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RESEARCH ARTICLES

Tools for the identification of bioactives impacting the metabolic syndrome: screening of a botanical extract library using subcutaneous and visceral human adipose-derived stem cell-based assays☆

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Abstract

Plant extracts continue to represent an untapped source of renewable therapeutic compounds for the treatment and prevention of illnesses including chronic metabolic disorders. With the increase in worldwide obesity and its related morbidities, the need for identifying safe and effective treatments is also rising. As such, use of primary human adipose-derived stem cells represents a physiologically relevant cell system to screen for bioactive agents in the prevention and treatment of obesity and its related complications. By using these cells in a primary screen, the risk and cost of identifying artifacts due to interspecies variation and immortalized cell lines is eliminated. We demonstrate that these cells can be formatted into 384-well high throughput screens to rapidly identify botanical extracts that affect lipogenesis and lipolysis. Additionally, counterscreening with human primary stem cells from distinct adipose depots can be routinely performed to identify tissue specific responses. In our study, over 500 botanical extracts were screened and 16 (2.7%) were found to affect lipogenesis and 4 (0.7%) affected lipolysis.
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1. Introduction

The prevalence of obesity, metabolic syndrome, diabetes and their associated comorbidities has increased significantly across the world over the recent past. As such, it is imperative that we implement successful and viable strategies in order to address the global epidemic. In this regard, and in an historical perspective, extracts from plants, i.e., botanicals, have proven to be a rich resource for the discovery of therapeutic compounds and the sources of many current medicinal drugs [1,2]. Throughout the world, traditional cultures have identified specific plant species for the treatment of multiple disorders, including the metabolic syndrome and diabetes [1]. In particular, plant extracts proposed to contain effective "bioactives" are marketed routinely as therapeutic agents for metabolic syndrome and diabetes, often with limited understanding of mechanism or proof of efficacy. Recently, the World Health Organization and the NIH have supported international efforts to develop comprehensive libraries of botanical extracts for therapeutic drug discovery. Studies have begun to screen these libraries for candidate molecules directed toward the prevention and treatment of the metabolic syndrome using cell-based in vitro assays [3–9].

Given the importance of insulin resistance in peripheral tissues such as adipose tissue and muscle in the etiology of metabolic syndrome and progression to type 2 diabetes, both adipocyte and skeletal muscle cell lines have been employed in these studies [4,8]. In most analyses of adipocytes, investigators have chosen to use the 3T3-L1 murine preadipocyte cell line [4,10–15]. This in vitro model of adipogenesis is characterized by a homogeneous morphology and a robust lipogenic and lipolytic response to known agonists and antagonists [12–15]. However, since the 3T3-L1 cell line is of murine origin, its utility as a drug discovery tool for eventual human conditions has been questioned due to interspecies variations. A

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number of laboratories, including those in the pharmaceutical industry, have begun to employ primary human adipose-derived stromal/stem cells (ASCs) as an alternative in vitro model for adipocyte differentiation and function [16–23]. The ASC can be derived from many adipose depots in human subjects, including subcutaneous and omental/visceral tissue. There is a growing body of literature indicating that the metabolic function and drug response of adipocytes in these depots are distinct. This has potential implications with respect to the pathology, diagnosis and treatment of metabolic syndrome and diabetes for the human condition [24,25]. The current study reports the use of both primary human subcutaneous- and visceral-derived ASCs to screen a library of botanical extracts for both lipogenic and lipolytic agonists and antagonists. These preliminary studies have the potential to yield novel pharmaceuticals of plant origin for the treatment or prevention of metabolic syndrome.

2. Materials and methods

2.1. Chemicals

2.1.1. Botanical extract library

The source of plant extracts was prepared and obtained from the John S. McIlhenny Laboratory of Botanical Research as part of the NIH-funded Center for the study of Botanicals and Metabolic Syndrome. The extracts were originally obtained as part of the International Cooperative Biodiversity Group program. Field-collected plant samples were prepared by air drying and extraction with 80% ethanol (1:5 w/v) three times, infused each time for 24 h and evaporated in a rotary evaporator. The obtained extracts were dried under vacuum and stored in amber glass vials at −20°C. The yield of air-dried extract as the percent weight of the dried plant tissue varied among the tested samples based on the plant part used. Before tests, concentrates were dissolved by sonication in DMSO for a final concentration of 20 mg/ml.

2.1.2. Chemicals and reagents

Type I collagenase was obtained from Worthington Biochemical (Lakewood, NJ). Bovine serum albumin (BSA), dexamethasone, biotin, pantothenate, isoproterenol and isobutyl methylxanthine were purchased from Sigma (St. Louis, MO). Human recombinant insulin was obtained from MP Biomedicals (Solon, OH). The fetal bovine serum, Dulbecco modified Eagle's medium, phosphate-buffered saline and Ham's F-12 nutrient broth were from Zen-Bio (RTP, NC). Tumor necrosis factor α was from Roche Diagnostics (Indianapolis, IN). All tissue culture flasks and plates were obtained from Corning (Corning, NY).

2.2. Preparation and culture of primary ASC from subcutaneous and visceral depots

2.2.1. Isolation of human primary ASCs from subcutaneous and omental depots

Human adipose tissue samples were procured from consenting donors undergoing elective surgeries under IRB-approved protocols. Subcutaneous and omental adipose tissue was from surgical waste material derived from subcutaneous lipoaspirate or omentum tissue.

Human ASCs were isolated from lipoaspirate or omental waste tissue as previously described [26,27]. Adipose tissue was extensively washed with PBS prior to processing. Omental tissue samples were hand-minced while washed lipoaspirate material was directly transferred for dissolution with an equal volume of Krebs Ringer buffer containing 0.1% collagenase and 1% BSA. Following a 15- to 45-min incubation at 37°C, floating primary adipocytes and most of the collagenase buffer were removed and PBS was added. The remaining stromal vascular cell suspension was subjected to centrifugation for 5 min at 300×*g* and the resulting cell pellet washed several times with PBS. Finally, the cell pellet was suspended in preadipocyte medium (PM-1, Zen-Bio) and plated in a culture flask for expansion. ASCs were subcultured prior to reaching confluence and plated at a density of 1.4 million cells per T-225 flask (6300 cells/cm^2) for further expansion in PM-1 containing epidermal growth factor and fibroblast growth factor.

2.2.2. Preparation of subcutaneous and omental ASC super-lots

Mixed patient lots of either subcutaneous or omental ASCs were prepared from cryopreserved ASCs derived from four to six donor lots each. The subcutaneous super-lot was derived from six female donors with an average age of 40±3.3 (S.E.) and average BMI of 27.9±0.6. The omental super-lot was derived from four female donors with an average age of 40±3.8 (S.E.) and average BMI of 45.6±4.9. Equal numbers of Passage 1 ASCs were combined in preadipocyte medium and plated for expansion at a density of 2.2 million cells per chamber (3500 cells/cm^2) in CellSTACK chambers (Corning). The super-lot cells were expanded to near confluence and harvested by trypsinization for cryopreservation and later use.

To ensure the quality of the prepared cell super-lots, both the omental and subcutaneous lots were tested for differentiation and lipolytic response. Both lots met the required cutoff values for triglyceride accumulation and free fatty acid (FFA) release under standard differentiation procedures. Cell surface markers for both lots of ASCs were confirmed using flow cytometry and antibodies to positive markers (CD29, CD44, and CD105) and negative markers (CD14, CD31, and CD45).

2.3. Screen botanical extracts for human adipocyte lipolysis

Mature adipocytes in clear bottom 384-well plates were prepared by seeding ASCs at a density of 6000 cells/well and differentiating them to adipocytes by adding differentiation medium (DM-2, Zen-Bio) for 7 days. After differentiation, the medium was partially replaced with adipocyte medium (AM-1, Zen-Bio) and the cells incubated for a further 7 days at 37°C with 5% CO_2. This procedure results in greater than 80% adipocyte differentiation [28]. Medium was aspirated and the cells washed twice with 50 µl PBS using a cell washer (ELx-405 Select CW, BioTek) prior to adding 25 µl of assay buffer to each well. Botanical extracts were diluted in a two-step process by using a Matrix PlateMate 2×3 (ThermoScientific); first a 50-fold dilution into assay buffer was performed, followed by a similar 10-fold dilution. Twenty-five microliters of the 500-fold dilutions were added to the assay plates in quadruplicate for a final dilution of 1000-fold (20 µg/ml). Isoproterenol (100 nM) and DMSO (0.1%) were added to quadruplicate wells on every plate and served as the positive and negative controls, respectively. Cells and extracts were incubated for 4 h at 37°C with 5% CO_2 to accumulate FFA in the conditioned assay buffer.

For follow-up experiments in 96-well plates, preadipocytes were seeded at a density of 13,000 cells/well and differentiated as described above. Cells were washed twice with PBS and treated with extracts in a final volume of 100 µl of assay buffer for 4 h.

2.3.1. Measuring fatty acid release in human cultured adipocytes

FFAs released into the conditioned assay buffer were quantified using Lipolysis Assay Kit reagents (Zen-Bio, Inc.). For the initial screen, 15 µl of conditioned assay buffer was removed from each well and transferred to new 384-well plate for determination of fatty acid release. Fifty microliters of FFA Solution A was added to 15 µl of conditioned assay buffer and incubated at 37°C for 10 min. Twenty-five microliters of FFA Solution B was added to each well and incubated for an additional 10 min at 37°C. Total FFAs released were determined by measuring the optical density at 540 nm and comparison to a standard curve ranging from 1.5 to 333 µM. Quadruplicate values were averaged, and standard deviations were used to determine statistical differences between controls. Follow-up assays were performed in 96-well plates essentially as described but with 30 µl of conditioned assay buffer, 100 µl FFA Solution A and 50µl FFA Solution B.

To determine if positive values resulted from extract interference with the assay reagents, we performed fatty acid assays using extracts diluted into assay buffer without exposure to cells. If the resulting value was above 10 µM, it was concluded that the extract interfered with the assay.

2.4. Screen botanical extracts for lipogenic effects in human adipocytes

ASCs were seeded at 6000 cells/well in clear bottom 384-well plates using PM-1. The next day, a 40% mixture of DM-2 and PM-1 was added and incubated for 7 days at 37°C with 5% CO_2. Four wells were given 100% DM-2 to serve as a positive control. An additional four wells were given PM-1 to serve as an uninduced control. Botanical extracts were added on Day 7 through a two-step dilution process in AM-1 to give a 50 µg/ml final concentration. 0.25% DMSO was added to four wells to serve as a vehicle control and 10 ng/ml TNFα served as a positive control of inhibition. The cells and extracts were incubated at 37°C with 5% CO_2 for an additional 7 days prior to quantifying total accumulate triglyceride.

2.4.1. Triglyceride measurement in subcutaneous and omental adipocytes

The total accumulated triglyceride was determined using Total Triglyceride Kit reagents (Zen-Bio, Inc.). The medium was aspirated and the cells were washed once with 50 µl wash buffer using an automated cell washer (BioTek). Five microliters of lysis buffer was added to each well and cells were incubated at 37°C for 20 min to complete the cellular lysis. Forty-five microliters of wash buffer was added to each well before adding 6.7 µl Reagent B (lipase) and incubating for an additional 2 h at 37°C. 12.5 µl of each lipase treated sample was transferred to a new 384-well plate containing 12.5 µl wash buffer. An equal volume of Glycerol Reagent A (25 µl) was added to each well and incubated for 15 min at room temperature. Glycerol content was determined by measuring the optical density at 540 nm and comparing the values to a glycerol standard curve. There is a 1:1 molar ratio between the amount of glycerol detected and cellular triglyceride content.

Treatments and assays performed in 96-well plates followed a similar protocol, but the volumes were adjusted for the larger plate format. Preadipocytes were seeded at 13,000 cells per well prior to differentiation and extract treatment in 150 µl final volume. Volumes were changed to 15 µl lysis buffer, 135 µl wash buffer and 20 µl of Reagent B. Ten microliters of each sample was diluted into 40 µl of wash buffer and 50 µl of Reagent A added to initiate quantification of glycerol.

2.5. Cytotoxicity analysis of botanical extracts

Extract-induced cytotoxicity was assessed in the 384-well format using conditions for the lipogenic assay. Instead of performing the triglyceride assay, 25 µl of

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