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Methods development

Screening native botanicals for bioactivity: An interdisciplinary approach

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ABSTRACT

Objective: Plant-based therapies have been used in medicine throughout recorded history. Information about the therapeutic properties of plants often can be found in local cultures as folk medicine is communicated from one generation to the next. The aim of this study was to identify native Louisiana plants from Creole folk medicine as a potential source of therapeutic compounds for the treatment of insulin resistance, type 2 diabetes, and related disorders.
Methods: We used an interdisciplinary approach combining expertise in disciplines ranging from cultural anthropology and botany to biochemistry and endocrinology to screen native southwest Louisiana plants. Translation of accounts of Creole folk medicine yielded a list of plants with documented use in treating a variety of conditions, including inflammation. These plants were collected, vouchered, and catalogued before extraction of soluble components. Extracts were analyzed for bioactivity in regulating inflammatory responses in macrophages or fatty acid–induced insulin resistance in C2C12 skeletal muscle cells.
Results: Several extracts altered gene expression of inflammatory markers in macrophages. Multiplex analysis of kinase activation in insulin-signaling pathways in skeletal muscle also identified a subset of extracts that alter insulin-stimulated protein kinase B phosphorylation in the presence of fatty-acid–induced insulin resistance.
Conclusion: An interdisciplinary approach to screening botanical sources of therapeutic agents can be successfully applied to identify native plants used in folk medicine as potential sources of therapeutic agents in treating insulin resistance in skeletal muscle or inflammatory processes associated with obesity-related insulin resistance.

Introduction

The prevalence of metabolic syndrome (MetS), characterized by abdominal obesity, dyslipidemia, hypertension, insulin resistance (IR), and chronic inflammation, contributes greatly to development of type 2 diabetes mellitus (T2DM) and remains a significant public health concern [1–3]. In addition to lifestyle management as the cornerstone of management, a suggested treatment for MetS and the first suggested treatment for T2DM is metformin [4], a synthetic compound based on the hypoglycemic

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properties of galegine (isoamylene guanidine) found in *Galega officinalis*, a plant commonly known as French lilac or goat's rue [5]. Extracts from French lilac were used in folk medicine as early as the Middle Ages, and accounts of using French lilac extracts for treating symptoms typical of T2DM appeared in the 17th century [6]. Thus, the efficacy of metformin in treating hyperglycemia points to the value of botanicals as an important source of therapeutic compounds, even in an era of molecular medicine.

Plants from the genus *Artemisia* also have been used traditionally to treat diabetes, including the perennial herb *Artemisia dracunculus* or Russian tarragon [7]. Several recent studies demonstrate that an ethanolic extract of *A. dracunculus* lowers blood glucose and enhances insulin signaling in skeletal muscle in murine models of diabetes and in human skeletal muscle cells *in vitro* [8–11]. These studies support the idea that characterizing botanical sources of therapeutic compounds continues to be a viable approach to developing novel treatments for insulin resistance (IR), T2DM, and other chronic diseases. However, identifying new plant sources of botanical material requires cultural knowledge of traditional uses of medicinal plants as well as adherence to scientific nomenclature, plant classification, and accepted standards of plant vouchering before characterization of plant preparations. We assembled a team of individuals with expertise in cultural anthropology, botany, ecology, biochemistry, and endocrinology to identify native medicinal plants from southwestern Louisiana with properties having potential therapeutic applications in treating IR and T2DM.

Our initial results indicate that our interdisciplinary approach can be successfully used to identify potential botanical sources of therapeutic agents in the treatment of MetS and T2DM.

Materials and methods

Identification and cataloguing of medicinal plants from southwest Louisiana

Medicinal native plants were identified from a translation [12–14] of the original Creole-language accounts of plants used in Creole folk medicine recorded by Charles Bienvenu in 1933 [15]. Many of those plants are currently present in southwest Louisiana and were identified and collected by the National Wetlands Research Center; a voucher was made for each specimen and deposited at the National Wetlands Research Center herbarium in Lafayette, Louisiana.

Preparation of plant extracts

Plants were lyophilized and extracted in 80% ethanol 10:1 by sonication in a 50°C water bath for 1 h. Extracts were filtered through Miracloth (Calbiochem, Billerica, MA, USA) and centrifuged to obtain a clear solution. The clear extracts were then dried by rotary evaporation followed by lyophilization and stored at −20°C. The extracts were resuspended in dimethyl sulfoxide or ethanol and diluted to the indicated concentrations in cell culture media for use in the screening assays.

Cell culture

Macrophage

RAW 264.7 macrophages (American Type Culture Collection; Manassas, VA, USA; #TIB-71) were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose, L-glutamine, and sodium pyruvate (DML 10, Caisson, North Logan, UT, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. RAW macrophage cells were incubated in a humidified chamber at 37°C with 5% carbon dioxide and subcultured by cell scraping. For experiments, RAW cells were plated at a density of 4×10^5 cells/mL in 24-well plates. Cells were incubated overnight (18 h) and washed with phosphate-buffered saline and replaced with fresh media. Cells were treated with 10 or 100 μg/mL plant extract or vehicle (50% ethanol or dimethyl sulfoxide) for 8 h. To elicit inflammatory responses, 1 μg/mL lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO, USA) was added to macrophages after 2 h and incubated for an additional 6 h. To demonstrate induction of inflammation and to evaluate potential proinflammatory effects, extract treatments (100 μg/mL) without LPS were included. Cells were treated in duplicate or triplicate and experiments included vehicle controls. Media were collected and the cells were washed with phosphate-buffered saline before collection with Trizol Reagent. Samples were stored at −80°C.

Macrophage cell viability with each treatment was measured using MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide] (TCI, Portland, OR, USA) as described [16]. Cell viability of each treatment was normalized to media only control (100%).

Skeletal muscle

Murine C2C12 myoblasts (ATCC; #CRL-1771) were cultured in DMEM, high glucose (25 mM) with 10% fetal bovine serum, 2 mM glutamine, and antibiotics (100 units/mL penicillin G and 100 μg/mL streptomycin) in a humidified chamber at 37°C and 5% carbon dioxide. To obtain fully differentiated myotubes, the medium was exchanged for DMEM, high glucose with 2% horse serum, glutamine, and antibiotics when the myoblasts reached confluence. The medium was replaced every 48 h and the cells were maintained in this medium until fully differentiated, when the medium was exchanged for DMEM, low glucose (5 mM) with 2% horse serum. The myotubes were fully formed by the fourth day post-induction. For experiments, myotubes were incubated overnight (16 h) with fatty acid (FA)-free bovine serum albumin (BSA)-conjugated sodium palmitate (200 μM) alone or BSA-conjugated sodium palmitate and increasing concentrations of each plant extract (1, 10, or 50μg/mL). Thereafter, the cells were serum-deprived by exchanging the media for DMEM containing 0.3% FA-free BSA for 4 h before insulin stimulation (100 nM). Palmitate alone or in combination with the plant extracts remained in the media during the 4-h serum deprivation incubation. Ten minutes after adding insulin, the cells were rinsed with cold wash buffer and harvested in cell lysis buffer (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The lysates were stored at −80°C. Control lysates were harvested from myotubes that were incubated overnight in the presence or absence of palmitate and the presence or absence of the ethanolic extract from *A. dracunculus* termed PMI5011, as a positive control for plant extracts that enhance phosphorylation of protein kinase B (Akt) in the presence of palmitate-induced IR [10,11].

Gene expression analysis

Total RNA was extracted from RAW macrophage cells according to manufacturer's specifications (Life Technologies, Carlsbad, CA, USA) and as previously described [23]. RNA integrity was evaluated by agarose gel electrophoresis. Following treatment with DNase I Amplification grade (Invitrogen, Carlsbad, CA, USA), RNA quality was checked on the NanoDrop 1000 system (Thermo Fisher Scientific, Wilmington, DE, USA). A ratio of optical density (OD) 260/280 $\geq$ 2.0 and OD 260/230 $\geq$ 1.8 was acceptable. First-strand cDNA synthesis was performed using ABI High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) with RNAse I inhibitor, according to the manufacturer's instructions using 1 μg of RNA.

Synthesized cDNAs were diluted 25-fold and 5 μL of dilution was used for quantitative polymerase chain reaction (PCR) with 12.5 μL of Power SYBR Green PCR master mix (Applied Biosystems, Warrington, UK), 0.5 μL primers (6 μM) and biotechnology performance-certified grade water (Sigma-Aldrich, St. Louis, MO, USA) to a final reaction volume of 25 μL. Exon-spanning primer sequences [17] were as follows: β-*actin* forward 5′-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse: 5′-CAC AGC CTG GAT GGC TAC GT-3′, *IL*-1β forward 5′-CAA CCA ACA AGT GAT ATT CTC CAT-3′, reverse 5′-GAT CCA CAC TCT CCA GCT GCA-3′, *iNOS* forward 5′-CCC TCC TGA TCT TGT GTT GGA-3′, reverse 5′-TCA ACC CGA GCT CCT GGA A-3′, *COX-2* forward 5′-TGG TGC CTG GTC TGA TGA TG-3′, reverse 5′-GTG GTA ACC GCT CAG GTG TTG-3′, *TNF-α* forward 5′-TGG GAG TAG ACA AGG TAC AAC CC-3′, reverse 5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′.

All experimental samples were run in triplicate and each reaction plate included controls without template added. Quantitative PCR amplifications were performed on an ABI 7300 Real-Time PCR System (Applied Biosystems) with the following thermal cycler profile: 2 min, 50°C; 10 min, 95°C; 15 sec, 95°C for 40 cycles; 1 min, 60°C for the dissociation stage; 15 sec, 95°C; 1 min, 60°C; and 15 sec, 95°C.

Samples were analyzed by the comparative ΔΔCt method and normalized with respect to the average Ct value of β-*actin*. Vehicle with LPS served as the calibrator for ΔΔCt analysis and was assigned a relative quantitation (RQ) value of 1.0. Lower RQ values indicated inhibition of gene expression and therefore greater anti-inflammatory activity. Percent inhibition was determined by [(1-RQ)*100] and anti-inflammatory potential was denoted by percent inhibition (>90%, >60%, or >30% of selected targets).

Protein expression analysis

Whole-cell lysates were sonicated while on ice to ensure complete disruption of the cellular membranes. Cellular debris was removed via centrifugation at 4500*g* for 20 min at 4°C. Protein concentration of the supernatants was determined using a BCA assay (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's directions. Multiplex analysis (BioPlex, Bio-Rad, Hercules, CA,