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MOLECULAR METABOLISM

Fatty acid oxidation is required for active and quiescent brown adipose tissue maintenance and thermogenic programing

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ABSTRACT

Objective: To determine the role of fatty acid oxidation on the cellular, molecular, and physiologic response of brown adipose tissue to disparate paradigms of chronic thermogenic stimulation.

Methods: Mice with an adipose-specific loss of Carnitine Palmitoyltransferase 2 ($Cpt2^{A-/-}$), that lack mitochondrial long chain fatty acid β-oxidation, were subjected to environmental and pharmacologic interventions known to promote thermogenic programming in adipose tissue.

Results: Chronic administration of β3-adrenergic (CL-316243) or thyroid hormone (GC-1) agonists induced a loss of BAT morphology and UCP1 expression in $Cpt2^{A-/-}$ mice. Fatty acid oxidation was also required for the browning of white adipose tissue (WAT) and the induction of UCP1 in WAT. In contrast, chronic cold (15 °C) stimulation induced UCP1 and thermogenic programming in both control and $Cpt2^{A-/-}$ adipose tissue albeit to a lesser extent in $Cpt2^{A-/-}$ mice. However, thermoneutral housing also induced the loss of UCP1 and BAT morphology in $Cpt2^{A-/-}$ mice. Therefore, adipose fatty acid oxidation is required for both the acute agonist-induced activation of BAT and the maintenance of quiescent BAT. Consistent with this data, $Cpt2^{A-/-}$ BAT exhibited increased macrophage infiltration, inflammation and fibrosis irrespective of BAT activation. Finally, obese $Cpt2^{A-/-}$ mice housed at thermoneutrality exhibited a loss of interscapular BAT and were refractory to β3-adrenergic-induced energy expenditure and weight loss.

Conclusion: Mitochondrial long chain fatty acid β-oxidation is critical for the maintenance of the brown adipocyte phenotype both during times of activation and quiescence.

Keywords Fatty acid oxidation; Brown adipose tissue; Cold induced thermogenesis; Adrenergic signaling; Adipose macrophage

1. INTRODUCTION

The major function of adipose tissue is the storage of fatty acids as an energetic buffer during times of food scarcity. This is accomplished by white adipocytes that store triglyceride in large, unilocular lipid droplets throughout the body. Alternatively, brown adipocytes are tasked with maintaining body temperature by consuming fatty acids via nonshivering thermogenesis under cold environmental temperatures. Although white adipocytes contain few mitochondria, brown adipocytes are packed with mitochondria and generate heat by chemical energy fueled by increasing their cellular metabolic rate. These cells are important during the postnatal period and in times of acute cold stimulation to maintain body temperature. Cold stimulation or pharmacologic activation of adrenergic receptors on brown adipocytes dramatically increases mitochondrial respiration via the uncoupling of the mitochondrial electrochemical gradient via Uncoupling Protein 1 (UCP1). Fatty acid oxidation is critical for this process as it provides the mitochondrial bioenergetics as well as the biophysical activator of uncoupling [1–4]. Fatty acids are uniquely required for UCP1-induced uncoupling. While cold induces glucose uptake into brown adipocytes and the full oxidation of glucose generates reducing equivalents, brown adipose tissue (BAT) thermogenesis and glucose uptake can be experimentally uncoupled [5,6]. Consequently, mice with an adipose-specific deficit in fatty acid oxidation are severely cold intolerant, demonstrating an autonomous requirement for adipose fatty acid oxidation in cold-induced thermogenesis [7,8].

Previously, we generated mice with an adipose-specific defect in fatty acid oxidation by deleting Carnitine Palmitoyltransferase 2 (Cpt2), an obligate step in mitochondrial long chain fatty acid β-oxidation, specifically in adipocytes ($Cpt2^{A-/-}$) [7]. These mice are severely cold intolerant as expected, but did not exhibit an obesogenic phenotype following low or high fat feeding. Similarly, Ucp1KO mice are resistant, rather than prone, to diet-induced obesity under standard housing conditions [9,10]. Housing Ucp1KO mice at thermoneutrality (30 °C) acutely increases their adiposity, even though thermoneutrality suppresses Ucp1 in white and brown adipose tissue [11]. However, $Cpt2^{A-/-}$ mice did not show increased body weight gain or adiposity at thermoneutrality even though interscapular BAT was lost following housing at 30 °C [12]. This suggests adipose

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bioenergetics alone is not required for the homeostatic regulation of body weight.

The expression of genes required for thermogenesis are increased with cold and adrenergic stimulation and suppressed through thermoneutral housing. This thermogenic programing and plasticity is an important component of long-term cold tolerance. Although fatty acid oxidation is required for thermogenesis, we were surprised that $Cpt2^{A-/-}$ mice could not induce the expression of thermogenic genes such as *Ucp1*. Acute cold exposure (21 °C—4 °C) or the β3-adrenergic agonist, CL-316243, failed to induce *Ucp1*, *Pgc1α*, and *Dio2* among others in $Cpt2^{A-/-}$ BAT [7]. This deficiency in transcriptional programing was further exacerbated by acclimatizing mice at 30 °C [12]. Therefore, $Cpt2^{A-/-}$ mice represent a unique model to dissect the physiological functions of brown adipocytes *in vivo* because they exhibit molecular, cellular, and biochemical defects that prevented canonical BAT or beige cell function.

To understand the role of fatty acid oxidation to adipose tissue structure, function, and physiology, we subjected $Cpt2^{A-/-}$ mice to disparate thermogenic stimuli including a β3-adrenergic agonist (CL-316243), a thyroid hormone agonist (GC-1) and altered ambient temperature. Here we show that pharmacologic thermogenic agonists induced a loss of UCP1 and BAT morphology in $Cpt2^{A-/-}$ mice, and failed to induce UCP1 and thermogenic programing in white adipose tissue (WAT). However, chronic cold stimulation induced UCP1 and thermogenic programming albeit to a lesser extent in $Cpt2^{A-/-}$ adipose. Structural analysis of $Cpt2^{A-/-}$ BAT revealed increased macrophage infiltration, inflammation, and fibrosis. Finally, obese $Cpt2^{A-/-}$ mice housed at thermoneutrality exhibited a loss in interscapular BAT and were refractory to β-adrenergic-induced energy expenditure and weight loss. These data show that fatty acid oxidation is critical for the maintenance of the brown adipocyte phenotype, particularly under conditions of metabolic stress.

2. EXPERIMENTAL PROCEDURES

2.1. Animals and diets

$Cpt2^{A-/-}$ and $Cpt2^{lox/lox}$ littermate control mice were generated as previously described [7,12]. For temperature acclimation studies, 12-week old male and female $Cpt2^{A-/-}$ and $Cpt2^{lox/lox}$ mice were housed in an animal incubator (Key Scientific) at the indicated temperatures for 10 days on a 12 h light/12 h dark cycle and fed a standard chow diet (Teklad Global Rodent Diets). For studies using GC-1 (Tocris; 4554) and CL-316243 (Tocris; 1499), 12-week old male and female $Cpt2^{A-/-}$ and $Cpt2^{lox/lox}$ mice were housed in a facility with ventilated racks on a 12 h light/12 h dark cycle with access to a standard chow diet (Teklad Global Rodent Diets) and were subjected to an intraperitoneal injection with vehicle (0.9% NaCl), CL-316243 (1 mg/kg), or GC-1 (0.3 mg/kg) for 10 consecutive days. Tissue depots for both respective studies were collected on day 11, and were either snap frozen in liquid nitrogen, or stored in Formalin (Sigma) for H&E staining (AML Laboratories).

For the diet study, male $Cpt2^{A-/-}$ and $Cpt2^{lox/lox}$ mice were housed at room temperature on a 12 h light/12 h dark cycle and fed a 60% high-fat diet (Research Diets; D12492) starting at 6-weeks of age (12 weeks on diet). At 12-weeks of age, mice were transferred to an animal incubator at 30 °C on a 12 h light/12 h dark cycle. At 17-weeks of age, the same mice were subjected to an intraperitoneal injection with CL-316243 (1 mg/kg) for 10 consecutive days. Body weights were measured on a weekly basis and on a daily basis during injections with CL-316243. At approximately 18-weeks of age, following the last injection with CL-316243 on day 10, the same mice were individually housed in Comprehensive Laboratory Animal Monitoring System (Columbus Instruments) cages on a 12 h light/12 h dark cycle. O_2 and CO_2 consumption and production, respectively, food, and water intake, and home—cage activity were measured continuously. Data were collected for 96 h for ad libitum and a 24 h fasting period. At the end of the study, the same mice were injected with CL-316243 (10 mg/kg) and were monitored for 3 h. Body fat and lean mass of the same mice was measured via magnetic resonance imaging analysis (Minispec MQ10). BAT, iWAT, and gWAT depots were collected for H&E staining.

For the CL-316243 time course experiments, 12-week old male and female $Cpt2^{A-/-}$ and $Cpt2^{lox/lox}$ mice were housed at room temperature on a 12 h light/12 h dark cycle with access to a standard chow diet (Teklad Global Rodent Diets) and subjected to an intraperitoneal injection with CL-316243 (1 mg/kg) at the indicated time points. BAT depots were collected 24 h following the last injection with CL-316243 (days 3, 5, 7, 9, or 11). All procedures were performed in accordance with the NIH's *Guide for the Care and Use of Laboratory Animals* and under the approval of the Johns Hopkins Medical School Animal Care and Use Committee.

2.2. Analysis of gene expression by quantitative PCR

Total RNA was isolated using Trizol followed by the RNeasy Mini Kit (QIAGEN). RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences). 1—2 ug of cDNA was diluted to 2 ng/ml and was amplified by specific primers in a 20 μl reaction using SsoAdvanced SYBR Green Supermix (Bio-Rad). Analysis of gene expression was carried out in a CFX Connect Real-Time System (Bio-Rad). For each gene, mRNA expression was calculated as 2deltaCT relative to Rpl22 and 18s expression [13]. Primers and gene information are provided in Table S1.

2.3. Western blot

BAT, iWAT, and gWAT depots were homogenized with 300—500 ul of RIPA buffer (50 mM Tris—HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% deoxycholate) containing protease inhibitor cocktail (Roche) and phospho-STOP cocktail (Roche), followed by pelleting of the insoluble debris at 13,000×*g* for 15 min at 4 °C. The protein concentrations of lysates were determined by BCA assay (Thermo Scientific), and 30 μg of lysate was separated by Tris-glycine SDS-PAGE (10% polyacrylamide). Proteins were transferred to nitrocellulose membranes (Protran BA 83, Whatman), blocked in 3% BSA in 1X TBST (Tris-buffered saline with Tween 20), and incubated with primary antibodies overnight. The blots were probed with the following antibodies: Ucp1 (Sigma; U6382), Ndufb8, Sdhb, Uqcrc2, Atp5a (MitoProfile total OXPHOS, Abcam; ab110413), Aco2 (Cell Signaling; 6922), Mcad (GeneTex; GTX32421), Pcx (Abcam; ab128952), Vdac (Calbiochem; PC548), Pdh E2/E3bp (Abcam; ab110333), Acot2 (Sigma; SAB2100030), beta-Actin (Sigma; A2228), and Hsc-70 (Santa Cruz; sc-7298). Cy3-conjugated anti-mouse (Invitrogen), or HRP-conjugated anti-mouse (GE Healthcare) or anti-rabbit (GE Healthcare) secondary antibodies were used appropriately. Images were collected and analyzed using an Alpha Innotech FluorChemQ.

2.4. Transmission electron microscopy

Samples for TEM were collected and processed as described with minor modifications [14]. BAT was collected and immersed in 3% glutaraldehyde PBS solution and cut into ~0.5 mM sections with a Mcilwain tissue chopper. The tissue was then fixed in 3%

