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Dietary restriction increases skeletal muscle mitochondrial respiration but not mitochondrial content in C57BL/6 mice

Sarah Hempenstall, Melissa M. Page, Katrina R. Wallen, Colin Selman *

Integrative and Environmental Physiology, Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK

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ABSTRACT

Dietary restriction (DR) is suggested to induce mitochondrial biogenesis, although recently this has been challenged. Here we determined the impact of 1, 9 and 18 months of 30% DR in male C57BL/6 mice on key mitochondrial factors and on mitochondrial function in skeletal muscle, relative to age-matched *ad libitum* (AL) controls. We examined proteins and mRNAs associated with mitochondrial biogenesis and measured mitochondrial respiration in permeabilised myofibres using high resolution respirometry. 30% DR, irrespective of duration, had no effect on citrate synthase activity. In contrast, total and nuclear protein levels of PGC-1α, mRNA levels of several mitochondrial associated proteins (*Pgc-1α*, *Nrf1*, *Core 1*, *Cox IV*, *Atps*) and cytochrome c oxidase content were increased in skeletal muscle of DR mice. Furthermore, a range of mitochondrial respiration rates were increased significantly by DR, with DR partially attenuating the age-related decline in respiration observed in AL controls. Therefore, DR did not increase mitochondrial content, as determined by citrate synthase, in mouse skeletal muscle. However, it did induce a PGC-1α adaptive response and increased mitochondrial respiration. Thus, we suggest that a functionally 'efficient' mitochondrial electron transport chain may be a critical mechanism underlying DR, rather than any net increase in mitochondrial content *per se*.

1. Introduction

It is well established that dietary restriction (DR) robustly extends healthy lifespan in most organisms (Masoro, 2009; Weindruch and Walford, 1988), with health benefits seen in both non-human primates (Colman et al., 2009) and humans (Fontana et al., 2004, 2007). Whilst the effects of DR on healthy lifespan are clear, it is still uncertain as to what is the mechanism in driving these effects, although many putative mechanisms have been proposed (Gesing et al., 2011; Mair and Dillin, 2008; Masoro, 2009). One prominent candidate mechanism is mitochondrial biogenesis (Lopez-Lluch et al., 2006, 2008; Nisoli et al., 2005). DR has been shown to alter several molecular markers indicative of mitochondrial biogenesis in different organisms. These include increased mitochondrial DNA content, increased expression of mitochondrial associated genes (*e.g.* peroxisome proliferator-activated receptor gamma co-activator (*Pgc-1α*), nuclear respiratory factor-1 (*Nrf-1*), mitochondrial transcription factor A (*Tfam*)), and increased cytochrome c oxidase (COX IV) and cytochrome c protein levels in a range of mouse tissues (Nisoli et al., 2005). Mitochondrial biogenesis has also been reported in human skeletal muscle following DR, with increased mitochondrial DNA content and expression of several mitochondrial related genes reported, although citrate synthase (CS) and COX IV activities were unchanged (Civitarese et al., 2007). Critically, it appears that mitochondrial function may adapt to DR through PGC-1α regulation (Anderson et al., 2008), and age-related declines in both skeletal muscle and heart *Pgc-1α* in *ad libitum* (AL) rats have been shown to be attenuated by DR (Hepple et al., 2006).

Whilst mitochondrial biogenesis is widely accepted as a cellular response to DR, this belief has recently been challenged. Hancock et al. (2011) reported no effect on PGC-1α protein or in mRNA levels of several mitochondrial associated genes in triceps muscle, heart or liver of DR male Wistar rats. In addition, whilst DR increased *Pgc1-α* mRNA levels significantly in liver and skeletal muscle (but not heart) of male B6D2F1 mice, it had no effect on PGC-1α protein levels or on mRNA of *Nrf-1*, *Tfam* and various mitochondrial proteins (Miller et al., 2011). CS activity in liver, heart and skeletal muscle, a mitochondrial matrix protein used as a marker of mitochondrial content, was also unaffected by DR (Hancock et al., 2011). Similarly, DR had no effect on CS activity in skeletal muscle of rats (Sreekumar et al., 2002) and was reduced in liver of DR mice (Weindruch et al., 1980), although other studies have reported DR-induced increases

Abbreviations: DR, dietary restriction; AL, *ad libitum*; FI, food intake; BM, body mass; CS, citrate synthase; COX IV, cytochrome c oxidase; HRR, high resolution respirometry; BIOPS, biopsy preservation solution; PGC-1α, peroxisome proliferator-activated receptor gamma co-activator; ETS, electron transport system; OXPHOS, oxidative phosphorylation.

* Corresponding author at: Integrative and Environmental Physiology, Institute of Biological and Environmental Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, UK. Tel.: +44 01224 272399; fax: +44 01224 272396.
E-mail address: c.selman@abdn.ac.uk (C. Selman).

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(*e.g.* Lopez-Lluch et al., 2006). At the functional level, the effects of DR on mitochondria are also ambiguous. Reduced mitochondrial respiration, primarily state 4, was reported in isolated mitochondria from both rats (Bevilacqua et al., 2004, 2005; Sohal et al., 1994) and mice (Lal et al., 2001; Weindruch et al., 1980) following DR. In an elegant study, Lopez-Lluch et al. (2006) demonstrated that HeLa cells incubated in serum derived from DR rats had decreased mitochondrial respiration, lower membrane potential, increased proton leak and reduced reactive oxygen species (ROS) production. In addition, no effect on ATP production was reported, leading the authors to suggest that DR leads to a state of bioenergetic efficiency (Lopez-Lluch et al., 2006). However, state 4 respiration was unaltered in several tissues from DR rats ((Gredilla et al., 2001; Lambert et al., 2004b), and see also Bevilacqua et al., 2005). In addition, state 3 respiration was increased in liver mitochondria from DR mice (Weindruch et al., 1980), state 4 respiration increased in brown adipose tissue from DR rats (Lambert et al., 2004a), and total oxygen (O_2) consumption was elevated in several tissues in DR mice (Nisoli et al., 2005).

The precise reasons for these incongruous findings are unclear, although gender-, strain- and species-specific differences may be important (Hunt et al., 2006). In addition, the intensity, duration and age of DR onset may be critical (Bevilacqua et al., 2005; Johnson et al., 2006), as might tissue-specific responses to DR (Lambert et al., 2004b; Miller et al., 2011; Zangarelli et al., 2006). Historically, isolated mitochondria have been used to examine the effects of DR and/or ageing on mitochondrial function. As discussed elsewhere (*e.g.* Picard et al., 2010, 2011), the isolation methods themselves may exaggerate mitochondrial phenotypes and may introduce experimental artefacts by disrupting the complex cellular environment experienced by mitochondria *in vivo*. Measurements of respiration in permeabilised tissue using high resolution respirometry (HRR) have recently been employed in an attempt to mitigate some of these potentially confounding factors (Aragones et al., 2008; Boushel et al., 2007; Picard et al., 2010; Rabol et al., 2009). It has been suggested that this approach may help maintain mitochondrial morphology and better reflect the *in vivo* conditions experienced by mitochondria (Picard et al., 2010). Indeed, the age-related deteriorations in skeletal muscle mitochondrial function observed in rats appeared significantly exaggerated in isolated mitochondria when compared to permeabilised myofibres (Picard et al., 2010).

In light of current uncertainty on exactly how DR and ageing impact on mitochondrial biology, we examined firstly whether mitochondrial biogenesis occurred in hindlimb (gastrocnemius) skeletal muscle of male C57BL/6 mice following 1, 9 or 18 months of 30% DR (4, 12 or 21 months of age respectively). We hypothesised that DR would induce adaptive changes to mitochondrial function *via* PGC-1α (Anderson et al., 2008), leading to a predicted attenuation of an age-related decline in mitochondrial function. Our experimental design enabled us to examine both the effects of DR and age, by comparing our DR animals to age-matched AL controls. Skeletal muscle was studied as it is particularly prone to age-related declines in its oxidative and functional capacities (Baker et al., 2006; Hepple et al., 2005; Jang and Van Remmen, 2011; Marzetti et al., 2009). Initially we determined the levels of several key proteins and genes linked to mitochondrial biogenesis (Anderson et al., 2008; Civitarese et al., 2007; Nisoli et al., 2005). Secondly, we extended current knowledge by examining mitochondrial respiration in detail following DR and ageing using HRR in permeabilised myofibres (Kuznetsov et al., 2008; Picard et al., 2010).

2. Materials and methods

2.1. Animals

Male C57BL/6N mice were purchased from a commercial breeder (Charles River Laboratories, UK) at 4 weeks of age. Mice were maintained in pairs from 8 weeks of age onwards in shoebox cages (48 cm × 15 cm × 13 cm). Initially all animals had *ad libitum* (AL) access to water and standard chow (D12450B, Research Diets Inc., New Brunswick, NJ, USA; protein 20 kcal%, carbohydrate 70 kcal%, fat 10 kcal%) and maintained on a 12L/12D cycle (lights on 0700–1900 h) at 22 ± 2 °C. At 10 weeks of age, weight matched pairs were assigned to the AL or dietary restricted (DR) group, with no difference in body mass observed between the experimental groups at this time (AL = 25.1 ± 0.5 g, DR = 25.3 ± 0.5 g; $F = 3.354$; $p = 0.137$). Mice were then maintained in these same pairs throughout the experiment. DR mice underwent an incremental step-down protocol as previously described (Hempenstall et al., 2010; Selman et al., 2006). In brief, daily food intake of DR mice was reduced to 90% of AL levels at 10 weeks of age, 80% of AL levels at 11 weeks of age and held at 70% of AL levels (30% DR) from 12 weeks of age onwards. Total food intake of paired AL mice was measured weekly (±0.01 g) and 30% DR calculated from the average AL mice intake over the preceding week. DR mice were fed daily between 1630 and 1730 h. No evidence of hierarchies or fighting was seen between paired mice within a cage, as previously reported by us (Selman et al., 2006) and others (*e.g.* Ikeno et al., 2005). DR mice fed simultaneously at the hopper, with no evidence that one individual interfered with the feeding of the other individual within a cage. Following 1, 9 or 18 months of 30% DR (equivalent to 4, 12 or 21 months of age), 8 mice per experimental group were culled by cervical dislocation and the gastrocnemius (hindlimb) muscle was dissected out. All experiments were carried out under local ethical review (University of Aberdeen, UK), under a licence from the UK Home Office and followed the "principles of laboratory animal care" (NIH Publication No. 86-23, revised 1985).

2.2. Citrate synthase activity and cytochrome c oxidase levels

Citrate synthase (a marker for mitochondrial content) activity was determined spectrophotometrically following the protocol of Srere (Srere, 1969). Cytochrome c oxidase (Complex IV) content was also determined spectrophotometrically, as previously described (Balaban et al., 1996). All high resolution respiration measurements were expressed as O_2 flux (picomoles O_2 per s^{-1} per mg wet weight) and corrected for citrate synthase (CS) activity (Rabol et al., 2009, 2010).

2.3. Protein extraction

A portion of hindlimb muscle was powdered in liquid nitrogen and then suspended in ice cold homogenisation buffer (0.25 M monobasic potassium phosphate, 0.5 M EDTA, 0.5 M potassium chloride, 10% Triton X-100, 50% glycerol). Protease inhibitor cocktail (Merck KGaA, Darmstadt, Germany) was added for each sample (final concentration in 1 ml homogenisation buffer; 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 8 μm aprotinin, 50 μm bestatin, 1.5 μm E-64 protease inhibitor, 20 μm leupeptin, 10 μm pepstatin A). Samples were vortexed, incubated on ice for 45 min, homogenised in three 15 s^{-1} pulses at maximum setting using a polytron homogeniser (Fisher Scientific, Loughborough, UK) and subsequently centrifuged for 10 min^{-1} (4 °C) at 9000 × *g* (PeqLab, PerfectSpin, Erlangen, Germany). Nuclear and cytoplasmic isolation followed previously published protocols (Cabelof et al., 2002; Thomashevski et al., 2004), with protein concentration quantified by the Bradford method (Bradford, 1976).

2.4. Western blot analysis

Equal loading (20 μg) of muscle protein extract in Laemmli buffer were loaded onto Tris–HCl acrylamide gels. Following resolution, proteins were transferred to polyvinylidene difluoride membranes (PeqLab), using a semi-dry blotter (PeqLab). Ponceau staining was used to ensure equal transfer (data not shown). Membranes were incubated in Tris-buffered saline Tween 20 (TBST) containing 5% powdered milk for 1 h^{-1}. Blots were then washed in TBST (3 × 10 min^{-1}), incubated with primary antibody for 24 h^{-1} (4 °C), washed again (TBST) and incubated with secondary antibody for 1 h^{-1} (room temperature). Blots were visualised using enhanced chemiluminescent and HRT (Pierce Thermo Scientific, Rockford, IL, USA). Primary (peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1α), mitochondrial transcription factor A (TFAM)) and secondary (anti-rabbit) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.5. Quantitative PCR

RNA extraction from muscle, quantitative-PCR (qPCR) and analysis was carried out as previously described (Selman et al., 2006, 2008). Briefly, RNA was extracted using TRIzol® (Invitrogen, Life Technologies Ltd., Paisley, UK) and quantified spectrophotometrically (Nanodrop ND-1000, Thermo Scientific, East Sussex, UK). RNA purity was determined by the 260/280 and 260/230 ratios using a Nanodrop ND-1000, and by gel electrophoresis. qPCR was carried out using KAPA SYBR fast qPCR universal fluorescence dye (Anachem Ltd., Luton, UK) and Ct values were detected using the Lightcycler 480 2.0 qPCR system (Roche, West Sussex, UK). Expression levels of *Pgc-1α*, *Atps*, *Nrf1*, *Cox IV* and *Complex III* (for primer sequences see Table 1) were determined (following melting curve analysis), as these genes are associated with mitochondrial biogenesis (see Hancock et al., 2011; Nisoli et al., 2005). Changes in mRNA expression levels were calculated as fold change expressed relative to transcription elongation factor A (SII)-1 (*Tcea1*) using the delta-delta CT method. *Tcea1* was selected as our reference gene as its expression is unaffected by DR (Selman et al., 2006).

Table 1
Primer sequences for qPCR.

Primer	Sequence	
	Forward	Reverse
Cox IV	5′-CTGCCCGGAGTCTGGTAATG-3′	5′-CAGTCAACGTAGGGGGTCATC-3′
Pgc-1α	5′-TATGGAGTGACATAGAGTGTGCT-3′	5′-CCACTTCAATCCACCCAGAAAG-3′
Nrf1	5′-AGCACGGAGTGACCCAAAC-3′	5′-TGTACGTGGCTACATGGACCT-3′
Atps	5′-CCCCTTCTACGACCGCTAC-3′	5′CCACTGGCTGCTTTCGGAA-3
Complex III	5′-CCTACAGCTTGTCGCCCTTT-3′	5′-GATCAGGTAGACCACTACAAACG-3′
Tcea1	5′-TGATGCTGTACGAAACAAATGCC-3′	5′-CCGCACCCGATTCTTGTACT-3′

2.6. Muscle permeabilisation

Gastrocnemius muscle was placed in ice-cold biopsy preservation solution (BIOPS; pH 7.1). BIOPS contained 10 mmol/L Ca^{2+} ethylene glycol tetra-acetic acid, 0.1 μmol/L free calcium, 20 mmol/L imidazole, 50 mmol/L potassium 4-morpholinoethanesulfonic acid, 0.5 mmol/L dithiothreitol, 6.56 mmol/L magnesium chloride, 5.77 mmol/L disodium-adenosine-triphosphate and 15 mmol/L phosphocreatine. Connective tissue and fat was removed and then muscle bundles manually teased apart and weighed using analytical scales. Fibre bundles were permeabilised in BIOPS solution containing 0.05 mg/ml saponin, with gentle rocking following previously described methods (Boushel et al., 2007; Kuznetsov et al., 2008; Picard et al., 2010). After permeabilisation, fibres were placed in ice-cold respiration medium (mitochondrial respiration medium #5; MiR05) for 10 min^{-1} prior to use (Boushel et al., 2007).

2.7. High resolution respirometry

Mitochondrial respiration measurements were performed in duplicate on permeabilised gastrocnemius muscle fibres using a polarographic oxygen sensor (Oxygraph-2k, Oroboros® Instruments GmbH Corp., Innsbruck, Austria). Standard calibrations were performed to correct for residual background oxygen (O_2) flux, and then ~5–10 mg (wet weight) permeabilised muscle fibres were added to one of two glass respirometry chambers containing air-saturated MiR05 (37 °C). The chambers were subsequently sealed to exclude oxygen exchange with the external environment. Baseline respiration was determined initially and then O_2 flux measured using sequential titration of the following substrates. Glutamate (19 mmol/L) and malate (1.5 mmol/L) were added to stimulate complex 1 driven respiration, and then ADP (4.8 mmol/L) added to stimulate oxidative phosphorylation. Succinate (9.5 mmol/L) was then titrated to measure oxidative phosphorylation with convergent electron input from complexes I and II. Outer mitochondrial membrane integrity was determined through addition of cytochrome c (19 μmol/L) following the induction of convergent (maximal) oxidative phosphorylation (Kuznetsov et al., 2004). Rotenone (0.1 μmol/L; complex I inhibitor) was added to measure complex II driven oxidative phosphorylation. Carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP; 0.7 μmol/L) was added to induce uncoupled respiration. Mitochondrial leak respiration was measured by adding the ATP synthase inhibitor oligomycin (2 μg/ml) following glutamate and malate. The respiration data is presented in the aforementioned sequence of titrations as previously reported (Boushel et al., 2007), with the exception of LEAK respiration.

2.8. Statistical analysis

All statistical analyses were performed using SPSS (SPSS Inc., Armonk, NY, USA, version 18) and GraphPad Prism (GraphPad Inc., La Jolla, CA, USA, version 5) software. Data were checked for normality using the Shapiro–Wilks test, and analysed using general linear modelling (GLM) with treatment (AL or CR) and age (4, 12 or 21 months of age) introduced as fixed factors. All non-significant interaction effects ($p > 0.05$) were removed to obtain the best-fit model in each case, with only significant interactions reported. *Post hoc* Tukey tests were performed to examine differences between age groups, although it should be noted that these analyses do not take into account the treatment or treatment*age interaction effects. Results are reported as mean ± standard error of the mean (SEM), with $p < 0.05$ regarded as statistically significant. Significant treatment effects are denoted by $^{t}p < 0.05$, $^{tt}p < 0.01$, $^{ttt}p < 0.001$; and significant age effects denoted by $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$.

3. Results

DR mice were significantly lighter than AL mice at all timepoints (Fig. S1; $F = 196.977$, $p < 0.001$). A significant age-associated increase in body mass was also observed in both groups ($F = 33.802$, $p < 0.001$).

DR had no effect ($F = 0.786$, $p = 0.381$) on citrate synthase (CS) activity (Fig. 1), implying that mitochondrial content was unchanged. However, a significant age effect ($F = 12.460$, $p < 0.001$; *post hoc* 4 month *vs* 12 month $p = 0.001$, 12 month *vs* 21 month $p = 0.002$) and significant treatment*age interaction were observed ($F = 5.431$, $p < 0.01$).

Mice undergoing DR had significantly higher total peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) protein levels, the 'master' regulator of mitochondrial biogenesis, compared to AL mice (Fig. 2A; $F = 40.690$, $p < 0.001$), although by 21 months of age this difference was lost. In addition, there was also a highly significant age effect ($F = 20.231$, $p < 0.001$; *post hoc* 4 month *vs* 21 month $p < 0.001$, 12 month *vs* 21 month $p < 0.001$), with levels in both groups decreased by 21 months of age and a significant treatment*age interaction also observed ($F = 18.407$, $p < 0.001$). As PGC-1α is a key transcriptional co-activator we also examined both nuclear (Fig. 2B) and cytoplasmic (Fig. 2C) protein levels. Nuclear PGC-1α levels were increased significantly by DR ($F = 25.373$; $p < 0.001$), with, perhaps surprisingly, an age-related increase ($F = 17.556$, $p < 0.001$; *post hoc* 4–21 month $p = 0.001$, 12 month *vs* 21 month $p < 0.001$). A treatment*age interaction was also observed ($F = 4.223$, $p < 0.05$). Cytoplasmic PGC-1α levels were unaffected by either treatment ($F = 0.852$, $p = 0.362$) or age ($F = 0.854$, $p = 0.434$). The protein levels of mitochondrial transcription factor A (TFAM), which regulates mitochondrial DNA expression, was unaltered by DR (Fig. 2D; $F = 0.723$, $p = 0.400$), but a highly significant age-related decline was seen in both AL and DR mice ($F = 9.740$, $p < 0.001$; *post hoc* 4 month *vs* 12 month $p = 0.005$, 4 month *vs* 21 month $p < 0.001$). Fig. S2 shows representative blots of total PGC-1α, nuclear PGC-1α, cytoplasmic PGC-1α, TFAM and GAPDH.

The expression of *Pgc-1α* was significantly higher in DR mice (Fig. 3A; $F = 126.151$, $p < 0.001$), with expression decreasing in an age-related manner ($F = 29.653$, $p < 0.001$; *post hoc* 4 month *vs* 12 month $p = 0.031$, 4 month *vs* 21 month $p < 0.001$, 12 month *vs* 21 month $p < 0.001$), which was much more apparent in AL mice. This different age-related pattern explained the significant treatment*age interaction ($F = 5.027$, $p < 0.001$). Similarly, DR significantly increased the mRNA levels of *Nrf1* (Fig. 3B; $F = 39.443$, $p < 0.001$)), *Core 1* (Complex 3; Fig. 3C; $F = 45.651$, $p < 0.001$), *Cox IV* ((Fig. 3D; $F = 51.700$, $p < 0.001$) and mitochondrial *Atps* (Fig. 3E; $F = 24.079$,

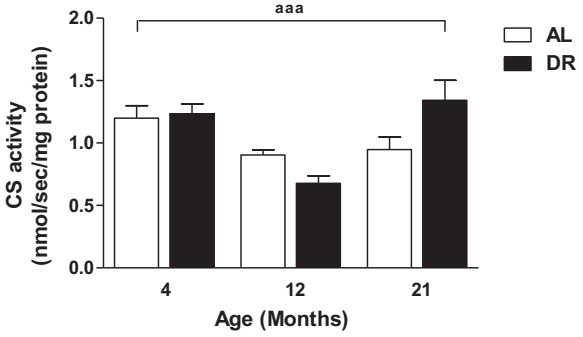

Fig. 1. Citrate synthase activity was unaltered by DR in hindlimb skeletal muscle, although activity levels were significantly altered by age. Values are expressed as mean ± SEM for $N = 7$ per group. Age effects $^{aaa}p < 0.001$.

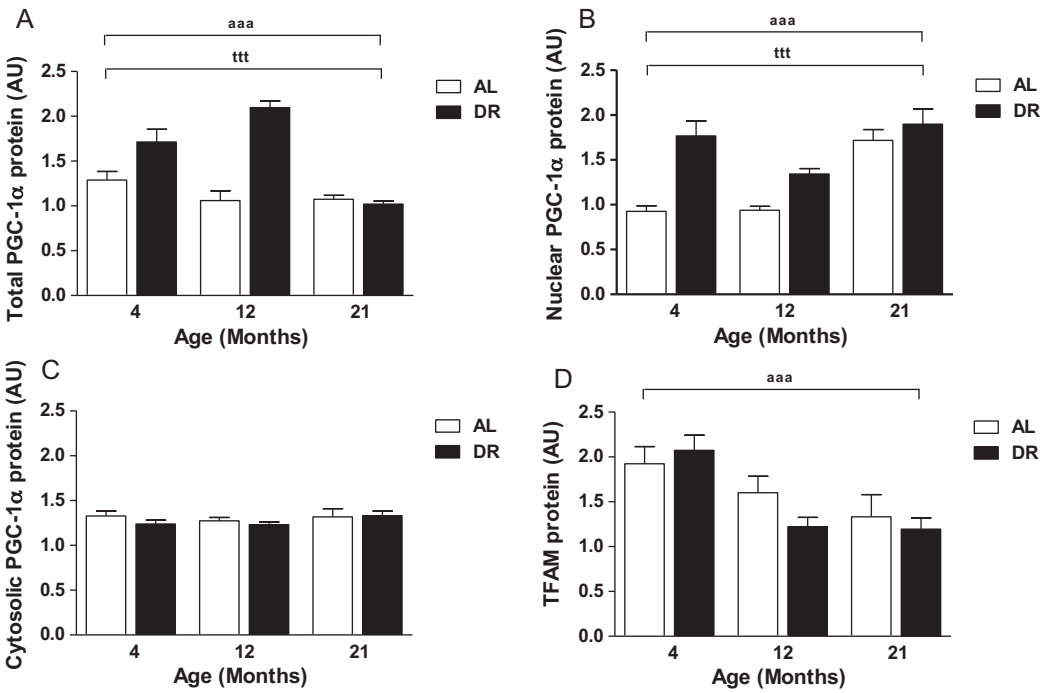

Fig. 2. DR increased PGC-1α protein levels in total (A), nuclear fraction (B), but not in the cytoplasmic (C) fraction, from mouse hindlimb skeletal muscle. DR had no effect on TFAM levels, although a significant age-related decline was observed (D). Values for A–D are arbitrary units (AU) relative to GAPDH. Mice were 4, 12 or 21 months of age (equivalent to 30% DR for 1, 9 or 18 months). All values are expressed as means ± SEM for $N = 8$ per group. Treatment effects $^{ttt}p < 0.001$; age effects $^{aaa}p < 0.001$. Figure S2 shows representative blots for A-D and for GAPDH.

$p < 0.001$). Neither *Nrf1* nor *Atps* showed any age-related change in expression levels ($F = 1.739$, $p = 0.188$ and $F = 0.676$, $p = 0.514$ respectively), although a significant age effect was observed for *Core 1* ($F = 7.169$, $p < 0.01$; *post hoc* 4 month *vs* 12 month $p = 0.002$, 12 month *vs* 21 month $p = 0.037$) and *Cox IV* ($F = 3.773$, $p < 0.05$; *post hoc* 12 month *vs* 21 month $p = 0.024$). Significant treatment*age interactions were detected for *Nrf1* ($F = 4.200$, $p < 0.05$), *Cox IV* ($F = 3.768$, $p < 0.05$), and *Atps* ($F = 7.519$, $p < 0.01$).

Mitochondrial respiration (Fig. 4A) in permeabilised gastrocnemius myofibres, in the resting state (without any substrate addition) was significantly elevated in DR mice ($F = 9.782$, $p < 0.01$), particularly at 21 months of age. A significant age effect was also observed ($F = 6.277$, $p < 0.01$; *post hoc* 4 month *vs* 21 month $p = 0.019$, 12 month *vs* 21 month $p = 0.006$), with a clear age-related increase in DR mice that was not apparent in AL mice leading to a significant treatment*age interaction ($F = 5.085$, $p < 0.05$). Leak respiration was also significantly increased in DR mice (Fig. 4B; $F = 9.976$, $p < 0.01$), which was particularly noticeable at 12 months of age (9 month of 30% DR). A significant age related decline was also observed in leak respiration ($F = 9.689$, $p < 0.001$; *post hoc* 4–21 month $p < 0.001$, 12 month *vs* 21 month $p = 0.013$; treatment*age interaction $F = 4.296$, $p < 0.05$), with this decline occurring later in DR mice compared to AL mice (*i.e.* after 12 months of age rather 4 months of age). Complex 1 driven OXPHOS (Fig. 4C) was increased by DR ($F = 24.335$, $p < 0.001$). An age-related decline in complex 1 driven OXPHOS was also seen in AL mice from 4 months of age but this was delayed in DR mice (age: $F = 21.511$, $p < 0.001$, treatment*age $F = 12.891$, $p < 0.001$ respectively; *post hoc* 4 month *vs* 12 month $p = 0.030$, 4 month *vs* 21 month $p = 0.003$, 12 month *vs* 21 month $p < 0.001$). Maximal respiration rate (simultaneous complex I and II OXPHOS, Fig. 4D) showed a significant treatment ($F = 26.411$, $p < 0.001$), age ($F = 12.436$, $p < 0.001$; *post hoc* 4 month *vs* 21 month $p = 0.006$, 12 month *vs* 21 month $p < 0.001$) and treatment*age ($F = 8.183$, $p < 0.001$) interaction, being higher in DR than AL mice and showing a delay in an age-related decline in DR mice. Complex II driven OXPHOS respiration (observed following the titration of complex I inhibitor rotenone) was similarly increased by DR (Fig. 4E; $F = 26.918$, $p < 0.001$), showing a similar age-related decline ($F = 7.334$, $p < 0.01$; *post hoc* 4 month *vs* 21 month $p = 0.049$, 12 month *vs* 21 month $p = 0.002$) that was partially attenuated in the DR mice (treatment*age interaction, $F = 10.309$, $p < 0.001$). Finally we examined uncoupled respiration (Fig. 4F), following the addition of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). Uncoupled respiration was also elevated in DR mice at all ages ($F = 33.194$, $p < 0.001$). An age-related decline in uncoupled respiration was also observed ($F = 10.980$, $p < 0.001$; *post hoc* 4 month *vs* 21 month $p = 0.018$, 12 month *vs* 21 month $p < 0.001$), which occurred earlier in AL mice than DR mice (treatment*age interaction, $F = 11.462$, $p < 0.001$). These data demonstrate that DR increased mitochondrial respiration in permeabilised skeletal muscle myofibres. In addition, the age-related declines in several respiratory states observed in AL mice were delayed by DR. Respiratory control ratios (Fig. S3) were unaffected by either treatment ($F = 0.605$, $p = 0.441$) or age ($F = 1.070$, $p = 0.353$) in this study.

4. Discussion

It is well established that an individual's mitochondrial phenotype is malleable, responding to energetic requirements and/or substrate delivery to maintain bioenergetic efficiency (Brand, 2005; Hancock et al., 2011). Consequently, impairments in this system can lead to profound health consequences, with mitochondrial dysfunction appearing to play a central role in ageing (Finley and Haigis, 2009; Hunt et al., 2006). As discussed earlier, mitochondrial biogenesis is proposed as a key mechanism underlying DR (Civitarese et al., 2007; Lopez-Lluch et al., 2006; Nisoli et al., 2005). In agreement, several long-lived mutant mice have increased (mRNA and protein) levels of mitochondrial-associated proteins (Katic et al., 2007; Selman et al., 2008), suggesting that alterations in mitochondrial biology may be a

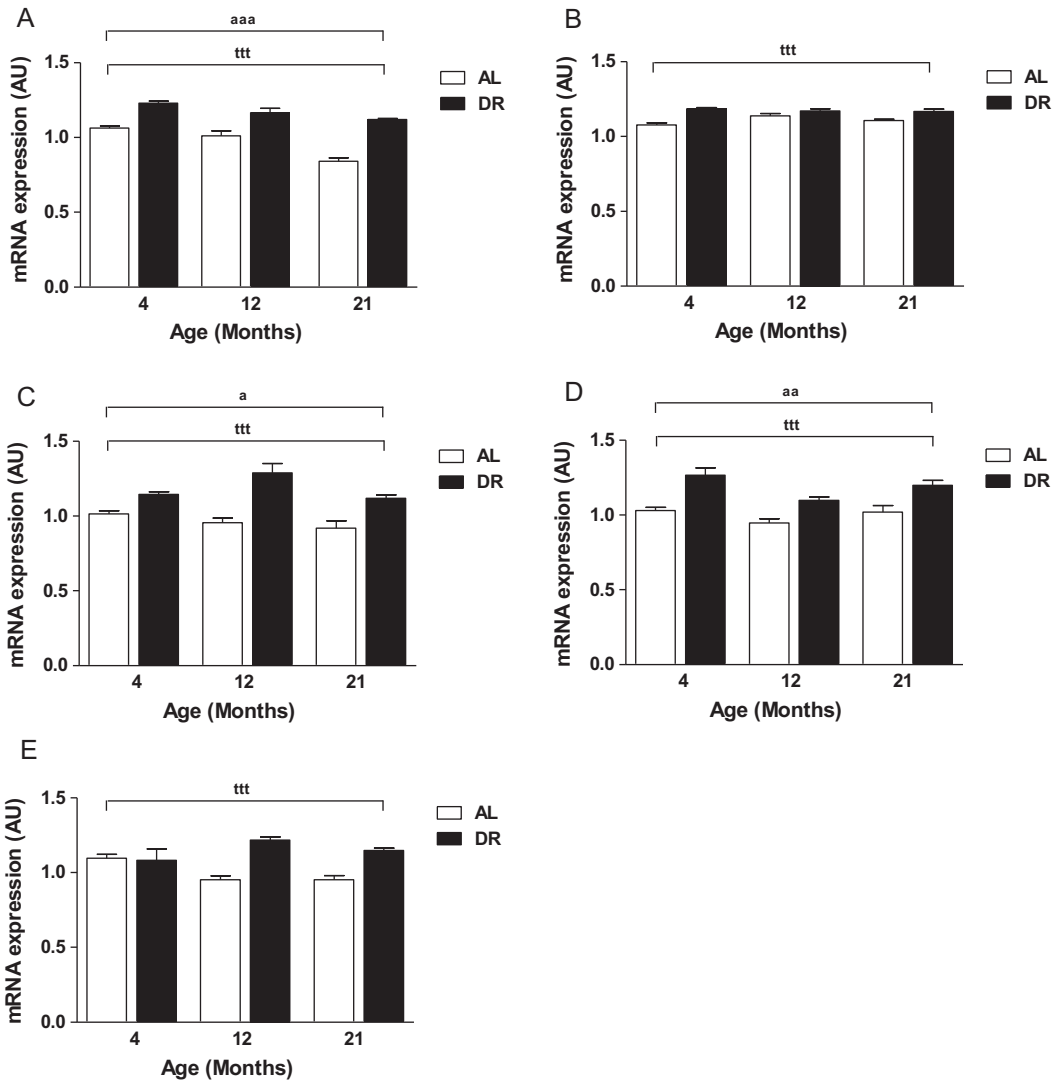

Fig. 3. DR significantly increased the mRNA expression levels of (A) *Pgc-1α*, (B) *Nrf1*, (C) *Core 1* (Complex 3), (D) *Cox IV* and (E) mitochondrial *Atps*. Mice were 4, 12 or 21 months of age (equivalent to 30% DR for 1, 9 or 18 months). Values (AU = arbitrary units relative to *Tcea1*) are expressed as means ± SEM for $N = 8$ per group. Treatment effects $^{ttt}p < 0.001$; age effects $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$.

conserved lifespan determinant. However, recently the notion that mitochondrial biogenesis underlies DR was not supported in a study of rats (Hancock et al., 2011). In accordance with this study, we show that CS activity, a mitochondrial matrix enzyme used as a marker of mitochondrial content, was unaltered between AL and DR mice. However, a mid-life decline in CS activity in both AL and DR mice was partially reversed by 21 months of age in DR mice (30% DR for 18 months).

Despite CS activity in skeletal muscle being unaffected by DR, several protein and transcriptional markers associated with mitochondrial biogenesis were increased significantly by DR. DR also attenuated many of the age-related declines in these parameters observed in AL mice. Strikingly PCG-1α (total and nuclear) protein and *Pgc-1α* mRNA levels were significantly increased by DR, although with the exception of mRNA, levels were comparable in 21 month old AL and DR mice. These findings also suggest that alterations in PCG-1α protein during ageing cannot be completely explained by changes at the transcript level, again supporting the idea that post-transcriptional turnover of PCG-1α leading to adaptive changes in mitochondrial function are critical during DR and ageing (Anderson et al., 2008). PCG-1α acts as a 'master regulator of mitochondrial biogenesis', with additional roles in energy metabolism, metabolic health and muscular function (Anderson and Prolla, 2009; Kraft et al., 2006; Lopez-Lluch et al., 2008; Puigserver and Spiegelman, 2003; Wu et al., 1999). The increase in PCG-1α levels in the nuclear, but not cytosolic, fraction following DR is interesting as its presence in the nucleus appears central to its ability to regulate mitochondrial function (Anderson et al., 2008). Ectopic expression of PGC-1α in muscle cells increased mitochondrial DNA content and increases expression of genes associated with oxidative phosphorylation (Wu et al., 1999). Increased PGC-1α expression also increased mitochondrial membrane potential (Valle et al., 2005) and increased myofibre O_2 consumption (Wu et al., 1999), whilst PGC-1α knockout mice have lower COX IV activity and reduced mitochondrial respiration in skeletal muscle compared to controls (Adhihetty et al., 2009). Perhaps surprisingly, TFAM protein levels were unaffected by DR, although a significant age-related decline was observed in both AL and DR mice. TFAM, which is co-activated by PGC-1α and NRF-1, plays a key role in regulating mitochondrial transcription and in the maintenance of mitochondrial copy number (Arany et al., 2005; Joseph et al., 2006). Genes associated with the respiratory chain (*Nrf-1*, *Core 1* (Complex 3), *Cox IV* and mitochondrial *Atps*) were all significantly increased following DR, in common with previous findings (Civitarese et al., 2007; Nisoli et al., 2005). However, it should be noted that whilst Nisoli et al.

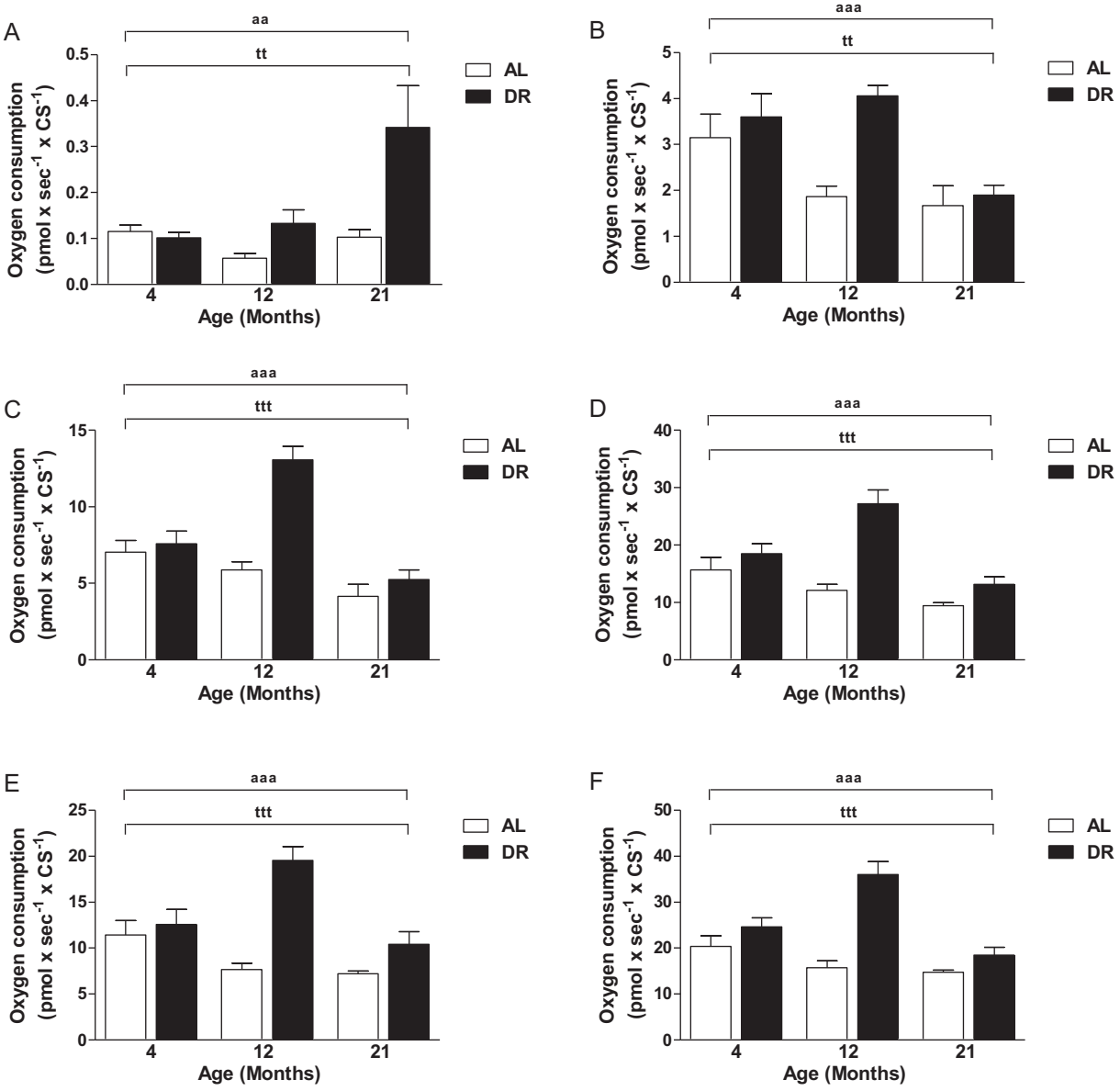

Fig. 4. DR increased mitochondrial respiration in permeabilised gastrocnemius myofibres in mice and partially attenuated age-related declines in mitochondrial respiration observed in AL mice. Resting state (A; without any substrate addition). (B) Leak respiration, (C) complex 1 driven oxidative phosphorylation (OXPHOS), (D) maximal respiration rate (simultaneous complex I and II driven OXPHOS, (E) complex II driven OXPHOS respiration and (F) uncoupled respiration (see Section 2 for the specific substrates used). Mice were 4, 12 or 21 months of age (equivalent to 30% DR for 1, 9 or 18 months). Values are expressed as mean ± SEM for $N = 7$ per group. All values are expressed relative to citrate synthase activity. Treatment effects $^{tt}p < 0.01$, $^{ttt}p < 0.001$; age effects $^{aa}p < 0.01$, $^{aaa}p < 0.001$.

(2005) observed significant increases in CS levels in a range of tissues, they did not examine skeletal muscle. Whilst Pgc-1α, *Core 1* and *Cox1V* showed a similar age-related decline to total and nuclear PGC-1α, neither *Nrf1* nor *Atps* showed any such decline, perhaps suggesting that the specific relationship between transcription factors and their target genes may alter during ageing. Interestingly, we also report that COX IV content, a subunit of the electron transport system (ETS), in contrast to CS, was significantly increased in our DR group (Fig. 5). COX IV content was also significantly reduced with ageing, with this enzyme previously reported to be particularly prone to age-related decreases in skeletal muscle relative to other mitochondrial enzymes (Hepple et al., 2005). Despite this age-related decline, the DR-related increase meant that at 21 months of age DR mice still had COX IV levels similar to 4 month old AL mice. Hancock et al. (2011) reported no effect of DR on total PGC-1α levels in contrast to our findings. The reason for this lack of agreement is unclear, but differences in the species used (rat *vs* mouse), the tissue studied (fore *vs* hindlimb skeletal muscle) and the duration of DR (3 *vs* 1, 9 and 18 month) may be important.

Whilst mitochondrial content was unaltered, our protein and transcriptional data showed clear changes following DR, in agreement with other studies (*e.g.* Civitarese et al., 2007). To examine whether these changes impacted on mitochondrial function, we employed high resolution respirometry (HRR) in permeabilised myofibres. In common with other studies (for review see Lopez-Lluch et al., 2008), a general decline in mitochondrial respiration was seen with advancing age that was partially rescued by DR. DR also significantly increased mitochondrial respiration in skeletal muscle across a range of mitochondrial respiratory states relative to AL mice. In particular, striking differences between AL and DR mice was observed during the mid-time-point (12 months of age, equating to 9 months of 30% DR). As discussed earlier, the directional effects of DR on mitochondrial

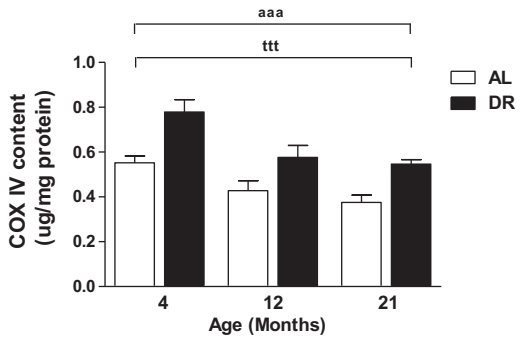

Fig. 5. Cytochrome c oxidase (COX IV) content was significantly increased by DR in hindlimb skeletal muscle ($F = 28.170$, $p < 0.001$). An age-related decline was also observed across both the AL and the DR groups ($F = 13.076$, $p < 0.001$; *post hoc* 4–12 month $p = 0.001$, 4–21 month $p < 0.001$). Values for COX IV levels (E) are expressed as μg/mg protein. All values are expressed as means ± SEM for $N = 8$ per group. Treatment effect $^{ttt}p < 0.001$; age effect $^{aaa}p < 0.001$.

respiration appear ambiguous (Bevilacqua et al., 2004, 2005; Gredilla et al., 2001; Hagopian et al., 2011; Lal et al., 2001; Lambert et al., 2004b; Lopez-Lluch et al., 2006; Nisoli et al., 2005; Sohal et al., 1994; Weindruch et al., 1980). The lack of consensus may be due to several factors, *e.g.* model organism, tissue-specificity, isolation protocol, DR duration, which are reviewed in detail elsewhere (Hunt et al., 2006; Picard et al., 2010). However, to our knowledge, ours is the first study employing HRR to simultaneously examine the impact of both DR and ageing on mitochondrial function in permeabilised myofibres of mice. This approach may give a better perspective on the *in situ* conditions encountered by mitochondria during DR and ageing (Picard et al., 2010). An increase in respiration during a period of reduced energy consumption (*i.e.* DR) may be difficult to reconcile (see Hancock et al., 2011), although there are some caveats to this perceived bottleneck. The impact of DR on metabolic rate is confounded by associated changes in body mass and composition (see Even et al., 2001; Ferguson et al., 2008; Hempenstall et al., 2010; Selman et al., 2005). Indeed, whilst total energy consumption is reduced following DR, total energy expenditure was higher than that predicted in DR rats from their altered body composition (Selman et al., 2005). In addition, resting metabolic rate was not significantly altered in male C57BL/6 mice following appropriate corrections for body mass changes (Hempenstall et al., 2010). We suggest that the energetic constraints experienced during DR may not be universally similar across all tissues during DR. That is, individual tissues that contribute proportionately more or less to total metabolism, may not respond metabolically to DR in exactly the same manner (see Lambert et al., 2004b).

Our data strongly supports other studies (Baker et al., 2006; Hepple et al., 2005, 2006) suggesting that DR may attenuate age-related declines in skeletal muscle physiology through altering mitochondrial function. However, we saw no evidence that mitochondrial content was increased (CS activity), in common with earlier studies (Civitarese et al., 2007; Hancock et al., 2011), despite a clear increase in mitochondrial respiration in following DR in our study. However, such an uncoupling of mitochondrial content and function has been reported in other studies. For example, mitochondrial mass in wild type *C. elegans* was unaltered by age, despite a reduction in key mitochondrial proteins and a decline in energy production (Brys et al., 2010). In addition, changing requirements for ATP can occur *via* specific alterations in the synthesis and/or activities of specific respiratory chain components (Herzig et al., 2000). We suggest that DR may increase the number of available entry points for electrons through targeted biogenesis of ETS components, rather than mitochondrial biogenesis *per se*. An increase in ETS components per mitochondria without an increase in mitochondrial content was recently reported in long-lived yeast (Mittal et al., 2009). The authors went on to suggest that this strategy was more efficient as it reduced both 'electron stalling' and ROS production. In support, an age-related decline in complex IV high affinity sites that was attenuated by DR has been reported in skeletal muscle of mice (Feuers, 1998), leading to the suggestion that age-associated obstruction of ETS binding sites would inhibit electron flow, resulting in mitochondrial dysfunction and increased ROS. Similarly, increased complex IV turnover at maximal O_2 consumption resulting in higher O_2 flux was seen in skeletal muscle from DR rats (Hepple et al., 2005). In terms of DR and the energetic constraints argument discussed earlier, Mittal et al. (2009) propose that up-regulation of specific ETS subunits will be energetically less costly than the synthesising of new mitochondria, although this 'ETS biogenesis' will still maintain optimal and efficient mitochondria. Indeed, mitochondrial turnover to maintain optimal efficiency may also be important without any need for an overall increase in mitochondrial content, with fractional synthesis rates of protein in some muscles (Zangarelli et al., 2006) and mitochondrial turnover in liver (Miwa et al., 2008) being increased by DR. DR was also recently reported to maintain mitochondrial protein synthesis (fractional synthesis) in mice over a 6-week period in liver, heart and skeletal muscle but decreased cellular proliferation (DNA synthesis) over this same period (Miller et al., 2011). This may help explain our finding that whilst CS activity did not alter with DR, COX IV content, a subunit of the electron transport system (ETS), was significantly increased by DR.

5. Conclusions

We demonstrate that DR does not increase mitochondrial content, as determined by CS activity, in skeletal muscle of mice. However, DR increased PGC-1α levels, increased mRNA levels of several mitochondrial-associated genes, increased COX IV content and increased mitochondrial respiration in permeabilised myofibres. DR also attenuated the age-related decline in several of these parameters that was observed in AL mice. Thus, we suggest that DR induces an adaptive response *via* PGC-1α (Anderson et al., 2008) that helps maintain a functionally 'efficient' ETS and hence mitochondria in skeletal muscle, possibly through increased turnover of mitochondria rather than any increase in mitochondrial number *per se*. We propose that these changes are critical for the ability of DR to attenuate the age-related declines in mitochondrial respiration observed in AL mice. We also propose that studies examining the turnover of mitochondrial *in vivo* will be critical to our further understanding of how DR and ageing impact on mitochondria and on mitochondrial function.

Conflict of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mad.2011.12.002.

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