

Skeletal muscle mitochondrial oxidative capacity and uncoupling protein 3 are differently influenced by semistarvation and refeeding

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Abstract We investigated, in skeletal muscle mitochondria isolated from semistarved and refed rats, the relation between the protein expression of uncoupling protein 3 (UCP3) and mitochondrial oxidative capacity, assessed as state 4 and state 3 respiration rates in presence of substrates that are either non-lipids (glutamate, succinate) or lipids (palmitoyl CoA, palmitoylcarnitine). During semistarvation, when whole-body thermogenesis is diminished, state 3 respiration was lower than in fed controls by about 30% independently of substrate types, while state 4 respiration was lower by 20% only during succinate oxidation, but UCP3 was unaltered. After 5 days of refeeding, when thermogenesis is still diminished, neither state 4, state 3 nor UCP3 were lower than in controls. Refeeding on a high-fat diet, which exacerbates the suppression of thermogenesis, resulted in a two-fold elevation in UCP3 but no change in state 4 or state 3 respiration. These results during semistarvation and refeeding, in line with those previously reported for fasting, are not in support of the hypothesis that UCP3 is a mediator of adaptive thermogenesis pertaining to weight regulation, and underscore the need for caution in interpreting parallel changes in UCP3 and mitochondrial oxidative capacity as the reflection of mitochondrial uncoupling by UCP3.

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1. Introduction

Uncoupling protein 3 (UCP3), a member of the family of mitochondrial carrier proteins, is predominantly expressed in skeletal muscle [1]. It was identified in 1997 on the basis of its close sequence homology to UCP1, the uncoupling protein that mediates thermogenesis in brown adipose tissue by abolishing the proton gradient needed to drive adenosine triphosphate (ATP) synthesis. There is, however, considerable uncer-

tainty and debate about whether UCP3 has physiologically relevant uncoupling properties, amid proposals that it might play an important role in the regulation of thermogenesis [2], in the regulation of lipids as fuel substrate [3], in controlling the export of fatty acids out of the mitochondria during rapid fatty acid oxidation [4], and/or in controlling the production of reactive oxygen species [5,6]. First, its overexpression in cell culture systems [7,8] and in mouse skeletal muscle [9] alters mitochondrial membrane potential in a direction that is consistent with uncoupling properties, but it has been argued that this uncoupling is the expression of artifacts arising from the disruption of mitochondrial membrane integrity [10]. Second, an uncoupling role for UCP3 has also been proposed on the basis that fasting, which increases UCP3 expression in muscle, decreased its capacity to produce heat in response to the uncoupler FCCP, i.e. carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone [11]. This hypothesis of a UCP3-mediated state of partial uncoupling in fasted muscle is, however, at odds with the findings that: (i) fasting-induced upregulation of UCP3 is not associated with an increase in basal proton conductance in isolated muscle mitochondria [12,13], and (ii) thermogenesis is reduced both at the whole-body level and in hind-leg muscle of the rat during fasting [14].

A physiological role for UCP3 in mitochondrial energy coupling is nonetheless supported by a number of studies in laboratory rodents and in humans, in which skeletal muscle mitochondrial oxidative capacity (MOC) was assessed during respiration in presence of ADP (state 3) and/or during state 4 (non-phosphorylating) respiration. In particular, the demonstration that state 4 respiration (which may reflect proton leak) is lower in mitochondria from skeletal muscle of mice lacking UCP3 [15] has led to the proposal that UCP3, expressed at physiological levels in muscle, has proton transport activity and consequently might function as a genuine uncoupling protein. In line with this proposal are the findings of increased state 4 and state 3 respiration, as well as UCP3, in mitochondria isolated from skeletal muscle of overfed rats resisting obesity by enhanced diet-induced thermogenesis [16,17]. Furthermore, it has been reported that during weight stabilisation following a hypocaloric slimming therapy, both state 4 mitochondrial respiration and UCP3 mRNA levels were higher in skeletal muscle from patients with the greatest weight losses than those with the least weight losses [18]. Taken together, these studies in mice, rats and humans raise the possibility that the functional role of UCP3 as an uncoupling protein might be reflected in changes in MOC, i.e. in mito-

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Abbreviations: UCP3, uncoupling protein 3; MOC, mitochondrial oxidative capacity; ATP, adenosine triphosphate; ADP, adenosine diphosphate

chondrial state 4 and/or in state 3 respiration, and the state of fasting might be the exception to the rule, for reasons that are not presently understood.

To test this hypothesis, we have investigated the extent to which this association between UCP3 and MOC in skeletal muscle mitochondria persists under conditions of dietary manipulations other than fasting and overfeeding, namely in response to semistarvation, refeeding on a low-fat diet or to refeeding on a high-fat diet rich in lard, all of which (like fasting) are nutritional states characterised by a reduction in whole-body thermogenesis [3,19–22]. In the assessment of skeletal muscle MOC, we have utilised four types of substrates, the oxidation of each of which reflects the activity of specific enzyme systems and complexes of the mitochondrial electron transport chain, namely:

1. *glutamate* – an NADH-linked substrate which enters through complex I of the respiratory chain;
2. *Succinate* – an FAD-linked substrate which is used to specifically test the tricarboxylic acid cycle and mitochondrial complexes II–IV of the electron transport chain while complex I is inhibited by rotenone;
3. *palmitoyl CoA* – a lipid substrate whose oxidation reflects the activity of carnitine palmitoyltransferase (CPT) I and the intramitochondrial β -oxidation pathway; and
4. *palmitoylcarnitine* – a lipid substrate which bypasses the step catalysed by CPT I and represents an index of fatty acid oxidation per se, and is hence used to test β -oxidation pathway and all complexes of the electron transport chain.

2. Materials and methods

2.1. Animals and diets

Male Sprague Dawley rats, aged 6 weeks, were adapted to room and cage environments for at least 5 days prior to the start of each experiment; they were caged singly in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) with a 12-h light/dark cycle. They were maintained on a commercial pelleted chow diet (Kliba, Cossonay, Switzerland) consisting, by energy, of 24% protein, 66% carbohydrates, and 10% fat, and had free access to tap water. The experiments were conducted after this period of adaptation in rats selected on the basis of body weight being within ± 5 g of the mean body weight (200 g). Animals used in the present studies were maintained in accordance with our institute's regulations and guide for the care and use of laboratory animals.

2.2. Experimental designs

In a first experiment, two groups of rats ($n=6-8$) were either fed ad libitum on a pelleted chow diet or food restricted on a daily basis for 2 weeks at approximately 50% of the chow intake of ad libitum fed rats, i.e. they were semistarved so as to arrest growth – a condition that leads to diminished energy expenditure due to suppressed thermogenesis, as previously reported [19,20]. At the end of the semistarvation period, all semistarved rats as well as their ad libitum fed controls were killed by decapitation, and skeletal muscle dissected out and processed for the isolation of mitochondria. In a second experiment, rats ($n=6-8$) were semistarved, as described above, and subsequently refed the chow diet at a level approximately equal in metabolisable energy content to the spontaneous food intake of rats matched for weight at the onset of refeeding. The refed group therefore consumed, on a day-to-day basis, the same amount of food energy as their weight-matched controls fed ad libitum. The refeeding experiments performed here utilised a design similar to that previously described in establishing a rat model for studying adjustments in energy expenditure which, via suppressed thermogenesis, favours fat deposition during refeeding [3,21]. At the end of 5 days of controlled refeeding, animals in refed and control groups were killed by decapitation, and skeletal muscle dissected out and processed for the isolation of mitochondria. In a third experiment, two groups of rats ($n=6$) were semistarved, as described above. They were then refed

at the same level of energy intake found previously for the control group above, either a low-fat diet or an isocaloric amount of a high-fat diet with lard providing about 50% of metabolisable energy content of the diet; details of diet preparation and composition have been reported previously [22]. Under these conditions, the high-fat refed group shows significantly lower energy expenditure and thermogenesis (even after adjusting for differential cost of fat synthesis on the two diets) and greater body fat gain than the low-fat refed group [22,23]. At the end of 5 days of controlled refeeding, animals in both refed groups were killed by decapitation, and skeletal muscle dissected out and processed for the isolation of mitochondria.

2.3. Isolation of skeletal muscle mitochondria

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, pH 7.5, 5 mM MgCl_2 , 1 mM EDTA, 5 mM EGTA, 0.1% (w/v) fatty acid-free bovine serum albumin (BSA). Tissue fragments were treated with protease nagarse (EC 3.4.21.14; 8.3 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 through sterile gauze. Homogenate was centrifuged at $3000 \times g$ for 10 min, the precipitate was resuspended and subsequently centrifuged at $500 \times g$ for 10 min. The supernatant was then centrifuged at $3000 \times g$ for 10 min and the resulting pellet containing mitochondria was washed twice and gently resuspended in a medium containing 250 mM sucrose, 50 mM Tris, pH 7.5, 0.1% (w/v) fatty acid-free BSA. Aliquots of isolated mitochondria were then used for measurements of respiration rate, total protein content and UCP3 assay.

2.4. Measurement of mitochondrial respiration

Oxygen consumption of mitochondria was measured polarographically with a Clark-type electrode (Dual digital model 20, Rank Brothers, Cambridge, UK) with the chambers maintained at 30°C and magnetically stirred, using the same protocol as detailed by Iossa et al. [16]. Mitochondria (about 0.5 mg protein/ml) were incubated in a medium containing 30 mM KCl, 6 mM MgCl_2 , 75 mM sucrose, 1 mM EDTA, 20 mM KH_2PO_4 , pH 7.0, 0.1% (w/v) fatty acid-free BSA. Substrates used were as follows: (i) glutamate 10 mM+malate 2.5 mM, (ii) succinate 10 mM+rotenone 3.75 μM , (iii) palmitoyl CoA 40 μM +carnitine 2 mM+malate 2.5 mM and (iv) palmitoyl-L-carnitine 40 μM +malate 2.5 mM. State 4 respiration was measured in the absence of ADP, while state 3 respiration measurements were performed in presence of 0.6 mM ADP. Mitochondrial protein concentration was determined by a modification of the Lowry method that gives a linear photometric response [24]. Data on oxygen consumption are expressed as $\text{nmols O min}^{-1} \text{mg protein}^{-1}$.

2.5. Western blot analysis of UCP3

Mitochondrial protein (40 μg) was denatured in Sample buffer (60.0 mM Tris pH 6.8, 10% saccharose, 2% sodium dodecyl sulphate (SDS), 4% β -mercaptoethanol) and loaded onto a 12% SDS–polyacrylamide gel together with a prestained protein marker (Benchmark, Life Technologies). After a 2-h run in electrode buffer (50 mM Tris pH 8.3, 384 mM glycine, 0.1% SDS) gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) at 0.8 mA/cm^2 for 90 min. The membranes were preblocked in blocking buffer (1 \times PBS; 5% milk powder; 0.5% Tween 20) for 1 h and then incubated overnight at 4°C with UCP3 polyclonal antibodies (Chemicon) diluted 1:3000 in blocking buffer; the specificity of the antibody for UCP3 has been validated [12]. Membranes were washed (3 \times 15 min) in 1 \times PBS/0.5% Tween 20, 3 \times 15 min in 1 \times PBS and then incubated for 1 h at room temperature with alkaline phosphatase-labelled antibodies (Promega). The membranes were washed as described above, rinsed in distilled water and incubated at room temperature for 30 min with a 1:500 dilution of a chemiluminescence substrate (CDP star, Roche). Signals were detected by exposing autoradiography films (Kodak) to the membranes, acquired by densitometry (Bio-Rad, model GS-700 Imaging Densitometer) and quantified using Molecular Analyst Software (Bio-Rad).

2.6. Chemicals

All chemicals utilised were of analytical grade and, except where indicated, were purchased from Fluka (Buchs, Switzerland) or from Sigma (St. Louis, MO, USA).

Table 1

Respiration rate in skeletal muscle mitochondria from rats fed ad libitum or semistarved for 2 weeks

		Ad lib fed controls	Semistarved	<i>t</i> -test
Glutamate (10 mM)+malate (2.5 mM)	State 3	484 ± 76	337 ± 48	<i>P</i> = 0.06
	State 4	49 ± 6	40 ± 3	NS
	RCR ^a	10 ± 2	9 ± 2	
Succinate (10 mM)+rotenone (3.75 μM)	State 3	507 ± 34	367 ± 40	<i>P</i> ≤ 0.05
	State 4	127 ± 6	104 ± 7	<i>P</i> ≤ 0.05
	RCR	4.0 ± 0.2	3.5 ± 0.2	
Palmitoyl CoA (40 μM)+carnitine (2 mM)+malate (2.5 mM)	State 3	168 ± 14	120 ± 9	<i>P</i> ≤ 0.05
	State 4	34 ± 1	36 ± 4	NS
	RCR	4.9 ± 0.4	3.3 ± 0.6	
Palmitoylcarnitine(40 μM)+malate (2.5 mM)	State 3	125 ± 9	84 ± 8	<i>P</i> ≤ 0.01
	State 4	37 ± 3	37 ± 3	NS
	RCR	3.4 ± 0.2	2.2 ± 0.3	
UCP3 (arbitrary units)		3.88 ± 1.0	4.17 ± 0.5	NS

All values are mean ± S.E.M. (*n* = 6–8). State 3 and state 4 respirations are expressed as natoms O min⁻¹ mg protein⁻¹.^aRCR is respiratory control ratio (i.e. ratio of state 3 to state 4 respiration).

2.7. Statistical analysis

Data are provided as means ± S.E.M. Statistical analyses were performed using two-tailed unpaired Student's *t*-test, using the computer software STATISTIK, version 4.0 (Analytical Software, St. Paul, MN, USA).

3. Results and discussion

Using a previously described rat model of semistarvation and controlled refeeding, characterised by sustained reductions in whole-body thermogenesis [3,19–23], we now show that after 2 weeks of semistarvation, skeletal muscle mitochondrial state 3 respiration rate is lower than in fed controls by about 30%, irrespective of the substrates utilised (Table 1). These differences in respiration in the presence of ADP are statistically significant for the two lipid substrates and for succinate, and close to achieving statistical significance (*P* = 0.06) for glutamate. State 4 (non-phosphorylating) respiration rate is also significantly lower in mitochondria from

semistarved animals during succinate oxidation (−20%, *P* ≤ 0.05).

What these reductions in muscle MOC, assessed as state 4 or state 3 respiration, signify is not clear. Although the diminished state 4 respiration during succinate oxidation in muscle mitochondria from semistarved animals might reflect increased mitochondrial energy coupling [25] and hence contribute to their diminished thermogenesis during semistarvation, UCP3 in their mitochondria was not lower than in controls. After 5 days of controlled refeeding – a time-point at which diminished thermogenesis has been shown to persist [3,21] – no difference is observed between mitochondria from muscles in refed and control animals in UCP3 nor in MOC, whether assessed during state 3 or state 4 respiration, and irrespective of the substrate being oxidised (Table 2), the notable exception being a significant increase in state 3 respiration during glutamate oxidation. By contrast, after 5 days of refeeding on a diet high in saturated fat, which was previously shown to exacerbate the suppressed thermogenesis found during refeed-

Table 2

Respiration rate in skeletal muscle mitochondria from rats fed ad libitum and rats refed on chow (low-fat) diet for 5 days after semistarvation for 2 weeks

		Ad lib fed controls	Refed	<i>t</i> -test
Glutamate (10 mM)+malate (2.5 mM)	State 3	547 ± 24	656 ± 38	<i>P</i> ≤ 0.05
	State 4	55 ± 6	56 ± 3	NS
	RCR ^a	10 ± 1	12 ± 2	
Succinate (10 mM)+rotenone (3.75 μM)	State 3	784 ± 57	830 ± 52	NS
	State 4	154 ± 16	172 ± 6	NS
	RCR	5.1 ± 0.4	4.8 ± 0.4	
Palmitoyl CoA (40 μM)+carnitine (2 mM)+malate (2.5 mM)	State 3	209 ± 17	220 ± 17	NS
	State 4	41 ± 2	47 ± 3	NS
	RCR	5.1 ± 0.3	4.7 ± 0.4	
Palmitoylcarnitine(40 μM)+malate (2.5 mM)	State 3	216 ± 31	220 ± 24	NS
	State 4	38 ± 4	45 ± 1	NS
	RCR	6.5 ± 1.5	4.8 ± 0.5	
UCP3 (arbitrary units)		3.33 ± 0.7	2.72 ± 0.8	NS

All values are mean ± S.E.M. (*n* = 6–8). State 3 and state 4 respirations are expressed as natoms O min⁻¹ mg protein⁻¹.^aRCR is respiratory control ratio (i.e. ratio of state 3 to state 4 respiration).

Table 3

Respiration rate in skeletal muscle mitochondria from rats refed isocaloric amounts of a low-fat and high-fat diet for 5 days after semistarvation for 2 weeks

	Refed low-fat	Refed high-fat	<i>t</i> -test
Glutamate (10 mM)+malate (2.5 mM)			
State 3	576 ± 57	534 ± 54	NS
State 4	39 ± 7	39 ± 10	NS
RCR ^a	17 ± 5	19 ± 9	
Succinate (10 mM)+rotenone (3.75 μM)			
State 3	697 ± 44	671 ± 71	NS
State 4	139 ± 19	109 ± 16	NS
RCR	4.9 ± 0.7	5.5 ± 0.4	
Palmitoyl CoA (40 μM)+carnitine (2 mM)+malate (2.5 mM)			
State 3	228 ± 22	229 ± 7	NS
State 4	27 ± 5	33 ± 5	NS
RCR	9 ± 1	6 ± 1	
Palmitoylcarnitine(40 μM)+malate (2.5 mM)			
State 3	277 ± 36	265 ± 4	NS
State 4	34 ± 5	28 ± 4	NS
RCR	9 ± 1	12 ± 4	
UCP3 (arbitrary units)	1.15 ± 0.2	2.43 ± 0.3	<i>P</i> ≤ 0.02

All values are mean ± S.E.M. (*n* = 6). State 3 and state 4 respirations are expressed as natoms O min⁻¹ mg protein⁻¹.

^aRCR is respiratory control ratio (i.e. ratio of state 3 to state 4 respiration).

ing on low-fat diets [22,23], UCP3 is significantly elevated (+two-fold, *P* ≤ 0.02), but neither state 3 nor state 4 respiration is significantly altered, irrespective of the substrates being oxidised (Table 3).

It might be argued that a two-fold increase in UCP3 during high-fat refeeding may not be high enough to induce a measurable increase in state 4 respiration, which under our *in vitro* conditions might be limited by the availability of fatty acids which have been shown to be required for UCP3-mediated uncoupling [26]. In fact, in the latter study, a role for UCP3 in thermoregulatory uncoupling has been proposed on the basis that 24 h cold exposure results in a three-fold elevation in UCP3 protein in rat skeletal muscle mitochondria and a pronounced lowering of the membrane potential in isolated rat or mouse skeletal muscle mitochondria [26]. How can these results, linking UCP3 upregulation and thermoregulatory uncoupling, be reconciled with our refeeding data here indicating that an increase in UCP3 (even if it were to be reflected in an increase in state 4 respiration in the presence of greater fatty-acid availability) is occurring in a state of suppressed thermogenesis. One explanation might reside in the proposal by Skulachev [5] that mitochondria possess a mechanism called 'mild uncoupling' which serves to reduce the production of reactive oxygen species. The possibility arises that the functional significance of UCP3 upregulation during high-fat refeeding is primarily to limit the production of fatty-acid-related reactive oxygen species, and that the associated increase in heat production resulting from such mild uncoupling is masked by other (unknown) mechanisms that suppressed thermogenesis during high-fat refeeding. In response to severe cold exposure, however, the recruitment of UCP3-induced uncoupling [26] would assume greater importance for thermoregulatory thermogenesis. In other words, the extent to which the uncoupling function of UCP3 is recruited might differ according to whether the stimulus is from cold exposure or from dietary manipulation.

Within the context of the current debate about whether UCP3 is a physiologically relevant uncoupling protein for adaptive thermogenesis pertaining to weight regulation, the earlier demonstrations – from studies in UCP3 KO mice [15] and in overfed rats resisting obesity [16,17] – that changes

in UCP3 content correlate positively with changes in MOC *in vitro*, would tend to favour the notion that UCP3 might have genuine uncoupling properties. However, as our studies here reveal, these associations do not extend to other dietary manipulations, since in response to semistarvation, skeletal muscle MOC is reduced without any change in protein expression of UCP3, and in response to refeeding on a high-fat diet, UCP3 is elevated but not MOC. This is in sharp contrast, using exactly the same experimental protocols, to the association observed between a similarly two-fold elevation in UCP3 and increased state 4 (and/or state 3 respiration) during glutamate or palmitoyl CoA oxidation in skeletal muscle mitochondria from young rats resisting obesity when overfed on a high-fat diet [16,17].

Taken together, our present results, revealing an overall poor correlation between skeletal muscle MOC and UCP3 in response to semistarvation and refeeding, are in line with the lack of association between UCP3 and basal proton conductance in isolated muscle mitochondria in response to fasting [12,13], and suggest that either UCP3 has no physiological role in the regulation of mitochondrial energy coupling or that MOC *in vitro* does not reflect the extent to which mitochondria are coupled *in vivo*. In addition, it can always be argued that changes (or lack of them) in the expression of UCP3 protein do not reflect changes in the activity of the protein. To date, therefore, the most direct evidence that UCP3 has physiologically relevant uncoupling properties rests a priori upon the report that state 4 respiration – which may (or may not) reflect basal proton leak respiration – is lower in UCP3-knockout mice. The implications of these findings, however, have been questioned following the failure of two other groups to confirm these same observations in another UCP3-knockout mouse model [27,28]. Whatever the explanation(s) for these discrepancies or for the physiological significance of changes in UCP3 (gene vs. protein vs. activity) or in MOC (*in vitro* vs. *in vivo*), the current findings of a lack of correlation between UCP3 protein expression and MOC in response to dietary manipulations that alters adaptive thermogenesis considerably weaken the plausibility of a role for UCP3 in mediating adaptive changes in thermogenesis pertaining to weight regulation. They also underscore the need

for caution in interpreting parallel changes in UCP3 and in state 4 respiration, or in MOC in general, as evidence that UCP3 has physiologically relevant mitochondrial uncoupling properties.

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