (HRMS) analyses. LRMS and HRMS were performed with Micromass Quattro Micro API (Waters, USA) and LTQ Orbitrap XL (Thermo Scientific, USA), respectively, using electron spray ionization (ESI). Additionally, the sulfuretin was identified by the spectrum of ^1H and ^{13}C NMR. The ^1H and ^{13}C NMR spectra were recorded on Avance III 700 [Bruker Biospin, Germany]. NMR chemical shifts were measured in parts per million (ppm) from specific solvent (DMSO-d_6) signal. Multiplicities were indicated as follows: d (doublet); dd (doublet of doublet). Coupling constants were reported in Hz.

Butein, (*E*)-1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)prop-2-en-1-one (Supplemental Fig. 2). Butein was purchased from Tokyo Chemical Industry (Tokyo, Japan). The purchased butein was identified based on ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra[1] and by LC/MS along with chemical identifications for sulfuretin. A significant impurity from commercially available butein was not detected in liquid chromatography (LC).

2.3. Cell culture

The preadipocyte 3T3-L1 cell line (passage; 8) was purchased from the American Type Culture Collection (Rockville, MD). 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT) supplemented with 10% fetal calf serum (FCS) (Hyclone) and antibiotics (penicillin and streptomycin, Hyclone). Cells were maintained at 37 °C under a 5% CO_2 atmosphere. For experiments, cells were seeded (5×10^4 cells/ml) in 6-well tissue culture plates. To induce adipocyte differentiation, confluent cells were incubated in medium containing DMEM, 10% FBS, 1 μM dexamethasone (Sigma, St. Louis, MO, USA), 0.5 mM isobutyl-1-methylxanthine (Sigma), and 5 μg/ml insulin (Sigma) for 2 days. The medium was refreshed with DMEM containing 10% FBS and 5 μg/ml insulin every 3 days in the presence of the compounds.

2.4. Oil red O staining

At 5 days after the induction of differentiation, cells were fixed with 3% para-formaldehyde in PBS at room temperature for 4 h and then stained with 0.5% Oil Red O (Sigma) in a mixture of isopropanol and distilled water (3:2 vol:vol) for 1 h. After washing with water, the cells were photographed under a microscope. To measure lipid accumulation, 100% isopropanol was added to the cells, and the resultant absorbance at 520 nm was measured.

2.5. Real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated from 3T3-L1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA (cDNA) was synthesized from 0.5 μg of total RNA using ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan) with random primers. After cDNA synthesis, THUNDERBRID® SYBR® qPCR Mix (TOYOBO, Osaka, Japan), primers, and cDNA were mixed in a final reaction volume of 25 μl. Polymerase chain reaction (PCR) thermocycling consisted of 40 amplification cycles in a Thermal Cycler Dice instrument (Takara, Shiga, Japan) according to the manufacturer's instructions. Expression levels were normalized to that of 36B4. All reactions were performed at least twice. The oligonucleotide primer (Integrated DNA Technologies, San Diego, CA) sequences used for PCR were as follows: peroxisome proliferator-activated receptor γ (Pparγ): Pparγ F, 5′-CCATTCTGGCCCACCAAC-3′ and Pparγ R, 5′-AATGCGAGTGGTCTTCCATCA-3′; fatty acid binding protein 4 (Fabp4): Fabp4 F, 5′-CACCGCAGACGACAGGAAG-3′ and Fabp4 R, 5′-GCACCTGCACCAGGGC-3′; CCAAT/enhancer binding protein alpha (C/ebpα): and C/ebpα F, 5′- GCGGGCAAAGCCAAGAA −3′ and C/ebpα R, 5′- GCGTTCCCGCCGTACC −3′. The Δcycle threshold (CT) method was used to calculate the differences between the target CT value and the control (36B4) CT value for each sample: ΔCT = CT (target) - CT (control). The relative expression level was calculated using the 2-ΔCT formula.

2.6. Ethics statement

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Sungkyunkwan University School of Medicine (Approval Number: SKKUIACUC-20150037). All methods were carried out in accordance with the approved guidelines.