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Uncoupled respiration, ROS production, acute lipotoxicity and oxidative damage in isolated skeletal muscle mitochondria from UCP3-ablated mice

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ABSTRACT

The function of uncoupling protein 3 (UCP3) is still not established. Mitochondrial uncoupling, control of ROS production, protection against lipotoxicity and protection against oxidative stress are functions classically discussed. To establish a role for UCP3 in these functions, we have here used UCP3 (−/−) mice, backcrossed for 10 generations on a C57Bl/6 background. In isolated skeletal muscle mitochondria, we examined uncoupled respiration, both unstimulated and in the presence of fatty acids. We did not observe any difference between mitochondria from wildtype and UCP3 (−/−) mice. We measured H_2O_2 production rate and respiration rate under reactive oxygen species-generating conditions (succinate without rotenone) but found no effect of UCP3. We tested two models of acute lipotoxicity—fatty acid-induced oxidative inhibition and fatty acid-induced swelling—but did not observe any protective effect of UCP3. We examined oxidative stress by quantifying 4-hydroxynonenal protein adducts and protein carbonyls in the mitochondria—but did not observe any protective effect of UCP3. We conclude that under the experimental conditions tested here, we find no evidence for the function of UCP3 being basal or induced uncoupling, regulation of ROS production, protection against acute lipotoxicity or protection against oxidative damage.

1. Introduction

More than a decade after their identification, the "novel" uncoupling proteins—UCP2 and UCP3—remain without established functions. Although a series of suggestions for function have been formulated over the years, none have gained general acceptance. For most of this time, the discussions (e.g. [1]) concerning UCP3 function have concentrated on the following suggestions: that UCP3, similarly to UCP1, should be an uncoupling protein [2]; that it is an uncoupling protein but only when stimulated by certain activators [3]; that it controls ROS production [4]; that it is a fatty acid anion exporter [5]; that it could protect against lipotoxicity [6,7]; and that it could protect against oxidative damage (induced by ROS production). Particularly this latter suggestion is discussed and promoted in a large number of reviews (e.g. [6,8–11]).

The physiological function of a protein can be studied in several ways. Particularly the outcomes of gene ablation experiments are accepted as indications for the functional role of a given protein in a given process. Concerning UCP3, several studies examining the above statements in UCP3(−/−) mice have been published but there is variation in the results. Inspection of the literature indicates that few of the investigations of UCP3(−/−) mice have been performed with backcrossed mice, and effects observed may therefore be related to strain differences and not due to functions of UCP3 as such.

In the present investigation, we have therefore (re-)examined experimental models for these suggested functions in skeletal muscle mitochondria isolated from wildtype mice and from UCP3(−/−) mice, backcrossed for 10 generations to the C57Bl/6 (wildtype) strain.

It is the outcome of the investigation that none of the proposed functions, at least as examined under the conditions used here, withstood this experimental testing.

2. Material and methods

2.1. Animals

Male C57Bl/6 mice lacking UCP3 (UCP3(−/−)) and their wildtype controls, about 20 weeks of age, were derived from those generated

Abbreviations: ANT, adenine nucleotide translocase; DNPH, dinitrophenylhydrazine; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol tetraacetic acid; FCCP, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; GDP, guanosine 5′-diphosphate; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; 4-HNE, 4-hydroxynonenal; PMSF, phenyl methyl sulfonyl fluoride; SDS, sodium dodecyl sulfate; PBS, phosphate buffered saline; UCP, uncoupling protein; WT, wildtype

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by Gong et al. [12]. The UCP3(−/−) mice used in this study were backcrossed for 10 generations onto a C57Bl/6 background. Animals were maintained on a 12:12 h light–dark cycle (light from 07.00 to 19.00 hours), at 21–22 °C and were allowed unlimited access to standard laboratory chow and tap water. All experiments were approved by the Animal Ethics Committee of the North Stockholm region and of the Maastricht University and complied with the principles of laboratory animal care.

2.2. Tissue collection and mitochondrial isolation

Mice were anesthetized by a mixture of 79% CO_2 and 21% O_2 and killed by decapitation. Isolation of skeletal muscle mitochondria, 2 mice per isolation, and determination of protein concentration were performed as previously described [13]. Briefly, hind limb skeletal muscle (~2.0 g) was dissected and immediately placed into ice-cold isolation buffer (100 mM sucrose, 50 mM KCl, 20 mM K^+-TES, 1 mM EDTA, and 0.2% (w/v) fatty acid-free bovine serum albumin). Tibialis anterior muscle was held separate for western blot analysis and the remaining tissue was finely minced with scissors, followed by mechanical Potter homogenization in the presence of nagarse (Fluka Chemie, GmbH, Germany; 1 mg/g tissue). Homogenates were centrifuged (8500*g* for 10 min at 4 °C) and the resulting pellets were resuspended in isolation buffer. After hand-homogenization in a Potter homogenizer, the suspensions were centrifuged (800*g* for 10 min at 4 °C). The pellet was discarded and the supernatant was again centrifuged (8500*g* for 10 min at 4 °C). The final resulting mitochondrial pellets were gently resuspended by hand-homogenization in a small glass homogenizer with a Teflon pestle.

Mitochondrial protein concentration was determined using fluorescamine (Fluram®, Fluka Chemie, Gmbh, Germany) with albumin as a standard.

2.3. Levels of mitochondrial proteins

Western blotting was performed in isolated skeletal muscle mitochondria and in tissue homogenates. Coomassie Brilliant Blue staining was used to determine the total protein content of every sample. Subsequently, western blotting was performed using 10% polyacrylamide SDS-gels as described before [14,15]. Gels were loaded with 30 μg of protein per sample and equal protein loading was confirmed by western blotting of actin (for tissue homogenates) and porin (for mitochondria). Rabbit polyclonal antibody against UCP3 (code 1338, kindly provided by LJ Slieker, Eli Lilly, dilution 1:10000), as well as monoclonal antibodies against actin (A2172; Sigma-Aldrich, St. Louis, MO, USA, dilution 1:250000), adenine nucleotide translocase (ANT) (MSA02; Mitosciences, OR, USA, dilution 1:2000), porin (sc-58649; Santa Cruz, CA, USA, dilution 1:10000), and a mixture of monoclonal antibodies against some of the structural components of oxidative phosphorylation (Oxphos) complexes I, II, III and V (MS601; Mitosciences, OR, USA, dilution 1:10000) were used. The UCP3 protein band was visualized by chemiluminescence and analyzed by densitometry using Image Master (Pharmacia Biotech, Roosendaal, The Netherlands), whereas bands of other proteins were detected and quantified with Odyssey Infrared Imager (LI-COR; Wesburg, Leusden, The Netherlands).

2.4. Mitochondrial respiration studies

Skeletal muscle mitochondria (0.25 mg/ml) were incubated in a medium consisting of 100 mM sucrose, 20 mM K^+-Tes (pH 7.2), 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 4 mM KH_2PO_4, and 0.1% (w/v) fatty acid-free bovine serum albumin. Oxygen consumption rates were monitored with a Clark-type oxygen electrode (Yellow Springs Instrument Co., USA) in a sealed chamber at 37 °C as described [13]. The substrates used were either 3 mM malate plus 5 mM pyruvate, or 5 mM succinate (here used in the absence of rotenone (if not otherwise stated)); oligomycin (1 μg/ml) was added to block ATP synthesis ("state-4 respiration"). For measurements of fatty acid-stimulated respiration, the mitochondria were titrated with increasing levels of palmitate (dissolved in 50% ethanol). The free concentrations of palmitate were calculated using the equation described in Ref. [16] for the binding of palmitate to albumin at 37 °C. Data for fatty acid concentration–response curves were analyzed with the four parameter logistic curve fit option of the Sigmaplot 8.0 application for adherence to simple Michaelis–Menten kinetics.

To assess the effect of fatty acids on maximal oxidative capacity, a submaximal concentration of palmitate (100 μM, based upon the palmitate-induced respiratory measurements) was added to the mitochondria in state-4 (pyruvate + malate present), while respiration was monitored. Subsequently, the maximal capacity of the electron transport chain was determined by addition of an amount (indicated) of the chemical uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) that in additional experiments had been established to be optimal (i.e. not inhibitory). In the control condition, palmitate was replaced by its solvent (50% ethanol).

2.5. ROS production and effects

Mitochondrial H_2O_2 net production was determined fluorometrically by the use of the Amplex Red reagent (Invitrogen). Oxidation of Amplex Red coupled by horseradish peroxidase to reduction of H_2O_2 produces the red fluorescent oxidation product resorufin [17]. Mitochondria (0.1 mg of mitochondrial protein ml^{-1}) were incubated at 37 °C under magnetic stirring in the same buffer as that used for respiration studies. All incubations also contained 5 μM Amplex Red, 12 units ml^{-1} horseradish peroxidase and 45 units ml^{-1} superoxide dismutase. The reaction was initiated by addition of substrate (5 mM succinate, or 5 mM pyruvate plus 3 mM malate); this was followed after 3–4 min by addition of rotenone (2 μM). The increase in fluorescence at an excitation wavelength of 545 nm and emission via Band Pass Filter 600 ± 20 nm was followed in a 3 ml cuvette for 8–10 min with a Sigma spectrofluorometer. The rate of H_2O_2 production was calculated as the change in fluorescence intensity during the linear increase (as described before [18]). Calibration curves were obtained by adding known amounts of freshly diluted H_2O_2 (concentration of stock solution was checked at 240 nm using a molar extinction coefficient of 43.6) to the assay medium. The standard curve was linear in a range up till 900 nM H_2O_2.

2.6. Fatty acid-induced mitochondrial swelling

Fatty acid-induced swelling of freshly isolated mitochondria (0.25 mg) was monitored as change in absorbance at 540 nm using an Aminco DW-2 UV-VIS spectrophotometer as described before [19]. The incubation medium and experimental conditions were the same as described for oxygen consumption experiments with pyruvate (5 mM) and malate (3 mM) as substrates. An initial addition of oleate (200 μM) was followed by 20 μM titrations. Titrations with solvent (50% ethanol) were performed as control to check for spontaneous swelling.

2.7. Lipid peroxidation and oxidative modification of mitochondrial proteins

The level of 4-HNE-adducts was determined by Western blot (principally as described before [20,21] using rabbit polyclonal antibodies detecting mouse 4-HNE-Michael adducts (Calbiochem, San Diego, CA, USA, dilution 1:1000). 4-HNE adduct bands between 30 and 100 kDa were detected and quantified as total absorbance with Odyssey Infrared Imager (LI-COR; Wesburg, Leusden, The Netherlands). To show in-vitro 4-HNE adduct formation, wildtype skeletal

muscle mitochondria were incubated with 0, 64 and 640 μM 4-HNE for 20 min at 37 °C in the medium used for the oxygen consumption measurements.

The amount of protein carbonyls was determined using Oxyblot protein oxidation detection kit (Millipore, Billerica, MA, USA). Mitochondria were 10 times diluted in a buffer containing HEPES (50 mM, pH 7.4), triton-X-100, EGTA (3.6 mM, pH 7.5), EDTA (9 mM, pH 7.5), DTT (50 mM), PMSF (1 mM) and complete protease inhibitor cocktail (EDTA free, Roche Diagnostics, Mannheim, Germany). After 2 h incubation at 4 °C, the samples were centrifuged for 15 min at 17,500*g* at 4 °C. Subsequently, the supernatant was removed and used for Bradford protein determination (Bio-Rad laboratories, Veenendaal, The Netherlands). Five microliters diluted sample (3 μg sample/μl buffer) was added to 5 μl SDS (12%) for denaturation and vortexed. Samples were derivatized by a 10 μl addition of 1 × DNPH, mixed and incubated for 15 min at room temperature where after 7.5 μl of neutralization solution was added. Samples (3 μg protein) were loaded onto a 12% polyacrylamide gel and blotted for 60 min at 100 V. Membranes were blocked in PBS:LI-COR buffer (LI-COR, Wesburg, Leusden, The Netherlands, dilution 1:1) for 1 h and then incubated with primary rabbit antibody against DNPH moieties (Millipore, dilution 1:150) for 1 h. Donkey anti rabbit antibody conjugated with Alexa 488 (LI-COR, dilution 1:10.000) was used as secondary antibody and incubated for 1 h. Protein carbonyl bands between 30 and 100 kDa were detected and quantified with the Odyssey Infrared Imager. Negative control reactions were performed with derivatization control solution, as opposed to the reaction with 2,4-dinitrophenylhydrazine (DNPH). To show in-vitro protein carbonylation, wildtype skeletal muscle mitochondria were incubated in the absence or presence of 25 mM ascorbate and 400 μM Fe^{2+} for 1 h at 37 °C in the same medium as that used for the oxygen consumption measurements.

2.8. Investigating in-vitro endogenous 4-HNE adduct formation

Freshly isolated mitochondria (0.5 mg), wildtype and UCP3(−/−), were incubated for 1 h at 37 °C in mitochondrial respiration buffer supplemented with oligomycin (1 μg/ml) under 4 conditions: 1) succinate (5 mM) in the absence of rotenone. 2) succinate plus linolenic acid (100 μM). 3) and 4) as in 1) and 2) but in the presence of rotenone (2 μg/ml). After 1 h, the solution was centrifuged for 5 min at 15,000*g*. The mitochondrial pellet was washed once with phosphate buffered saline (PBS) and centrifuged again. The final mitochondrial pellet was diluted in PBS and Laemmli sample buffer (Bio-Rad Laboratories) in a 2:1 ratio, obtaining solutions of about 7 μg/μl of protein. Subsequently samples were heated at 100 °C for 4 min, centrifuged at 15,000*g* for 5 min and frozen at −20 °C for subsequent analysis of 4-HNE protein adducts as above. Five independent experimental series were executed.

2.9. Statistics

Statistical analyses were performed using SPSS for Windows 11.0 software (SPSS Inc., Chicago, IL, USA) by unpaired t-test with statistical significance threshold set at $P = 0.05$. Results are presented as means ± SEM.

3. Results

3.1. UCP3, adenine nucleotide transporter, and Oxphos protein expression

We first validated the phenotype of the mice. As seen in Fig. 1A, UCP3 was only detected in the skeletal muscle preparations from wildtype mice. To examine if the lack of UCP3 was compensated for by an increase in adenine nucleotide transporter (ANT) (as has been discussed [22]), we examined the ANT level by immunoblotting, as exemplified in Fig. 1B (upper panel). As seen from the compilation from five preparations from each genotype (lower panel), there was no increase in ANT amount in the UCP3(−/−) mice. Protein levels of the oxidative phosphorylation complexes (Oxphos), a marker for mitochondrial content, were similar in skeletal muscle homogenates of both genotypes (Fig. 1C and Table 1).

3.2. Mitochondrial respiration in the absence or presence of fatty acids

Functionally, we first examined the effect of UCP3 ablation on uncoupled respiration under different conditions. The basal rate of mitochondrial respiration, i.e. the rate that is not coupled to ATP synthesis (i.e. state-4 respiration), was examined in mitochondria incubated with pyruvate plus malate as substrate, in the presence of oligomycin, as exemplified in the first part of the oxygen electrode trace in Fig. 2A. The presence (versus the absence) of UCP1 in (brown-fat) mitochondria leads to a high rate of uncoupled respiration [23–25]. If UCP3 had a similar direct uncoupling effect, a higher respiration rate would have been anticipated in the mitochondria from the wildtype mice than in those from UCP3(−/−) mice. However, we found state-4 respiration rate to be similar in skeletal muscle mitochondria from UCP3(−/−) and wildtype mice (Fig. 2B). Thus, in contrast to UCP1, UCP3 does not seem to possess an innate uncoupling effect—or the capacity is so small that it is not discernable.

All mitochondria respond to fatty acids with increased basal respiration (uncoupling), likely due to fatty acid interaction with the isoform 2 of the ANT [25]. The presence of UCP1 endows mitochondria with a much enhanced response to fatty acids, even when UCP1 is GDP-inhibited [24]. In extension of this, fatty acids have been suggested to be activators of UCP3 [26,27]. We therefore tested whether the presence of UCP3 endowed the skeletal muscle mitochondria with enhanced fatty acid sensitivity with respect to uncoupling.

As expected, palmitate stimulated uncoupled respiration in skeletal muscle mitochondria in a dose-dependent manner (Fig. 2A and C). Calculated EC_{50} values (140 ± 16 vs 147 ± 21 nmol free palmitate, in UCP3(−/−) and wildtype, respectively) and V_{max} values (281 ± 20 vs 284 ± 16 nmol O_2/min/mg, in UCP3(−/−) and wildtype, respectively) were similar in UCP3(−/−) and wildtype mice. Thus, fatty-acid-induced uncoupling was similar in UCP3(−/−) and wildtype mice, excluding a role for fatty acids in inducing an uncoupling activity of UCP3.

3.3. Rate of ROS production

It has been suggested that a function of UCP3 is to diminish the rate of ROS production [4,28]. We therefore measured ROS (in the form of H_2O_2) production rate in wildtype and UCP3(−/−) mice, as exemplified in Fig. 3A.

We first examined ROS production with pyruvate plus malate as mitochondrial substrate. As seen in the compilation in Fig. 3B, we were unable to observe any difference in ROS production rate between mitochondria from wildtype and UCP3(−/−) mice.

We then examined ROS production under conditions of reverse electron flow, i.e. in the presence of succinate without rotenone, is a condition known to lead to a high net superoxide release [19,29]. We both measured H_2O_2 production from Complex I (i.e. succinate in the absence of rotenone) and from Complex III (after rotenone). As seen both in the trace in Fig. 3A and in the compilation in Fig. 3B, we induced a high rate of H_2O_2 production with succinate minus rotenone—but we obtained exactly the same H_2O_2 production rate in muscle mitochondria from wildtype and UCP3(−/−) mice. We find therefore no evidence for a protective role of UCP3 against ROS production.

Formally, this result is in contrast to that of Toime and Brand [28] who reported that they could detect an ≈12% higher rate of H_2O_2 production in muscle mitochondria from backcrossed UCP3(−/−) mice than that from wildtype mice. The experiments of Toime and Brand were conducted under experimental conditions very similar to

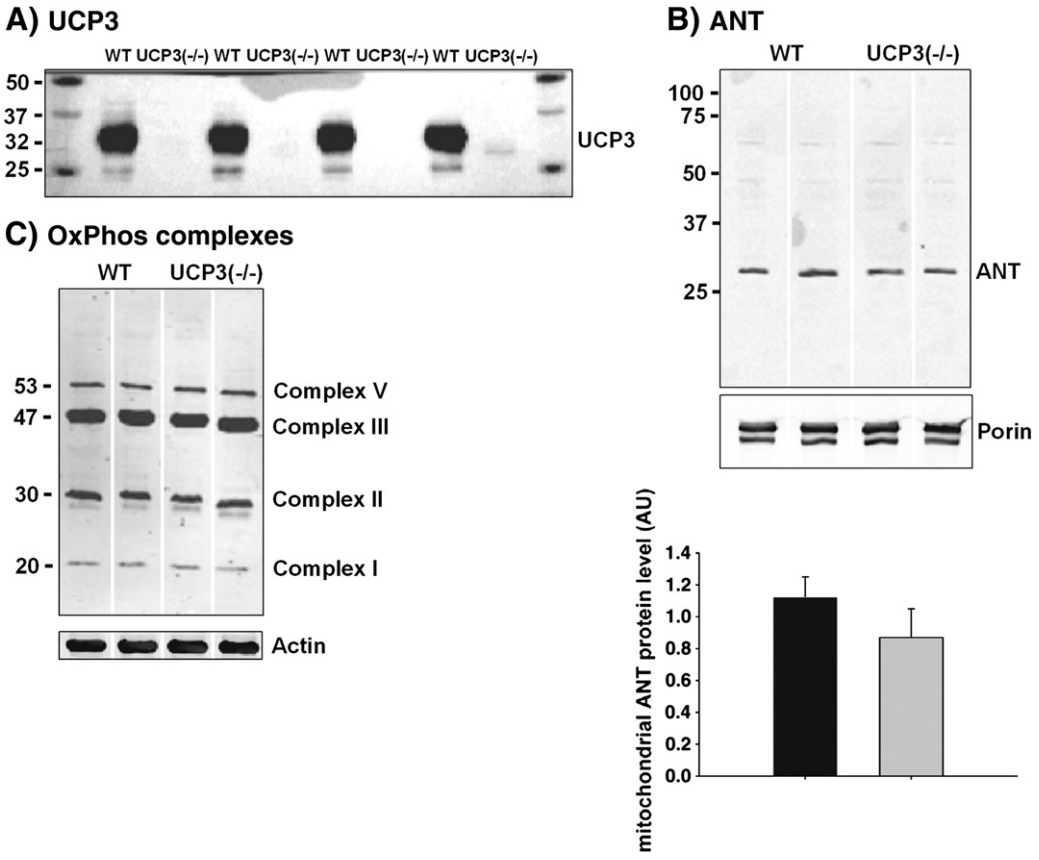

Fig. 1. UCP3 and adenine nucleotide transporter (ANT) protein levels and levels of structural components of the oxidative phosphorylation in skeletal muscle of wildtype (WT) and UCP3(−/−) mice. A) Representative western blot of UCP3 protein levels in skeletal muscle homogenates of WT and UCP3(−/−) mice. As expected, UCP3 was only present in the WT mice and could not be detected in UCP3(−/−) mice. B) Representative western blot (upper panel) and quantification (lower panel) of ANT protein levels in skeletal muscle mitochondria from WT and UCP3(−/−) mice ($n=5$). Black bar represents WT mice and grey bar UCP3(−/−) mice. C) Representative western blot of structural components of oxidative phosphorylation in skeletal muscle homogenates from WT and UCP3(−/−) mice. (Quantification of this western blot is shown in Table 1).

those used here, and whether it is the absence of an effect (as reported here) or a small effect (as reported by Toime and Brand) that is a statistical coincidence is not possible to establish.

3.4. Effect of ROS on UCP3 activity

As mentioned above, it has been suggested that UCP3 may only function as a proton channel when it is activated [3]. In addition to fatty acids tested above as activators (Section 3.2), another suggested activator is superoxide [29,30]. To examine whether the endogenously released superoxide was able to activate UCP3, we incubated the mitochondria under the conditions above, i.e. in the presence of succinate without rotenone, creating a high net superoxide release (as verified in Fig. 3). However, we did not under these conditions detect a difference in mitochondrial respiration rate between UCP3(−/−) and wildtype mice (Fig. 4A and B). Thus, despite equal and high rates of H_2O_2 production, there was no indication that an uncoupling activity of UCP3 was induced.

It has been stated that whereas the suggested activating role of ROS on UCP3 demands the presence of fatty acids if the ROS is *exo*genously produced [30], this is not the case when superoxide is *endo*genously produced [29]. According to this, fatty acids would not be needed in the experiments described above (Fig. 4). To examine the possibility that the reason that we could not observe an enhancing effect of the presence of UCP3 in Fig. 3 anyhow was the absence of dual stimulation by fatty acids and ROS, we tested the effect of further respiratory stimulation with addition of fatty acids, principally as in Fig. 2A, but with succinate (without rotenone) as substrate (Fig. 4A). With palmitate additions corresponding to free palmitate concentrations of 10 and 30 nM, we were again able to stimulate respiration (as in Fig. 2A and C), but the effect of the palmitate additions was (if anything) lower than under the previous conditions (Fig. 2C) where ROS was not present (thus there was no "dual stimulation effect"), and there was no difference between the response in wildtype and UCP3(−/−) mitochondria (Fig. 4C). With higher palmitate concentrations we started to observe confounding inhibitory effects of fatty acids on succinate oxidation, as expected.

Table 1
Muscle content of structural components of oxidative phosphorylation.

	Wildtype	UCP3(−/−)
Complex I	0.24 ± 0.03	0.31 ± 0.03
Complex II	0.53 ± 0.08	0.67 ± 0.09
Complex III	1.27 ± 0.20	1.41 ± 0.15
Complex V	0.16 ± 0.02	0.19 ± 0.03
Total complexes	2.20 ± 0.31	2.55 ± 0.21

Quantification of western blot signal intensity of structural components of the oxidative phosphorylation from skeletal muscle homogenates of wildtype and UCP3(−/−) mice. Values are arbitrary units. Although all complexes showed a tendency to a 10–20% higher content in the preparation from UCP3(−/−), this difference was not significant ($n=5$).

3.5. Acute lipotoxicity

It has been suggested that UCP3 may protect against lipotoxicity [31]. We have here examined two conditions which may be said to exemplify acute lipotoxicity.

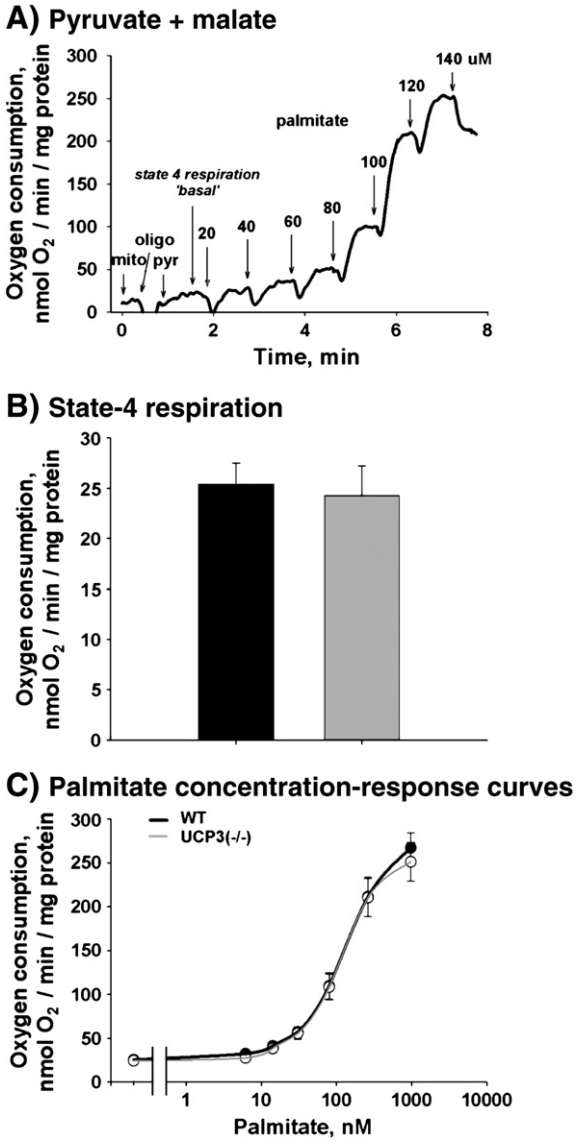

Fig. 2. Mitochondrial respiration in skeletal muscle mitochondria isolated from wildtype and UCP3(−/−). A) Representative recording of basal respiration in wildtype skeletal muscle mitochondria using pyruvate (5 mM) and malate (3 mM) as substrates in the presence of oligomycin (1 μg/ml) and fatty acids. B) The state-4 rate ($n=5$). C) Palmitate concentration-oxygen consumption response curves of wildtype and UCP3 (−/−) mice ($n=5$).

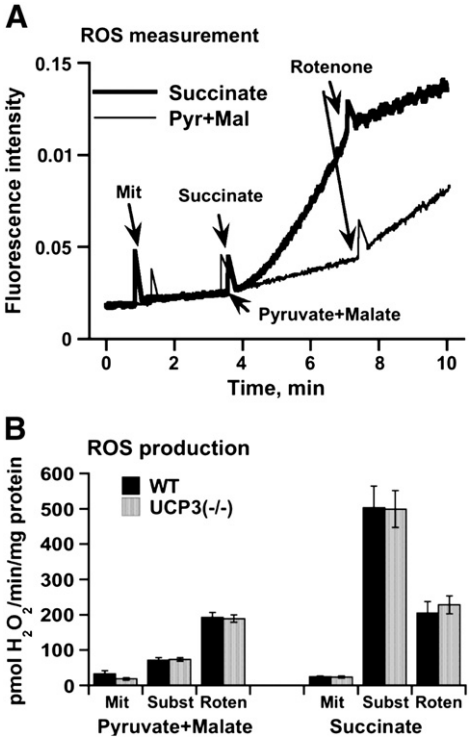

Fig. 3. The rate of ROS production in mitochondria from wildtype and UCP3(−/−) mice. A) Traces (from wildtype mitochondria) of H_2O_2 production after addition of different substrates (5 mM pyruvate plus 3 mM malate, or 5 mM succinate) and 2 μM rotenone. B) Compilation of data obtained from 4 preparations of wildtype and UCP3(−/−) mitochondria, examined as in A). Whereas a tendency to a somewhat longer lagtime after succinate addition could be discerned in UCP3(−/−) mitochondria (not shown), the stable rates of H_2O_2 production were not different between the two types of mitochondria.

3.5.1. Maximal mitochondrial oxidative capacity

First we tested the effect of direct addition of fatty acids on maximal mitochondrial oxidative capacity. We examined muscle mitochondria respiring on pyruvate. In the control condition we added the fatty acid solvent ethanol to the mitochondria and then measured the maximal respiratory capacity as observed after addition of the chemical uncoupler FCCP (Fig. 5A). We then repeated the experiment when FCCP was added after addition of a fairly high dose of fatty acid (Fig. 5B). The addition of this dose of fatty acid led to diminished maximal oxidative capacity (Fig. 5C). However, the absence of UCP3 did not aggravate the inhibitory effect of fatty acids on maximal oxidative capacity (Fig. 5C).

3.5.2. Mitochondrial swelling

The second condition for testing the ability of UCP3 to protect against acute lipotoxicity was fatty acid-induced mitochondrial swelling. We performed experiments as illustrated in Fig. 6A and B. As seen, successive additions of oleate led successively to high amplitude swelling; no spontaneous swelling was seen when mitochondria were incubated for 30 min at 37 °C with solvent (50% ethanol) only (Fig. 6A and B). As compiled in Fig. 6C, the mitochondria from wildtype and UCP3(−/−) mice were equally sensitive to oleate. Maximal swelling as induced by alamethicin was also similar in both groups (not shown). Thus, also in this test, the absence of UCP3 failed to aggravate the lipotoxicity.

3.6. Oxidative stress

To examine whether UCP3 protects against oxidative stress, we examined two measures of oxidative damage.

3.6.1. Lipid peroxidation products

Oxidative stress (ROS production) often leads to lipid peroxidation with 4-hydroxynonenal (4-HNE) being one of the major, highly reactive products [32]. 4-HNE would react irreversibly with certain amino acids on the mitochondrial proteins, leading to accumulation of 4-HNE protein adducts, which can thus be seen as a measure of oxidative damage. We initially demonstrated that oxidative damage can be observed in this way in the system here investigated. For this, we made in-vitro additions of 4-HNE to muscle mitochondria. As seen in Fig 7A, these additions lead to increased levels of adducts in the muscle mitochondria (just as it did in brown-fat mitochondria [20]), verifying that the adducts would be detectable. We have also earlier demonstrated in-vivo that in mice with enhanced levels of the mitochondrial superoxide dismutase, the 4-HNE levels were markedly lowered, demonstrating that levels of adducts in isolated mitochondria reflect in-vivo modifications [13]. That different physiological conditions can lead to altered levels of 4-HNE adducts has been shown both in experimental animals [33,34] and in humans [18].

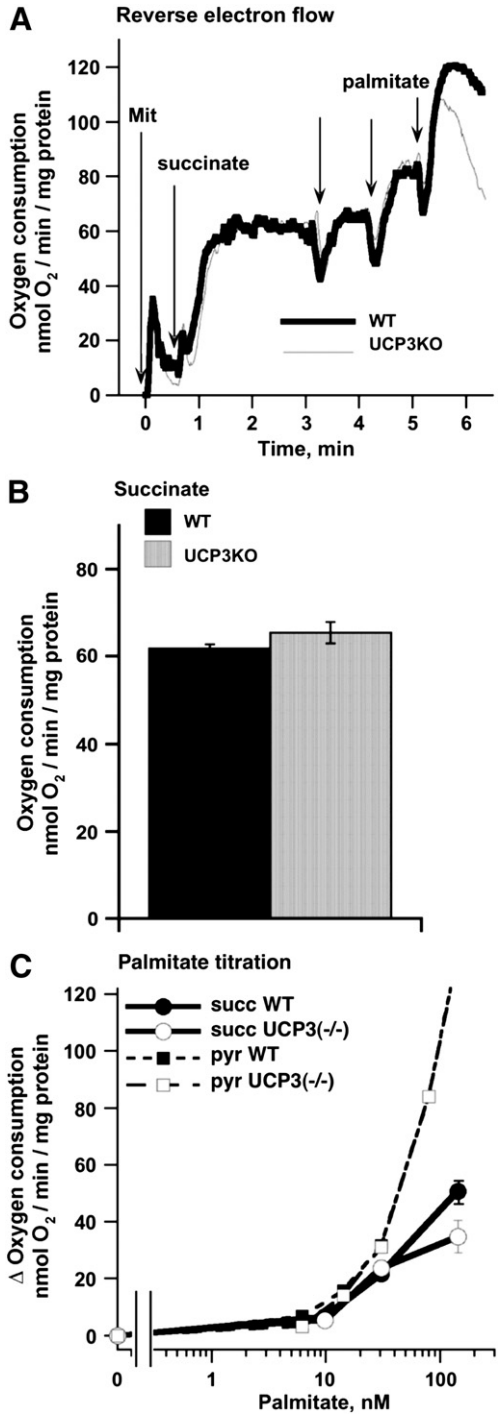

Fig. 4. Effect of ROS production on respiratory rates in mitochondria from wildtype and UCP3(−/−) mice. A) Respiratory traces under conditions of high endogenous ROS production. B) The rate of state-4 measured under reversed electron flow (high endogenous superoxide production) ($n=4$). C) The increase in oxygen consumption caused by added palmitate ($n=4$). The stippled curves indicated by "pyr" (pyruvate) are from Fig. 2C and thus show the uncoupling effect of palmitate in the absence of endogenously produced ROS.

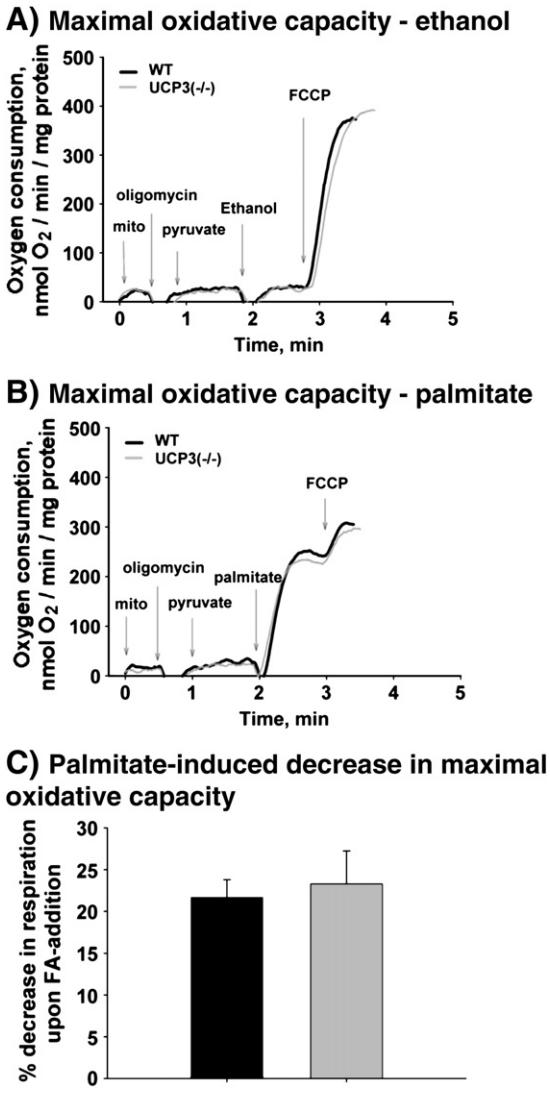

Fig. 5. The effect of palmitate on the maximal oxidative capacity of isolated skeletal muscle mitochondria from wildtype (WT) and UCP3(−/−) mice. FCCP was used to chemically induce uncoupling and to stimulate mitochondria to maximal respiration, at a concentration of 1 μM or 1.5 μM dependent on the amount necessary to induce maximal mitochondrial respiration. A) Representative recordings of maximal mitochondrial oxygen consumption, monitored in presence of pyruvate (5 mM) and malate (3 mM) as substrates. Ethanol (the palmitate vehicle) was used as a control. B) Representative recordings of the effect of palmitate on the maximal oxidative capacity of isolated mitochondria of UCP3(−/−) and wildtype mice. Maximal mitochondrial oxygen consumption was monitored upon addition of 100 μM palmitate in presence of pyruvate and malate as substrate. C) The percentage decrease upon palmitate addition as compared to ethanol was calculated per mitochondrial isolation and averaged per genotype. Black bars represent the percentage decrease for wildtype and gray bars that for UCP3(−/−) mice ($n=5$).

As expected, we observed the presence of 4-HNE protein adducts in skeletal muscle mitochondria from wildtype mice (Fig. 7B). However, the level of these adducts was not increased in the mitochondria from the UCP3(−/−) mice (Fig. 7C).

3.6.2. Protein carbonyl groups

Also formation of protein carbonyls is generally used as a marker of oxidative stress [35]. To verify that oxidative conditions can increase protein carbonylation in these mitochondria, we incubated mitochondria under such conditions (i.e. with Fe^{2+} and ascorbate). As seen in Fig. 7D, this increased the level of carbonylation. Also this marker of oxidative damage was clearly seen in mitochondria from wildtype mice (Fig. 7E), but again no significant increase was seen in mitochondria from UCP3(−/−) mice (Fig. 7F).

3.6.3. Effects of prolonged in-vitro endogenous oxidative stress

To examine whether a protective effect of UCP3 could be manifest under conditions where endogenous formation of 4-HNE adducts was enhanced, we incubated wildtype and UCP3(−/−) mitochondria for 1 h at 37 °C under the combined conditions of high endogenous superoxide production (succinate without rotenone) and added

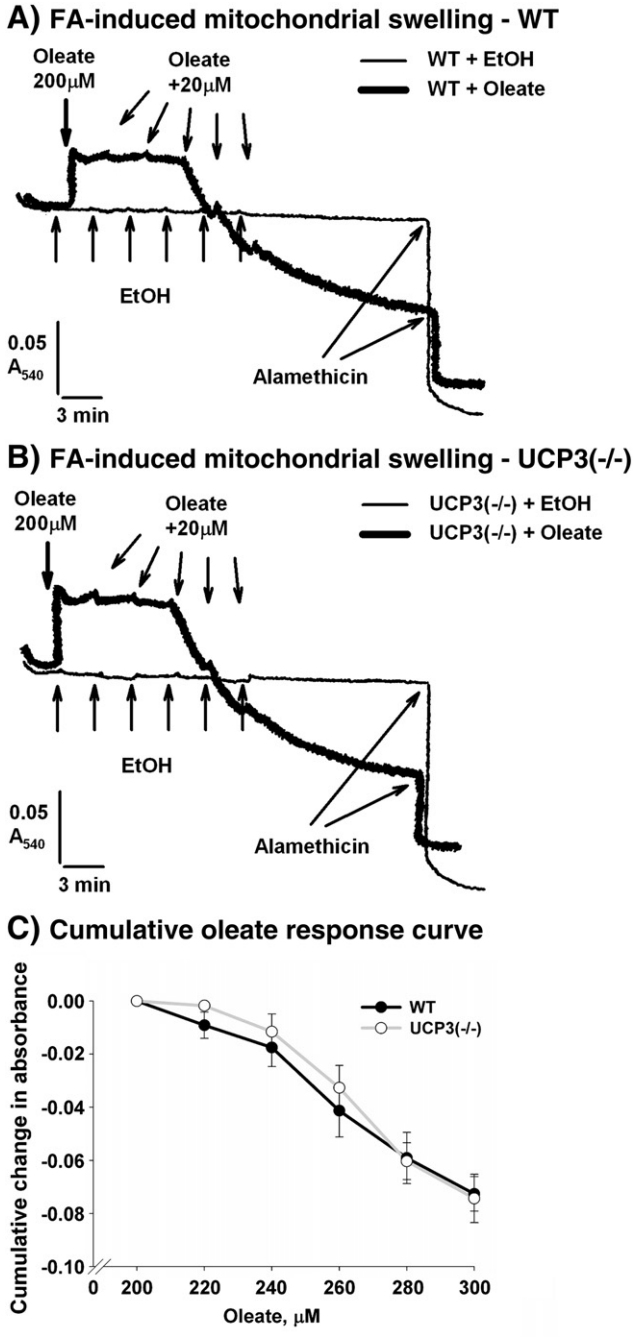

Fig. 6. Mitochondrial membrane swelling upon fatty acid addition. Oleate-induced swelling of skeletal muscle mitochondria from wildtype (WT) (A) and UCP3(−/−) (B) mice. Pyruvate (5 mM) and malate (3 mM) were used as substrates. Ethanol (EtOH, 50%) (solvent) was used as a control to check for spontaneous swelling. C) Cumulative oleate–response curves for wildtype (WT) and UCP3(−/−) mice. Oleate induced mitochondrial swelling, visible as a decrease in absorbance, in concentrations >200 μM. The cumulative decrease in absorbance upon oleate titration (20 μM additions) was calculated and depicted as a function of oleate concentration ($n=5$).

linoleic acid (the precursor of 4-HNE). However, we were not able to see any effect of the presence or absence of UCP3 on the formation of 4-HNE adducts under these conditions (not shown).

4. Discussion

In the present investigation, we have demonstrated that the presence of UCP3 in muscle mitochondria does not in itself increase the basal respiration of the mitochondria, nor the respiration seen after addition of fatty acids. We found that UCP3 did not alter the rate of endogenously produced superoxide and that even in the presence of such superoxide, no effect of the presence of UCP3 on respiratory rate was seen. We found no indication that UCP3 protected the mitochondria against acute lipotoxicity or against oxidative stress under our experimental conditions. Thus, the present experiments do not support a role for UCP3 either as an uncoupling protein, or as a protector against lipotoxicity or oxidative stress.

4.1. UCP3 and uncoupled respiration

We define here the basal respiration as state-4 respiration, i.e. respiration under conditions where ATP synthesis is not taking place. We did not find evidence that UCP3 in itself increased this basal respiration of isolated mitochondria (Fig. 2A and B). This is in contrast to what was observed in one of the original papers on the UCP3(−/−) mouse [36] but is in agreement with the other [12]. However, in these first reports, the UCP3(−/−) mice were not backcrossed. In later studies (where it is not clearly indicated whether the mice were backcrossed or not), an increased basal respiration has also not been observed [30,37–39]. Respiratory studies have also been performed where it has been stated that the muscle mitochondria were from backcrossed UCP3(−/−) mice but the data have not been analyzed for basal respiration [22]. We used a complex I-linked substrate (pyruvate) for this study, in contrast to the studies quoted above that all used succinate (thus, our reported mitochondrial respiratory rates are much lower than those reported elsewhere), but, taken together, it is clear that the absence of effect of UCP3 observed by us is not secondary to the substrate used (i.e. it is not an issue whether succinate could possibly induce a somewhat higher membrane potential than pyruvate).

We have not measured membrane potential here and we can therefore formally not state that UCP3 is not associated with a proton leak. However, in accepted Mitchellian bioenergetics, an increased respiration rate must necessarily be associated with an increased proton leak, everything else being equal. Thus, all these data indicate that UCP3 is not associated with an innate proton leak.

In agreement with the observations here of an absence of effect of ablating UCP3, there is a series of studies that demonstrate that physiologically induced increased levels of UCP3 are not associated with higher rates of basal uncoupled respiration [13,40–45]. Effects of increased UCP3 levels induced by transfection are difficult to evaluate [46] and will not be discussed here.

Earlier tenets that UCP3 (uncoupling) activities could be estimated indirectly as being equal to the inhibitory effect of addition of GDP cannot be discussed presently, as it is now clear that GDP interacts with ANT [39,47,48] (whereas an interaction with UCP3 actually presently is less clear, at least as judged from state-4 respiratory data [13,39,49]). (When calculated as proton leak at a fixed voltage, GDP has been reported to have a UCP3-related inhibitory effect [39]—but, bioenergetically confounding, the decreased proton leak estimated in this way was not associated with a decreased state-4 respiration, and it is therefore difficult to comprehend what such a proton leak measurement represents).

Thus, the conclusion from all the above studies must be that in contrast to what was originally assumed, and in contrast to what is the case for UCP1 [50] (or for ANT isoform 1 [25,51]), UCP3 is not an uncoupling protein, i.e. UCP3 in itself does not innately increase mitochondrial proton leak.

4.2. UCP3 and ROS production

One suggested function of UCP3 is protection against formation of reactive oxygen species (ROS) [28,52]. We have here measured ROS production in the form of H_2O_2 formation under different conditions: forward and reverse electron flow, Complex I- and Complex II-linked

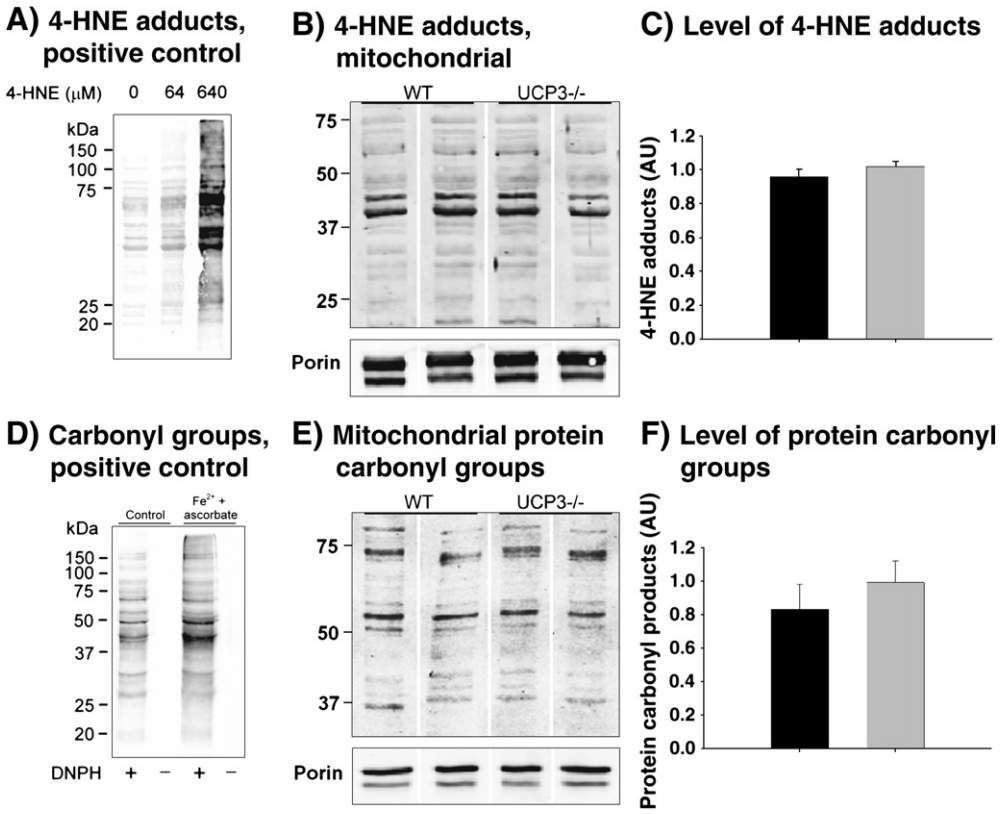

Fig. 7. Oxidative damage measured by Western blotting of skeletal muscle mitochondria from UCP3(−/−) and wildtype (WT) mice. A) Demonstration of in-vitro 4-HNE adduct formation. B) Representative 4-HNE Western blot. C) Quantification of Western blots ($n=4$) of the 4-HNE protein adducts. D) Demonstration of in-vitro protein carbonylation. E) Representative Western blot. F) Quantification of Western blots ($n=5$) of protein carbonyl products. Black bars represent wildtype mice and grey bars UCP3(−/−) mice.

substrates, and from Complex I or Complex III sites (Fig. 3). We have not observed any difference on ROS production between UCP3-containing or UCP3-lacking mitochondria. Seifert et al. [53] also observed identical levels of H_2O_2 production in muscle mitochondria from wildtype and UCP3(−/−) mice. Thus, these results do not support the tenet that UCP3 protects against ROS formation.

The mechanism underlying the suggested protective effect of UCP3 against ROS production has been formulated to be a “mild uncoupling", meaning a small decrease in membrane potential. There is good evidence that a minor decrease in membrane potential leads to reduction in succinate-supported ROS production [54]; such a decrease in membrane potential is necessarily, according to Mitchellian bioenergetics, also leading to increase in respiration (a 90% decrease in ROS production concomitant with a 300% increase in respiration was originally observed [54]). As our condition leading to high ROS production (Fig. 3) did not lead to activation of uncoupling (i.e. increased respiration) (Fig. 4), it cannot have led to decreased membrane potential and thus cannot have counteracted an (further) increase in ROS production. In general, as it does not seem that UCP3 has any uncoupling (membrane potential decreasing) effect, it is difficult to understand how it could reduce ROS production: a novel, not membrane-potential-linked mechanism would need to be formulated (as pointed out earlier [55]).

4.3. UCP3 and induced uncoupled respiration

Although initial views on UCP3 function implied a direct uncoupling effect of the protein, later suggestions have been that it only functions as a proton leak if induced by certain activators. We have here tested fatty acids and reactive oxygen species as activators but we were unable to identify a UCP3-mediated effect of these agents.

4.3.1. Fatty acids as potential UCP3 activity inducers

Particularly based on experiments in reconstituted systems (liposomes and black lipid membranes), it has been suggested that fatty acids could be activators of UCP3 [27]. We examined this possibility here by titrating fatty acids (palmitate) in muscle mitochondria from wildtype and UCP3(−/−) mice. We used pyruvate as substrate for respiration; under the conditions used here the palmitate cannot be a substrate as it cannot be oxidized in the mitochondria in the absence of CoA, ATP and carnitine (and after treatment of the mitochondria with nagarse). We found no differences between the uncoupling activity of fatty acids in wildtype and UCP3(−/−) mitochondria (Fig. 2A and C). These results are principally in agreement with those of Cadenas et al. [37] and those of Lombardi et al. [38]. As Lombardi et al. used the polyunsaturated arachidonic acid as fatty acid, the absence of effect observed by us cannot be ascribed to our use of a saturated fatty acid for the investigation.

In accordance with the present observations, studies of muscle mitochondria with physiologically induced increased levels of UCP3 [13,42] have not indicated an increased ability of fatty acids to uncouple when more UCP3 is present.

Thus, in general, we find there is presently no evidence that endogenous UCP3 can be induced by fatty acids to function as a proton leak over the mitochondrial membrane.

4.3.2. Reactive oxygen species as potential UCP3 activity inducers

Original observations indicated an ability of exogenous superoxide (artificially produced by addition of xanthine + xanthine oxidase) to induce increased proton leakage in mitochondria from wildtype mice, and that these effects were not seen in muscle mitochondria from UCP3(−/−) mice [30]. Superoxide should thus be an inducer of UCP3 uncoupling activity.

As the analysis of the experimental outcome of the xanthine + xanthine oxidase system is somewhat complex [56], we here tested whether induced endogenous superoxide production would display differences between wildtype and UCP3(−/−) mitochondria. Mitochondria respiring on succinate in the absence of rotenone have the highest known levels of endogenous superoxide production. That a high superoxide production is observed under these conditions is well demonstrated [54,57], earlier verified by us in skeletal muscle mitochondria [18,19] and also directly demonstrated here (Fig. 3). However, we did not see any difference in the basal respiratory rate between wildtype and UCP3(−/−) muscle mitochondria when examined under these conditions (Fig. 4B). This observation is principally in agreement with results by Lombardi et al. [38] and thus imply that endogenous superoxide cannot activate an uncoupling activity of UCP3.

Further in line with these observations of an absence of effect of ablation of UCP3, studies examining whether physiologically induced increased levels of UCP3 lead to higher ROS-increased respiratory rates have failed to observe such effects [13,39,58,59].

We conclude that superoxide is unable to activate UCP3-mediated mitochondrial uncoupling under our experimental conditions, suggesting that a function of UCP3 as a protector against oxidative damage via ROS-induced activation of UCP3 is not likely, in contrast to what has been suggested [3,4,60].

We also tested the possibility that a ROS effect would only manifest if ROS were present in combination with fatty acids. We did not observe any such synergic effect (Fig. 4); if anything the uncoupling effect of fatty acids was lower under conditions where ROS was produced (but this may be a technical limitation due to succinate oxidation inhibition by fatty acids).

4.3.3. 4-HNE as a potential UCP3 activity inducer

4-hydroxynonenal (4-HNE) has also been implied to be able to induce UCP3-mediated uncoupled respiration [61]. However, according to that paper, the presence of UCP3 is not essential for the 4-HNE-induced response, and a quantitative difference between the response of wildtype and UCP3(−/−) muscle mitochondria was not clearly discernable. Recent observations confirm this picture [49]. In the present paper, we did not examine 4-HNE as a potential UCP3-activator, but this has recently been done by Aguirre and Cadenas [39]. Their state-4 respiratory data do not show any effect of the presence of UCP3 on HNE-induced respiratory increase in muscle mitochondria, and in our opinion this means that an HNE-induced proton leak is not mediated by UCP3. (Even when estimated as calculated proton leak, it would seem that UCP3 only is responsible for ≈25% of the response to HNE in muscle (Figs. 3–5 in [39])—but, bioenergetically confounding, the proton leak calculated in this way does thus not correspond to an augmented state-4 respiration, and what such a leak measurement represents is therefore difficult to understand). There is no mediation at all of any HNE effect by UCP3 in heart mitochondria, neither measured as state-4 respiration rate, nor as calculated proton leak [39].

Thus, presently there are no data that confirm that UCP3 is an uncoupling protein, neither in its native state, nor when examined in the presence of suggested activators, such as fatty acids, endogenously produced ROS, combinations of these, or 4-HNE.

4.4. UCP3 and protection against lipotoxicity

UCP3 has been suggested to protect against lipotoxicity, e.g. by transporting fatty acids out of the mitochondria. Accordingly, UCP3 expression is elevated under lipotoxic conditions [6,62,63]. We tested here more directly two experimental models of acute lipotoxicity: fatty acid-induced inhibition of oxidation and fatty acid-induced mitochondrial swelling. In neither of these did we see an acute protective effect of the presence of UCP3.

It can clearly not be ruled out that UCP3 under other experimental conditions than those used here can protect against negative effects of fatty acids, but such conditions would need to be defined.

4.5. UCP3 and oxidative stress

UCP3 has been implicated in the protection against oxidative damage (e.g. [6,8–11]). However, we did not observe here any protective effect of UCP3 against oxidative damage. This may be considered remarkable in the context that practically any review on the function of UCP3 (or any introduction part in primary papers on UCP3) states that "UCP3 protects against oxidative damage".

Two original papers are normally quoted for demonstrating that the absence of UCP3 promotes oxidative damage. One is the paper by Vidal-Puig et al. [36] in which the UCP3(−/−) mitochondria also demonstrated a lower basal respiration; as discussed above this difference cannot be observed in backcrossed mice by any group today. Probably, the effects on lucigenin-derived chemiluminescense and on aconitase observed in that paper may also be related to the use of non-backcrossed mice. The second paper is that of Brand et al. [64] where different markers of oxidative damage were examined with CG/MS techniques. They reported that aminoadipic semialdehyde and N-(carboxymethyl)-lysine were significantly higher in the UCP3 underexpressers ($P<0.05$) than in the controls used, and that N-(carboxyethyl)-lysine ($P<0.10$) and glutamic semialdehyde and N-(malondialdehyde)-lysine (both $P<0.15$) tended to be increased in the UCP3 underexpressers, however without the differences reaching normal levels of statistical significance. These were early investigations and the mice were not backcrossed. As the access to mice at that time was limited, the UCP3 underexpressers ($n=6$) were a combined group of homozygotic UCP3(−/−) mice and heterozygotic UCP3(−/+) mice (these likely had diminished levels of UCP3 protein—but it was not absent [37]), and the control group used ($n=9$) was a combined group of the UCP3(+/+) homozygote littermates for these and a group of transgene-negative littermates from a strain of mice that were transgenic overexpressers of UCP3. This paper, which is widely quoted, constitutes the main direct evidence for a protective innate role of UCP3.

In contrast to expectations from these two papers we did not observe any protection by UCP3 against oxidative damage, as examined by three methods: 4-HNE adduct level and amount of protein carbonyls in isolated mitochondria (implied to reflect the level of oxidative damage in-vivo)—or 4-HNE adduct levels following an in-vitro incubation. In agreement with our observation of identical levels of 4-HNE adducts, Seifert et al. [53] observed no increase in 4-HNE adduct levels in UCP3(−/−) muscle mitochondria under normal conditions, although ROS-related effects assigned to UCP3 have been observed in backcrossed UCP3(−/−) mice under certain conditions [28,53].

We are not aware of any studies showing that physiologically induced increases in UCP3 amount are associated with diminished levels of oxidative damage. There are papers that—in their title—imply that a protection against oxidative damage by UCP3 has been observed [65,66]. These papers show that a ROS increase generally is associated with an increased UCP3 gene expression. Thus these papers cannot as such be seen as support for a role for UCP3 against oxidative stress (in reality, these papers may equally well be quoted as indicating a function for UCP3 as an inducer, not protector, of ROS production).

5. Conclusions

Early suggestions for UCP3 function have mainly been based on experiments performed with mice that have not been backcrossed. There is increasing awareness that differences between observations performed on genetically modified mice that are not backcrossed to a single strain may include erroneous outcomes, caused not by true

action of the protein under study but by other strain differences. We have therefore felt it necessary to re-examine some of the basic observations relating to UCP3 function in backcrossed UCP3(−/−) mice. Among the tenets we examined (basal respiration, ROS production, reactive oxygen species- or fatty acid-induced respiration, and protection against acute lipotoxicity and against oxidative damage), we could not confirm that any of these could withstand the tests when performed on isolated mitochondrial from back-crossed mice. Clearly, it cannot be excluded that we may not have chosen conditions that allow the effects studied to be revealed. We conclude that the currently available data do not support a function for UCP3 in mitochondrial uncoupling or protection against acute lipotoxicity or against oxidative damage.

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