Biochimica et Biophysica Acta 1797 (2010) 324–330

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

UCP1 ectopically expressed in murine muscle displays native function and mitigates mitochondrial superoxide production

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ARTICLE INFO

Article history:
Received 19 June 2009
Received in revised form 9 October 2009
Accepted 25 November 2009
Available online 1 December 2009

Keywords:
Uncoupling protein
Mitochondrial respiration
Superoxide production
Skeletal muscle
Proton conductance

ABSTRACT

Mitochondrial uncoupling in skeletal muscle has raised a major interest as a therapeutic target for treatment of obesity, insulin sensitivity, and age-related disease. These physiological effects could be demonstrated in several mouse models ectopically expressing uncoupling protein 1 (UCP1). Here, we investigated whether UCP1 expressed under the control of the human skeletal actin (HSA) promoter in mouse skeletal muscle can be regulated, and whether it affects mitochondrial superoxide production. We show that the skeletal muscle UCP1 can be fully inhibited by a purine nucleotide (GDP) and reactivated by fatty acids (palmitate). During mitochondrial resting state (State 4), mitochondrial superoxide production is about 76% lower in transgenic mice. We suggest that this reduction is due to uncoupling activity as the administration of GDP restores superoxide production to wildtype levels. Our study confirms native behaviour of UCP1 in skeletal muscle and demonstrates beneficial effects on prevention of mitochondrial reactive oxygen species production which may reduce age-related deleterious processes.

1. Introduction

Uncoupling protein 1 is usually located in the mitochondrial inner membrane of brown adipose tissue where it uncouples substrate oxidation by the respiratory chain from ATP synthesis [1]. Substrate oxidation by the respiratory chain builds up a proton motive force by proton pumping of the respiratory complexes. UCP1 allows the return of protons into the matrix without ATP synthesis and thereby dissipates proton motive force as heat. UCP1-mediated heat production plays an important role in non-shivering thermogenesis in small rodents, hibernators and human infants [2].

Random energy dissipation may cause severe problems for the energy budget of an animal and therefore, UCP1 activity is tightly regulated. UCP1 can be activated by free fatty acids, superoxide and reactive alkenals, and potently inhibited by purine nucleoside di- and triphosphates [3–5].

The uncoupling activity by UCP1 may also have a physiological role in the regulation of energy metabolism and has attracted studies as a potential therapeutic target for obesity treatment. Recently, it has been demonstrated that UCP1 ablation itself induced obesity [6]. In this study, UCP1-ablated compared to wildtype mice gained more weight even when kept at thermoneutrality, fed on either control or high fat diet. In other studies, an increase of uncoupling activity by pharmacological manipulation such as administration of 2,4-Dinitrophenol reduced obesity and improved insulin sensitivity in mice [7]. Dinitrophenol treatment, however, has a narrow dose range, and overdosing led to illness or death [8]. Considering these observations, major interest arises as to whether ectopic UCP1 expression in tissues other than BAT may serve as a potential therapeutic target for anti-obesity treatment. The detrimental attempt to use DNP in humans in the past emphasizes the importance to understand the effect of uncoupling on cellular mechanisms and physiological effects in animal models.

In order to control UCP1 function in tissues other than BAT, its native behaviour towards activators (e.g. fatty acids) and inhibitors (e.g. purine nucleotides) is mandatory. Functional studies on mouse UCP1, overexpressed in yeast-mutants, displayed artifactual effects on mitochondrial uncoupling at high protein levels, seen as GDP insensitive uncoupling activity [9]. UCP1-containing cells showed a growth defect suggesting that a proportion of UCP1-mediated uncoupling is uncontrolled and ATP-synthesis capacity is compromised. In accordance with this, in another study investigating yeast expressing the UCP1-paralogue UCP3, UCP3 could neither be stimulated by the known UCP3 activators palmitate and superoxide nor inhibited by the purine nucleotide GDP [9]. These studies show the lack of evidence for native uncoupling behaviour of mammalian UCPs expressed in the yeast system. Although such studies should be interpreted with caution due to significant differences in species and mitochondrial membrane composition, UCP3 also failed to be activated by superoxide or inhibited by GDP in skeletal muscle of transgenic mice overexpressing UCP3 [10].

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doi:10.1016/j.bbabio.2009.11.008

Taken together, previous work on ectopically expressed UCPs shows the importance to demonstrate native function of any uncoupling protein that is introduced in different cell-types.

Skeletal muscle may represent a primary tissue for displaying native protein function as a recent study shows that brown fat and skeletal muscle cells, but not white adipose tissue (WAT) cells, differentiate from a common precursor [11,12] suggesting that brown fat cells are more similar to skeletal muscle cells than to WAT. So far, several different transgenic mouse models with ectopic expression of UCP1 in skeletal muscle have been generated independently and were shown to display reduced adiposity and increased energy expenditure [13,14]. In a mouse line expressing rat UCP1 in heart and skeletal muscle under the control of the mouse muscle creatine kinase (MCK), regulated control of UCP1 activity was shown in isolated mitochondria [15]. In this mouse line, there was a focus on obesity research, while a recent study addressed beneficial effects of UCP1 in skeletal muscle on age-related diseases [16]. Aging is strongly related to oxidative damage which accumulates with time, caused by reactive oxygen species (ROS). Mitochondria are the main source of cellular superoxide production leading to the generation of ROS. It has been suggested that respiratory rates play an important role in the amount of ROS production [17,18]. Superoxide production is very sensitive towards a decrease in proton motive force suggesting that uncoupling activity and UCPs may play a role in decreasing ROS concentration in the cell. Treatment with low doses of the uncoupler 2,4-dinitrophenol promoted a decrease of ROS levels and subsequently, a decrease of tissue DNA and protein oxidation [7]. Pertaining to UCPs, superoxide and products of lipid peroxidation activate UCPs promoting a feedback down-regulation of mitochondrial ROS production, although the concentration of activators used in these studies were beyond physiological levels [19]. This model is disputed as other studies reported no involvement of UCP1 in 4-hydroxynonenal activation and observed no altered UCP1 activation with manipulated matrix superoxide levels [20,21]. The observation of decreased superoxide induced DNA double strand break in UCP1 overexpressing endothelial cells, however, indirectly indicates that UCP1 may alter mitochondrial superoxide levels [22].

Therefore, UCP1 may have a beneficial impact on oxidative stress in skeletal muscle.

In the present study, we investigated a HSA (human skeletal muscle actin promoter)-transgenic mouse model expressing murine UCP1 in skeletal but not heart muscle. Previously we have shown that the HSA-mUCP1 mice had improved glucose tolerance and altered substrate oxidation in muscle and adipose tissue [13,23]. UCP1 protein levels in skeletal muscle of these mice were approximately 14-fold lower compared to levels normally found in BAT [23].

Functionality of UCP1 was judged by inhibition with purine nucleotides and by re-activation with free fatty acids. To test whether uncoupling affects mitochondrial superoxide production in skeletal muscle, we measured mitochondrial hydrogen peroxide release by fluorescent detection.

2. Methods

2.1. Animal experiments

Experiments were performed in adult (male, female) heterozygous HSA-UCP1 transgenic mice and their wildtype littermates established as described previously [24] . Mice were maintained at 22 °C a 12 h:12 h dark–light cycle with food and water ad libitum. Mean body weights of wildtype and transgenic mice were 29.1 $\pm$ 3.2 g, and 21.3 $\pm$ 1.8 g respectively. Prior to experiments, mice were killed by cervical dislocation. The animal experiments were approved by the animal welfare committee of the Ministry of Agriculture and Environment (State of Brandenburg, Germany).

2.2. Isolation of mitochondria

Mitochondrial preparations of the two genotypes were done in parallel to correct for day-by-day variations in mitochondrial quality. Skeletal muscle was obtained, minced and placed in ice-cold CP1 medium (0.1 M KCl, 0.05 M Tris–HCL, 2 mM EGTA, pH 7.4). The tissue homogenate was then transferred to CP2 medium [CP1 to which was added, 0.5% BSA, 5 mM $MGCl_2$, 1 mM ATP, Protease Type VIII (Sigma P5380)], digested for 3 min and homogenized using a Polytron (Kinematica) followed by another 3 min digestion step. The homogenate was centrifuged for 10 min at 490 *g*. The resulting supernatant was filtered through layers of muslime and centrifuged for 10 min at 10,400 *g*. The resulting pellet was resuspended in CP1. The high speed spin cycle was repeated twice and the final pellet resuspended in a minimum volume of CP1 medium.

The protein concentration of mitochondrial suspensions was determined by the Biuret method using fatty acid free bovine serum albumin (BSA; Sigma 3803) as standard.

2.3. Mitochondrial respiration

Oxygen consumption was measured using a Clarke-type electrode (Rank Brothers Ltd, Cambridge, UK) maintained at 37 °C and calibrated with air-saturated medium [120 mM KCl, 5 mM KH_2PO_4, 3 mM EGTA, 0.3% BSA, 5 μM rotenone (to inhibit complex I of the respiratory chain) pH 7.2] which was assumed to contain 406 nmol O ml^{-1} [25]. Mitochondria were resuspended to a concentration of 0.3 mg protein ml^{-1} in the assay medium. Mitochondrial respiration was started by adding 4 mM succinate.

The hydrophobic fatty acid was equilibrated in BSA-containing buffer for the proton leak assays, and dissolved in ethanol for injections seen in Fig. 3. The final concentration of the FFA was calculated according to Richieri et al. 1993 [26].

2.4. Proton leak kinetics

The mitochondrial membrane potential was measured simultaneously with mitochondrial respiration by using an electrode sensitive to the potential-sensitive probe, $TPMP^+$ (triphenylmethylphosphonium) in the presence of 100 nM nigericin to dissipate the pH gradient, as described previously [27]. The $TPMP^+$ sensitive electrode was calibrated with sequential addition of $TPMP^+$ up to 2.5 μM, and succinate was added to initiate mitochondrial respiration. Membrane potential and respiration were progressively inhibited by successive steady states imposed by the competitive inhibitor, malonate, up to 2 mM.

Finally 0.3 μM FCCP was added to dissipate the membrane potential and release all the $TPMP^+$ from the mitochondria, allowing correction for small baseline drifts. Leak respiration at each steady state was plotted against the corresponding membrane potential to illustrate the dependence of proton leak rate on membrane potential.

2.5. Measurement of mitochondrial superoxide production

The assay is based on the detection of H_2O_2 using Amplex red fluorogenic substrate (10-acetyl-3.7-dihydroxyphenoxazine; Invitrogen). The amplex red molecule reacts with H_2O_2 by a 1:1 stoichiometry to resorufin, a fluorescent molecule. In the presence of superoxide dismutase, superoxide is converted to hydrogen peroxide by a 1:2 stoichiometry. Measurements were performed similar to Lambert et al. [31,40]. 10–20 μg skeletal muscle mitochondria were incubated in assay buffer (50 mM KCl, 5 mM TES, 2 mM $MgCl_2 \times 6H_2O$, 4 mM KH_2PO_4, 1 mM EGTA, BSA 0.4% (w/v), pH 7.2 at RT) containing a cocktail of 50 μM Amplex Red, 30 U ml^{-1} superoxide dismutase (to convert extra-mitochondrial superoxide to hydrogen peroxide), 6 U ml^{-1} horseradish peroxidase (catalyzing the

reaction of hydrogen peroxide with Amplex red resulting in fluorescent resorufin) and 2 μg ml^{-1} oligomycin (to inhibit ATP synthesis).

H_2O_2 formation was initiated by addition of succinate (4 mM) and fluorescence was detected at 37 °C in a microplate reader (BMG Labtech, FLUOstar OPTIMA) in 96-well microplates (GREINER 96-Well μClear, F-Bottom, black). The excitation wavelength was set to 550 nm, and the fluorescence emission was detected at 585 nm. Fluorescence was calibrated using known amounts of H_2O_2 at each experimental day. Superoxide production was measured in the presence of rotenone (2 μM, inhibiting complex I-derived ROS production) and GDP (1 mM, to inhibit UCP1).

2.6. Statistical analysis

Data are given with means ± SEM. Statistical significance was assessed by two-tailed Students *t*-test, and differences of $P<0.05$ were considered significant.

3. Results

3.1. Mitochondrial proton conductance of skeletal muscle from transgenic vs wildtype mice

To address the question whether ectopically expressed UCP1 changes mitochondrial proton conductance in skeletal muscle, and whether UCP1 displays native behavior, we determined proton conductance in isolated skeletal muscle mitochondria. We measured the full kinetic response of proton leak at 37 °C, measured as oxygen consumption rate in the presence of oligomycin, to stepwise changes in its driving force, membrane potential (Fig. 1). In contrast to wildtype mice, the proton leak titration of transgenic mice was shifted upwards demonstrating higher proton conductance. To quantify UCP1-mediated proton leak, we determined oxygen consumption as a measure for proton leak rate at the highest common potential. A common potential for quantitative curve comparisons is necessary as proton motive force is the driving force of the proton leak with pronounced non-ohmic nature at higher potentials. At the highest common potential of ~154 mV, oxygen consumption was about 4-fold ($P<0.0001$, $N=7$) increased in transgenic mice (100.15 nmol O min^{-1} mg^{-1} +/− 7.75) as compared to wildtype controls (27.07 nmol O min^{-1} mg^{-1} +/− 2.25). We confirmed the genotypes of the mice by blotting skeletal muscle protein and probing against mouse UCP1. Furthermore, we also analyzed UCP3 levels which were found to be unchanged (Supplement 1a).

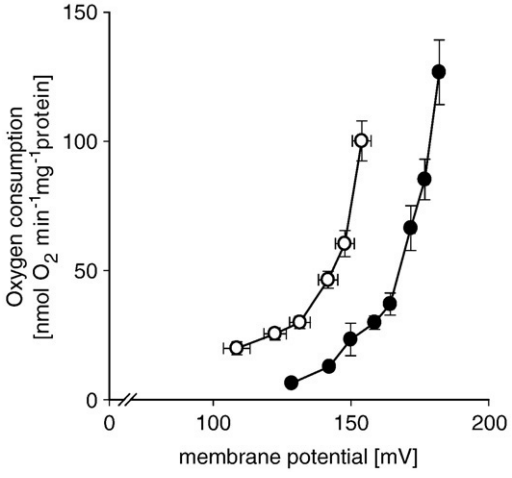

Fig. 1. Proton leak kinetics showing basal proton conductance of isolated skeletal muscle mitochondria from HSA-mUCP1 transgenic mice (open symbols; $n=6$) and wt littermate controls (black symbols; $n=3$). The proton conductance of transgenic mitochondria was significantly elevated compared to wildtype mitochondria.

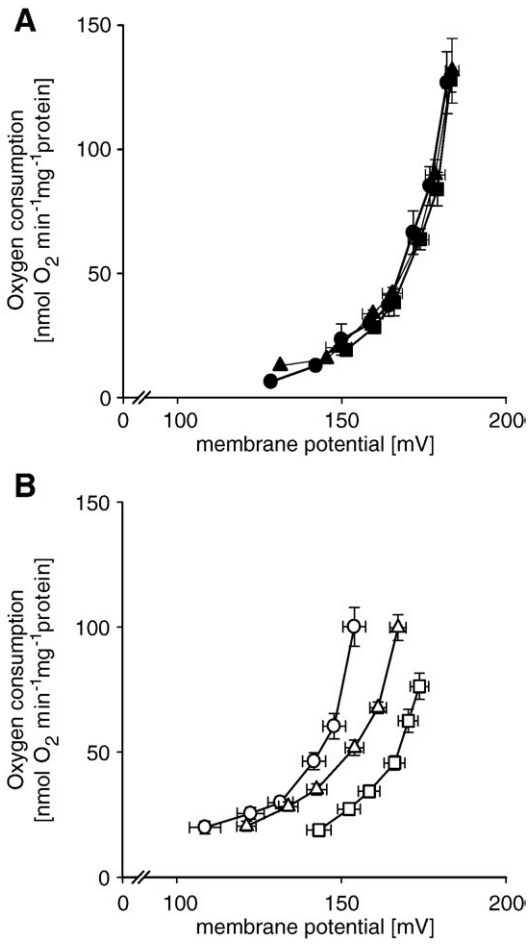

Fig. 2. Effect of GDP and palmitate on proton conductance of skeletal muscle mitochondria in wt (A black symbols) and HSA-mUCP1 transgenic mice (B open symbols) after GDP inhibition (squares) and after re-activation with 100 μM palmitate (triangle) at 37 °C in the presence of 0.3% palmitate. While GDP and palmitate had no effect on wildtype skeletal muscle mitochondria (A), in transgenics, GDP-inhibited proton leak resulted in a downshift of the curve that could be partially restored by the addition of palmitate.

3.2. Effect of the UCP1-inhibitor GDP and the activator palmitate on proton conductance

We further explored UCP1 function in skeletal muscle mitochondria by adding an inhibiting purine nucleotide, GDP, and an activating free fatty acid, palmitate, respectively, and monitored the effect on proton conductance (Fig. 2).

In transgenic mice, the addition of GDP shifted oxygen consumption at 154 mV back to wildtype level (transgenic GDP: 29.31 nmol O min^{-1} +/− 2.72) while the compound GDP had no effect in wildtype mice (26.65 nmol O min^{-1} +/− 1.18). Comparing transgenic and wildtype mice in the presence of GDP, there were no significant genotype effects apparent ($P=0.09$, $N=3$–7).

Palmitate is known to overcome GDP inhibition by simple competitive kinetics [28]. 100 μM palmitate, approximately resulting in 11 nM free fatty acids in the presence of 0.3% BSA [26] shifted the proton leak curve of GDP–inhibited UCP1 upwards. Comparing at the highest membrane potential of ~167 mV, oxygen consumption rate increased from 50.31 nmol O min^{-1} +/− 6.73 to 99.81 nmol O min^{-1} +/-5.60. Palmitate-induced uncoupling was not apparent at state 4 potential in

wildtype mitochondria (GDP: 183.18 mV, 128.05 nmol O min^{-1} +/−4.95; Palmitate: 183.59 mV, 131.63 nmol O min^{-1} +/−6.48).

3.3. Controlling for effects putatively mediated by the adenine nucleotide translocase (ANT)

It has been demonstrated that uncoupling activity mediated by the ANT can also be increased by fatty acids [29] and recently, that GDP is able to inhibit ANT uncoupling [30]. To control for potential misinterpretation of the data, we first determined the amount of ANT by CAT titration in skeletal muscle mitochondria of transgenic and wildtype mice. ANT contents were similar in transgenic (2.56 nmol ANT/mg protein) and wildtype (2.73 nmol/mg protein) mice. An illustration of the CAT titration can be found in supplement 1b. Furthermore, we repeated the effects of palmitate and GDP in the presence of CAT, an exemplified measurement is shown in Fig. 3. Also, no effect of CAT by palmitate and GDP was found in proton conductance (graphs are shown in supplement 1c).

3.4. Genotypic differences in substrate oxidation measured as mitochondrial respiration on succinate

Although differences in proton conductance between genotypes can be explained conclusively by the presence of UCP1 activity, we initially found differences in state 4 respiration which may be explained by different substrate oxidation rates between genotypes. Therefore, we also measured state 2 respiration in the presence of succinate and state 3 respiration induced by 600 μM ADP (Fig. 4). The reduced oxygen consumption in transgenic mice suggests a decrease in substrate oxidation capacity. This assumption is further corroborated when plotting oxygen consumption rate against membrane potential obtained from Fig. 3 (see supplement 1d).

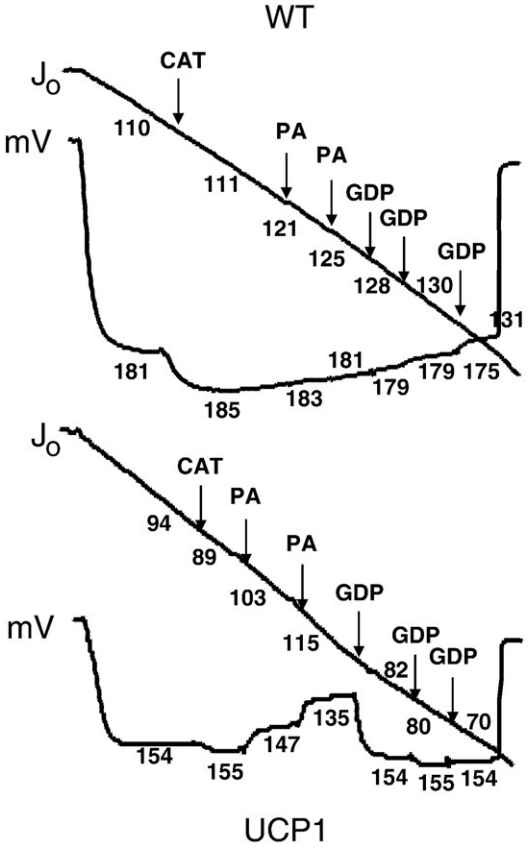

Fig. 3. Representative parallel measurement of mitochondrial oxygen consumption illustrated as decline of oxygen concentration (Jo) and membrane potential as the uptake of the membrane potential dependent uptake of TPMP+ (mV) along a time scale. The emphasis is to illustrate the effects of palmitate and GDP in the presence of CAT (inhibiting the ANT) in wildtype and HSA-mUCP1 transgenic mouse. In contrast to Fig. 2, UCP1 was activated first with fatty acid and then inhibited with GDP.

3.5. Measurement of mitochondrial hydrogen peroxide release as a measure of superoxide production

It has been hypothesized that mild uncoupling of the respiratory chain leads to prevention of mitochondrial ROS production possibly reducing age-related dysfunctions. Here we estimated mitochondrial superoxide production by a standard Amplex red assay measuring mitochondrial hydrogen peroxide release. To assist the conversion of escaping superoxide to hydrogen peroxide, saturating amounts of superoxide dismutase were added, similar to Lambert et al. [31]. All measurements were performed in the presence of oligomycin, shifting mitochondria to state 4 and getting maximum membrane potential, also mimicking a state close to resting of muscle.

When comparing transgenic with wildtype mitochondria (Fig. 5), we found that superoxide production levels were about 76% lower in transgenics (wt: 2.79 nmol superoxide min^{-1} mg^{-1} mitochondrial protein vs transgenic: 0.66 nmol superoxide min^{-1} mg^{-1} mitochondrial protein). The administration of 1 mM GDP increased superoxide production to almost wildtype levels (2.77 nmol superoxide min^{-1} mg^{-1} mitochondrial protein) while in wildtypes, 1 mM GDP almost doubled the basal value to 6.69 nmol superoxide min^{-1} mg^{-1} mitochondrial protein, notably without detectable differences in proton conductance (compare to Fig. 2). In the presence of rotenone, preventing reverse electron transfer, superoxide production decreased significantly in wildtype mice, while the low levels from transgenics were slightly elevated.

4. Discussion

In this study we demonstrate native behavior of mouse UCP1 ectopically expressed in skeletal muscle of our transgenic mouse line [24]. Furthermore, UCP1 mitigated superoxide production by reverse electron transfer as determined by detection of mitochondrial hydrogen peroxide release which is indicative of mitochondrial superoxide production.

4.1. Uncoupling protein 1 activity in skeletal muscle of transgenic mice displays native behavior

In order to unambiguously relate physiological and biochemical parameters to the presence or absence of a functional UCP1 in skeletal muscle, it is of major importance to characterize UCP1-mediated proton conductance. In the light of problems that occurred during UCP expression in other cell systems [32], here we aimed to identify any arbitrary proton leak activity which can be caused by proteins not properly folded and inserted into the mitochondrial inner membrane. Our data showed that UCP1 expressed in skeletal muscle catalyzes a proton leak which could be fully regulated with free fatty acids (palmitate) and purine nucleotides (GDP). In the presence of the purine nucleotide GDP, we found no significant difference in proton conductance between wildtype and transgenic mice and therefore excluded any artificial uncoupling. The typical activator of UCP1, a fatty acid, palmitate, overcame GDP-inhibition in transgenic mice and increased proton conductance while there were no effects of 100 μM palmitate in wildtype.

Although purine nucleotides and fatty acids are generally regarded as typical activators/inhibitors of UCP1, another abundant mitochondrial carrier protein, the adenine nucleotide translocase (ANT), has been shown to also uncouple the respiratory chain from ATP synthesis which can be modulated by fatty acids [33] and GDP [30]. It was therefore necessary to re-investigate the activity of mouse UCP1 in

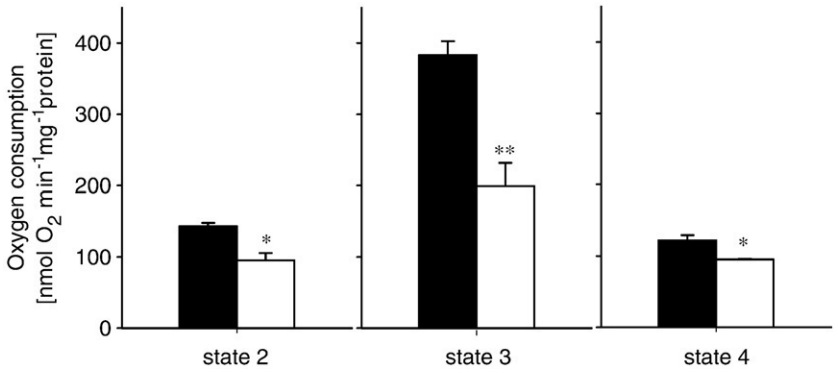

Fig. 4. Respiration rates of wildtype (black columns) and HSA-UCP1 transgenic (white columns) skeletal muscle mitochondria. Mitochondria respired on succinate, state 2 respiration was performed in the presence of rotenone, state 3 respiration induced by 600 μM ADP, and mitochondria shifted into state 4 with oligomycin. *P<0.02; **P<0,01).

skeletal muscle in the presence of carboxyatractylate which inhibits uncoupling activity of the ANT. In these experiments, we could show that all effects on UCP1 activity were still preserved. In fact, we also determined the ANT concentration per milligram mitochondrial protein which was not affected by ectopic UCP1 expression.

4.2. UCP1 gene expression in skeletal muscle reduces the risk of reverse electron transfer and the production of reactive oxygen species

It has been hypothesized that mild uncoupling prevents mitochondrial superoxide production [34]. Using succinate as substrate, we investigated whether skeletal muscle UCP1 has an effect on superoxide production, analyzing the production of forward and reverse electron transfer in mitochondrial state 4. We found that superoxide production was about 76% lower in UCP1 expressing mice. In the presence of rotenone, preventing reverse electron transfer at complex I when energized with complex II substrate, superoxide production in transgenic mice was slightly elevated, while there was a significant decrease in wildtype mice as described previously for other rodent species [35]. The negligible elevation of signal caused by rotenone in transgenic mitochondria may be caused by residual endogenous substrates feeding into complex I. However, this had apparently no significant impact. Administration of GDP increased superoxide production in transgenics to almost wildtype level. GDP also had an effect on wildtype mitochondria, doubling the production rate. Although we detect no apparent mitochondrial uncoupling between proton leak curves in absence or presence of GDP in wildtype mitochondria, it may be that other uncouplers also contribute to the prevention of superoxide with UCP3 being one of the candidates. In fact, Talbot et al. showed strong UCP3 dependent effects on superoxide production in the presence of GDP resulting in a 25% decrease on aconitase activity [36]. Considering that the reduction state of the respiratory chain increases with membrane potential exponentially at high potentials, small activities of UCP3 may cause this effect in wildtype mice having higher potentials than transgenics. We found, however, no differences in skeletal muscle UCP3 protein content between transgenic and wildtype mice. On the other hand, our results clearly show that UCP1 has a preventive effect on superoxide production in transgenic mice which in turn may slow down the accumulation of damage in the muscle. In accordance to our observations, previous studies observed an increase of median survival in transgenic mice and lowered age-related disease [16]. The manipulation of mitochondrial superoxide production by uncoupling activity in skeletal muscle may therefore represent an interesting therapeutic target to decrease oxidative stress, not only in muscle.

4.3. Does UCP1 cause a depression of substrate oxidation in skeletal muscle mitochondria?

During proton leak titration we observed that although proton conductance in transgenic mouse mitochondria was elevated there was a decrease in state 4 respiration indicating a depression in substrate oxidation (Fig. 1). We found that state 2, 3 and state 4 respiration were decreased in transgenic muscle mitochondria (Fig. 4) strongly suggesting that succinate dehydrogenase activity in mitochondria of transgenic mice is decreased. In this study, we did not further explore the nature of this secondary effect of UCP1 expression in skeletal muscle. We found, however, two other studies investigating mitochondrial respiration of UCP1-containing mitochondria: in a study by Couplan et al. [15], no depression of mitochondrial respiration was apparent in their mouse model. Unfortunately, the other study investigating UCP1 muscle gene transfer [37] "normalised" their respiration data to chemical uncoupler values, which masks any effects on substrate oxidation.

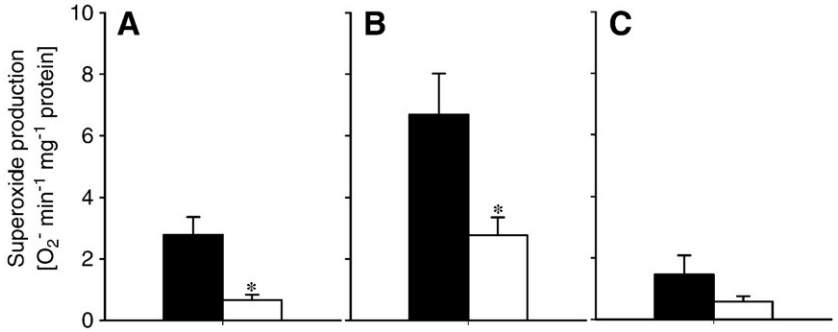

Fig. 5. Rates of superoxide production (calculated by 1:2 stoichiometry of H_2O_2 production) of isolated skeletal muscle mitochondria from transgenic mice (white columns) and wildtype mice (black columns). (A) Control medium; (B) addition of 1 mM GDP to inhibit UCP1 activity; (C) addition of 4 μM rotenone to inhibit complex I-derived ROS production. Bars show means +/− SEM (*P<0.05).

That mitochondria may change substrate oxidation behavior can be noticed in Couplan et al.: presumably due to a switch from fast to slow muscle fibres, the response towards alpha-glycerol phosphate in transgenic mice was blunted [15].

Interestingly, over-expression of UCP3 in skeletal muscle of mice led to a decrease in state 3 respiration in isolated mitochondria when using succinate as a substrate but increased when using palmitate [38]. Here we only used succinate, it thus remains to be investigated if other substrates might have different effects on maximum respiration rates in our model.

In cultured neurons, mild uncoupling by FCCP decreased spare respiratory capacity, similar to our observations of depressed substrate oxidation capacity in skeletal muscle mitochondria [39]. Mild uncoupling in neurons increased toxicity to glutamate and oxidative stress, presumably due to mismatching energetic demands. Therefore, mild uncoupling may not be beneficial in all tissues, further refuting the use of general uncouplers such as DNP but promoting research on tissue-specific uncoupling.

4.4. Relating physiological observations to skeletal muscle UCP1 function

Interestingly, the presence of UCP1 in muscle leads to physiological effects (e.g. reduction in body weight) that can be explained by increased energy dissipation in skeletal muscle. Our data show that UCP1 can be fully inhibited with purine nucleotides. Given the intracellular ATP/ADP concentrations, UCP1 in the muscle cell should be inhibited at all times, similar to the situation in the brown adipocyte without a cold stimulus, when levels of free fatty acids and lipolysis are low. In fact, the muscle cell artificially hosting UCP1 does presumably not possess the same cellular machinery that assists the uptake and breakdown of lipids at high throughput rates as found for brown adipose tissue. Furthermore, the activation of UCP1 by cold-induced noradrenaline is probably also not present in skeletal muscle.

If UCP1 is inhibited, the question arises why there are obvious phenotypes associated with skeletal muscle uncoupling. Recently, it has been found that brown adipose tissue UCP1 deficiency induces obesity and abolishes diet-induced thermogenesis under thermoneutral conditions, even when fed on control diet [6]. This emphasizes that UCP1 under physiological conditions is either not fully inhibited and may conduct protons below detection levels. It may also well be that the increased fatty acid metabolism and circulation induced by moderate cold (normal animal maintenance is between 22 and 24 °C) is sufficient to activate UCP1 in skeletal muscle.

The importance of this study is the demonstration of potent regulation of UCP1 protein function in skeletal muscle that further corroborates the beneficial effects of skeletal muscle mitochondrial uncoupling on obesity [14,24], insulin resistance [13,14,23], and aging [16].

Acknowledgements

This work was supported by a grant of the German research foundation (DFG). S. Klaus is a member of EU COST action Mitofood (FA0602).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.11.008.

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