Original Article

MOLECULAR METABOLISM

Degradation of brown adipocyte purine nucleotides regulates uncoupling protein 1 activity

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ABSTRACT

Objective: Non-shivering thermogenesis in mammalian brown adipose tissue depends on thermogenic uncoupling protein 1. Its activity is triggered by free fatty acids while purine nucleotides mediate inhibition. During activation, it is thought that free fatty acids overcome purine-mediated inhibition. We measured the cellular concentration and the release of purine nucleotide metabolites to uncover a possible role of purine nucleotide degradation in uncoupling protein 1 activation.

Methods: With mass spectrometry, purine nucleotide metabolites were quantified in cellular homogenates and supernatants of cultured primary brown adipocytes. We also determined oxygen consumption in response to a β-adrenergic agonist.

Results: Upon adrenergic activation, brown adipocytes decreased the intracellular concentration of inhibitory nucleotides (ATP, ADP, GTP and GDP) and released the respective degradation products. At the same time, an increase in cellular calcium occurred. None of these phenomena occurred in white adipocytes or myotubes. The brown adipocyte expression of enzymes implicated in purine metabolic remodeling is altered upon cold exposure. Pharmacological and genetic interference of purine metabolism altered uncoupling protein 1 mediated uncoupled respiration.

Conclusion: Adrenergic stimulation of brown adipocytes lowers the intracellular concentration of purine nucleotides, thereby contributing to uncoupling protein 1 activation.

Keywords Purine nucleotides; Uncoupling protein 1; Brown adipose tissue; Non-shivering thermogenesis; HILIC-MS/MS; Guanosine monophosphate reductase

1. INTRODUCTION

Mammalian non-shivering thermogenesis in brown adipose tissue is a heat dissipation mechanism of immense specific capacity. As such, it has to be tightly controlled to prevent wasting stored energy reserves. Mitochondrial uncoupling protein 1 (UCP1) is essential for the thermogenic process and a target of the crucial regulatory signals. In the resting state, UCP1 is inhibited by the binding of free (i.e. non-complexed to magnesium or calcium) purine di- and triphosphate nucleotides (GDP, ADP, GTP, ATP). Upon β-adrenergic activation, lipolytically liberated free fatty acids directly interact with UCP1 to overcome inhibition by purine nucleotides (reviewed in [1]).

The cytosolic concentration of free ATP alone (0.25–10 mM) by far exceeds its binding constant to UCP1 (<1 μM) as determined in proteoliposome experiments [2]. According to the established model of UCP1 regulation, modulating the concentration of purine di- and triphosphate nucleotides is thus not a means of UCP1 activation.

As already noted by others, this assessment seems inappropriate given the substantial evidence for a flexible, different binding constant of purine nucleotides to UCP1 *in vivo* [3,4]. Firstly, the mitochondrial inner membrane lipid cardiolipin reduces purine nucleotide binding to UCP1 in a dose-dependent manner [3]. At the 12% molar amount cardiolipin expected in BAT mitochondria [5,6], the KD is increased more than 10-fold. Secondly, purine nucleotide inhibition is pH dependent [7]. Upon activation of thermogenesis in a brown fat cell, the cytosolic pH increases to up to 8.0 [8]. In a conservative estimate, in the presence of 12% cardiolipin and at a moderate pH of 7.5, the calculated KD is not <1 μM, but already as high as 80 μM. Thirdly, the concentration of free nucleotides depends on the concentration of divalent cations in the cytosol [7]. The cytosolic concentrations of calcium and magnesium increase following activation of a brown adipocyte as a consequence of

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both uptake from the extracellular medium and release from intracellular stores [9–12]. Free purine nucleotide concentrations will decrease accordingly.

For all these reasons, the concentration of purine nucleotides capable of inhibiting UCP1 can be assumed to be well in a range, in which a further degradation may actively contribute to activation of UCP1 and/or sensitize to fatty acid activation. Indeed, brown adipose tissue mRNA and protein abundance of central enzymes of purine nucleotide metabolism are strongly regulated upon cold exposure [13,14].

In this study, we investigated concentration changes of purine nucleotides in activated brown adipocytes by hydrophilic interaction chromatography and mass spectrometry. We found a decrease in purine nucleotide di- and triphosphate concentration upon adrenergic stimulation of primary brown adipocytes and present evidence that alterations in purine nucleotide metabolism are involved in the activation of UCP1-mediated thermogenesis.

2. MATERIAL & METHODS

2.1. Cell culture and transfection

Primary brown adipocytes were prepared from the interscapular brown adipose tissue of male C57BL/6J mice as previously described [15]. Mice originated from our specific pathogen free barrier colony that operates according to the German Animal Welfare Law.

Proliferating cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). Confluent cells were induced by addition of 0.5 mM isobutylmethylxanthine (IBMX), 125 nM indomethacin, 1 μM dexamethasone, 850 nM insulin and 1 nM T3 for 2 days and differentiated for a further 6 days in the presence of 850 nM insulin and 1 nM T3. Immortalized, primary brown adipocytes were a gift from Bruce Spiegelman's lab, generated by a published method [16], and cultured by the above protocol.

3T3-L1 white adipocytes were proliferated in DMEM plus 10% FBS, induced for 3 days in the presence of 5 μg/ml insulin, 250 nM dexamethasone and 0.5 mM IBMX, and differentiated for a further 6 days with 5 μg/ml insulin. C2C12 myoblasts were proliferated in DMEM plus 10% FBS and differentiated for 8 days in DMEM plus 2% horse serum. Human embryonic kidney cells (HEK293) were cultured in DMEM plus 10% FBS. The generation and validation of HEK293 cells stably expressing UCP1 has been described previously [17]. Transient transfection of a GMPR overexpression plasmid (pCMV-Sport6) was performed by the calcium phosphate method (ProFection Kit, Promega) using 10 μg of vector.

We detected cytosolic protease activity in cell culture supernatants as a marker of ruptured cells with a commercial kit system (CytoTox Glo, Promega).

2.2. Respirometry

Oxygen consumption of primary brown adipocytes was determined at 37 °C with an XF96 extracellular flux analyzer (Seahorse Bioscience) following a published protocol [18]. Briefly, cells were fully differentiated on appropriate multiwell plates and changed to unbuffered DMEM with 2% bovine serum albumin and a CO_2-free incubator 1 h before the measurement. ATP synthesis was blocked by injection of 5 μM oligomycin A, UCP1 mediated respiration was activated by addition of 0.5 μM isoproterenol, maximal respiration by 1 μM Carbonyl-cyanide p-[trifluoromethoxy]-phenyl-hydrazone (FCCP) and finally non-mitochondrial respiration by 5 μM antimycin A. Results are shown as mean values ± SD of 10–12 replicate wells.

Oxygen consumption of HEK293 cells was determined by a Clark-type electrode (Model 10, Rank Brothers Ltd) at 37 °C in DMEM medium. Cells were trypsinized, pelleted and resuspended in fresh DMEM. After measurement of basal respiration, ATP synthesis was inhibited by 1.2 μM oligomycin A and the basal leak was quantified. A bolus of 300 μM palmitate was used to activate UCP1-mediated uncoupling and 3 μM FCCP to fully uncouple to maximal respiration rate.

2.3. Quantitation of purine metabolites, fatty acids and cations

We harvested metabolites by washing cultured cells with ice-cold phosphate buffered saline (pH 7.4), placing the dish on ice and scraping cells in ice-cold 15 mM tetrabutylammonium hydrogen sulfate (TBAHS, pH 2.0) including a 5 μM internal standard mix of 15N-labeled ATP, GTP, AMP, GMP (Silantes, Munich, Germany) and 13C-labeled adenosine (Cambridge Isotope Laboratories, Andover, USA). Samples were centrifuged and clear supernatants analyzed by HILIC-MS/MS using a ZIC pHILIC 5 μM, 150 × 4.6 mm column (Merck) to quantify nucleotides and by RP-LC–MS/MS using a Synergi Polar-RP 100 A, 2.5 μm, 2.0 × 100 mm column (Phenomenex, Aschaffenburg, Germany) to quantify fatty acids (detailed method in the Supplement). Cations were quantified in the same samples by ion exchange chromatography (Chromatograph: Dionex ICS-2000, Thermo Fischer) using Dionex Ion Pac CS19 (Thermo Fisher) column with 0.1 M methanol sulfonic acid as solvent with a 250 μL/min flowrate. One brown adipocyte outlier sample was excluded from the divalent cation analysis due to >5-fold content as compared to the next highest sample. This removal had no effect on statistically significant group differences. All analytical data were normalized to DNA concentration measured by bis-benzimide fluorometry as a surrogate measure for cell number.

2.4. Transcript quantification

Primer sequences for transcript detection with quantitative PCR are given in Suppl. Table 4. We employed cDNA from a first strand synthesis from total RNA (Quantitect cDNA synthesis Kit, Qiagen) in a qPCR reaction mix including SYBR Green (Sensimix, Bioline) on a multiwell PCR machine (Mastercycler, Eppendorf). Transcript abundance of the analyzed genes shown was normalized to the mean standardized expression of all six housekeeping genes listed in the primer table.

We analyzed a publicly available transcriptomic dataset (GEO accession GSE63031; by Q Hao and HB Hansen, Copenhagen, Denmark) generated by deep-sequencing of transcripts isolated from murine adipose tissues [19]. Original data was downloaded, mapped and reads per kilobase per million mapped reads calculated by a dedicated software platform (Mining Station and Genome Analyzer, Genomatix).

2.5. Western Blot

Western blot detection of UCP1 was performed with a primary rabbit antibody raised against hamster UCP1 [20]. GMPR was detected with a rabbit polyclonal antibody raised against a peptide representing amino acids 2–16 of human GMPR, generated by Ernest Beutler (San Diego, CA, USA) and kindly provided by Reed Larsen (Boston, MA, USA) [13,21]. We detected pan-actin with a commercial mouse monoclonal antibody (MAB1501, Merck Millipore). Secondary antibodies were coupled to an infrared fluorophore (goat-anti-rabbit IRDye 800CW, 925-32210; donkey-anti-mouse 680RD, 925-68072, Licor) and visualized on an imaging system (Odyssey, Licor).

3. RESULTS

3.1. Cold exposure altered brown fat expression of purine nucleotide metabolic enzymes

Cold exposure leads to a drastic increase in guanosine monophosphate reductase (GMPR) in activated brown adipose tissue (BAT) [13]. This

