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Original Contribution

Uncoupling protein-3 lowers reactive oxygen species production in isolated mitochondria

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ABSTRACT

Mitochondria are the major cellular producers of reactive oxygen species (ROS), and mitochondrial ROS production increases steeply with increased proton-motive force. The uncoupling proteins (UCP1, UCP2, and UCP3) and adenine nucleotide translocase induce proton leak in response to exogenously added fatty acids, superoxide, or lipid peroxidation products. “Mild uncoupling” by these proteins may provide a negative feedback loop to decrease proton-motive force and attenuate ROS production. Using wild-type and *Ucp3*$^{-/-}$ mice, we found that native UCP3 actively lowers the rate of ROS production in isolated energized skeletal muscle mitochondria, in the absence of exogenous activators. The estimated specific activity of UCP3 in lowering ROS production was 90 to 500 times higher than that of the adenine nucleotide translocase. The mild uncoupling hypothesis was tested by measuring whether the effect of UCP3 on ROS production could be mimicked by chemical uncoupling. A chemical uncoupler mimicked the effect of UCP3 at early time points after mitochondrial energization, in support of the mild uncoupling hypothesis. However, at later time points the uncoupler did not mimic UCP3, suggesting that UCP3 can also affect ROS production through a membrane potential-independent mechanism.

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Introduction

In cells, mitochondria are the major producers of the reactive oxygen species (ROS) that can result in the oxidative stress and mitochondrial dysfunction found in many age-related and degenerative diseases [1–3]. Different components of the electron transport chain generate ROS under different conditions, although complexes I and III are thought to be among the important sites [4]. The rate of ROS production increases steeply with increased proton-motive force [5,6], and it has been hypothesized that partial dissipation of the proton-motive force by mild uncoupling of oxidative phosphorylation is a mechanism used by mitochondria to attenuate ROS production [7,8].

The uncoupling proteins (UCPs) and adenine nucleotide translocase (ANT) are members of the mitochondrial carrier family, and can provide regulated pathways for uncoupling [9]. UCP1 is the best characterized of these proteins, mediating nonshivering thermogenesis in brown adipose tissue by catalyzing proton leak activated by long-chain fatty acids and inhibited by purine nucleotides [10]. UCP3 is expressed predominantly in skeletal muscle and brown adipose mitochondria, at concentrations 200- to 700-fold lower than UCP1 in brown adipose tissue [11]. Its physiological function has remained controversial. Suggested functions include mild uncoupling, for which there is good empirical evidence [1,12,13]. It has also been proposed that UCP3 is involved in increasing fatty acid oxidation, a hypothesis supported primarily by correlative studies and transgenic UCP3-overexpression experiments; however, no impairment in fatty acid oxidation has been found in mice lacking UCP3 [13–17].

The mild uncoupling hypothesis for UCPs proposes that they partially uncouple mitochondria in the presence of ROS or downstream peroxidation products, resulting in a negative feedback loop that decreases ROS production by lowering both the proton-motive force and local oxygen concentration [7,8]. This idea has been supported by several experiments in which UCP3-dependent uncoupling in isolated mitochondria was activated by exogenously generated superoxide or exogenous lipid peroxidation products such as HNE (4-hydroxynonenal) [18–20]. Furthermore, mice lacking UCP3 had increased levels of oxidative damage markers and decreased activity of aconitase, a protein sensitive to damage by superoxide [14,21,22]. However, whether UCP3 functions to attenuate ROS production by simply catalyzing mild uncoupling remains to be directly tested.

In the present study we use wild-type (WT) and *Ucp3* knockout (KO) transgenic mice to test the hypothesis that UCP3 lowers ROS production in isolated mitochondria by catalyzing mild uncoupling. We find that native UCP3 actively lowers the rate of ROS production in isolated energized skeletal muscle mitochondria, in the absence of exogenous activators. From estimates of specific activity, UCP3 is 90 to 500 times more effective at lowering ROS production than ANT. We

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further show that the effect of UCP3 in lowering ROS production can be mimicked by a chemical uncoupler, consistent with a simple uncoupling mechanism for UCP3, although another more complex activity develops over time.

Materials and methods

Animals

Mice were housed at 21 ± 2 °C, 57 ± 5% humidity, 12/12 h light/dark, with standard chow and water ad libitum, following UK Home Office Guidelines for the Care and Use of Laboratory Animals. Male and female *Ucp3* knockout mice (*Ucp3*KO) [23] were crossed 10 times into the C57BL/6 background, and were sacrificed at age 12–15 weeks along with wild-type (WT) sex- and sibling-paired controls. *Ucp3* ablation was confirmed by PCR analysis of genomic *Ucp3* and Western blot analysis in skeletal muscle mitochondria.

Mitochondria

Four mice per preparation were killed by stunning followed by cervical dislocation, and mitochondria were isolated from total hind limb skeletal muscle [19]. WT and *Ucp3*KO mitochondria were assayed in parallel in 96-well plates using a fluorescence microplate reader (Spectramax Gemini XPS, Molecular Devices) set at 37 °C, which measured fluorescence of each well every 25 s. Mitochondria were suspended at 0.35 mg mitochondrial protein/ml in assay medium comprising 120 mM KCl, 3 mM HEPES, 1 mM EGTA, 5 mM KH_2PO_4, pH 7.2, supplemented with 0.3% (w/v) defatted bovine serum albumin (BSA), 6 U/ml horseradish peroxidase (HRP), 30 U/ml superoxide dismutase (SOD), 1 μg/ml oligomycin, and 80 ng/ml nigericin. Either 50 μM Amplex Red or 5 μM safranin O was included for measurement of H_2O_2 or membrane potential, respectively, and all conditions were measured in triplicate for each independent experiment. The reaction was initiated by addition of 10 mM succinate.

Measurement of H_2O_2 in isolated mitochondria

Mitochondrial H_2O_2 production was measured fluorescently using Amplex Red (Invitrogen) [24]. HRP catalyzed reaction between Amplex Red and H_2O_2, in the presence of exogenously added SOD, to form the fluorophore resorufin, with excitation and emission wavelengths at 563 and 587 nm, respectively. H_2O_2 standard curves were generated for each condition for each independent experiment, to calculate the cumulative mitochondrial H_2O_2 production from the resorufin signal at each measurement time point. The rate of H_2O_2 production at each time point was then determined by calculating the rate of change of H_2O_2 concentration over the following 2 min, in nmol H_2O_2/min/mg mitochondrial protein. Background rates of fluorescence change in the absence of added succinate were very small, but were subtracted for each experiment.

Measurement of membrane potential using safranin O

Mitochondrial membrane potential was measured using the positively charged dye safranin O, which changes fluorescence in a manner linearly proportional to the mitochondrial membrane potential [25]. The safranin O signal for each condition was measured at excitation and emission wavelengths of 533 and 576 nm, respectively, before mitochondrial energization with succinate, throughout energization, and then for 10 min after dissipation of the membrane potential by addition of 0.3 μM FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone). The relative decrease in fluorescent signal on energization of the mitochondria is proportional to the membrane potential (above about 60 mV), and results are reported as the absolute magnitude of this change in fluorescence, with larger changes in relative fluorescence units (RFU) indicating higher membrane potentials. Comparison of the safranin O signal before energization and after dissipation of the membrane potential with FCCP allowed correction for any small drift in the baseline fluorescent signal.

Statistics

Data shown are means ± SEM. Student's *t* tests were used as appropriate to compare two averages. Graphpad Prism (Version 5) was used for statistical analysis of data using analysis of variance (ANOVA), to find best-fit regressions, or to test for differences between curves.

Results

Reactive oxygen species production by energized skeletal muscle mitochondria from wild-type and UCP3KO mice

To investigate the role of UCP3 in mitochondrial ROS production, the rate of H_2O_2 production was measured in isolated mouse skeletal muscle mitochondria respiring on succinate in the absence of rotenone. Under these conditions, ROS production rate is high and originates mostly from the Q-binding site of complex I [26]. Over the 10-min course of the assay, *Ucp3*KO mitochondria produced significantly more ROS than WT mitochondria (Fig. 1A). The rate of ROS production by both WT and *Ucp3*KO mitochondria was relatively stable for the first 2 min after energization, but then decreased by more than 50% over the course of the assay (Fig. 1B). Addition of carboxyatractylate, an inhibitor of fatty acid-mediated proton transport through the adenine nucleotide translocase (ANT), largely eliminated this decrease in ROS production in *Ucp3*KO mitochondria (and also in WT, not shown), indicating a time-dependent involvement of the ANT in lowering ROS production rates (Fig. 1B). The average ROS production rate over the first 2 min was 17% higher in *Ucp3*KO mitochondria than in WT mitochondria (Fig. 1C), suggesting a significant role for UCP3 in the regulation of ROS generation. Critically, addition of 500 μM GDP eliminated the difference between WT and *Ucp3*KO mitochondria, indicating that UCP3 was actively lowering ROS production, and that secondary changes in other proteins (such as antioxidant defences) resulting from UCP3 knockout in the mice did not account for the differences in ROS generation between WT and *Ucp3*KO mitochondria. Addition of 500 μM GDP to *Ucp3*KO mitochondria also resulted in a small but nonsignificant increase in the ROS production rate over the first 2 min, which was likely due to inhibition of uncoupling through the ANT by GDP.

Activity of UCP3 and ANT in lowering ROS

UCP3 lowered the rate of ROS production by an average 0.0824 ± 0.0002 nmol H_2O_2/min/mg protein over the course of the assay, as shown by the difference between the ROS production rates of WT and *Ucp3*KO mitochondria (Fig. 2). The activity of UCP3 remained relatively stable, even though the overall rate of ROS production was decreasing (Fig. 1B), such that UCP3 had a proportionately greater effect on the rate of ROS production over time, accounting for an average 39% decrease in ROS production 8 to 10 min after energization.

The effect of ANT in lowering ROS production over time is shown as the difference between *Ucp3*KO H_2O_2 production rates in the presence and absence of carboxyatractylate. These data show that ANT proton transport activity increased steeply over the first 5 min of energization, before peaking at 0.480 ± 0.005 nmol H_2O_2/min/mg protein. Assuming the abundance of UCP3 is 4 pmol/mg protein [11] and that of ANT is 500-fold higher at 2 nmol/mg protein [27], this implies that each UCP3 molecule was 500 times more effective than ANT at lowering ROS at the start of the assay, and 90 times more

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