FEBS Letters 582 (2008) 4147–4152

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

Miranda Nabben[a], Joris Hoeks[a], Jacob J. Briedé[b], Jan F.C. Glatz[c], Esther Moonen-Kornips[a], Matthijs K.C. Hesselink[d], Patrick Schrauwen[a,*]

[a] *Departments of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, P.O. Box 616, 6200 MD, Maastricht, The Netherlands*
[b] *Departments of Health Risk Analysis and Toxicology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, The Netherlands*
[c] *Department of Molecular Genetics, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, The Netherlands*
[d] *Human Movement Sciences, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, The Netherlands*

Received 26 September 2008; revised 4 November 2008; accepted 12 November 2008

Available online 27 November 2008

Edited by Vladimir Skulachev

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 4o respiration was higher in UCP3Tg animals. To conclude, UCP3 overexpression leads to increased state 4o respiration and, in presence of 4-HNE, blunts the age-induced increase in ROS production.

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, H_2O_2 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].

*Corresponding author. Fax: +31 43 3670976.
E-mail address: p.schrauwen@hb.unimaas.nl (P. Schrauwen).

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-pyrolline N-oxide; FCCP, *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone; SOD, superoxide dismutase

0014-5793/$34.00
doi:10.1016/j.febslet.2008.11.016

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 21–22 °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. Slieker, 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· signals were measured on a Bruker EMX 1273 and quantification of the spectra was performed by peak area measurements of DMPO-OH· 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 μg/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 ± 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.