Original Article

MOLECULAR METABOLISM

Small molecule PGC-1α1 protein stabilizers induce adipocyte Ucp1 expression and uncoupled mitochondrial respiration

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ABSTRACT

Objective: The peroxisome proliferator-activated receptor-γ coactivator-1α1 (PGC-1α1) regulates genes involved in energy metabolism. Increasing adipose tissue energy expenditure through PGC-1α1 activation is potentially beneficial for systemic metabolism. Pharmacological PGC-1α1 activators could be valuable tools in the fight against obesity and metabolic disease. Finding such compounds has been challenging partly because PGC-1α1 is a transcriptional coactivator with no known ligand-binding properties. While, PGC-1α1 activation is regulated by several mechanisms, protein stabilization is a crucial limiting step due to its short half-life under unstimulated conditions.

Methods: We designed a cell-based high-throughput screening system to identify PGC-1α1 protein stabilizers. Positive hits were tested for their ability to induce endogenous PGC-1α1 protein accumulation and activate target gene expression in brown adipocytes. Select compounds were analyzed for their effects on global gene expression and cellular respiration in adipocytes.

Results: Among 7,040 compounds screened, we highlight four small molecules with high activity as measured by: PGC-1α1 protein accumulation, target gene expression, and uncoupled mitochondrial respiration in brown adipocytes.

Conclusions: We identify compounds that induce PGC-1α1 protein accumulation and show that this increases uncoupled respiration in brown adipocytes. This screening platform establishes the foundation for a new class of therapeutics with potential use in obesity and associated disorders.

Keywords Small molecule screening; PGC-1a; PGC-1alpha; PGC-1alpha1; Protein stabilization; UCP1; Mitochondrial respiration; Brown adipose tissue

1. INTRODUCTION

Proteins of the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) family of transcriptional coactivators regulate genes involved in energy metabolism and are expressed in energy-demanding tissues like fat, skeletal muscle, liver, and brain [1]. Interestingly, a single PGC-1α gene can be differently regulated by alternative promoter usage and alternative splicing to generate discrete PGC-1α variants with different biological activities [2–4]. For example, PGC-1α1, the founding member of the family [5] is a strong regulator of mitochondrial biogenesis and oxidative metabolism, whereas PGC-1α4 regulates muscle mass and strength [2,3,5,6]. In skeletal muscle, induction of PGC-1α1 promotes a more oxidative phenotype, efficient fuel handling, and increases resistance to fatigue [7]. In addition, skeletal muscle PGC-1α1 activates local kynurenine detoxification, thereby positively impacting on mental health [8,9]. In the brain, PGC-1α1 has been implicated in reactive oxygen species (ROS) detoxification and protection from neurodegenerative disease [10]. However, it is in brown adipose tissue that PGC-1α1 shows the highest expression and has some of its most well-established roles. Initially discovered for

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Abbreviations: C/EBP, CCAAT-enhancer-binding proteins; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; HEK, human embryonic kidney; HNF, hepatocyte nuclear factor; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IDP, intrinsically disordered protein; MEF2, myocyte enhancer factor 2; NRF2, nuclear respiratory factors 2; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; PGC, peroxisome proliferator-activated receptor-γ coactivator; PTM, post-translational modifications; UCP1, uncoupling protein 1; SREBP1, sterol regulatory element-binding protein 1; XBP1, X-box binding protein 1

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its ability to induce mitochondrial biogenesis and adaptive thermogenesis in brown adipocytes, PGC-1α1 has been shown to coordinate the expression of thermogenic genes, of which uncoupling protein 1 (*Ucp1*) is of central importance [5,6,11]. This mechanism is also inducible in white adipose depots, through an adipocyte population called beige, brite, or recruitable (hereon referred to as beige adipocytes) [12–15]. Moreover, PGC-1α1 expression correlates with typical markers of beige-selective genes in human brown adipose tissue [16]. Therefore, elevated PGC-1α1 levels in adipose tissue could have beneficial effects on systemic metabolism, making PGC-1α1 an interesting target in the treatment of obesity and type 2 diabetes [17,18]. Genetic deletion of PGC-1α1 (knockout) in adipose tissue perturbs the mitochondrial and thermogenic gene programs rendering mice carrying the knockout alleles more sensitive to high-fat diet-induced insulin resistance [19]. In addition, diabetic humans have reduced PGC-1α levels in adipose tissue, which may contribute to the pathogenesis of metabolic disease [20,21]. For these reasons, there are great therapeutic promises in finding ways to activate PGC-1α1 and its target genes.

PGC-1α1 activation can be achieved through several mechanisms including gene expression, protein stabilization, and post-translational modifications. Previous attempts to screen for regulators of PGC-1α1 action have focused on identifying inducers of PGC-1α1 gene transcription (potential activators) or of PGC-1α1 acetylation (potential inhibitors) [22–26]. However, the short protein half-life makes protein stabilization a limiting step in the activation process of PGC-1α1 and downstream target gene transcription [4,27–32]. Indeed, PGC-1α1 levels are tightly controlled by several E3 ubiquitin-ligases that target the protein for ubiquitin-proteasome-mediated degradation [27,29,30,33]. Additionally, it has been suggested that PGC-1α1 is an intrinsically disordered protein, which makes it susceptible to default degradation by the 20S proteasome [34].

Here, we report the development of a cell-based high-throughput screening system that allows for the identification of agents that activate PGC-1α1 by increasing protein stability. Using this system, we were able to identify several small molecule PGC-1α1 activators that are biologically active in brown adipocytes. Treatment of adipocytes with select compounds leads to PGC-1α1 protein accumulation, increased *Ucp1* expression, and higher mitochondrial respiration rates.

2. EXPERIMENTAL PROCEDURES

2.1. Plasmids and cell lines

2.1.1. Establishment of the screening cell line

To generate a pEGFP-C1-mousePGC-1α1 plasmid (containing a neomycin selection cassette), the cDNA for mouse PGC-1α1 (mPGC-1α1) was excised from a previously published pcDNA3.1-Flag-mPGC-1α1 plasmid [2] using XhoI and NotI. The pEGFP-C1 was opened with BglII and the cDNA for mouse PGC-1α1 was subsequently blunt-end ligated downstream of EGFP. To efficiently produce EGFP-PGC-1α1 fusion protein the A in the start codon for PGC-1α1 was point mutated to a G (generating valine instead of methionine) using QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, #200523).

To generate the screening cell line (293-EGFPmPGC-1α1), human embryonic kidney (HEK) 293-T cells were transfected with the pEGFP-C1-mPGC-1α1 plasmid using Lipofectamine® 2000 (Thermo Fisher Scientific). Stable cell lines were generated by culturing cells in the presence of 500 μg/ml G418 (also termed Geneticin®, Thermo Fisher Scientific, #11811). Single cell-derived colonies were obtained using a dilution protocol. Cells were subsequently cultured according to standard protocols for HEK 293 cells in DMEM high glucose (Gibco, #31966021) containing 10% Fetal Bovine Serum (FBS, Sigma–Aldrich, #F7524) and penicillin/streptomycin (pen/strep) (Gibco, #15140122) in the presence of 500 μg/ml G418. Cells were grown at 37 °C in 5% CO_2.

2.1.2. Brown adipocyte cell line

Validation of compound screening was done in an immortalized brown preadipocyte line of mouse origin and was a kind gift from Dr. Bruce Spiegelman (Dana-Farber Cancer Institute and Harvard Medical School, Boston, USA) and has been described previously [35]. Preadipocytes were cultured in DMEM high glucose (Gibco, #31966021) containing 20% FBS (Sigma–Aldrich, #F7524), 20 mM Hepes (Gibco, #15630056), and pen/strep (Gibco, #15140122). For differentiation, cells were seeded and grown to confluence in differentiation medium (DMEM high glucose supplemented with 10% FBS, 20 nM insulin, and 1 nM triiodothyronine, T3). Cells were subsequently induced to differentiate with an induction medium (i.e. differentiation medium supplemented with 0.125 mM indomethacin, 0.5 μM dexamethasone and 0.5 mM isobutyl methylxanthine) for 2 days, after which, medium was changed to differentiation medium for 3–4 additional days. Fully differentiated brown adipocytes were exposed to 10 μM of compound or 0.1% DMSO for 8 h. Isoproterenol (10 μM) was included as a positive control (as previously reported [35]). MG132 treated cells were included to control for general proteasome inhibition. Following treatment, cells were used for cellular respirometry or harvested for isolation of protein and RNA. Cells were grown at 37 °C in 5% CO_2.

2.2. High-throughput screening, image acquisition and analysis

The compound library used in this study was a collection of compounds from the Enamine Ltd Drug-Like Set and Pharmacological Diversity Set, which constitutes a total of 28,160 compounds. Compounds in the Drug-Like Set are selected using diversity sorting from the combined file of high-throughput screening and historical collections and strictly conform to rules of Lipinski and Veber [36,37], and do not bear undesired reactive functional groups. Compounds from the Pharmacological Diversity Set are particularly recommended for research on new targets because only biologically relevant chemical space has been searched. Moreover, the design is based on the use of predicted pharmacological properties of compounds. Each compound from Enamine collection was profiled by over 3000 activities. Compounds predicted to be toxic are excluded; the others are clustered by their activities.

293-EGFPmPGC-1α1 cells, stably expressing the EGFP-PGC-1α1 fusion protein, were seeded with laminin (L2020, Sigma–Aldrich) 0.2 μg/cm^2 in 384-well optical bottom plates (BD Falcon Optilux #353962 plates) using a Multidrop. Prior to the experiment 50 nl of the library compounds were transferred using an ECHO 550 (Labcyte Inc.) acoustic liquid handler to destination plates and stored at −20 °C. Twenty-four hours after seeding the cells the compound plates were thawed and 20 μl of cell culture medium was added to the plates using a Multidrop. Compound plates were shaken for 1 h at RT after which 20 μl compound solution was transferred to the cell plate using a PerkinElmer Janus 384 MTD system. Cells were incubated in a final concentration 10 μM of each compound, 0.1% DMSO (=negative control) or 10 μM of a proteasome inhibitor, MG132 (=positive control) for 8 h in 37 °C, 5% CO_2. After 8 h treatment, cells were fixed in 4% paraformaldehyde and stained with Hoechst 33342 (nuclear staining). Fluorescence was detected in a fully automated system using

