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The effect of UCP3 overexpression on mitochondrial ROS production in skeletal muscle of young versus aged mice

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Abstract Uncoupling protein 3 (UCP3) is suggested to protect mitochondria against aging and lipid-induced damage, possibly via modulation of reactive oxygen species (ROS) production. Here we show that mice overexpressing UCP3 (UCP3Tg) have a blunted age-induced increase in ROS production, assessed by electron spin resonance spectroscopy, but only after addition of 4-hydroxynonenal (4-HNE). Mitochondrial function, assessed by respirometry, on glycolytic substrate was lower in UCP3Tg mice compared to wild types, whereas this tended to be higher on fatty acids. State 4_o respiration was higher in UCP3Tg animals. To conclude, UCP3 overexpression leads to increased state 4_o respiration and, in presence of 4-HNE, blunts the age-induced increase in ROS production.

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Keywords: UCP3; ROS; Aging; Mitochondria; 4-HNE; Mild uncoupling

1. Introduction

Based on an inverse correlation between the rate of oxygen consumption and longevity in mammals found in 1908 [1], ‘the free radical theory of aging’ [2–5] suggests that oxidative damage derived from mitochondrial ROS production might play a key role in cellular aging. Although mitochondria are essential in the production of ATP inside the body, they are also a primary source of reactive oxygen species (ROS), which makes them important players in the mammalian aging process. The free radical theory of aging hypothesizes that free oxygen radicals, produced by mitochondria when electrons divert from the mitochondrial electron transport chain and react with molecular oxygen, cause oxidative damage, leading to

mitochondrial and ultimately cellular dysfunction. Thus, higher rates of metabolism are proposed to result in higher amounts of ROS and thereby shorten lifespan.

In contrast to this hypothesis, Skulachev [6] has suggested that mitochondria possess the capacity for so-called ‘mild uncoupling’ a process that would slightly reduce the mitochondrial proton gradient. Since mitochondrial ROS production displays a steep positive relationship to mitochondrial proton gradient [7], a mild reduction of the proton gradient – with only a modest effect on ATP production (mild uncoupling) – would already reduce ROS production markedly. Indeed, already in 1973 it has been shown that reducing the proton gradient by chemical uncouplers and ADP, decreases ROS production in isolated mitochondria [8]. Recent in vivo human measurements underpin the suggestion that the level of mitochondrial coupling rather than respiration rate per se has greatest impact on cellular aging [9].

Uncoupling proteins are likely candidates for mediating mild uncoupling. They are related to the mitochondrial transport proteins and since the discovery of the first uncoupling protein (UCP1) in the 1970s [10], several other isoforms have been reported [11,12]. Since UCP1, which is involved in regulation of adaptive thermogenesis, is only expressed in brown adipose tissue, it was suggested that the skeletal muscle homologue UCP3 might regulate energy expenditure in muscle. However, research conducted so far does not point towards a major role for UCP3 in the regulation of energy expenditure, but rather suggests a role for UCP3 in the protection of mitochondria against lipid-induced oxidative damage, either by facilitating mitochondrial fatty acid export or by affecting ROS production [13–15]. Indeed, H₂O₂ production, measured with a fluorogenic probe, was decreased in L6-muscle cells overexpressing UCP3 [16] whereas UCP3 knockout mice showed decreased aconitase activity, indirectly indicating increased ROS production [17]. Additionally, mice lacking UCP3 in skeletal muscle showed higher oxidative damage to proteins and phospholipids compared to their wild type (WT) controls [18]. Thus, indirect measures of ROS production indicate that UCP3 may affect the level of ROS. In that context it is also interesting to note that marked decreases in UCP3 mRNA and protein expression have been reported with aging [19–21].

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Abbreviations: ROS, reactive oxygen species; UCP, uncoupling protein; ESR, electron spin resonance; UCP3Tg, mice overexpressing human skeletal muscle UCP3; WT, wild type; BSA, bovine serum albumin; TA, tibialis anterior; 4-HNE, 4-hydroxynonenal; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; FCCP, *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone; SOD, superoxide dismutase

Taken together, as ROS production may play a role in aging and could be affected by UCP3 function, we here compare mitochondrial ROS production and respiration in young and aged UCP3 overexpressing mice with WT littermates. We have previously shown that mice overexpressing UCP3 had lower body weights and increased AMPK activity when compared to WT littermates. In addition, the same strain of mice have been shown to have elevated metabolic rate, lower fasting plasma glucose and insulin levels, and improved glucose clearance compared to WT control animals [22,23]. Here we focused on the measurement of ROS production by means of electron spin resonance (ESR) spectroscopy, which allows the direct measurement of superoxide production from isolated mitochondria. The data indicate that UCP3 overexpression blunts the age-related increased ROS production and mitochondrial dysfunction.

2. Materials and methods

2.1. Animals

Twelve young male C57Bl6 mice overexpressing human skeletal muscle UCP3 (UCP3Tg) together with 10 WT littermate control animals (aged: 25 ± 5 weeks) and 5 UCP3Tg mice together with 5 WT littermates (aged: 75 ± 5 weeks) were used. Creation of UCP3Tg mice has been previously described [22]. Animals were housed individually on a 12:12 h light-dark cycle (light from 0700 to 1900 h), at $21\text{--}22^\circ\text{C}$ and allowed unlimited access to standard chow and tap water. All experiments were approved by the Institutional Animal Care and Use Committee of the Maastricht University and complied with the principles of laboratory animal care.

2.2. Tissue collection

Mice were anaesthetized by a mixture of 79% CO_2 and 21% O_2 and sacrificed by cervical dislocation. Skeletal muscle from both hind limbs (~ 2.0 g) was rapidly dissected and placed into ice-cold mitochondrial isolation medium (10 ml) containing 100 mM sucrose, 50 mM KCL, 20 mM K^+ -TES, 1 mM EDTA, and 0.2% (w/v) bovine serum albumin (BSA) [24]. The tibialis anterior (TA) muscle was held separate and frozen for further analysis as described previously [25], whereas remaining muscle was used for mitochondrial ROS production and respiration measurements.

2.3. UCP3 protein expression

Endogenous and human UCP3 protein content in TA-muscle was determined by Western blotting, using a rabbit polyclonal antibody detecting both mouse and human UCP3 (code 1338, kindly provided by L.J. Sliker, Eli Lilly) [26]. The UCP3 protein band was visualized by chemiluminescence and analyzed by densitometry using Image Master (Pharmacia Biotech, Roosendaal, The Netherlands).

2.4. 4-HNE adducts protein expression

Protein adducts of the lipid peroxidation byproduct 4-hydroxynonenal (4-HNE) were determined as marker of lipid peroxidation. Western blotting was performed in TA-muscle using a rabbit polyclonal antibody detecting 4-HNE-Michael adducts (Calbiochem, VWR International BV, Amsterdam, The Netherlands). Protein bands between 30 and 100 kDa were quantified using an Odyssey Near Infrared Scanner (Licor).

2.5. Mitochondrial isolation

Skeletal muscle mitochondria were isolated as described earlier [24]. Shortly, tissue was kept in isolation medium and cooled on ice at all times. First, tissue was finely minced with scissors and a mechanical Potter homogenizer in the presence of a proteinase (Subtilisin, 0.7 mg/g tissue, Sigma–Aldrich, St. Louis, MO, USA). Volumes were adjusted to ~ 35 ml with isolation medium. Then, homogenates were centrifuged at $8500 \times g$ for 10 min at 4°C using a Beckman J2-MC

centrifuge. The resulting pellets were resuspended, homogenized by hand in a Potter homogenizer and centrifuged at $800 \times g$ for 10 min at 4°C . Subsequently, the supernatant was centrifuged at $8500 \times g$ for 10 min at 4°C . The final concentrated mitochondrial pellets were gently resuspended by hand-homogenization in a small glass homogenizer with a Teflon pestle in a small volume of isolation medium. Mitochondrial protein concentrations were determined using fluorescamine (Fluram[®], Fluka, Zwijndrecht, The Netherlands) with BSA as a standard [27]. Subsequently, the freshly isolated mitochondria were used immediately for both ESR spectroscopy and respirometry.

2.6. Mitochondrial ROS production

ESR spectroscopy in combination with the spin trappings technique was used to measure mitochondrial ROS production as described previously by Hoeks et al. [24]. Freshly isolated mitochondria were diluted (0.2 mg/ml protein concentration) in respiration medium (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% of BSA) and incubated for 5 min at 37°C . Mitochondria treated with 30 μM of a potential UCP3 activator, 4-HNE, were pre-incubated for 5 min on ice before incubation at 37°C . Immediately after incubation, 100 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (Sigma–Aldrich, St. Louis, MO, USA) and complex I and complex II substrates (3 mM malate, 10 mM glutamate and 10 mM succinate) were added. DMPO- OH^\bullet signals were measured on a Bruker EMX 1273 and quantification of the spectra was performed by peak area measurements of DMPO- OH^\bullet spectrum using the WIN-EPR spectrum program. Values are expressed as percentage of the average radical signal of the 25-week-old WT mice.

2.7. Mitochondrial oxygen consumption

Mitochondrial respiratory rates were measured as described before [24] using a two-chamber Oxygraph (Oroboros[®] Instruments, Innsbruck, Austria). Briefly, freshly isolated mitochondria (0.2 mg mitochondrial protein for pyruvate/glutamate + succinate and 0.5 mg for carnitine + palmitoyl-CoA) were incubated in a respiration medium with malate (3 mM). Parallel to the ESR experiments, in the same isolated mitochondrial population, glutamate (10 mM) + succinate (10 mM) were added in an attempt to mimic formation of intermediates of the citric acid cycle as is naturally the case in vivo. Additionally, experiments with pyruvate (5 mM), as a glycolytic substrate, were performed and a combination of carnitine (2 mM) + palmitoyl-CoA (50 μM) was used as fatty acid substrate. Addition of ADP (450 μM) initiated state 3 respiration whereas state 4o respiration was measured as oligomycin (1 $\mu\text{g}/\text{ml}$) blocked respiration. Finally, maximal oxygen flux (state uncoupled) was obtained by titration of the chemical uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The oxygen concentration was recorded at 0.5 s intervals using the acquisition software DatLab4 (Oroboros[®] Instruments). The first derivative of the oxygen tension changes in time was displayed as oxygen flux and mean values during about 1 min were obtained from these recordings for calculation of stable oxygen flux rates. Oxygen flux per mg mitochondria was expressed as percentage of maximal mitochondrial uncoupling by FCCP.

2.8. Statistical analyses

Results are presented as means \pm S.E. Statistical analysis was performed using SPSS for Windows 11.0 software (SPSS Inc., Chicago, IL, USA) with statistical significance set at $P < 0.05$. Interaction (age * genotype) effects were performed using a two-way ANOVA (2×2 factorial experiment) with univariate analysis of variance.

3. Results

3.1. UCP3 overexpression

UCP3Tg mice showed ~ 12 -fold higher levels of total UCP3 protein (human + endogenous) compared to their WT controls (Fig. 1, 0.15 ± 0.05 versus 1.82 ± 0.14 AU, $P < 0.001$) irrespective of age.

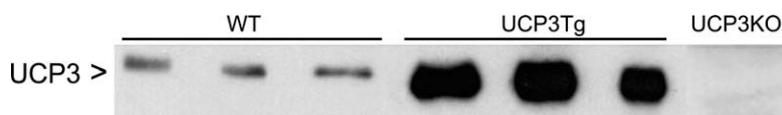


Fig. 1. Total UCP3 protein levels in tibialis anterior muscle of mice overexpressing UCP3 and WT mice. A 12-fold increase in total UCP3 levels is found in the overexpressing mice, compared to their WT littermates. Skeletal muscle from a UCP3 knock out mice (UCP3KO) is used as negative control.

3.2. Lipid peroxidation

Western blotting showed that UCP3Tg mice had lower levels of 4-HNE protein adducts compared to Wt mice, however this did not reach significance (Fig. 2, 0.87 ± 0.33 versus 1.05 ± 0.46 in UCP3Tg and Wt resp., $P = 0.27$). In addition, a significant age-induced increase in 4-HNE protein adducts was found (0.85 ± 0.40 versus 1.17 ± 0.36 in young versus old, respectively, $P < 0.05$).

3.3. Mitochondrial superoxide anion radical production

Mitochondrial ROS production was measured by ESR spectroscopy. In Fig. 3A, a representative spectrum for a superoxide anion radical derived DMPO-OH[•] signal is depicted. Experiments performed in the presence of superoxide dismutase (SOD) (1000 U/ml) completely inhibited the signal, showing that the signal was derived from O₂ (data not shown). Quantification of all spectra showed that ROS production increases with age, both when measured in the presence (Fig. 3B, $P < 0.01$) or absence (Fig. 3C, $P < 0.01$) of 4-HNE. UCP3 overexpression alone did not lead to significant differences in ROS production. However, in the presence of 4-HNE, UCP3Tg mice showed significantly decreased ROS production ($P < 0.01$). Additionally, a significant genotype * age effect interaction ($P < 0.05$) was found, with a blunted increase in ROS production with age in UCP3Tg mice compared to their WT-controls.

3.4. Mitochondrial respiration

Mitochondria of UCP3Tg mice, fuelled by glutamate and succinate, showed decreased ADP-driven state 3 respiration

compared to WT-controls ($P = 0.01$), but no age related changes were found (Table 1). In contrast, state 3 respiration on palmitoyl-CoA and carnitine tended to be higher in transgenic mice ($P = 0.08$), again without an age effect. State 3 respiration upon pyruvate did not show a genotype effect ($P = 0.18$), but tended to decrease with age ($P = 0.06$). State 4o respiration (Table 2) was found to be increased in UCP3Tg mice, both on glutamate and succinate ($P < 0.01$) and palmitoyl-CoA + carnitine ($P < 0.05$), while the increase in state 4o on pyruvate did not reach statistical significance ($P = 0.13$). Again no age related effects on state 4o respiration were found.

4. Discussion

Mild uncoupling has been suggested as a way to lower mitochondrial ROS production, thereby preventing lipid peroxidation and mitochondrial damage. UCP3, an uncoupling protein mainly present in skeletal muscle, has been suggested to mediate this mild uncoupling and the further detrimental effects of these ROS. Several studies already indicated that UCP3 is able to lower ROS production. Furthermore, in vitro evidence suggests the existence of a negative feedback mechanism in which UCP3 can be activated by lipid peroxide intermediates, such as 4-HNE. Of note, UCP3 levels have been reported to decline with aging, a condition characterized by increased ROS production and oxidative damage. In the present study, we therefore examined the effect of UCP3 overexpression on mitochondrial ROS production and mitochondrial function and in young versus aged mice as compared to their WT littermates. We here show that overexpression of UCP3, after activation by 4-HNE, may be able to blunt the age-induced increase in mitochondrial ROS production.

To determine ROS production, levels of O₂^{•-} production were measured via ESR spectroscopy. We found that ROS production increases with age, which is in line with the age-related increases in H₂O₂ production [28–30], frequency of cytochrome c oxidase deficient human muscle fibers and cardiomyocytes [31,32] and accumulation of mtDNA mutations [33] that have been shown before. Interestingly, the present study also shows that UCP3 overexpression alone does not lead to significant differences in ROS production compared to WT mice, but that the presence of UCP3-activator 4-HNE resulted in a significantly decreased ROS production in the overexpressing mice. In addition, a significant genotype * age effect interaction was found, with a blunted increase in ROS production with age in mice overexpressing UCP3. These results are in accordance with previous studies that found negative correlations between the amount of UCP3 and ROS production, although none of these studies directly measured ROS production. Studies in UCP3 knockout mice showed decreased activity of aconitase, an enzyme sensitive to ROS-induced inactivation [17]. Furthermore, increased oxidative damage

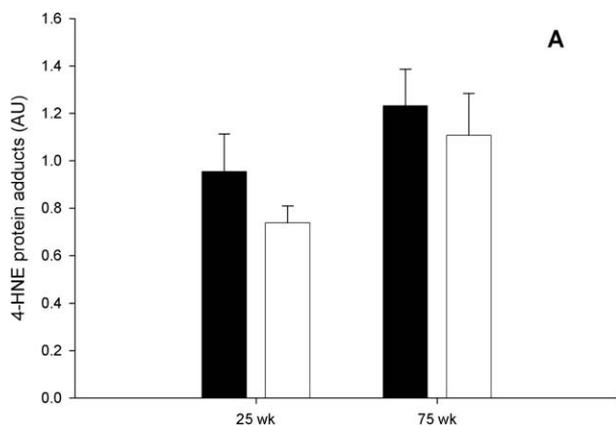


Fig. 2. Quantification of western blots of 4-HNE protein adducts in tibialis anterior muscle of 25 and 75-week-old animals. No significant differences in 4-HNE protein adducts between genotypes were found (0.87 ± 0.33 versus 1.05 ± 0.46 in UCP3Tg and Wt, respectively, $P = 0.27$), however, a significant age-induced increase (A) was found (0.85 ± 0.40 versus 1.17 ± 0.36 in young versus old, respectively, $P < 0.05$). Values are expressed as means \pm S.E.

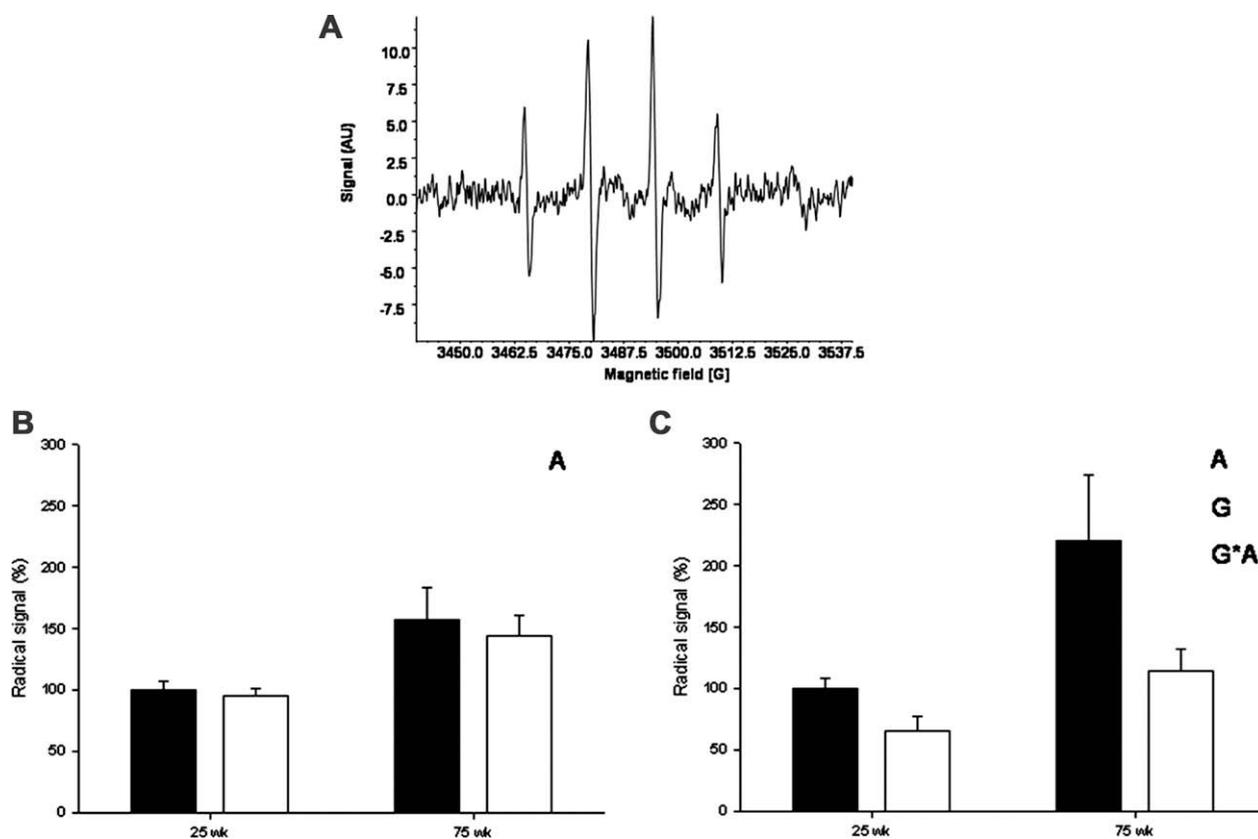


Fig. 3. Mitochondrial ROS production in isolated skeletal muscle mitochondria of 25 and 75-week-old animals: (A) representative example of a superoxide anion radical derived DMPO-OH[•] spectrum, (B) mitochondrial superoxide anion production on glutamate + succinate without, and (C) with addition of 4-HNE in WT mice (black bars) and UCP3 overexpressing mice (white bars). A significant age-effect (A) on ROS production was found without ($P < 0.01$) and with ($P < 0.01$) 4-HNE. ROS production with 4-HNE was lower in the UCP3Tg animals (G) ($P < 0.01$) with a significant genotype * age effect (G * A) ($P < 0.05$). The radical signal of the 25-week-old WT mice is set to 100%. Values are expressed as means \pm S.E.

Table 1
State 3 respiration in isolated mitochondria.

	25 weeks		75 weeks		P-value		
	WT	UCP3Tg	WT	UCP3Tg	Age	Genotype	Genotype * age
Glutamate + succinate	89.8 \pm 2.1	79.8 \pm 2.0	91.6 \pm 4.5	81.8 \pm 3.7	0.56	0.01	1.00
Pyruvate	75.5 \pm 3.4	74.2 \pm 2.4	72.2 \pm 2.2	65.3 \pm 1.3	0.06	0.18	0.35
PalmCoA + carnitine	71.8 \pm 1.4	77.6 \pm 1.1	73.0 \pm 2.7	75.5 \pm 3.3	0.91	0.08	0.43

State 3 respiration on different substrates in UCP3 overexpressing and wild type mice at 25 and 75 weeks of age. Values are expressed as percentage of maximally uncoupled respiration (means \pm S.E.).

to proteins and phospholipids has been found in mice lacking UCP3 [18]. The same study did not show altered oxidative damage in UCP3 overexpressing mice, whereas cell-studies with UCP3-infected L6 myotubes did show decreased H₂O₂ production, as measured with fluorescent probes [16]. Interestingly, we only find a lowering effect of increased levels of UCP3 on ROS production in the presence of 4-HNE. 4-HNE has been suggested to activate UCP3 in a negative feedback mechanism [34]. Thus, ROS would interact with polyunsaturated fatty acids, forming reactive aldehydes such as 4-HNE. These aldehydes would then activate UCP3 reducing further peroxidation of lipids and thereby preventing further downstream detrimental effects. Our finding that the age related increase in ROS production is blunted by overpres-

sion of UCP3 upon addition of 4-HNE, fits this hypothesis and suggests that UCP3 indeed has the ability to prevent lipid-induced oxidative damage. Additionally, we measured 4-HNE protein adducts as a marker for lipid peroxidation in TA muscle. Indeed, in agreement with ROS production data, lipid peroxidation also increased with age. However, UCP3 overexpression did not blunt the age-induced increase in 4-HNE levels, although 4-HNE levels seemed to be somewhat lower in mice overexpressing UCP-3. It should be noted that we could only determine 4-HNE levels in whole muscle homogenates, whereas the effect of UCP3 overexpression might be more specific for mitochondrial fractions.

Additionally, we assessed mitochondrial function of isolated skeletal muscle mitochondria using high-resolution

Table 2
State 4o respiration in isolated mitochondria.

	25 weeks		75 weeks		P-value		
	WT	UCP3Tg	WT	UCP3Tg	Age	Genotype	Genotype * age
Glutamate + succinate	13.8 ± 0.2	19.8 ± 1.1	15.8 ± 0.6	18.5 ± 0.1	0.58	<0.001	0.04
Pyruvate	4.2 ± 0.3	4.8 ± 0.3	4.8 ± 0.4	5.0 ± 0.0	0.44	0.13	0.73
PalmCoA + carnitine	13.5 ± 0.3	14.4 ± 0.9	13.6 ± 0.4	15.3 ± 0.5	0.30	0.03	0.49

State 4o respiration on different substrates in UCP3 overexpressing and wild type mice at 25 and 75 weeks of age. Values are expressed as percentage of maximally uncoupled respiration (means ± S.E.).

respirometry. With respect to aging, we show a tendency to decreased mitochondrial state 3 respiration on pyruvate in mitochondria of aged mice, but not when glutamate and succinate, or palmitoyl-CoA was used as substrates. This might suggest that the age-related decrease in mitochondrial state 3 respiration is substrate dependent, which would be in accordance with earlier findings that show decreased mitochondrial enzymatic activity with aging at complex I and IV, but not at complex II and III (reviewed by [35]).

State 4o respiration was increased in the UCP3 overexpressing mice as shown before [22]. Furthermore, overexpression of human UCP3 tended to increase mitochondrial state 3 respiration on a fatty acid substrate, but decreased state 3 respiration on glutamate and succinate and remained unchanged on pyruvate. Earlier cell studies also showed that overexpression of UCP3 increased fatty acid oxidation capacity, whereas glucose oxidation rates remained unaffected, thereby suggesting a role for UCP3 in fatty acid metabolism [16,36]. In addition, UCP3 overexpressing animals showed significantly reduced respiratory exchange ratio with increased expression of proteins involved in fatty acid metabolism [37]. Taken together, these studies point towards a role for UCP3 in preferential stimulation of fat oxidation.

Despite an age-induced increase in ROS, we did not find any age-induced changes in state 4o respiration. This might suggest that other processes next to mitochondrial uncoupling are able to contribute to the process of aging and ROS production. However, it should also be noted that state 4o is possibly not a sensitive measure of mitochondrial uncoupling, which would require the simultaneous measurement of proton gradient. Using such methodologies, other studies did report increased proton leak in UCP3 overexpressing mice [22] and decreased proton leak in isolated mitochondria from UCP3 knockout mice [17,38]. Since only a small change in membrane potential can already result in large changes in ROS production [7], it might very well be that UCP3 lowers the mitochondrial membrane potential via mild uncoupling.

Next to aging, UCP3 also seems to be involved in several diseases. We have previously shown that UCP3 is reduced by ~50% in human type 2 diabetic patients [39] and patients with COPD [40]. In both diseases, mitochondrial dysfunction has been suggested to play a prominent role. Our finding that UCP3 – when activated by 4-HNE – may lower ROS production may suggest that the lower levels of UCP3 in COPD and T2DM may lead to an accelerated aging process in skeletal muscle of these patients. However, further studies are warranted to prove this hypothesis.

In conclusion, in the present study, we showed that mitochondrial ROS production increases with age and that UCP3 overexpression, but only in the presence of HNE, is able to

diminish this age-induced increase. This might suggest that UCP3 induces mild uncoupling, when activated by 4-HNE.

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