Biochimica et Biophysica Acta 1797 (2010) 968–980

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Cold tolerance of UCP1-ablated mice: A skeletal muscle mitochondria switch toward lipid oxidation with marked UCP3 up-regulation not associated with increased basal, fatty acid- or ROS-induced uncoupling or enhanced GDP effects

Irina G. Shabalina [a], Joris Hoeks [a,b,1], Tatiana V. Kramarova [a,1], Patrick Schrauwen [b], Barbara Cannon [a], Jan Nedergaard [a,*]

[a] The Wenner-Gren Institute, Stockholm University, SE-106 91 Stockholm, Sweden
[b] Department of Human Biology, NUTRIM - School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Centre, 6200 MD Maastricht, The Netherlands

ARTICLE INFO

Article history:
Received 12 January 2010
Received in revised form 24 February 2010
Accepted 27 February 2010
Available online 19 March 2010

Keywords:
Uncoupling protein
Skeletal muscle mitochondria
Cold acclimation
Thermogenesis
Oxidative stress
Proton leak

ABSTRACT

Mice lacking the thermogenic mitochondrial membrane protein UCP1 (uncoupling protein 1) - and thus all heat production from brown adipose tissue - can still adapt to a cold environment (4 °C) if successively transferred to the cold. The mechanism behind this adaptation has not been clarified. To examine possible adaptive processes in the skeletal muscle, we isolated mitochondria from the hind limb muscles of cold-acclimated wild-type and UCP1(−/−) mice and examined their bioenergetic chracteristics. We observed a switch in metabolism, from carbohydrate towards lipid catabolism, and an increased total mitochondrial complement, with an increased total ATP production capacity. The UCP1(−/−) muscle mitochondria did not display a changed state-4 respiration rate (no uncoupling) and were *less* sensitive to the uncoupling effect of fatty acids than the wild-type mitochondria. The content of UCP3 was increased 3-4 fold, but despite this, endogenous superoxide could not invoke a higher proton leak, and the small inhibitory effect of GDP was unaltered, indicating that it was not mediated by UCP3. Double mutant mice (UCP1(−/−) plus superoxide dismutase 2-overexpression) were not more cold sensitive than UCP1(−/−), bringing into question an involvement of reactive oxygen species (ROS) in activation of any alternative thermogenic mechanism. We conclude that there is no evidence for an involvement of UCP3 in basal, fatty-acid- or superoxide-stimulated oxygen consumption or in GDP sensitivity. The adaptations observed did not imply any direct alternative process for nonshivering thermogenesis but the adaptations observed would be congruent with adaptation to chronically enhanced muscle activity caused by incessant shivering in these mice.

1. Introduction

Unexpectedly, mice that lack UCP1, the mitochondrial carrier that is the molecular mechanism behind brown-fat-derived thermogenesis, can survive in the cold provided that they are successively adapted to decreasing temperatures [1,2]. In the cold, these UCP1(−/−) mice still produce the same amount of "extra" heat that is necessary to compensate heat loss [1], but the mechanism behind this is debated.

According to one view, the UCP1(−/−) mice in the cold not only shiver initially, as wild-type mice do, but they continue to shiver in the cold for weeks and months (i.e. during the time when nonshivering thermogenesis develops in the wild-type due to recruitment of brown adipose tissue [3]). In this view, mice lack any possibility to develop any alternative means of nonshivering thermogenesis except that emanating from the activity of UCP1 in brown adipose tissue [1,4] and therefore have to chronically rely on shivering for producing the heat necessary.

According to alternative views, alternative mechanism(s) of heat production may develop in these mice, which will replace the heat from brown adipose tissue/UCP1. These mechanisms are thought to reflect abilities normally found in the animals, but the very special situation found in the UCP1(−/−) mice in the cold means that such mechanisms will be highly induced and thus become experimentally evident. The UCP1(−/−) mice in the cold are thus unique models to identify such processes.

Two different organs have been suggested to be responsible for this putative alternative nonshivering thermogenesis. One is white adipose tissue [2,5]; this possibility will not be further examined here.

Abbreviations: ANT, adenine nucleotide translocase; BSA, bovine serum albumin; COX1, cytochrome c oxidase subunit 1; CPT, carnitine palmitoyl transferase; EDTA, ethylenediamine tetraacetic acid; FA, fatty acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; GDP, guanosine 5'-diphosphate; HNE, 4-hydroxy nonenal; ROS, reactive oxygen species; SOD, superoxide dismutase; UCP, Uncoupling protein

* Corresponding author. Arrhenius Laboratories F3, The Wenner-Gren Institute, Stockholm University, SE-106 91 Stockholm, Sweden. Tel.: +46 8 164128; fax: +46 8 156756.
E-mail address: jan@metabol.su.se (J. Nedergaard).

[1] These authors contributed equally to the article.

0005-2728/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2010.02.033

The other organ is skeletal muscle. Classically, skeletal muscle has been suggested to possess means of (alternative) nonshivering thermogenesis, even before brown adipose tissue and UCP1 were introduced in this respect [6–13]. Particularly, uncoupling (increased proton permeability) in skeletal muscle mitochondria has been suggested as a source of heat production [6,7,9,10,13], especially for physiological systems where brown adipose tissue is absent (e.g. birds [9,14]). The UCP1(−/−) mice are an additional such system, and suggestions that alternative mechanisms may develop in the muscle of these mice have been forwarded [8,15,16].

We have here undertaken to examine skeletal muscle mitochondria from cold-acclimated UCP1(−/−) and wild-type mice, to investigate whether the UCP1(−/−) mice demonstrate metabolic alterations indicative of alternative thermogenic mechanisms (certain features concerning skeletal muscle in UCP1(−/−) mice have already been described [1,15,17,18]). We found alterations in substrate preference (a switch toward lipid metabolism) but no indications of basal or induced uncoupling. Particularly, we observed a highly enhanced UCP3 level in these mitochondria, and this allowed us to examine a series of hypotheses concerning UCP3 function in a physiologically relevant system. We could therefore additionally conclude that UCP3 does not mediate basal or fatty-acid- or endogenous superoxide-induced uncoupling, and is not the mediator of GDP effects, conclusions contrasting to predominating hypotheses concerning UCP3 function and regulation.

2. Materials and Methods

2.1. Animals

UCP1(−/−) mice (progeny of those described in [19]) were back-crossed to C57Bl/6 or to FVB/N mice for 10 generations and after intercrossing were maintained as UCP1(-/-) (UCP1(−/−)) and UCP1 (+/+) (wild-type) genotypes on a C56Bl/6 or on a FVB/N background. Routinely mice on a C57Bl/6 background were used here. Mice on the FVB/N background were used in this study only for obtaining double mutants, UCP1(−/−) and SOD2-overexpressing (UCP1(−/−)/hSOD2tg) and WT/hSOD2tg mice by crossing UCP1(-/-) and UCP1(+/+)mice on the FVB/N background with hSOD2/+ mice on the FVB/N background [20]. UCP3-overexpressing mice (UCP3-Tg) and wild-type littermates, original breeding pairs a gift from GlaxoSmithKline (Harlow, UK) [21], were also used. The mice were fed ad libitum (R70 Standard Diet, Lactamin), had free access to water, and were kept on a 12:12 h light: dark cycle, routinely at normal (24 °C) animal house temperature.

For experiments on cold-acclimated animals, adult female mice were divided into age-(7-8-week-old) and body weight (17-18 g)-matched groups, one per cage, and acclimated at 24 °C, or successively acclimated to cold by first placing them at 18 °C for 4 weeks with the following 4-6 weeks at 4 °C (the intermediate 18 °C step is required to allow for survival of the UCP1(−/−) animals at 4 °C [1]). Adult female mice age-(10-12-week-old) and body weight (22-23 g)-matched, one per cage, were used for experiments on short-term cold exposure. The experiments were approved by the Animal Ethics Committee of the North Stockholm region.

The body temperature of the mice was measured with a rectal probe for mice (RET-3) plugged to amplifier BAT-12 (Physiterm Instruments Inc., NJ, USA).

2.2. Skeletal muscle collection and mitochondrial isolation

Mice were anaesthetised for 1 min by a mixture of 79 % CO_2 and 21 % O_2 and decapitated. Combined skeletal muscles from the hind limbs of one mouse were placed into ice-cold medium containing 100 mM sucrose, 50 mM KCl, 20 mM K-TES, 1 mM EDTA and 0.1 % (w/v) fatty-acid-free bovine serum albumin and were freed of white fat and connective tissue, weighed and used for isolation of skeletal muscle mitochondria. The wet weights of the combined skeletal muscles from the hind limbs were not different between UCP1(−/−) and WT mice acclimated at any temperature. Lean body mass estimated using DEXA also indicated no signs of muscle hypertrophy in the UCP1(−/−) mice despite constant muscle activity in shivering (not shown).

The skeletal muscles were finely minced with scissors and homogenized in a Potter homogeniser with a Teflon pestle. During mincing and homogenising, the skeletal muscle fragments were treated with nagarse, added to the medium at a concentration of 1 mg per g of tissue. Throughout the isolation process, tissues were kept at 0 - 2 °C.

Mitochondria were isolated by differential centrifugation. Skeletal muscle homogenates were centrifuged at 8 500 *g* for 10 min at 2 °C using a Beckman J2-21 M centrifuge. The resulting supernatant, containing floating fat and nagarse, was discarded. The pellet was resuspended in ice-cold medium containing 100 mM sucrose, 50 mM KCl, 20 mM K-TES, 1 mM EDTA and 0.2 % (w/v) fatty-acid-free bovine serum albumin (BSA). The resuspended homogenate was centrifuged at 800 *g* for 10 min, and the resulting supernatant was centrifuged at 8 500 *g* for 10 min. The resulting mitochondrial pellet was resuspended in the same buffer (but albumin-free) and centrifuged again at 8 500 *g* for 10 min. The final mitochondrial pellets were resuspended by hand homogenisation in a small glass homogeniser in the appropriate final centrifugation medium. The concentration of mitochondrial protein was measured using fluorescamine [22] with BSA as a standard. Mitochondrial suspensions were kept on ice for no longer than 4 h during measurements of oxygen consumption and membrane potential.

2.3. Oxygen Consumption

Skeletal muscle mitochondria (0.25 mg protein/ml) were incubated in a medium consisting of 100 mM sucrose, 20 mM K^+-Tes (pH 7.2), 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 4 mM KP_i, 0.1 % fatty-acid-free BSA. The substrates were 3 mM malate plus 5 mM pyruvate or plus fatty acid-derived substrate (20 μM palmitoyl-L-carnitine or 5 mM carnitine plus 30 μM palmitoyl-CoA). Reverse electron flow was induced using 5 mM succinate in the absence of rotenone.

Oxygen consumption rates were monitored with a Clark-type oxygen electrode (Yellow Springs Instrument Co., USA) in a sealed chamber at 37 °C as described [23].

The free concentration of oleate and palmitate was calculated using the equations in [24] for the binding of fatty acid to bovine serum albumin at 37 °C: [Free oleate] (nM) = 6.5n − 0.19 + 0.13 exp (1.54n) and [Free palmitate] (nM) = 4.4n − 0.03 + 0.23 exp (1.16n), where n is the molar ratio of fatty acid to albumin. Concentration-response curve data were analysed with the general fit option of the KaleidaGraph application for Macintosh for adherence to simple Michaelis-Menten kinetics, $V(x) = \text{basal} + \Delta\text{Vmax} \cdot (x/(\text{Km} + x))$, where x is the concentration of free fatty acid.

2.4. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential measurements were performed with the dye safranin O [25]. The changes in absorbance of safranin O were followed at 37 °C in an Aminco DW-2 dual-wavelength spectrophotometer at 511 − 533 nm with a 3-nm slit. Signals were recorded every 0.5 s via a PowerLab/ADInstrument. The data were stored and analyzed using the Chart v4.1.1 program. Calibration curves were made for each mitochondrial preparation in K^+-free medium and were obtained from traces in which the extramitochondrial K^+, $[K^+]_{out}$, was altered by addition of KCl in a 0.1-20 mM final concentration range. The change in absorbance then caused by the addition of 3 μM valinomycin was plotted against $[K^+]_{out}$. The intramitochondrial K^+, $[K^+]_{in}$, was estimated by extrapolation of the line to the zero uptake point, as described in [25]. The absorbance readings were used to calculate the membrane potential (mV) by the Nernst equation according to: $\Delta\Psi_m = 61\ \text{mV} \cdot \log([K^+]_{in}/[K^+]_{out})$.