Nutrition 30 (2014) S31–S36

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Novel mechanisms for botanical effect

Artemisia extracts activate PPARγ, promote adipogenesis, and enhance insulin sensitivity in adipose tissue of obese mice

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ARTICLE INFO

Article history:
Received 16 January 2014
Accepted 14 February 2014

Keywords:
Fat cells
Insulin action
Botanicals
Diet-induced obesity
3T3-L1 adipocytes

ABSTRACT

Objective: Studies have shown that the inability of adipose tissue to properly expand during the obese state or respond to insulin can lead to metabolic dysfunction. *Artemisia* is a diverse group of plants that has a history of medicinal use. The aim of this study was to examine the ability of ethanolic extracts of *Artemisia scoparia* (SCO) and *Artemisia santolinifolia* (SAN) to modulate adipocyte development in cultured adipocytes and white adipose tissue (WAT) function *in vivo* using a mouse model of diet-induced obesity.
Method: Adipogenesis was assessed using Oil Red O staining and immunoblotting. A nuclear receptor specificity assay was used to examine the specificity of SCO- and SAN-induced PPARγ activation. C57BL/6J mice, fed a high-fat diet, were gavaged with saline, SCO, or SAN for 2 wk. Whole-body insulin sensitivity was examined using insulin tolerance tests. WAT depots were assessed via immunoblotting for markers of insulin action and adipokine production.
Results: We established that SCO and SAN were highly specific activators of PPARγ and did not activate other nuclear receptors. After a 1-wk daily gavage, SCO- and SAN-treated mice had lower insulin-induced glucose disposal rates than control mice. At the end of the 2-wk treatment period, SCO- and SAN-treated mice had enhanced insulin-responsive Akt serine-473 phosphorylation and significantly decreased monocyte chemotactic protein-1 levels in visceral WAT compared with control mice; these differences were depot specific. Moreover, plasma adiponectin levels were increased following SCO treatment.
Conclusion: Overall, these studies demonstrate that extracts from two *Artemisia* species can have metabolically favorable effects on adipocytes and WAT.
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Introduction

Adipocytes are dynamic, insulin-sensitive cells that have endocrine properties and contribute to whole-body energy homeostasis. Obesity is the primary disease of fat cells and significantly contributes to the development of type 2 diabetes mellitus (T2DM), cardiovascular disease, and certain cancers. Many researchers have investigated antiadipogenic agents, including some botanicals, as potential therapeutics for decreasing or preventing obesity. However, the prevailing current hypothesis is that disruption of adipocyte differentiation limits adipose tissue expansion and leads to insulin resistance and the development of T2DM [1–4].

Botanical extracts represent an alternative approach for the treatment or prevention of a disease. Plant extracts are used by many cultures and have resulted in the development of many drugs, including metformin. Metformin is a widely used T2DM drug that is derived from French lilac. In a blinded screening study to investigate the effects of botanicals on adipocyte differentiation, we identified plant extracts with substantial effects on adipogenesis. Two of these extracts were *Artemisia* species,

NCAAM and ODS funded NIH grant, 2P50AT002776-06. AJR was supported by an NIH T-32 postdoctoral training fellowship in botanicals granted to Pennington Biomedical Research Center (T32 AT004094). AJR, TPB, and JMS designed the experiments. AJR, YW, and DS-I processed and analyzed data. DMR provided the *Artemisia* extracts. AJR and JMS interpreted the data and wrote the manuscript. All authors contributed to preparation of the final manuscript. The authors have no potential conflicts of interest to disclose.
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0899-9007/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.nut.2014.02.013

Artemisia scoparia (SCO) and *Artemisia santolinifolia* (SAN). Many *Artemisia* species have been used in traditional medicine in East Asia, and are reported to demonstrate antihyperglycemic [5,6], antiobesity [7,8], and antidiabetic activities [5,9]. Our studies revealed that both SCO and SAN could promote adipocyte development in differentiating murine adipocytes. Additionally, these extracts specifically activate PPARγ, but did not modulate the activity of other nuclear receptors. We also observed that SCO and SAN could increase adiponectin (ADPN) secretion *in vitro* and *in vivo*. In a 2-wk gavage study, SCO and SAN enhanced insulin action in epididymal adipose tissue. These studies demonstrate that SCO and SAN have a positive effect on adipocyte-related diseases by promoting adipocyte development and increasing ADPN levels and insulin action in adipose tissue.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine and fetal bovine sera were purchased from HyClone (Thermo Scientific, Logan, UT, USA). For immunoblotting, STAT5A, MAPK/ERK, and PPARγ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-APDN antibody was obtained from Thermo Scientific (Rockford, IL, USA). Antiphospho-Akt-Ser^{473} (AktpS[473]), total Akt, and monocyte chemotactic protein-1 (MCP-1) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). With the exception of the mouse monoclonal anti-PPARγ immunoglobulin IgG, all other antibodies were rabbit polyclonal IgGs, and peroxidase-conjugated secondary antibodies to both species were purchased from Jackson ImmunoResearch (West Grove, PA, USA). The bicinchoninic acid and enhanced chemiluminescence kits were from Thermo Scientific.

Preparation, source, and characterization of the extracts

Artemisia scoparia Waldst. & Kit and *Artemisia santolinifolia* Turcz. ex Besser ethanolic extracts were prepared at Rutgers University. Briefly, the herb was greenhouse grown from seed and periodically harvested at the flowering stage, freeze-dried and stored at −20°C. The dried herb was extracted in 80% ethanol (1:20 w/v) at 50°C with sonication for 1 h followed by shaking at room temperature for 24 h. The solid material was removed by centrifugation at 3000*g* and the solvent was subsequently removed by evaporation. For *in vitro* and cell culture experiments, the dried extracts were solubilized in 100% dimethyl sulfoxide (DMSO) at a concentration that was 1000-fold higher than experimental concentrations and then diluted into the media. For animal studies, the extracts were solubilized in 20% Labrasol® (Gattefosse, Lyon, France) and administered via gavage.

Nuclear receptor specificity assay

SCO and SAN were assessed for their ability to modulate the activities of all 48 human nuclear receptors using a previously described Gal4 cotransfection assay system [10–12]. Both extracts were tested at 50 μg/mL and positive controls were included for characterized ligands. DMSO was used as the vehicle control.

Cell culture

Murine 3T3-L1 preadipocytes were plated and grown to 2 d postconfluence in DMEM containing 10% bovine serum. Medium was changed every 48 to 72 h. Cells were induced to differentiate using a standard 1-methyl-3-isobutylxanthine, dexamethasone, and insulin (MDI) induction protocol [13]. Mature adipocytes were maintained in DMEM supplemented with 10% fetal bovine serum until used for experimentation. For adipogenesis assays, the botanical extracts were added at the time of MDI induction and at each media change until the cells were stained with Oil Red O (ORO) or harvested for Western blot analysis.

Oil Red O staining

An ORO stock was prepared as previously described [14]. Cell monolayers were aspirated, rinsed with phosphate-buffered saline (PBS), fixed in 10% formaldehyde/phosphate-buffered saline, and rinsed under tap water. The remaining water was aspirated, and the cells were incubated for 1 h in the working ORO solution (0.3% in isopropanol). After incubation, stain aspiration, and rinsing, cells were examined by microscopy and scanned to produce the figures shown here.

Animals and gavage

Thirty-six 16-wk-old male C57BL/6J diet-induced obese (DIO) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained on a purified high-fat diet (HFD) containing 20 kcal% protein, 20 kcal% carbohydrate, and 60 kcal% fat (D12492; Research Diets, Inc., New Brunswick, NJ, USA) for the entire study. After 1 wk in quarantine, mice were housed four per cage in a temperature- and humidity-controlled room with a 12-h light–dark cycle. Mice were handled daily for 2 wk and non-fasting body weights were recorded daily. Non-fasting nuclear magnetic resonance (NMR; Bruckner Minispec) was recorded on day 14. Animals were gavaged (22-gauge plastic gavage needle, Instech Laboratories, Inc. Plymouth Meeting, PA, USA) with 20% Labrasol daily for 7 d. On day 21, another non-fasting NMR measurement was recorded and submandibular blood samples were collected. Animals were randomized by non-fasting body weight (day 21) to one of three groups: 20% Labrasol (CTL; n = 12), SCO (n = 12), and SAN (n = 12) and gavaged daily for another 2 wk. Botanical extracts were prepared in 20% Labrasol to increase bioavailability. All animal studies were performed with approval from the Pennington Biomedical Research Center Institutional Animal Care and Use Committee.

Animal study procedures

Non-fasted body weight was measured daily during the 2-wk gavage study. Body composition (NMR), fasting body weight, fasting plasma glucose, insulin, and ADPN levels were measured at the beginning (baseline measurements) and end of the 2-wk study period. One week after the initiation of experimental gavaging, and 4 h after mice received their last gavage, intraperitoneal insulin tolerance tests were performed on all animals. Briefly, a baseline blood glucose measurement was obtained via tail nick (0 min), and animals were then injected with 1 U/kg insulin (Humulin R, Eli Lilly, Indianapolis, IN, USA). Blood glucose measurements were obtained via tail nick at 10, 20, 40, and 60 min postinjection. All blood glucose measurements were performed using a Bayer Breeze 2 glucometer. After 2 wk of experimental gavage, mice were fasted overnight; fasted body weight, submandibular blood collection, and NMR measurement were obtained for each mouse. Immediately after these procedures, mice were gavaged with the appropriate extract or vehicle. Four hours after the gavage, mice were injected with either 1 U/kg insulin or saline and sacrificed via cervical dislocation 10 to 15 min after injections. Animals were then decapitated and trunk blood collected. Retroperitoneal white adipose tissue (rWAT), inguinal adipose tissue (iWAT), and epididymal adipose tissue (eWAT) depots were collected and immediately frozen in liquid nitrogen.

Whole cell extract and tissue preparation

Adipocyte whole cell extracts and WAT lysates were prepared by either harvesting adipocyte monolayers or homogenizing WAT in a non-denaturing extraction buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, 1 mM 1, 10-phenanthroline, and 0.2 mM sodium vanadate. Protein content was determined by bicinchoninic acid protein assay.

Gel electrophoresis and immunoblotting

Samples were separated on 7.5%, 10%, or 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels (PA) and transferred to nitrocellulose membranes. Results were visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. For native gel electrophoresis, 5% PA gels were prepared without SDS. Additionally, SDS and reducing agents were eliminated from the running and sample loading buffers.

Statistical analyses

Data were graphed as mean ± SEM. The differences between treatment means were tested using an analysis of variance. Pairwise *t* tests were performed to determine which means were different when the analysis of variance was statistically significant. A mixed model was used to model blood glucose. Time, as a categorical variable, treatment, and time by treatment interaction were included in the model. *t* Tests were performed at each time point on the least square means from the model to determine if there were any statistically significant differences between the treatments. All tests used $P < 0.05$ as the level of statistical significance.

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