UCP2 and UCP3 rise in starved rat skeletal muscle but mitochondrial proton conductance is unchanged

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Abstract The relationship between UCP2 and UCP3 expression and mitochondrial proton conductance of rat skeletal muscle was examined. Rats were starved for 24 h and the levels of UCP2 and UCP3 mRNA and UCP3 protein were determined by Northern and Western blots. Proton conductance was measured by titrating mitochondrial respiration rate and membrane potential with malonate. Starvation increased UCP2 and UCP3 mRNA levels more than 5-fold and 4-fold, respectively, and UCP3 protein levels by 2-fold. However, proton conductance remained unchanged. These results suggest either that Northern and Western blots do not reflect the levels of active protein or that these UCPs do not catalyse the basal proton conductance in skeletal muscle mitochondria.

Key words: Uncoupling protein; UCP2; UCP3; Fasting; Proton leak; Skeletal muscle

1. Introduction

Protons ejected from mitochondria during cellular substrate oxidation return through the ATP synthase, driving ATP synthesis. However, some of the energy is dissipated as protons return through natural leak pathways [1–3]. Proton leak in rat skeletal muscle causes 10–15% of basal metabolic rate [2–4], making it an attractive target for modulation of body mass, as demonstrated by the successful treatment of obesity in the 1930s using artificial uncoupling by dinitrophenol [5]. The natural proton leak pathway is undefined, but there are proposals that it is catalysed by uncoupling proteins: UCP1 in adipose, UCP2 in several tissues, UCP3 in muscle and BMCP1 and UCP4 in brain [6–12]. There is excellent evidence that UCP1 uncouples mitochondria in brown adipose tissue, resulting in facultative thermogenesis [6]. The evidence that other UCPs uncouple relies mostly on their strong sequence homology to UCP1; experimental evidence comes only from expression of exogenous genes in yeast or mammalian cells [7–14]. Here we monitor natural large changes in expression of endogenous UCP2 and UCP3 in rat skeletal muscle [13–15] and show that they are not accompanied by changes in the basal proton conductance of isolated skeletal muscle mitochondria.

2. Materials and methods

2.1. Rat treatments

Female Wistar rats (120 g) were starved for 24 h with free access to water and compared to paired controls fed ad libitum. The rats were killed by cervical dislocation. Total hind limb skeletal muscle was diced, and a random mixture of muscle cubes was frozen in liquid N_2 then used for Northern determination of UCP2 and UCP3 mRNA, and Western determination of UCP3 protein. Mitochondria were isolated from the remaining tissue [16,17]. Some were used immediately for assay of proton leak; the remainder were stored at −20/−80°C for Western assay of UCP3.

2.2. Northern blot analysis

Northern blot analysis of UCP2 and UCP3 mRNA was performed as described elsewhere [18].

2.3. Western blot analysis

Samples were solubilised with SDS, their protein concentration was determined, then they were subjected to reducing SDS-PAGE. UCP standards were HEK293-EBNA cells stably transfected with plasmids mediating inducible expression of hUCP2, hUCP3 or vector. After blotting to nitrocellulose, UCP3 was detected with a rabbit polyclonal antibody to a 14 amino acid human UCP3 C-terminal peptide (Chemicon #3046, diluted 1:1000 in a skimmed-milk blocking buffer), followed by goat anti-rabbit horseradish peroxidase-labelled secondary antibody (Bio-Rad #170-6515, diluted 1:50 000), and developed with a standard ECL kit (Amersham RPN2106). Chemiluminescence was detected with a FujiFilm LAS-1000 camera and quantified with FujiFilm Science Lab 97 ImageGauge software. No image enhancing procedures were used. For quantitation, the linear dynamic range was >10. Rat UCP3 signal was normalised to the UCP3/HEK293 signal on the same gel. UCP3 was 5–8-fold enriched in mitochondria compared to whole muscle. Ab #3046 was specific for UCP3 relative to UCP2, since it did not cross-react with UCP2 in extracts from HEK293/UCP2 cells. However, weak interactions with unidentified bands were sometimes seen. Another antibody (NN2096B) raised against a 20 amino acid N-terminal peptide of human and rat UCP2 did detect human UCP2 in HEK293/UCP2 cells and rat UCP2 in white fat, but it could not detect UCP2 in rat muscle or isolated mitochondria, indicating low UCP2 levels in muscle (data not shown).

2.4. Proton leak measurements

Respiration rate and membrane potential were measured simultaneously [19] using electrodes sensitive to oxygen and to the potential-dependent probe $TPMP^+$. Assays contained 0.5 mg of mitochondrial protein/ml, 120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, 2 mM $MgCl_2$ and 0.3% defatted bovine serum albumin (BSA), pH 7.2. The electrode was calibrated with sequential additions

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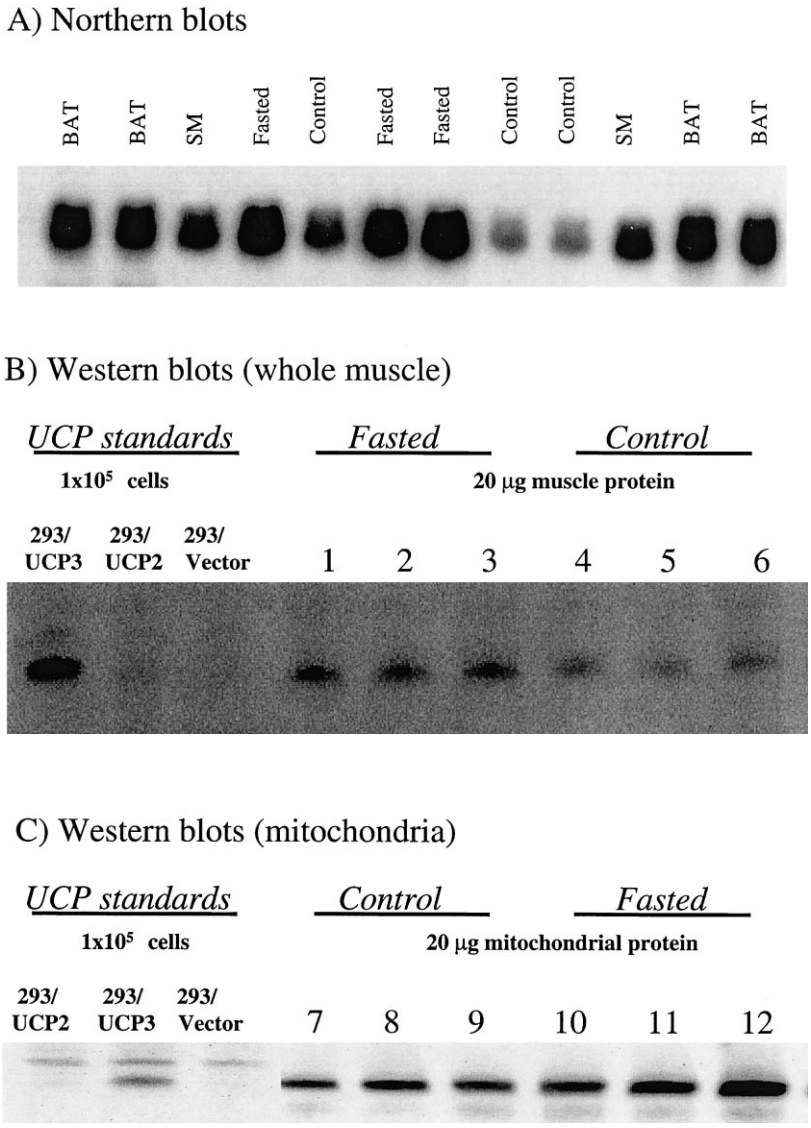

Fig. 1. Effect of 24 h starvation on UCP3 in rat skeletal muscle. A: Northern blots of UCP3 mRNA in diced muscle. Brown adipose tissue (BAT) and other skeletal muscle (SM) samples were internal controls. B: Western blots of UCP3 protein in diced muscle. C: Western blots of UCP3 in isolated mitochondria. Numbers 1–12 indicate different rats.

up to 2 μM TPMP. 5 μM rotenone, 1 μg/ml oligomycin, 80 ng/ml nigericin and 4 mM succinate were added. Malonate was sequentially added up to 2 mM to change mitochondrial potential. After each run, 0.2 μM FCCP was added to release TPMP for baseline correction. TPMP binding correction for skeletal muscle was taken to be 0.35 (μl/mg protein)$^{-1}$.

3. Results and discussion

UCP2 and UCP3 mRNA were measured by densitometry of Northern blots and compared to control, fed rats (Figs. 1 and 2). Fig. 2 shows that UCP2 mRNA was more than 5-fold higher in starved animals than in fed controls, and UCP3 mRNA was more than 4-fold higher, as expected [13–15]. Mitochondria were prepared from the same muscle samples, and both muscle and mitochondria were assayed for UCP3 protein by Western blotting (Fig. 1). Fig. 2 shows that UCP3 protein in starved animals was double that in fed controls.

We measured the proton conductance of the same mitochondria using published protocols [19] in the presence of BSA to chelate contaminating fatty acids. Fig. 3 shows that the rate of proton leak across the inner membrane at any given membrane potential was the same in mitochondria from starved animals as in mitochondria from fed animals. We obtained the same result if BSA was omitted from the medium. When the BSA-containing medium was supplemented with 150 μM oleate, state 4 respiration rates increased by 30%, showing the expected uncoupling effect of fatty acids, but once again there was no difference in proton leak between mitochondria from fed and starved animals (not shown).

Thus, starvation increases UCP2 and UCP3 mRNA in muscle, and UCP3 protein in whole muscle and in muscle mitochondria, but does not alter the mitochondrial proton conductance. There are two possible explanations of these findings. First, UCP3, which is the major UCP in muscle, catalyses the endogenous proton conductance. However, the proton leak catalysed by UCP3 does not change when there are increases in UCP3 mRNA and UCP3 protein as detected by Western blotting. This could happen if the newly synthe-

sised UCP3 protein was inactive or 'masked' as has been reported for UCP1 [20], or because of the presence of limiting amounts of some unknown essential cofactor in our isolated mitochondrial preparations. This cofactor is not a fatty acid such as oleate, since addition of oleate or removal of BSA did not affect the results. We should point out that there is no evidence for 'masking' or for any other cofactor of UCP3. Similar arguments apply to UCP2, but we have not measured UCP2 protein so cannot be sure that it changes when UCP2 mRNA increases. The second, simpler explanation is that UCP3 (and UCP2) do not catalyse the basal proton conductance of muscle mitochondria that we measure in our standard assay, but have some other function.

Several reports support a lack of correlation between UCP2 or UCP3 mRNA levels and mitochondrial proton conductance.

1. Tissue levels of UCP2 and UCP3 mRNA correlate better with regulation of lipids as fuel substrates than with thermogenesis [18,21]: they rise in muscle of food-deprived rats, when this tissue is thermogenically depressed [22]. Heat production from soleus muscle did not increase above basal in starved mice despite a 5-fold increase in UCP3 mRNA content [13].
2. In brown adipose tissue mitochondria from UCP1 knockout mice, UCP2 mRNA increased 5-fold, but basal proton conductance did not change [23,24].
3. UCP3 mRNA was increased in skeletal muscle from hyperthyroid compared to hypothyroid rats [25]. However, in the presence of bovine serum albumin (which should always be present, to prevent fatty acid-mediated uncoupling through other pathways such as the adenine nucleotide transporter) the muscle mitochondria showed 'no clear cut change in proton leak kinetics'. Thus the basal proton leak varied little despite 25-fold changes in UCP3 mRNA.
4. No UCP homologues have been found in hepatocytes from normal adult rats [26], but hepatocyte mitochondria have normal basal proton conductance [1,2].

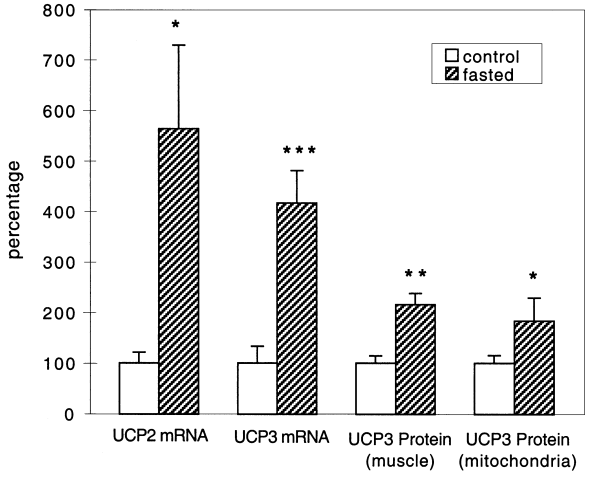

Fig. 2. Quantitative effect of 24 h starvation on UCP2 and UCP3 in rat skeletal muscle. Northern blots for UCP2 and UCP3 mRNA and Western blots for UCP3 protein were quantified and are shown as percentage of fed control. Data are mean ± S.E.M. for three experiments. Significances were determined using an unpaired *t*-test for mRNA and weighted ANOVA for protein. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

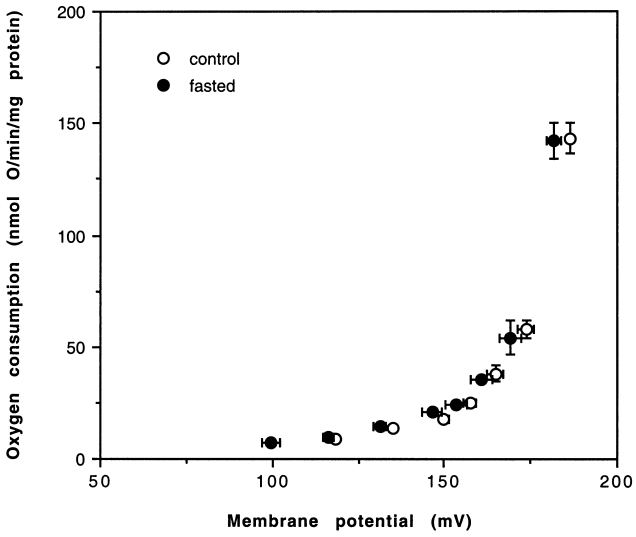

Fig. 3. Kinetic response of proton leak to potential in skeletal muscle mitochondria from control (open circles) and 24 h starved rats (closed circles). Data are mean ± S.E.M. of three experiments done in triplicate.

5. Clear UCP homologues are absent from the genomes of yeast (*Saccharomyces cerevisiae*) despite normal basal proton conductance [2,3], and the nematode *Caenorhabditis elegans* [3], the only fully sequenced multicellular organism. However, UCP2 is present in ectothermic vertebrates (carp and zebrafish), implying that it has functions other than thermogenesis [27].
6. Observed changes in UCP2 or UCP3 mRNA are frequently opposite to the ones expected if the proteins are involved in proton conductance and thermogenesis, e.g. [28]. Clever secondary hypotheses may explain the apparent anomaly, but the simplest explanation is that UCP2 and UCP3 do not catalyse the proton leak. Sometimes the changes are as expected, but the presence of a correlation in some cases is less persuasive than the absence of a correlation in others.

In our system, there are large changes in UCP2 mRNA and UCP3 mRNA in muscle, but no change in mitochondrial proton conductance. Therefore, it is unsafe in any system to assume that mitochondrial proton leak will change based only on the observation that UCP2 or UCP3 mRNA levels change, despite numerous examples of this inference in the literature. We find that UCP3 protein level changes too, so we conclude that it is unsafe in any system to infer changes in mitochondrial proton permeability even from changes in Western measurements of UCP3 protein. The proton conductance that we measure with our standard assay is a major contributor to basal metabolic rate, but it does not change when UCP2 and UCP3 mRNA and UCP3 protein levels change. Our evidence therefore suggests that these UCPs do not catalyse the observed basal proton conductance in isolated skeletal muscle mitochondria. UCP1 catalyses an inducible proton conductance in brown adipose tissue mitochondria. In principle, UCP2 or UCP3 could catalyse such an additional inducible proton conductance in skeletal muscle, but there is no evidence in native expression systems that this is the case, and

no evidence for it in our experiments using skeletal muscle from starved rats.

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