Maastricht University

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

Citation for published version (APA):

Hoeks, J., Briede, J. J., de Vogel, J., Schaart, G., Nabben, M., Kornips, E., Hesselink, M. K., & Schrauwen, P. (2008). Mitochondrial function, content and ROS production in rat skeletal muscle: effect of high-fat feeding. *Febs Letters*, *582*(4), 510-6. https://doi.org/10.1016/j.febslet.2008.01.013

Document status and date:
Published: 01/01/2008

DOI:
10.1016/j.febslet.2008.01.013

Document Version:
Publisher's PDF, also known as Version of record

Document license:
Taverne

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
• The final author version and the galley proof are versions of the publication after peer review.
• The final published version features the final layout of the paper including the volume, issue and page numbers.

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy
If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Download date: 23 Mar. 2023

FEBS Letters 582 (2008) 510–516

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

Joris Hoeks[a,b,*], Jacob J. Briedé[c], Johan de Vogel[a,b], Gert Schaart[d], Miranda Nabben[a], Esther Moonen-Kornips[a], Matthijs K.C. Hesselink[d,b], Patrick Schrauwen[a,b]

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

Received 12 November 2007; revised 3 January 2008; accepted 15 January 2008

Available online 28 January 2008

Edited by Berend Wieringa

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.**
© 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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.

*Corresponding author. Address: Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands. Fax: +31 (0) 43 3670976.
E-mail address: j.hoeks@hb.unimaas.nl (J. Hoeks).

0014-5793/$32.00 © 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2008.01.013

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.

To evaluate the relationship between variables, Pearson correlation coefficients were calculated. Outcomes were considered statistically significant if $P < 0.05$.

3. Results

3.1. Food intake and body weight

Analysis of food intake and faecal energy content revealed that over the last week of dietary intervention (Table 1) gross energy intake was comparable between the groups. This is reflected in the observation that body-mass increased similarly in the low-fat and the high-fat group (223.9 ± 8.9 vs. 214.1 ± 12.5 g/8 weeks, respectively, $P = 0.53$).

3.2. Mitochondrial function

Mitochondrial respiration upon both pyruvate and carnitine + palmitoyl-CoA as substrates was similar in both the low-fat and the high-fat fed rats (Fig. 1). As such, ADP-stimulated respiration (state 3), oligomycin-insensitive rate of respiration (state 4) and FCCP-stimulated respiration (state uncoupled) did not differ between the low-fat and high-fat group, respectively. As a result, the respiratory control ratios (RCR), calculated as state 3 divided by state 4 respiration, and indicating the tightness of the coupling between respiration and phosphorylation, was also similar between the groups (17.4 ± 0.7 vs. 16.7 ± 0.5, $P = 0.43$ for pyruvate and 5.8 ± 0.4 vs. 6.7 ± 0.4, $P = 0.12$ for carnitine + palmitoyl-CoA).

Along the same line, parallel electron input by glutamate + succinate, the substrate combination also used for the superoxide anion radical measurement, did not reveal any differences between low- vs. high-fat fed rats (data not shown).

3.3. Mitochondrial superoxide anion radical production

Mitochondrial superoxide anion radical production in the absence of ADP (where superoxide anion radical production is the highest because of a high proton gradient) was similar between low- and high-fat fed rats and averaged 28.6 ± 5.3 and 26.2 ± 5.3 AU, respectively, $P = 0.76$ ($n = 6$). Also in the presence of ADP, superoxide anion radical production was not different between the two dietary interventions (data not shown).

3.4. Intramyocellular lipid levels

After 8 weeks of high-fat feeding, IMCL were ~8-fold higher in comparison with the low-fat diet (Fig. 2, $P = 0.008$). The increase in intramuscular fat content occurred in both type 1 and type 2 muscle fibers (data not shown).

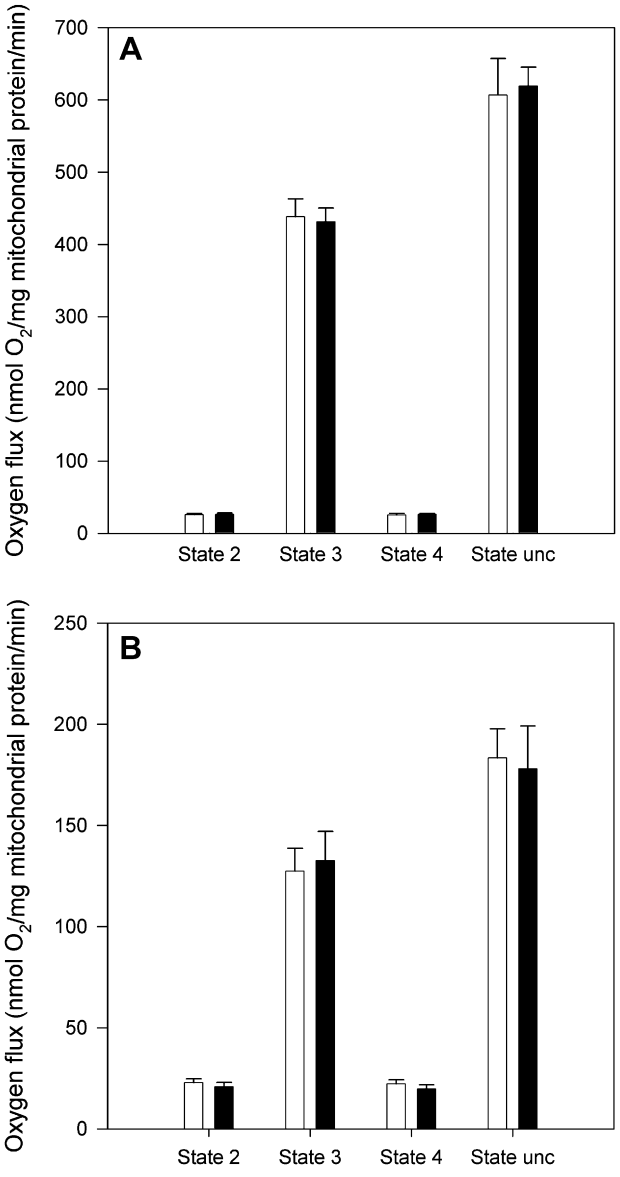

Fig. 1. Parameters of oxidative phosphorylation of skeletal muscle mitochondria respiring on (A) pyruvate and (B) palmitoyl-CoA + carnitine. White bars represent low-fat fed animals while black bars represent the high-fat group. Values are expressed as means ± S.E.M. ($n = 7$ per group).

Table 1
Energy metabolism during the last week of the dietary intervention ($n = 6$–7)

Parameters	Diet	
	Low-fat	High-fat
Gain of body mass (g/week)	17.5 ± 2.2	16.7 ± 1.7
Gross energy intake (kJ/week)	2570 ± 60	3051 ± 131*
Faeces (g/week)	17.2 ± 1.1	28.7 ± 1.3*
Energy lost in faeces (kJ/week)	322 ± 19	760 ± 34*
Net energy intake (kJ/week)	2220 ± 71	2429 ± 103
Energy intake per gram gain of body mass (kJ/g)	155 ± 17	161 ± 18

Values are means ± S.E.M.
*$P < 0.05$.

3.5. Parameters of mitochondrial biogenesis and -density

Interestingly, PPARGC1A protein levels (Fig. 3) were approximately doubled after the high-fat dietary intervention when compared to the control diet (3.85 ± 0.7 vs. 7.78 ± 1.6 AU, in low- vs. high-fat, respectively, $P = 0.042$). The protein content of structural subunits of different complexes of the respiratory chain in whole muscle homogenate was not statistically different between the diets (Complex I: 0.64 ± 0.21 vs. 1.96 ± 0.73 AU, $P = 0.12$; Complex II: 8.3 ± 1.1 vs. 13.4 ± 2.9 AU, $P = 0.13$; Complex III: 11.0 ± 1.9 vs. 17.5 ± 3.4 AU, $P = 0.11$; Complex V: 40.7 ± 5.3 vs. 45.9 ± 6.2, $P = 0.54$, in low- vs. high-fat, respectively). However, we

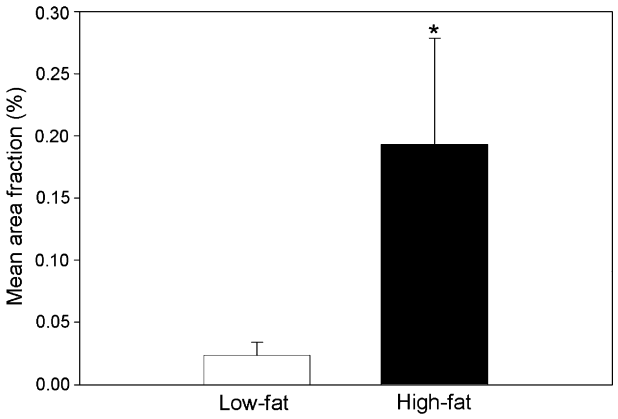

Fig. 2. Intramyocellular lipid levels after 8-weeks low-fat (white bar) vs. high-fat (black bar) feeding. Area fraction reflects the percentage of the total measured cell surface covered by lipid droplets ($n = 9$). $^{*}P < 0.05$.

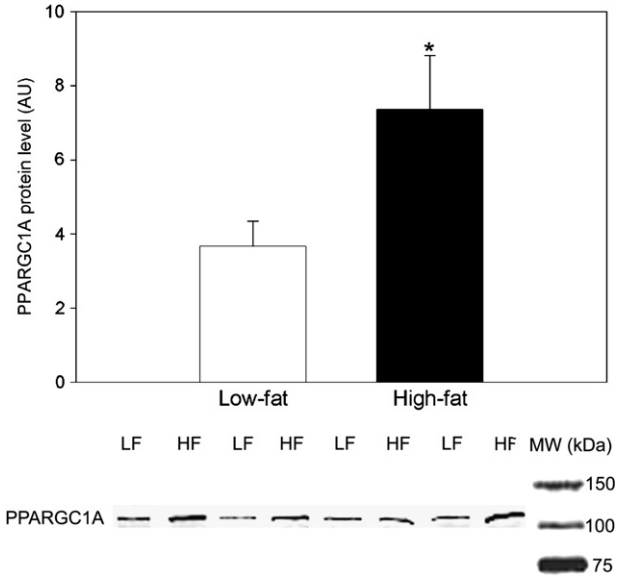

Fig. 3. PPARGC1A protein levels in rat gastrocnemius muscle homogenates after 8-weeks low-fat (white bar) vs. high-fat (black bar) feeding. Values are expressed as means ± S.E.M. ($n = 9$ per group). $^{*}P < 0.05$.

did observe strong positive correlations between PPARGC1A protein and the assessed complexes of the respiratory chain (Fig. 4). We also loaded gels with equal amounts of *mitochondrial* protein, derived from a subset of mitochondrial preparations and found that complexes I–V protein content was also not different between diets when measured per mitochondrion (Complex I: 32.4 ± 4.0 vs. 36.1 ± 1.8 AU, $P = 0.46$; Complex II: 47.1 ± 5.2 vs. 51.8 ± 3.1 AU, $P = 0.45$; Complex III: 52.4 ± 4.7 vs. 51.8 ± 3.3 AU, $P = 0.66$; Complex V: 29.7 ± 3.4 vs. 31.6 ± 5.6, $P = 0.79$, in low- vs. high-fat, respectively) and did not correlate to PPARGC1A levels.

Citrate synthase activity in whole muscle homogenate was similar between the low-fat and the high-fat group and averaged 19.1 ± 2.3 vs. 17.0 ± 2.0 μmol/min/g protein, respectively ($P = 0.52$).

Interestingly however, relative mitochondrial DNA copy number was significantly reduced in high-fat fed rats in comparison to the low-fat fed rats (Fig. 5) and averaged 1.22 ± 0.08 vs. 0.92 ± 0.08 in low-fat vs. high-fat, respectively ($P = 0.019$). Finally, the expression of mitochondrial transcription factor A (mTFAM), a target gene of PPARGC1A, remained unaffected (1.17 ± 0.22 vs. 1.05 ± 0.29 AU, in low- vs. high-fat, respectively, $P = 0.31$).

4. Discussion

Previous data suggest that a high intake of fat and high intramyocellular lipid levels might be involved in, at least part of the mitochondrial disturbances observed in aging and type 2 diabetes. However, where most papers concerning the relation between high intake in dietary fat and skeletal muscle mitochondrial dysfunction only addressed indirect parameters of mitochondrial function, we here determined intrinsic mitochondrial function by respirometry and mitochondrial ROS production in a direct manner by electron spin resonance spectroscopy.

We show that an 8 week, high-fat dietary intervention does not affect mitochondrial function in isolated skeletal muscle mitochondria, either on a carbohydrate (pyruvate)- or on a lipid (palmitoyl-CoA)-substrate (Fig. 1). In other words, the high-fat dietary intervention did not impede functioning of skeletal muscle mitochondria. If anything, the mitochondrial fat oxidative capacity improved upon high-fat feeding, as supported by a tendency towards a higher rather than lower respiratory control ratio on the lipid substrate ($P = 0.12$). In this context, a previous study revealed that a shorter, 2-week high-fat feeding regimen resulted in an enhanced mitochondrial capacity to use lipids as metabolic fuels in skeletal muscle of young Wistar rats [36] while a 7-week high-fat dietary intervention in adult rats showed a decreased oxidative capacity in (subsarcolemmal) mitochondria [37]. These findings indicate that both the duration of the dietary intervention and the age of the animals at onset may contribute to differences in metabolic responses.

It has been suggested that an impaired mitochondrial function is a prerequisite for the accumulation of fat in skeletal muscle on a high fat diet, which could subsequently impede proper cellular function. To that end, we also measured IMCL content in the present study. Despite the lack of intrinsic skeletal muscle mitochondrial dysfunction in the present study, the 8-week dietary intervention lead to an ~8-fold increase in intramyocellular lipid levels. Therefore, it can be concluded that the existence of intrinsic skeletal muscle mitochondrial dysfunction is not a prerequisite for the accumulation of muscular fat.

In past years, the formation of reactive oxygen species (ROS) has been implicated in the progression of aging and manifestation of type 2 diabetes and insulin resistance [18,38]. Since ROS can have detrimental effects on mitochondrial (membrane) lipids and proteins, their excessive production is a likely candidate in explaining the observed mitochondrial dysfunction. ROS production can be enhanced by excessive fuel supply or functional impairment of one or more complexes of the respiratory chain [23]. Here we show however, that the dietary intervention did not induce

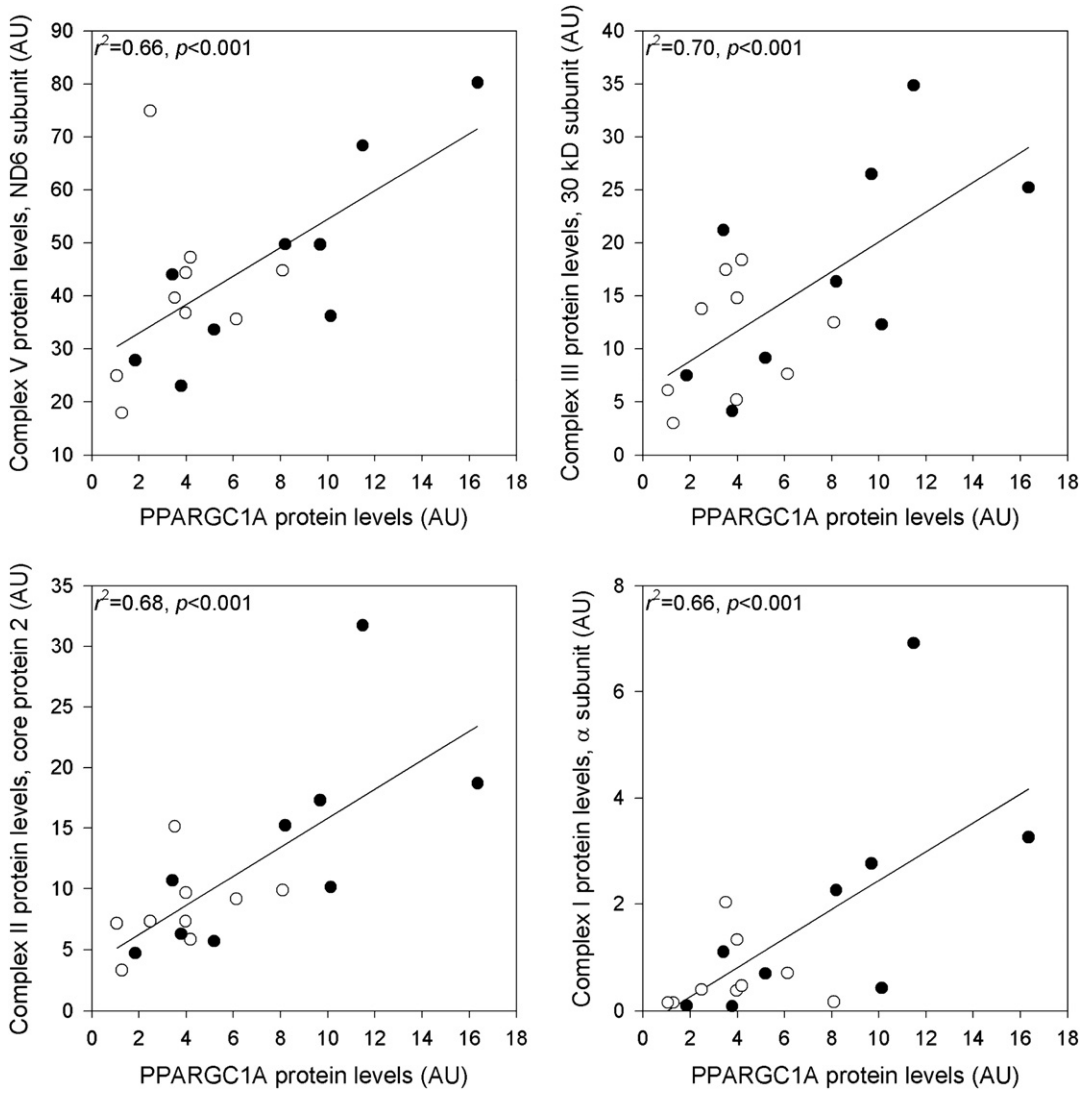

Fig. 4. Correlations between PPARGC1A protein levels and various subunits representative of Complexes I, II, III and the F_0/F_1-ATPase (complex V) of the respiratory chain, determined by Western blot in whole muscle homogenates ($n = 9$). White dots represent low-fat fed animals while black dots represent the high-fat group.

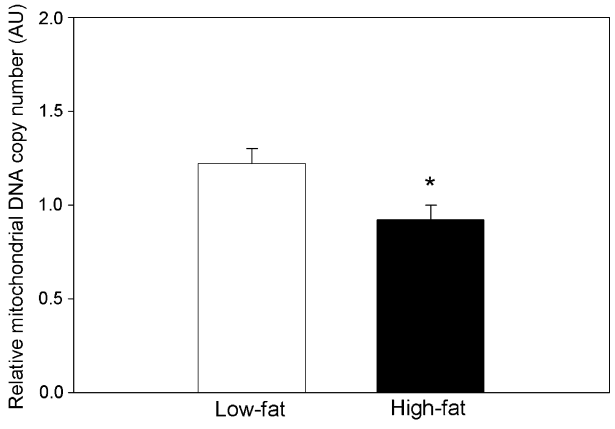

Fig. 5. Relative mitochondrial DNA copy number after 8-weeks low-fat (white bar) vs. high-fat (black bar) feeding, expressed as the ratio between the mitochondrial D-loop gene and the nuclear β-actin gene (see Section 2, $n = 9$). $^{*}P < 0.05$.

permanent changes to the mitochondria that lead to an increase in ROS production in vitro.

Alternatively to intrinsic mitochondrial defects, it can be hypothesized that a high supply of fat to the skeletal muscle hinders the proper maintenance of the mitochondrial pool, resulting in a decreased mitochondrial content and a lowered total oxidative capacity. To examine markers of mitochondrial biogenesis in the present study we determined protein levels of PPARGC1A and we found these levels to be significantly increased upon the high-fat diet (Fig. 3). Earlier findings in this context have rendered controversial results. Thus, Sparks et al. [22] showed a decrease in PPARGC1A mRNA and protein levels after high-fat feeding, both in humans and mice. In addition, we and others previously reported that the infusion of a lipid emulsion in humans leads to a decrease in *PPARGC1* gene expression [20,21], while suppression of plasma free fatty acids during exercise by a pharmacological agent increases the expression of *PPARGC1A* in skeletal muscle [39]. A recent study reported no difference in *PPARGC1A* after raising

plasma free fatty acid levels in rats by high-fat feeding combined with heparin administration [40]. In agreement with the present study however, Turner et al. [41] recently reported a significant increase in PPARGC1A protein in mice after both 5 weeks and 20 weeks of high-fat feeding. It should be mentioned that several studies only determined *PPARGC1* gene expression, which does not necessarily reflect protein content, let alone biological activity. Moreover, differences in studied species and the exact nature of the interventions (acute vs. chronic; single vs. combined interventions) further complicate comparison of the different outcomes.

To shed more light on this ongoing debate, we determined several markers for mitochondrial density to test whether the observed increase in PPARGC1A protein levels in the present study, was followed by an augmented mitochondrial biogenesis. Western blot analysis in whole muscle homogenates revealed that the increased PPARGC1A protein upon the high-fat diet was accompanied by higher though non-significant protein levels of structural subunits of four complexes of the respiratory chain. Although these differences remained only tendencies, we did observe strong and significant correlations between PPARGC1A and the assessed mitochondrial markers (Fig. 4). Moreover, we found that (1) levels of these structural proteins of the respiratory chain were similar when measured per mitochondria and (2) there was no correlation between PPARGC1A and these proteins per mitochondria. This finding could support the notion that the higher PPARGC1A levels lead to an increased number of mitochondria. However, the other assessed markers for mitochondrial density were similar (citrate synthase) between the experimental groups or interestingly, displayed a significant decrease (relative mitochondrial DNA copy number) after the high-fat dietary intervention and did not correlate with PPARGC1A. In addition, the unaltered mTFAM mRNA levels might suggest that PPARGC1A, despite its elevated levels, is in an inactive state.

Previous studies in humans and rodents also provided ambiguous results showing both lowered mitochondrial content after high-fat feeding in humans and mice [22] as well as increases in mitochondrial density upon lipid oversupply [40,41]. The discrepancy in results on markers for mitochondrial content in the present study is puzzling. The Western blot for OXPHOS proteins detects 1 structural protein of each of the different complexes in the respiratory chain. Although the protein levels of these structural proteins do not necessarily reflect the number of mitochondria, their level was constant when measured in the mitochondrial fraction (i.e. per mitochondria). On the other hand, the relative mitochondrial DNA copy number has been used frequently as a marker of mitochondrial density under the assumption that the number of mitochondrial DNA copies per mitochondrion is constant and similar between experimental groups. If the number of mitochondrial DNA copies per mitochondrion is constant in the present study is unknown. Therefore, the reason for the discrepancy between these markers cannot be deduced from the present study. For that reason, conclusions from the present study need to be restricted to a lack of effect of high-fat diet on intrinsic mitochondrial function and extrapolation to the whole muscle levels needs to be done with care.

In summary, we showed that an 8-week high-fat dietary intervention in rats, did not affect intrinsic mitochondrial functioning or mitochondrial ROS production. PPARGC1A protein levels increased in high-fat fed rats, while markers for mitochondrial density showed inconsistent results. These data indicate that despite normal intrinsic mitochondrial function, intramyocellular lipid levels did increase upon high fat feeding, illustrating that a reduced innate mitochondrial function is not a prerequisite for muscular fat accumulation.

Acknowledgements: This study was funded by TI Food and Nutrition (formerly Wageningen Centre for Food Sciences), an alliance of major Dutch food industries, TNO Quality of Life, Zeist, Maastricht University, Wageningen University and Research Centre, with financial support from the Dutch government. Dr. J. Hoeks was supported by a grant from the Netherlands Organization for Scientific Research (NWO) and the research of Dr. P. Schrauwen has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. Dr. M.K.C. Hesselink is supported by a VIDI Research Grant for innovative research from the Netherlands Organization for Scientific Research (Grant 917.66.359).

References

[1] Barazzoni, R., Short, K.R. and Nair, K.S. (2000) Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. J. Biol. Chem. 275, 3343–3347.
[2] Zahn, J.M. et al. (2006) Transcriptional profiling of aging in human muscle reveals a common aging signature. PLoS Genet. 2, e115.
[3] Mootha, V.K. et al. (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat. Genet. 34, 267–273.
[4] Patti, M.E. et al. (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. Proc. Natl. Acad. Sci. USA 100, 8466–8471.
[5] Rodriguez-Calvo, R. et al. (2006) PGC-1beta down-regulation is associated with reduced ERRalpha activity and MCAD expression in skeletal muscle of senescence-accelerated mice. J. Gerontol. A Biol. Sci. Med. Sci. 61, 773–780.
[6] Ling, C. et al. (2004) Multiple environmental and genetic factors influence skeletal muscle PGC-1alpha and PGC-1beta gene expression in twins. J. Clin. Invest. 114, 1518–1526.
[7] Petersen, K.F. et al. (2003) Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science 300, 1140–1142.
[8] Petersen, K.F., Dufour, S., Befroy, D., Garcia, R. and Shulman, G.I. (2004) Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. New Engl. J. Med. 350, 664–671.
[9] Petersen, K.F., Dufour, S. and Shulman, G.I. (2005) Decreased insulin-stimulated ATP synthesis and phosphate transport in muscle of insulin-resistant offspring of type 2 diabetic parents. PLoS Med. 2, e233.
[10] Drew, B., Phaneuf, S., Dirks, A., Selman, C., Gredilla, R., Lezza, A., Barja, G. and Leeuwenburgh, C. (2003) Effects of aging and caloric restriction on mitochondrial energy production in gastrocnemius muscle and heart. Am. J. Physiol. Regul. Integr. Comp. Physiol. 284, R474–R480.
[11] Trounce, I., Byrne, E. and Marzuki, S. (1989) Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. Lancet 1, 637–639.
[12] Mogensen, M., Sahlin, K., Fernstrom, M., Glintborg, D., Vind, B.F., Beck-Nielsen, H. and Hojlund, K. (2007) Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes 56, 1592–1599.
[13] Kelley, D.E., He, J., Menshikova, E.V. and Ritov, V.B. (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes 51, 2944–2950.
[14] Morino, K. et al. (2005) Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J. Clin. Invest. 115, 3587–3593.

[15] Simoneau, J.A. and Kelley, D.E. (1997) Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM. J. Appl. Physiol. 83, 166–171.
[16] Rooyackers, O.E., Adey, D.B., Ades, P.A. and Nair, K.S. (1996) Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. Proc. Natl. Acad. Sci. USA 93, 15364–15369.
[17] Liang, P., Hughes, V. and Fukagawa, N.K. (1997) Increased prevalence of mitochondrial DNA deletions in skeletal muscle of older individuals with impaired glucose tolerance: possible marker of glycemic stress. Diabetes 46, 920–923.
[18] Houstis, N., Rosen, E.D. and Lander, E.S. (2006) Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440, 944–948.
[19] Fukagawa, N.K., Li, M., Liang, P., Russell, J.C., Sobel, B.E. and Absher, P.M. (1999) Aging and high concentrations of glucose potentiate injury to mitochondrial DNA. Free Radic. Biol. Med. 27, 1437–1443.
[20] Richardson, D.K. et al. (2005) Lipid infusion decreases the expression of nuclear encoded mitochondrial genes and increases the expression of extracellular matrix genes in human skeletal muscle. J. Biol. Chem. 280, 10290–10297.
[21] Hoeks, J., Hesselink, M.K., Russell, A.P., Mensink, M., Saris, W.H., Mensink, R.P. and Schrauwen, P. (2006) Peroxisome proliferator-activated receptor-gamma coactivator-1 and insulin resistance: acute effect of fatty acids. Diabetologia 49, 2419–2426.
[22] Sparks, L.M., Xie, H., Koza, R.A., Mynatt, R., Hulver, M.W., Bray, G.A. and Smith, S.R. (2005) A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. Diabetes 54, 1926–1933.
[23] Lenaz, G. (2001) The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology. IUBMB Life 52, 159–164.
[24] Hoeks, J. et al. (2006) The effect of high-fat feeding on intramuscular lipid and lipid peroxidation levels in UCP3-ablated mice. FEBS Lett. 580, 1371–1375.
[25] Shabalina, I.G., Petrovic, N., Kramarova, T.V., Hoeks, J., Cannon, B. and Nedergaard, J. (2006) UCP1 and defense against oxidative stress. 4-Hydroxy-2-nonenal effects on brown fat mitochondria are uncoupling protein 1-independent. J. Biol. Chem. 281, 13882–13893.
[26] Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W. and Weigele, M. (1972) Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. Science 178, 871–872.
[27] O'Malley, Y., Fink, B.D., Ross, N.C., Prisinzano, T.E. and Sivitz, W.I. (2006) Reactive oxygen and targeted antioxidant administration in endothelial cell mitochondria. J. Biol. Chem. 281, 39766–39775.
[28] Han, D., Antunes, F., Canali, R., Rettori, D. and Cadenas, E. (2003) Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. J. Biol. Chem. 278, 5557–5563.
[29] Wang, Y., Fang, J., Leonard, S.S. and Rao, K.M. (2004) Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic. Biol. Med. 36, 1434–1443.
[30] Briede, J.J., Pot, R.G., Kuipers, E.J., van Vliet, A.H., Kleinjans, J.C. and Kusters, J.G. (2005) The presence of the cag pathogenicity island is associated with increased superoxide anion radical scavenging activity by *Helicobacter pylori*. FEMS Immunol. Med. Microbiol. 44, 227–232.
[31] Koopman, R., Schaart, G. and Hesselink, M.K. (2001) Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. Histochem. Cell Biol. 116, 63–68.
[32] Mensink, M., Hesselink, M.K., Russell, A.P., Schaart, G., Sels, J.P. and Schrauwen, P. (2007) Improved skeletal muscle oxidative enzyme activity and restoration of PGC-1 alpha and PPAR beta/delta gene expression upon rosiglitazone treatment in obese patients with type 2 diabetes mellitus. Int. J. Obes. (Lond). 31, 1302–1310.
[33] Shepherd, D. and Garland, P.B. (1969) Citrate synthase from rat liverMethods in Enzymology, Vol. XIII, pp. 11–16, Academic Press, New York.
[34] Nicklas, J.A., Brooks, E.M., Hunter, T.C., Single, R. and Branda, R.F. (2004) Development of a quantitative PCR (TaqMan) assay for relative mitochondrial DNA copy number and the common mitochondrial DNA deletion in the rat. Environ. Mol. Mutagen. 44, 313–320.
[35] Mandard, S. et al. (2007) Glycogen synthase 2 is a novel target gene of peroxisome proliferator-activated receptors. Cell Mol. Life Sci. 64, 1145–1157.
[36] Iossa, S., Mollica, M.P., Lionetti, L., Crescenzo, R., Botta, M. and Liverini, G. (2002) Skeletal muscle oxidative capacity in rats fed high-fat diet. Int. J. Obes. Relat. Metab. Disord. 26, 65–72.
[37] Lionetti, L., Mollica, M.P., Crescenzo, R., D'Andrea, E., Ferraro, M., Bianco, F., Liverini, G. and Iossa, S. (2007) Skeletal muscle subsarcolemmal mitochondrial dysfunction in high-fat fed rats exhibiting impaired glucose homeostasis. Int. J. Obes. (Lond). 31, 1596–1604.
[38] Harman, D. (1992) Free radical theory of aging: history. Exs 62, 1–10.
[39] Watt, M.J., Southgate, R.J., Holmes, A.G. and Febbraio, M.A. (2004) Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes. J. Mol. Endocrinol. 33, 533–544.
[40] Garcia-Roves, P., Huss, J.M., Han, D.H., Hancock, C.R., Iglesias-Gutierrez, E., Chen, M. and Holloszy, J.O. (2007) Raising plasma fatty acid concentration induces increased biogenesis of mitochondria in skeletal muscle. Proc. Natl. Acad. Sci. USA 104, 10709–10713.
[41] Turner, N., Bruce, C.R., Beale, S.M., Hoehn, K.L., So, T., Rolph, M.S. and Cooney, G.J. (2007) Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. Diabetes 56, 2085–2092.