Differences in proton leak kinetics, but not in UCP3 protein content, in subsarcolemmal and intermyofibrillar skeletal muscle mitochondria from fed and fasted rats

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Abstract We have investigated the effect of 24-h fasting on basal proton leak and uncoupling protein (UCP) 3 expression at the protein level in subsarcolemmal and intermyofibrillar skeletal muscle mitochondria. In fed rats, the two mitochondrial populations displayed different proton leak, but the same protein content of UCP3. In addition, 24-h fasting, both at 24 and 29°C, induced an increase in proton leak only in subsarcolemmal mitochondria, while UCP3 content increased in both the populations. From the present data, it appears that UCP3 does not control the basal proton leak of skeletal muscle mitochondria.

Key words: Proton leak; Subsarcolemmal; Intermyofibrillar mitochondrion; Fasting; Uncoupling protein 3

1. Introduction

Oxidation of reduced substrates and synthesis of ATP in mitochondria is not completely coupled. This is due to the presence of a proton leak pathway in the inner membrane [1,2], which has been demonstrated to be present in mitochondria from all tissues studied so far, both in isolated organelles and in the whole cells [1,2]. It has also been estimated that proton leak pathway makes an important contribution to resting energy expenditure of tissues [3], with values in the range of 20–50% being reported for the liver and skeletal muscle [3]. However, the nature of proton leak in tissues other than brown adipose tissue remains ill-defined [1,2] and there is to date no convincing evidence that it changes in response to physiological manipulations of energy expenditure [4,5]. With the discovery of uncoupling protein (UCP) UCP2 and UCP3 [6,7], two homologues of UCP1, which are well known to catalyse the proton leak in brown adipose tissue mitochondria during cold-induced or diet-induced thermogenesis [8], the possibility has arisen that these UCP1 homologues could also control the mitochondrial proton leak in a variety of tissues and organs. To test this hypothesis, experiments linking physiological changes in mitochondrial proton leak and UCP homologues protein levels measured in mitochondrial fractions should be carried out. One physiological condition known to modify skeletal muscle UCP3 expression is fasting, when both mRNA and protein levels increase [9]. Recently, it has been shown that in skeletal muscle from fasted rats, an increase in UCP3 protein content was associated with no change in mitochondrial proton leak kinetics [9]. However, it is known that skeletal muscle cell contains two mitochondrial populations (subsarcolemmal and intermyofibrillar mitochondria), which differ in their location, function, and response to environmental stimuli [10–13]. We have previously suggested a specific role of subsarcolemmal mitochondria in the regulation of cellular-energy coupling [14]. Therefore, it is possible that the above mitochondrial subpopulations have different proton leak and UCP3 levels as well as different responses to fasting. To test this hypothesis, we evaluated the effect of 24-h fasting on the kinetics of proton leak of the two mitochondrial populations and correlated the above kinetics with changes in their UCP3 protein content.

2. Materials and methods

2.1. Animals

Male Wistar rats (about 170 g) were acclimated at 24 or 29°C for 14 days before the sacrifice. Then, they were killed after 24-h fasting or without any previous food deprivation. Treatment, housing, and killing met the guidelines of Italian Health Ministry.

Hind-leg muscles were freed of excess fat and connective tissue, finely minced and washed in a medium containing 100 mM KCl, 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA, 0.1% (w/v) fatty acid-free bovine serum albumin (BSA), pH 7.5. Tissue fragments were then divided in two aliquots and used for the preparation of total mitochondria or intermyofibrillar+subsarcolemmal mitochondria. Immediately after isolation, an aliquot of mitochondria was stored at −80°C for assay of UCP3, while the remainder was used for measurement of proton leak.

2.2. Preparation of total mitochondria

Tissue fragments were treated with protease Nagarse (E.C. 3.4.21.62; 9 U/g tissue) for 5 min, washed, homogenised with the above medium (1:8, w/v) in a Potter Elvehjem homogeniser (Heidolph, Kelheim, Germany) set at 500 rpm (4 strokes/min) and filtered. Homogenate was then centrifuged at $3000 \times g_{av}$ for 10 min, the supernatant was rapidly discarded and the precipitate was centrifuged at $500 \times g_{av}$ for 10 min. The pellet, containing total mitochondria, was washed once and resuspended in suspension medium containing 250 mM sucrose, 50 mM Tris, 0.1% fatty acid-free BSA, pH 7.5.

2.3. Preparation of skeletal muscle intermyofibrillar and subsarcolemmal mitochondria

Tissue fragments obtained as described above were homogenised

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with the above medium (1:8, w/v) as before. Homogenate was then centrifuged at $500\times g_{av}$ for 10 min and the resulting precipitate was subsequently used for the preparation of the intermyofibrillar mitochondria. The supernatant was centrifuged at $3000\times g_{av}$ for 10 min and the resulting pellet, containing subsarcolemmal mitochondria, was washed twice and resuspended in suspension medium. The pellet from the $500\times g_{av}$ centrifugation was resuspended in a small amount of homogenisation solution and treated with protease Nagarse (9 U/g tissue) for 5 min. The suspension was then homogenised, filtered through sterile gauze and centrifuged at $3000\times g_{av}$ for 10 min. The resulting supernatant was rapidly discarded and the pellet was resuspended and centrifuged at $500\times g_{av}$ for 10 min. The supernatant containing the intermyofibrillar mitochondria was centrifuged at $3000\times g_{av}$ for 10 min, the pellet was washed once and resuspended in suspension medium.

2.4. Measurements of mitochondrial respiration and membrane potential

Titration of state 4 respiration was carried out by sequential additions of malonate up to 5 mM in a medium containing 30 mM LiCl, 6 mM $MgCl_2$, 75 mM sucrose, 1 mM EDTA, 20 mM Tris–PO_4, 0.1% (w/v) fatty acid-free BSA, pH 7.0, in the presence of succinate (10 mM), rotenone (3.75 μM), oligomycin (2 μg/ml), safranin O (83.3 nmol/mg) and nigericin (80 ng/ml, to collapse the pH difference across the mitochondrial inner membrane and allow the whole of the proton-motive force to be represented by mitochondrial membrane potential). Mitochondrial membrane potential recordings were performed in parallel with safranin O using a JASCO dual-wavelength spectrophotometer (511–533 nm) [15]. The absorbance readings were transferred to mV membrane potential using the Nernst equation: $\Delta\psi = 61$ mV·log ($[K^+]_{in}/[K^+]_{out}$). Calibration curves made for each preparation were obtained from traces in which the extramitochondrial K^+ level ($[K^+]_{out}$) was altered in the 0.1–20 mM range. The change in absorbance caused by the addition of 3 μM valinomycin was plotted against $[K^+]_{out}$. $[K^+]_{in}$ was then estimated by extrapolation of the line to the zero-uptake point.

2.5. Measurement of UCP3 protein content in isolated mitochondria

40 μg of mitochondria were denatured in 2× loading buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol) and loaded onto a 12% SDS–polyacrylamide gel together with a prestained protein marker (Benchmark, Life Technologies, Basel, Switzerland). After a 2-h run, these gels were transferred onto polyvinylidene fluoride membranes (Immobilon-P, Millipore, Schwerzenbach, Switzerland) at 0.8 mA/cm^2 for 90 min. The membranes were preblocked in blocking buffer (1×PBS/5% milk powder/0.5% Tween 20) for 30 min and then incubated overnight at 4°C with the UCP3 antibody (Chemicon #3046, Ruschlikon, Switzerland) diluted in the above buffer at 1 μg/ml; validation of the specificity of the antibody #3046 for UCP3 was previously reported [6]. Membranes were washed 3× 15 min in blocking buffer and then incubated for 2 h at room temperature with a secondary antibody labelled with alkaline phosphatase. After three 10 min rinses with blocking buffer, the membrane was drained of any residual liquid and a substrate (Western blue, Promega, Madison, USA) for alkaline phosphatase was added. Quantification of the results was obtained using scans of the membranes imported into the UN-SCAN-IT gel computer programme (Silk Scientific, Utah, TX, USA).

3. Results and discussion

In control rats acclimated to 24 or 29°C, UCP3 protein content was not significantly different in the two subpopulations, while subsarcolemmal mitochondria exhibited markedly lower basal proton leak kinetics than intermyofibrillar ones (Fig. 1). This result cannot be explained by differential damage of the inner membrane, since NADH oxidation by the different subpopulations of mitochondria was the same (about 10%). Control experiments, doubling the BSA amount used in the leak measurements, gave the same results, thereby suggesting no differential contamination by fatty acids. The above results show for the first time that mitochondria located in

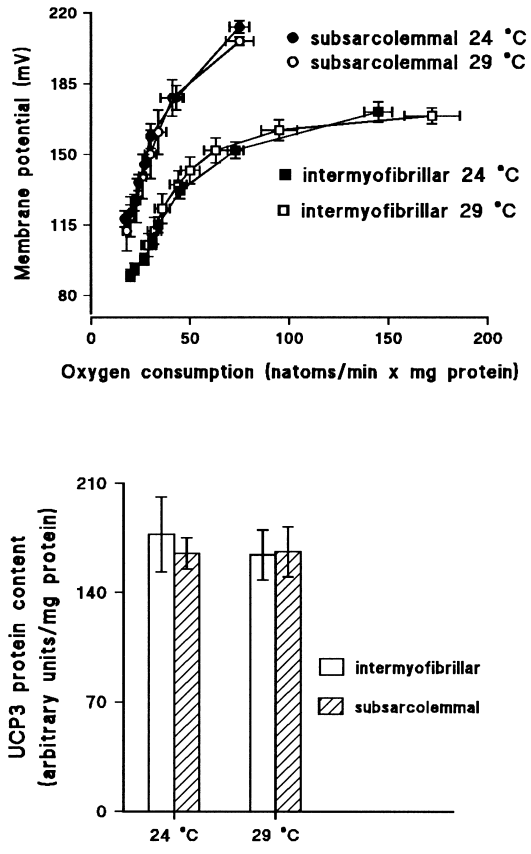

Fig. 1. Proton leak kinetics and UCP3 protein content in subsarcolemmal and intermyofibrillar skeletal muscle mitochondria from fed rats acclimated at 24 or 29°C. Data are means ± S.E.M. of four (29°C) or eight (24°C) experiments for proton leak, and the means ± S.E.M. of four (29°C) or three (24°C) experiments for UCP3 measurements.

different parts of skeletal muscle cells have different proton leak kinetics, and also that this difference cannot be attributed to UCP3. Inner membrane proton permeability adds to other functional parameters, which are different between the two subpopulations [10–14], while UCP3 content does not. The difference in proton permeability could be due to variations in the area and/or in phospholipid composition of the inner membrane. Subsarcolemmal mitochondria exhibit higher cardiolipin content than intermyofibrillar ones [11], while the area of the inner membranes is similar between the two subpopulations [16].

Fig. 2 shows that at 24°C, UCP3 protein levels significantly increase both in total mitochondria and in the two subpopulations after 24-h fasting. On the other hand, basal proton leak kinetics were unchanged in total and intermyofibrillar mitochondria, but increased in subsarcolemmal ones after fasting. The same results were obtained when fed and fasted rats acclimated at 29°C were compared (Fig. 3). Again, doubling BSA in the incubation medium had no effect. Our results from fasted rats strengthen the above conclusion that UCP3 does not catalyse basal proton conductance. This conclusion is in contrast with that of Gong et al. [17], who found decreased skeletal muscle mitochondrial proton leak from UCP3 knockout mice and claimed that UCP3 contributes to basal proton conductance. However, it cannot be excluded that complete removal of a protein from the inner

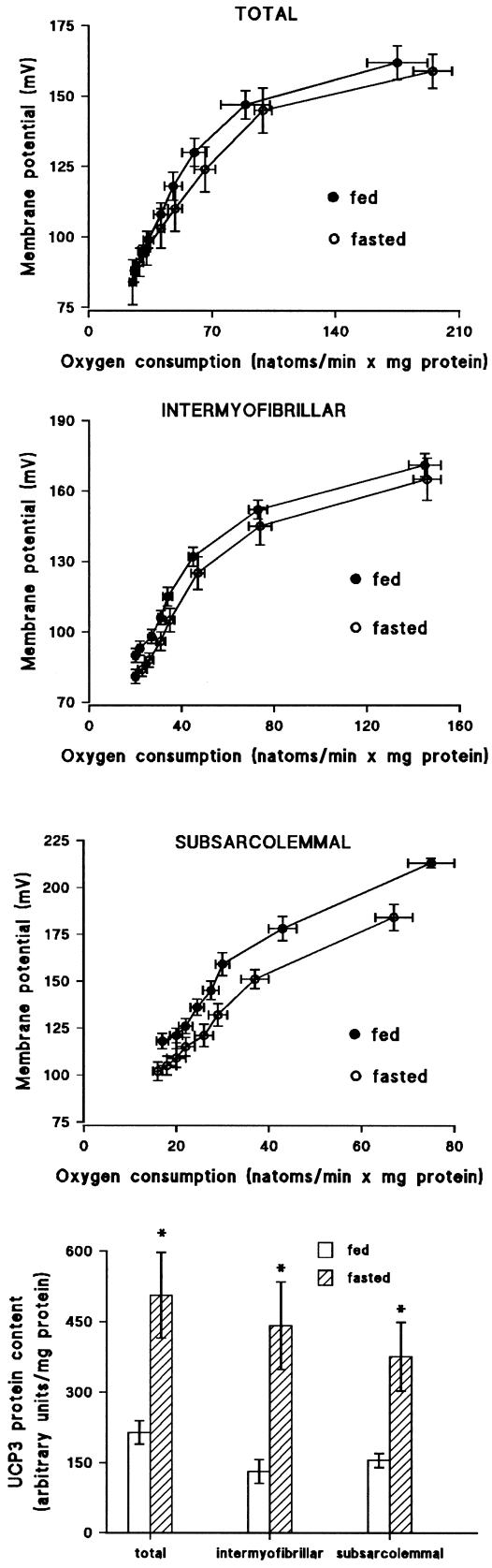

Fig. 2. Proton leak kinetics and UCP3 protein content in total, subsarcolemmal and intermyofibrillar mitochondria from fed and 24-h-fasted rats acclimated at 24°C. Data are means ± S.E.M. of eight (proton leak) or three (UCP3) experiments. *$P < 0.05$ compared to fed rats (Student's *t*-test).

membrane could alter its properties and therefore proton conductance.

The increased leak found in subsarcolemmal mitochondria from fasted rats at 24 and 29°C could have a role in protecting from reactive oxygen species (ROS) production. In fact, during fasting, skeletal muscle energy consumption decreases [18], due to lower ATP needs. Therefore, such a condition characterised by increased availability of fatty acid substrate with reduced ATP consumption could enhance the formation of ROS and oxidative damage in the cell [19]. Mild uncoupling by means of increased proton leak would counter this and avoid excess ROS production [20,21]. The different leak

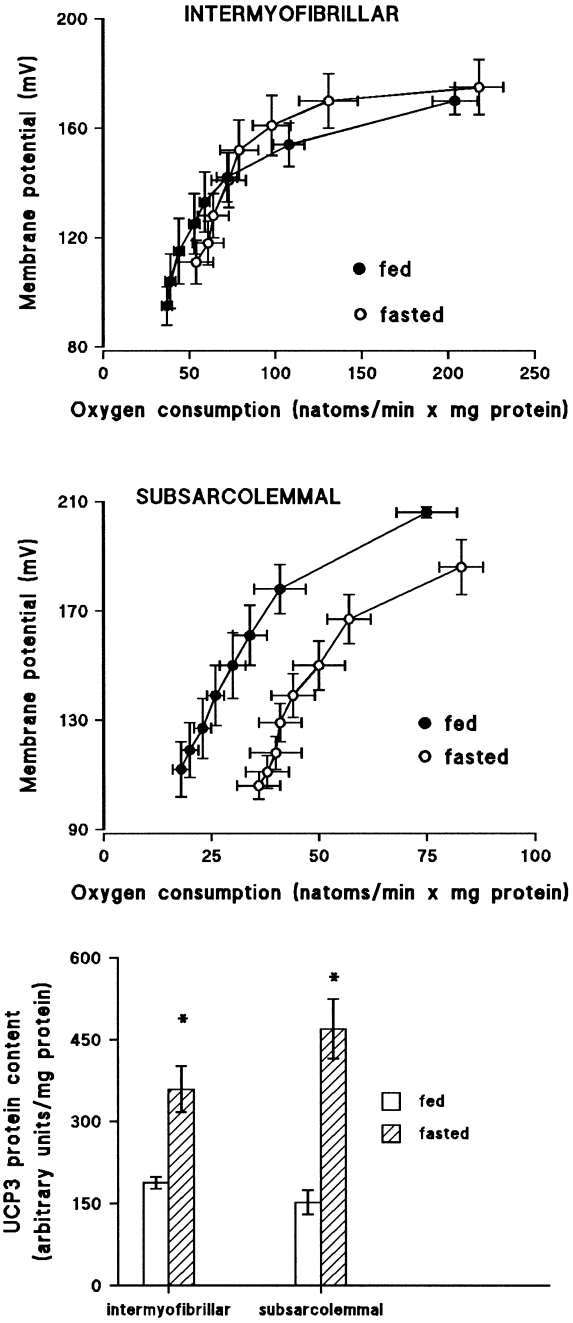

Fig. 3. Proton leak kinetics and UCP3 protein content in subsarcolemmal and intermyofibrillar mitochondria from fed and 24-h-fasted rats acclimated at 29°C. Data are means ± S.E.M. of four experiments. *$P < 0.05$ compared to fed rats (Student's *t*-test).

response to fasting in the two mitochondrial populations could be explained on the basis of their localisation. In fact, intermyofibrillar mitochondria could satisfy contractile element ATP requirements [11,12], which are not affected by fasting [22], while subsarcolemmal mitochondria could supply ATP to cytoplasmic reactions, such as protein synthesis and ATPase pumps [11,12], which are downregulated during fasting [23–25]. Therefore, increased ROS production during fasting should be more marked in subsarcolemmal mitochondria, which adapt by increasing proton leak. Finally, we cannot exclude that heat produced by increased leak could also try to compensate, at least in part, the decreased heat production of fasted rats. In fact, we have previously found a decrease in resting energy expenditure after 24-h fasting, which is partly due to decreased serum levels of free triiodothyronine (T_3), since it is absent in hypo- and hyperthyroid rats [26,27]. Therefore, increased leak could replace some of the T_3-mediated obligate thermogenesis, which decreases after 24-h fasting.

In conclusion, from our results it appears that the basal proton leak, which exists in isolated and 'in situ' skeletal muscle mitochondria [28,29] and is considered a contributor to the standard metabolic rate in mammals [29], is not under the control of UCP3. As shown here, mitochondria with different leak in the same cell have the same amount of UCP3, and the increase in UCP3 with fasting is associated with no variation or an increase in leak, depending on the mitochondrial population. It should be underlined, however, that what was measured in our experiments is basal proton leak, i.e. the inner membrane proton conductance, which can be evidenced also in mitochondria from tissues not expressing UCP3. Therefore, the possibility remains that UCP3 could contribute to inducible leak pathways, such as the uncoupling effect of fatty acids that involves also other mitochondrial proteins (ATP/ADP antiporter, glutamate/aspartate antiporter and probably UCP2) [30–32]. Accordingly, it has recently been shown that after 24-h exposure of rats to cold (5°C), an increase of the in vivo uncoupling of skeletal muscle mitochondria parallels the three-fold increase in the UCP3 protein level [32].

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