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Mitochondrial function, content and ROS production in rat skeletal muscle: Effect of high-fat feeding

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Abstract A high intake of dietary fat has been suggested to diminish mitochondrial functioning in skeletal muscle, possibly attributing to muscular fat accumulation. Here we show however, that an 8-week high-fat dietary intervention did not affect intrinsic functioning of rat skeletal muscle mitochondria assessed by respirometry, neither on a carbohydrate- nor on a lipid-substrate. Interestingly, PPARGC1A protein increased by ~2-fold upon high-fat feeding and we observed inconsistent results on different markers of mitochondrial density. Mitochondrial ROS production, assessed by electron spin resonance spectroscopy remained unaffected. Intramyocellular lipid levels increased significantly illustrating that a reduced innate mitochondrial function is not a prerequisite for intra-muscular fat accumulation.

Keywords: Skeletal muscle; Mitochondria; PGC-1; ROS; High-fat diet; Lipid

1. Introduction

Maintenance of a proper mitochondrial function is essential for cellular function. In recent years, the interest in skeletal muscle mitochondrial function has risen due to the findings of mitochondrial dysfunction in type 2 diabetes mellitus, as well as in the process of aging. Thus, both aging and type 2 diabetes have been associated with (1) decreases in the expression of genes involved in mitochondrial oxidative metabolism [1–4], (2) a decreased expression of PPARGC1, the major transcriptional coactivator regulating the expression of this OXPHOS gene set [3–6], (3) a decreased muscular ATP synthesis [7–10], (4) a functional impairment in mitochondrial respiration [11,12], (5) aberrations in mitochondrial morphology and -density [1,13,14], (6) reductions in mitochondrial enzymes and the activity of the electron transport chain [11,13–16] and (7) increased mitochondrial ROS production and mitochondrial DNA (mtDNA) damage [17–19]. Altogether these findings suggest that a reduction in mitochondrial function indeed has a negative impact on health.

The factors that impede mitochondrial function in skeletal muscle are incompletely understood. However, lifestyle factors such as physical inactivity and/or an increased supply of fat to the muscle have been suggested to underlie mitochondrial dysfunction. In this context, we and others have shown that an acute elevation of circulating plasma NEFA levels in humans reduces the gene expression of PPARGC1A [20,21]. In a more chronic approach, Sparks et al. [22] revealed that a 3-day high-fat diet decreased the expression of oxidative genes, as well as *PPARGC1*, in healthy human individuals. A separate experiment in mice showed similar results at the protein level after a 3-week high-fat dietary intervention [22]. These observations indeed suggest that high dietary fat intake may be at the basis of the reduced mitochondrial function that is frequently reported in aging and type 2 diabetes mellitus. However, the reports mentioned above have only used surrogate markers of mitochondrial function (also reflecting density) that do not address true intrinsic mitochondrial function upon chronic high-fat feeding. Therefore, the first aim of the present study was to examine the hypothesis that an 8-week high-fat dietary intervention causes intrinsic impairments in isolated rat skeletal muscle mitochondria.

In the process of mitochondrial ATP synthesis, formation of reactive oxygen species (ROS) is an inevitable event that can be significantly enhanced by excessive fuel supply or functional impairment of one or more complexes of the respiratory chain [23]. If a high fat supply to the muscle induces mitochondrial dysfunction, one may anticipate that mitochondrial ROS production increases, further aggravating mitochondrial damage and dysfunction. Therefore, the second aim of present study was to test the hypothesis that 8 weeks of high-fat feeding in rats enhances ROS production in skeletal muscle mitochondria, measured in a direct manner by electron spin resonance (ESR) spectroscopy.

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2. Methods

2.1. Animals and diets

Male Wistar rats ($n = 18$, 6 week, Charles River) were housed individually on a 12:12 h light–dark cycle at 21–22 °C and randomly subjected to either a low- or high-fat diet (10% vs. 47% energy from fat, respectively) for the duration of 8 weeks. Diets were purchased from Hope Farms (Woerden, The Netherlands, Low-fat: 4068.10; High-fat: 4031.17) and contained all essential nutrients. Diets and tap water were provided ad libitum and food intake and body mass were recorded weekly. To calculate net energy intake during the diet intervention, faecal samples were collected during the last week of the intervention period, freeze-dried and together with samples from the diet, analyzed for gross energy content using adiabatic bomb calorimetry (Ika-calorimeter system C4000 Heitersheim, Germany). 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

After the dietary intervention, rats were anaesthetized for 1 min by a mixture of 79% CO_2 and 21% O_2 and killed by cervical dislocation. The left gastrocnemius muscle (mostly comprised of type 2 muscle fibers) was rapidly dissected and placed into ice-cold mitochondrial isolation medium containing 100 mM sucrose, 50 mM KCl, 20 mM K^+-TES, 1 mM EDTA and 0.2% (w/v) bovine serum albumin (BSA). The midbelly region of the contralateral gastrocnemius muscle was dissected and frozen as described before [24]. Samples were stored at −80 °C until further analysis.

2.3. Mitochondrial isolation

Skeletal muscle mitochondria were isolated as described earlier [25] with slight modifications. Briefly, tissues were freed of adipose and connective tissue, finely minced with pre-cooled scissors and homogenized in a Potter homogenizer with a Teflon pestle in the presence of proteinase Nagarse (Fluka, Zwijndrecht, The Netherlands; 1 mg per g of tissue). Tissue homogenates were centrifuged at $8500 \times g$ for 10 min at 4 °C using a Beckman J2-MC centrifuge and the resulting pellets were resuspended and subsequently centrifuged at $800 \times g$ for 10 min. Then, the supernatants were centrifuged at $8500 \times g$ for 10 min after which the final mitochondrial pellets were resuspended by hand-homogenization in a small glass homogenizer in approximately 150 μl isolation medium. The concentration of mitochondrial protein was measured using fluorescamine (Fluram®, Fluka, Zwijndrecht, The Netherlands) with BSA as a standard [26] and the remaining mitochondria were stored as described before [25].

2.4. Oxidative phosphorylation in isolated mitochondria

Freshly isolated skeletal muscle mitochondria (0.2 mg of mitochondrial protein for pyruvate/glutamate + succinate and 0.5 mg for carnitine + palmitoyl-CoA) 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, 3 mM malate and 0.1% of BSA. The substrates used were 5 mM pyruvate, 2 mM carnitine plus 50 μM palmitoyl-CoA and 10 mM glutamate plus 10 mM succinate (state 2 respiration). State 3 respiration was initiated by addition of 450 μM ADP. State 4 respiration was measured as the residual respiration following addition of 1 μg/ml oligomycin. Maximal oxygen flux rates (state uncoupled) were obtained by titration with 0.25 μM additions of the chemical uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). All substrates were dissolved in double distilled water while FCCP and oligomycin were dissolved in 96% ethanol. Pilot experiments showed that ethanol in itself did not have any effects on the parameters measured. Mitochondrial respiratory rates were measured at 37 °C by polarographic oxygen sensors in a two-chamber Oxygraph (OROBOROS® Instruments, Innsbruck, Austria).

2.5. Electron spin resonance (ESR) spectroscopy for mitochondrial ROS detection

Detection of mitochondrial ROS production was based on previous publications [27–29]. Freshly isolated skeletal muscle mitochondria (0.1 mg/ml protein) in medium used for oxygen flux measurement without malate were incubated 5 min at 37 °C. Subsequently, 100 mM 5,5-dimethyl-1-pyrolline *N*-oxide (DMPO, Sigma–Aldrich, St Louis, MO, USA), further purified as previously described [30] and complexes I and II substrates were added (3 mM malate, 10 mM glutamate, 10 mM succinate). This combination was chosen to mimic formation of intermediates of the citric acid cycle as is naturally the case in vivo. From samples in glass capillaries (100 μl) (Brand AG, Wertheim, Germany), DMPO-OH signals were measured on a Bruker EMX 1273 utilizing settings and peak quantification method described before [30].

2.6. Histological analysis of intramyocellular lipids (IMCL)

Cryosections (5 μm) from the midbelly region of the gastrocnemius muscle were stained [31] and quantified [24] as previously described.

2.7. Western blot analyses

For PPARGC1A detection, 30 mg of frozen tissue-sections was homogenized in 250 μl ice-cold RIPA-buffer, containing 1% Nonidet-P40 (NP40, Fluka, Zwijndrecht, The Netherlands), 0.5% sodium dodecyl sulfate (SDS; Bio-Rad Laboratories, Veenendaal, The Netherlands), 0.1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma, Zwijndrecht; The Netherlands) and 10% Complete Protease Inhibitor (Roche Diagnostics, Mannheim, Germany) in PBS pH 7.4. The homogenates were rotated 'end-over-end' during 2 h at 4 °C and centrifugated for 15 min, $15000 \times g$ at 4 °C. Next, the supernatant was diluted (2:1) in Laemmli-sample buffer (Bio-Rad Laboratories) and heated for 4 min at 100 °C. After sample preparation, polyacrylamide gels containing 10% acrylamide and 0.1% SDS were loaded with equal amounts of protein from each sample, and electrophoresis and Western blotting were performed. Blots were blocked during 60 min at RT with Licor blockingbuffer (Westburg, Leusden, The Netherlands) and incubated overnight at RT with the primary antibodies against PPARGC1A (Calbiochem, Omnilabo, Etten-Leur, The Netherlands). After incubation with the appropriate secondary antibodies, specific protein bands were detected and analyzed with Odyssey Infrared Imager (Licor, Westburg, Leusden, The Netherlands). OXPHOS proteins in muscle homogenate were detected as described before [32]. For OXPHOS protein detection in mitochondrial preparations ($n = 6$), isolated mitochondria were directly diluted in Laemmli-sample buffer (2:1) and treated similarly. All proteins were expressed as arbitrary units (AU).

2.8. Citrate synthase (CS) activity, relative mitochondrial DNA copy number and mTFAM expression

CS activity was measured spectrophotometrically as described previously [33]. Muscle mitochondrial DNA copy number and mitochondrial transcription factor A (mTFAM) expression were measured with real-time PCR.

For mitochondrial DNA copy number, the D-loop gene was used to detect mitochondrial DNA and the β-actin gene was used to detect nuclear DNA (for primer and probe sequences see Ref. [34]). PCR amplification (cycling conditions of Ref. [34]) was carried out in a 25 μl reaction consisting of 1× Taqman Universal mix (Applied Biosystems), 400 nM β-actin forward and reverse primer (Sigma-genosys), 100 nM D-loop forward and reverse primer (Sigma-genosys) and 200 nM β-actin and D-loop probe (Applied Biosytems) and 5 ng of sample DNA. Each sample was processed in triplicate and fluorescence of the probes was detected with the 7000 ABI prism detector and analyzed using sequence detection software version 1.2.3 (Applied Biosystems). Standard curves were generated using serial dilutions of a pooled DNA mix from all samples ($n = 18$). The individual sample C_t values of the D-loop and β-actin gene were plotted on these standard curves and the ratio of the outcome values for the D-loop and β-actin gene is presented as relative mitochondrial DNA copy number.

Expression of mTFAM was determined as previously described [35] with the following oligonucleotides as forward and reverse primers: 5′-CCCAATCCCAATGACAACTC-3′ and 5′-GCTTCCAGGAGGCTAAGGAT-3′, respectively.

2.9. Statistical analysis

Results are presented as means ± S.E.M. Statistical analyses were performed with SPSS for Windows 13.0 software (SPSS Inc., Chicago, IL, USA). Differences between groups were determined with two-sided unpaired Student's *t*-tests. Because of their non-normal distribution, the values for IMCL were log-transformed prior to statistical testing.