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ORIGINAL ARTICLE

Minimal adaptation of the molecular regulators of mitochondrial dynamics in response to unilateral limb immobilisation and retraining in middle-aged men

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Abstract

Purpose Mitochondrial dynamics are regulated by the differing molecular pathways variously governing biogenesis, fission, fusion, and mitophagy. Adaptations in mitochondrial morphology are central in driving the improvements in mitochondrial bioenergetics following exercise training. However, there is a limited understanding of mitochondrial dynamics in response to inactivity.

Methods Skeletal muscle biopsies were obtained from middle-aged males ($n = 24$, 49.4 ± 3.2 years) who underwent sequential 14-day interventions of unilateral leg immobilisation, ambulatory recovery, and resistance training. We quantified v*astus lateralis* gene and protein expression of key proteins involved in mitochondrial biogenesis, fusion, fission, and turnover in at baseline and following each intervention.

Results PGC1α mRNA decreased 40% following the immobilisation period, and was accompanied by a 56% reduction in MTFP1 mRNA, a factor involved in mitochondrial fission. Subtle mRNA decreases were also observed in TFAM (17%), DRP1 (15%), with contrasting increases in BNIP3L and *PRKN* following immobilisation. These changes in gene expression were not accompanied by changes in respective protein expression. Instead, we observed subtle decreases in NRF1 and MFN1 protein expression. Ambulatory recovery restored mRNA and protein expression to pre-intervention levels of all altered components, except for BNIP3L. Resistance training restored BNIP3L mRNA to pre-intervention levels, and further increased mRNA expression of OPA-1, MFN2, MTFP1, and PINK1 past baseline levels.

Conclusion In healthy middle-aged males, 2 weeks of immobilisation did not induce dramatic differences in markers of mitochondria fission and autophagy. Restoration of ambulatory physical activity following the immobilisation period restored altered gene expression patterns to pre-intervention levels, with little evidence of further adaptation to resistance exercise training.

Keywords Muscle · Mitochondria · Immobilisation · Fissions · Fusion · Mitophagy

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Abbreviations

AIFM2	Apoptosis-inducing factor 2
AR	Ambulatory recovery
BL	Baseline
DRP1	Dynamin-1-like protein
Fis1	Mitochondrial fission 1 protein
IM	Immobilisation
LC3B	MAP1 light chain 3
MFF	Mitochondrial fission factor
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MTFP1	Mitochondrial fission process protein 1
NRF1	Nuclear regulatory factor 1
NRF2	Nuclear regulatory factor 2
OPA1	Optic atrophy 1
PINK1	PTEN-induced putative kinase 1
PGC1α	Peroxisome proliferator-activated receptor γ coactivator-1α
ROS	Reactive oxygen species
RT	Resistance training
TFAM	Mitochondrial transcription factor A

Introduction

Muscle atrophy is a common consequence of prolonged skeletal muscle disuse (resulting from bedrest, injury and inactivity) which ultimately leads to decreased muscle strength (Abadi et al. 2009). Activation of protein degradation pathways and inhibition of anabolic signalling contributes to the alterations in skeletal muscle architecture resulting from chronic muscle disuse (Goldspink 1999; Vazeille et al. 2008; Breen et al. 2013). Impaired mitochondrial function and oxidative stress contribute to the induction of proteolytic systems and atrogenic pathways in rodent models of hindlimb immobilisation (Krawiec et al. 2005; Talbert et al. 2013); however, human interventions have yielded conflicting results. While there is evidence to support immobilisation-induced decreases in mitochondrial respiratory capacity in humans (Gram et al. 2014; Miotto et al. 2019), there is also conflicting resulting where mitochondrial function is unchanged following immobilisation and inactivity (Salvadego et al. 2016; Pileggi et al. 2018; Edwards et al. 2020). Changes in mitochondrial networking and turnover regulate mitochondrial function, and may precede adaptions in skeletal muscle mitochondrial bioenergetics in response to exercise and inactivity.

Within skeletal muscle, mitochondria form as branched interconnected reticular networks (Kirkwood et al. 1986; Glancy et al. 2015). Maintenance of these networks is defined by the balance of four processes that control mitochondrial turnover: biogenesis, fusion, fission, and mitophagy. Mitochondrial biogenesis involves the synthesis of mitochondrial proteins and mRNAs that are imported into mitochondria for expansion of the mitochondrial electron transport chain (ETC). Mitochondrial biogenesis is transcriptionally controlled by peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) (Puigserver et al. 1998; Wu et al. 1999), the nuclear regulatory factors 1 and 2 (NRF1 and NRF2), estrogen-related receptor α (ERRα), and the mitochondrial transcription factor A (TFAM) (Virbasius and Scarpulla 1994; Schreiber et al. 2004; Handschin and Spiegelman 2006). Increases in mitochondrial biogenesis lead to elevations in mitochondrial mass, and prompt expansion of the mitochondrial reticulum by promoting fusion of adjacent mitochondria (Carter et al. 2015).

Activation of fission and fusion proteins allow for the networking of existing mitochondria by linking and dividing mitochondrial membranes, to facilitate reorganization of mitochondrial ultrastructure. At the cellular level, fusion allows for the exchanging of mtDNA (Ono et al. 2001), whereas fission is important for the adjustment of mitochondrial density as per metabolic requirements (Kissova et al. 2004), and to prime damaged mitochondria for elimination by mitophagy (Ashrafi and Schwarz 2013). Fusion is initiated by large mitochondrial GTPase proteins, mitofusin 1 and mitofusin 2 (MFN1 and MFN2), which tether opposing outer mitochondrial membranes (Koshiba et al. 2004). The optic atrophy 1 (OPA1) protein completes the fusion process to form a reticulum through fusion of the inner mitochondrial membranes (Meeusen et al. 2006). In contrast, fission is initiated when mitochondrial fission factor (MFF) or the mitochondrial fission 1 protein (Fis1), recruits dynamin-related protein 1 (DRP1) to constrict and sever the inner and outer mitochondrial membranes, dividing elongated mitochondria into smaller fragments (Elgass et al. 2013). The smaller fragments can subsequently be encapsulated by autophagosomes (Youle and Narendra 2011). The major pathway of degradation of mitochondria by autophagosomes, also termed mitophagy, involves activation of PTEN-induced putative kinase protein 1 (PINK1) (Yang et al. 2008) and parkin (Vives-Bauza et al. 2010), which ubiquitinate proteins on the mitochondrial membrane to allow binding of p62. The microtubule-associated proteins 1A/1B light chain (LC3) recognizes p62 and encapsulates mitochondria (Kim et al. 2007), which subsequently undergoes lysosomal degradation by proteolytic enzymes (Nakatogawa et al. 2009).

Within the context of physical activity, exercise has been shown to rapidly induce PGC1α expression (Baar et al. 2002; Norrbom et al. 2004), promote mitochondrial networking (Kirkwood et al. 1987), and enhance the expression of autophagic markers in skeletal muscle (He et al. 2012; Brandt et al. 2018; Memme et al. 2021). In contrast, inactivity leads to decreases in mitochondrial size, density, and fusion in rodent models of denervation-induced muscle disuse (Miledi and Slater 1968; Adhihetty et al. 2007;

Iqbal et al. 2013), and suppressed PGC1α mRNA expression in immobilised skeletal muscle from young adult males (Alibegovic et al. 2010). While alterations in mitochondrial dynamics offer an explanation for the plasticity of skeletal muscle mitochondria during chronic muscle disuse and exercise in rodents, there remains a paucity of data from human studies demonstrating how these processes are regulated following both immobilisation and resistance training.

The aim of the present study was to identify and characterize the alterations in mitochondrial dynamics in response to a period of immobilisation followed by two periods of remobilisation consisting of ambulatory recovery and supervised resistance training in middle-aged males. Changes in mitochondrial morphology and networking can drive changes in bioenergetic function, suggesting that changes in mitochondrial dynamics and turnover may precede the adaptions in bioenergetic function (Twig et al. 2008). Therefore, we hypothesised that a 2-week immobilisation period would decrease expression of fusion markers and increase expression of fission and mitophagy markers. Furthermore, we postulated that subsequent resumption of ambulation would partially restore mitochondrial dynamics similar to baseline levels, whereas a subsequent period of resistance exercise would favour expansion of the mitochondrial reticulum by promoting mitochondrial transcription and fusion.

Materials and methods

Participants

Participant inclusion/exclusion criteria for this study have previously been described elsewhere (Australia New Zealand Clinical Trial Registry No. ACTRN12615000454572) (Mitchell et al. 2018). Briefly, 30 healthy male participants were recruited to participate in the present study, with molecular data from 24 of the participants aged 49.43 ± 3.22 years being presented in the current study. Experimental procedures were approved by the Northern A New Zealand Health and Disability Ethics Committee (Ref# 14/NTA/146/AM02).

Experimental design

The intervention protocol used in this study has previously been described elsewhere (Mitchell et al. 2018). In brief, activity was tracked (FitBit Charge) throughout all intervention phases, excluding immobilisation, and meals provided. Participants were randomly assigned to receive either a daily protein supplement [20 g milk protein concentrate; amino acid composition reported in (Mitchell et al. 2015); Fonterra Co-operative Group Limited, New Zealand] or an isoenergetic placebo (maltodextrin). The supplement had no effect on any measures included in the current study; therefore, the results presented have been collapsed across supplement groups. Following the 2-week baseline period, the immobilised leg was fitted with a knee brace at a 60° angle (Donjoy IROM, Vista, CA, USA). Participants were provided with crutches and instructed to refrain from weight bearing activity with the immobilised leg (immobilisation). Immediately following the 2-week immobilisation period, participants were counselled to meet physical activity guidelines of 10,000 steps per day (ambulatory recovery). Following the ambulatory recovery phase, participants performed six supervised resistance training sessions over a 2-week period (resistance training), which the protocol is detailed previously (Mitchell et al. 2018).

Skeletal muscle sampling, tissue collection, and preparation

Participants reported to the laboratory for *vastus lateralis* muscle biopsies after an overnight fast prior to immobilisation (baseline), following the removal of the immobilising brace (immobilisation), after 2 weeks of restored ambulation (ambulatory recovery), and following 6-session of resistance exercise (resistance training). The contralateral leg was used for the baseline biopsy, whereas the immobilised leg was used for the remaining three biopsies (immobilisation, ambulatory recovery, and resistance training). Local anaesthetic (1% xylocaine) was injected subcutaneously into the area overlying the *vastus lateralis* and a small incision was made into the skin and underlying fascia. A 5 mm Bergstrom needle, modified for manual suction, was used to extract ~ 100 mg of *vastus lateralis* muscle, which was immediately divided for analysis. A fresh portion of the muscle was used for mitochondrial respiration analysis described elsewhere (Pileggi et al. 2018), and the remaining tissue was snap frozen in liquid nitrogen and stored at − 80 °C for protein and RNA expression, and enzyme activity analyses.

Protein extraction and quantification

Frozen skeletal muscle was weighed and homogenized in ice-cold modified lysis buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM ATP) supplemented with a commercially available protease and phosphatase inhibitor cocktail (Halt™ Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific, #78442, Waltham, MA, USA) using a Bullet Blender at 4 °C (Next Advance, Averill Park, NY, USA). Cell debris was removed by centrifugation at 800×*g* for 10 min at 4 °C, followed by a second 30 min spin at 9000×*g*. The total soluble protein concentration was determined using a BCA-protein kit according to the manufacturer's protocol

(Pierce BCA Protein Assay Kit; Thermo Fisher Scientific #23225, Rockford, IL, USA).

Immunoblotting

Sample aliquots containing 20 µg of protein were suspended in 1 × Laemmli buffer [10% glycerol, 2% SDS, 0.25% bromophenol blue, 400 mM dithiothreitol (DTT), 0.5 M Tris–HCl (pH 6.8)], boiled at 95 °C for 5 min, and subjected to separation by SDS/PAGE. Proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, US) using the semi-dry Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were incubated with blocking buffer [5% bovine serum albumin (BSA)/Tris buffered saline/0.1% Tween 20 (TBST)] for 2 h at room temperature, followed by overnight incubation at 4 °C with primary antibodies in blocking buffer under gentle agitation. Antibodies were obtained from Abcam (Cambridge, MA, USA) unless otherwise stated, and used at a 1:1000 dilution. Samples were probed for: NRF1 (ab55744), TFAM (ab131607), MFN1 (ab57602), MFN2 (ab50843), DRP1 (ab56788), OPA1 (ab42364), TTC11 (Fis1, ab156865), PGC1α (Millipore, ab3242). Membranes were washed for 25 min in TBST and probed with a goat anti-rabbit (H+L) or goat anti-mouse (H+L) IgG secondary antibody conjugated to horseradish peroxidase (HRP; Jackson Laboratories, PA) in 5% BSA/TBST for 1 h at RT. Following this, membranes were washed for 25 min in TBST and protein bands were visualised using Amersham ECL Select Western blotting detection reagent (GE Healthcare, Piscataway, NJ, USA). Signals were captured using a ChemiDoc™ MP Imaging System (Bio-Rad) and band densitometry analysis undertaken with ImageJ (NIH) software (Abramoff et al. 2004). To control for gel-to-gel variation, bands of interest for each sample were normalized to a pooled control sample, which was loaded on each gel. Equal protein loading was determined by stripping and re-probing membranes for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000, ab9485). Abundance of total proteins are presented normalized to GAPDH.

RNA extraction and cDNA synthesis

RNA was extracted using a Qiagen AllPrep DNA/RNA/miRNA universal extraction kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). Total RNA concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). 1000 ng of total RNA was synthesized into single stranded cDNA using a High-capacity RNA to cDNA Kit (Life Technologies, Carlsbad, CA).

Gene expression analysis

Primer pairs for target genes are outlined in Table 1. RT-qPCR was performed using the LightCycler 480 SYBR Green I Master (Roche Applied Science, Indianapolis, IN). As a control for between-sample variability, mRNA levels were normalized to the geometric mean of the chromosome 1 open reading frame 43 (*C1orf43*), charged multivesicular body protein 2A (*CHMP2A*), and ER membrane protein complex subunit 7 (*EMC7*). The relative expression of the gene of interest was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Results are reported as fold change relative to baseline expression for each subject.

Statistical analysis

Statistical analysis was performed using SigmaPlot for Windows version 14.5 (Systat Software Inc., San Jose, CA). Differences between intervention time points were determined using a one-way ANOVA with repeated measures. Where appropriate, multiple comparisons between each time point adjusted using Bonferroni post hoc tests. Normality was tested via Shapiro–Wilk test. When data were not normal distributed, data were log transformed prior to statistical analysis. As a result of missing biopsies or insufficient sample amounts, gene expression analyses were not conducted on biopsies from one participant (all time points), two participants after the ambulatory recovery intervention, and two participants after resistance training. Similarly, immunoblotting analyses were not conducted on biopsies from two participants after the resistance training period due to insufficient sample amounts. Occasionally, due to technical error, some samples were excluded from analysis and/or excluded as outliers (± 5 STD from the mean). Prism software (GraphPad Software Inc., La Jolla, California, USA) was used to generate graphs. Data are shown as means ± SD relative to baseline for each individual, in addition to the scatter dot plots for the individual points. Statistical significance was accepted at $p < 0.05$.

Results

Mitochondrial transcription factors

There was a 40% decrease in *PPARGC1A* (PGC1α) gene expression following the immobilisation period ($p < 0.001$), which was fully restored baseline levels with ambulatory recovery. *PPARGC1A* expression was not further induced following resistance training (Fig. 1a). Similarly, *TFAM* gene expression decreased by 17% following immobilisation ($p = 0.014$), and returned to baseline following ambulatory recovery (Fig. 1a). *NRF1* and *NFE2L2* gene expression did

Table 1 Primer sequences used for quantitative RT-PCR

Gene name	Primer sequence (5^1 to 3^1)
AIFM2	
Forward	GCAAAGCGTTTGAGAGCAGA
Reverse	CAATGGCGTAGACGTTGCTG
BAK1	
Forward	CCTGTTTGAGAGTGGCATC
Reverse	TCGTACCACAAACTGGCCCA
BNIP3	
Forward	CTGAAACAGATACCCATAGCATT
Reverse	CCGACTTGACCAATCCCA
BNIP3L	
Forward	AGCAGGGACCATAGCTCTCA
Reverse	AGTGGAACTCCTTGGGTGGA
DNM1L	
Forward	AGGAGAAGAAAATGGGGTGGAAG
Reverse	GATGAATTGGTTCAGGGCTTAC
FIS1	
Forward	ATCCGTAAAGGCATCGTGCT
Reverse	CGTATTCCTTGAGCCGGTAGT
MAP1LC3B	
Forward	TTCGAGAGCAGCATCCAACC
Reverse	TGAGCTGTAAGCGCCTTCTAA
MFF	
Forward	CTCTCAGCCAACCGCCTCTG
Reverse	GAGAGCCACTTTTGTCCCCCT
MFN1	
Forward	TGTTTTGGTCGCAAACTCTG
Reverse	CTGTCTGCGTACGTCTTCCA
MFN2	
Forward	ATGCATCCCCACTTAAGCAC
Reverse	CCAGAGGGCAGAACTTTGTC
TFAM	
Forward	AGATTCCAAGAAGCTAAGGGTGATT
Reverse	TTTCAGAGTCAGACAGATTTTTCCA
MTFP1	
Forward	GTGCTGCCTCTCTCTATGTCC
Reverse	GGAAATCCACCGACCTGTCAA
NRF1	
Forward	GGCACTGTCTCACTTATCCAGGTT
Reverse	CAGCCACGGCAGAATAATTCA
NFE2L2	
Forward	AAATTGAGATTGATGGAACAGAGAA
Reverse	TATGGCCTGGCTTACACATTCA
OPA1	
Forward	TGCCTGACATTGTGTGGGAA
Reverse	TCCGGAGAACCTGAGGTAAA
PRKN	
Forward	GTGTTTGTCAGGTTCAACTCCA
Reverse	GAAAATCACACGCAACTGGTC
PPARGC1A	
Forward	AGCCTCTTTGCCCAGATCTT
Reverse	GGCAATCCGTCTTCATCCAC
PINK1	
Forward	AGTGATTGACTACAGCAAGGCTGAT
Reverse	ATCTTGTCTAACTTCAGATTCTTCAGG

not differ between interventions ($p = 0.108$ and $p = 0.148$, respectively, Fig. 1a). Alterations in gene expression of *PPARGC1A* and *TFAM* were not accompanied by changes in their respective protein abundances (Fig. 1b). In contrast, protein abundance of NRF1 decreased by 19% following immobilisation ($p = 0.004$), and was restored to baseline levels with ambulatory recovery (Fig. 1b).

Markers of mitochondrial fusion

Gene expression of *OPA1* did not differ from baseline with immobilisation or ambulatory recovery; however, resistance training increased *OPA1* expression by 32% above baseline levels ($p < 0.001$; Fig. 2a). Expression of *MFN1* did not differ across interventions, whereas resistance training resulted in a small ~ 10% increase in *MFN2* gene expression compared to the immobilisation and ambulatory recovery periods ($p = 0.03$ and $p = 0.041$, respectively; Fig. 2a). Protein abundance of OPA1 followed a similar pattern to the alterations in *OPA1* gene expression, with no difference from baseline following immobilisation and ambulatory recovery, but increased by ~ 38% after the resistance training period compared to the immobilisation period (Fig. 2b). Similarly, protein abundance of MFN1 decreased by 29% following the immobilisation period ($p < 0.001$), and was restored to baseline levels with ambulatory recovery, with no further increase following resistance training (Fig. 2b). Protein abundance of MFN2 did not differ between interventions (Fig. 2b).

Markers of mitochondrial fission

Gene expression of *FIS1* and *MFF* did not differ following immobilisation or ambulatory recovery, but *FIS1* increased ~ 16% with resistance training compared to the immobilisation period ($p = 0.025$, Fig. 3a). In contrast, the immobilisation period resulted in a 15% decrease in *DNM1L* (DRP1) gene expression and a dramatic 56% decrease in *MTFP1* expression ($p = 0.014$ and $p < 0.001$, respectively, Fig. 3a). Ambulatory recovery restored expression of *MTFP1* to baseline levels. Resistance training restored the expression of *DNM1L* to baseline levels and further increased *MTFP1* expression by 77% (Fig. 3a). The decrease in *DNM1L* gene expression following immobilisation was

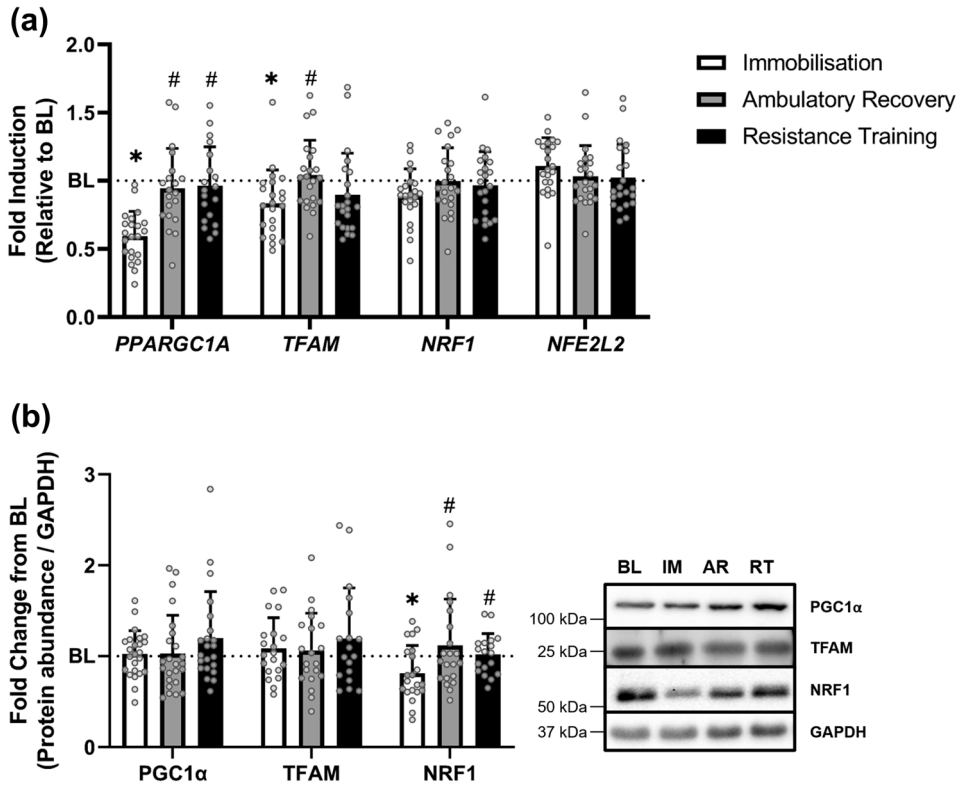

Fig. 1 Gene and protein expression of mitochondrial transcription factors modestly decrease with immobilisation, and recover with the resumption of ambulation. RT-qPCR and immunoblot analysis of vastus lateralis muscle at baseline, after 2 weeks of immobilisation, 2 weeks post-ambulatory recovery, and 2 weeks following supervised resistance training. **a** Gene expression of *PPARGC1A* (PGC1α) and *TFAM* decreased with immobilisation, but were restored with the restoration of activity. Gene expressions of *NRF1* and *NFE2L2* (NRF2) were unchanged across interventions ($n = 20$–23). **b** Protein abundance analysed by western blot demonstrates that PGC1α, TFAM, were unchanged across interventions. NRF1 protein abundance modestly decreased following immobilisation, and was recovered with the resumption of normal ambulation ($n = 20$–24). Data are expressed as mean ± SD relative to baseline for each individual. Representative blots are shown beside graph. $p < 0.05$ * vs. Baseline, # vs. Immobilisation

not reflected in DRP1 protein abundance; However, FIS1 protein abundance increased by 37% following resistance training compared to baseline levels (Fig. 3b).

Markers of autophagy and apoptosis

Gene expression of *ATG5* and *MAP1LCB* (LC3) did not differ across timepoints (Fig. 4a). There was a slight 16% increase in gene expression of *PRKN* (parkin) following the immobilisation period ($p = 0.021$). *PRKN* gene expression was recovered to baseline levels with ambulatory recovery and remained at similar levels with resistance training (Fig. 4a). Gene expression of *PINK1* did not differ with immobilisation or ambulatory recovery, but dramatically increased by 52% following resistance training ($p < 0.001$). BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 *(BNIP3)* gene expression did not differ across interventions (Fig. 4b). In contrast, immobilisation increased gene expression of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like *(BNIP3L)* and apoptosis-inducing factor 2 (*AIFM2*) by 28% and 33%, respectively ($p = 0.039$ and $p < 0.001$, respectively). Ambulatory recovery fully restored *AIFM2* expression to baseline values, whereas *BNIP3L* was recovered after the resistance training period (Fig. 4b). BCL2 antagonist/killer 1 (*BAK1*) gene expression paradoxically decreased following immobilisation ($p < 0.001$) and was restored with baseline levels with ambulatory recovery (Fig. 4b).

Discussion

We have previously demonstrated a decrease in mitochondrial biogenesis following immobilisation in skeletal muscle from middle-aged men (Mitchell et al. 2018), without alterations in mitochondrial respiration (Pileggi et al. 2018). Here, we sought to extend our investigation of the effects of unilateral limb immobilisation and subsequent periods of retraining with the analysis of key molecular components of mitochondrial networking and turnover. Our data

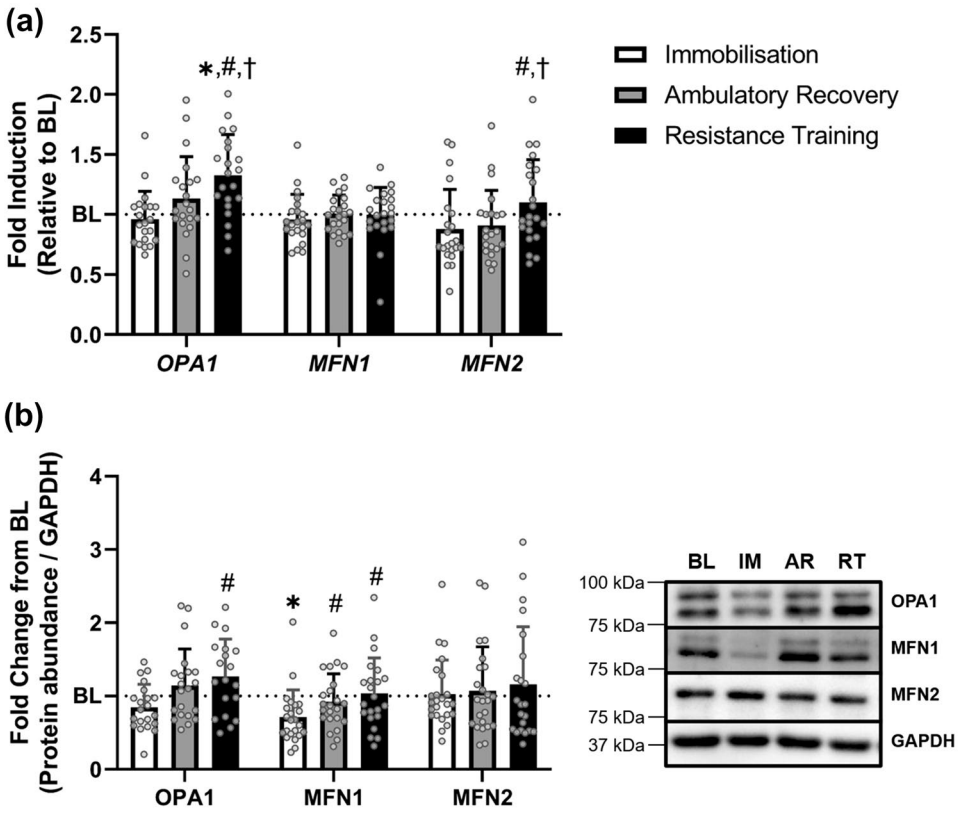

Fig. 2 Resistance training increases mitochondrial fusion gene and protein expression following immobilisation. **a** *MFN1, MFN2,* and *OPA1* gene expression did not differ from baseline following immobilisation or ambulatory recovery; however, supervised resistance training increased *MFN2,* and *OPA1* gene expression ($n = 21$–23). **b** MFN1 protein abundance decreased slightly following immobilisation, and was recovered following normal ambulation ($n = 21$–24). Data are expressed as mean ± SD relative to baseline for each individual. Representative blots are shown beside graph. $p < 0.05$ * vs. Baseline, # vs. Immobilisation, † vs. Ambulatory recovery

demonstrates that a 2-week period of immobilisation fails to elicit widespread suppression of gene expression necessary for encoding proteins involved in mitochondrial biogenesis, fission, fusion, and mitophagy. However, with the resumption of physical activity, first with a period of 2 weeks of normal ambulation and then a subsequent two-week period of resistance exercise training, a pattern of increased gene expression was observed, consistent with a restoration of mitochondrial biogenesis (summarised in Table 2).

Decreases in mitochondrial biogenesis may precede or accompany the loss of muscle mass following muscle disuse. Declines in activity level result in blunted PGC1α mRNA expression in young men (Alibegovic et al. 2010); whereas exercise, including resistance training, leads to robust increases in PGC1α mRNA expression (Baar et al. 2002; Norrbom et al. 2004; Ydfors et al. 2013). Consistent with these findings, the present study reported a decrease in *PPARGC1A* (PGC1α) gene expression following immobilisation, which was restored with ambulatory recovery and resistance training, supporting the concept that PGC1α is sensitive to physical activity level. Notably, bursts of mRNA transcription precede the protein adaptations of PGC1α protein expression (Pilegaard et al. 2003; Perry et al. 2010), and nuclear translocation of existing PGC1α occurs following exercise training (Wright et al. 2007). Consistent with previous research from bedrest studies (Alibegovic et al. 2010), expressions of the transcription factors NRF1 and *TFAM* were downregulated following immobilisation and restored with ambulatory recovery. These decreases in mitochondrial transcription factors following immobilisation explain our previously observed decline in mitochondrial protein synthesis, which was similarly restored following the resumption of physical activity (Mitchell et al. 2018).

The mitochondrial dynamics of fission and fusion pathways play essential quality control roles in maintaining mitochondrial structure and function (Chan 2006). Mitochondrial fusion and fission are disrupted in response to physical inactivity in rodents (Iqbal et al. 2013; Kang et al. 2016); however, the effects of inactivity on the balance of these processes are not well understood in humans. In the present study, we found that protein expression of MFN1 was decreased following immobilisation, accompanied by a small decrease in *DNM1L* (DRP1) gene expression. When considering our previous observation of normal mitochondrial respiration following 14 days of limb immobilisation (Pileggi et al. 2018), the muted effect on mitochondrial fusion and fission machinery suggests that there is a compensatory mechanism to preserve mitochondrial health. Consistent with this idea, in times of nutrient starvation, mitochondria can hyperfuse as an acute stress-response to protect against mitophagy and preserve mitochondrial function (Gomes et al. 2011). Mitochondrial hyperfusion occurs in response to perturbations in cellular redox status (Shutt et al. 2012), as a result of increased MFN2 oligomerization

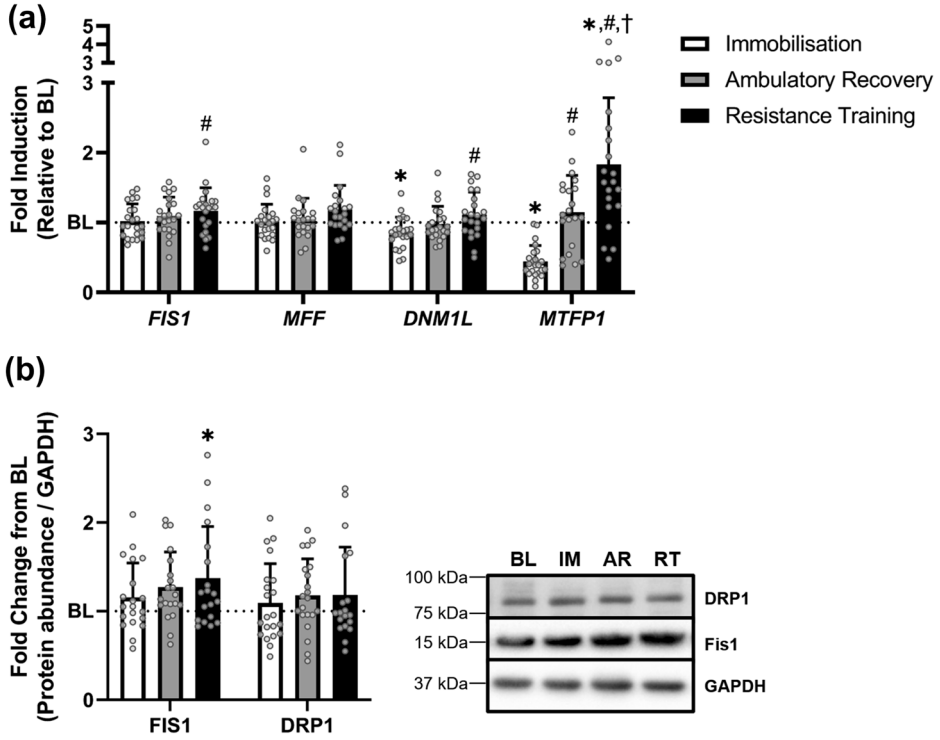

Fig. 3 Resistance training increases mitochondrial fission gene and protein expression following immobilisation. **a** *DNM1L* (DRP1) gene expression modestly decreased following immobilisation, which was restored with resistance training. *MFF* was unchanged across interventions; however, resistance training increased *FIS1* gene expression. Gene expression of *MTFP1* was drastically downregulated following immobilisation, and was restored to baseline levels with ambulatory recovery. Resistance training further increased *MTFP1* expression past baseline levels, ($n = 21$–23). **b** Resistance training increased FIS1 but not DRP1 protein abundance, ($n = 21$–24). Data are expressed as mean ± SD relative to baseline for each individual. Representative blots are shown beside graph. $p < 0.05$ *Baseline, # vs. Immobilisation, † vs. Ambulatory recovery

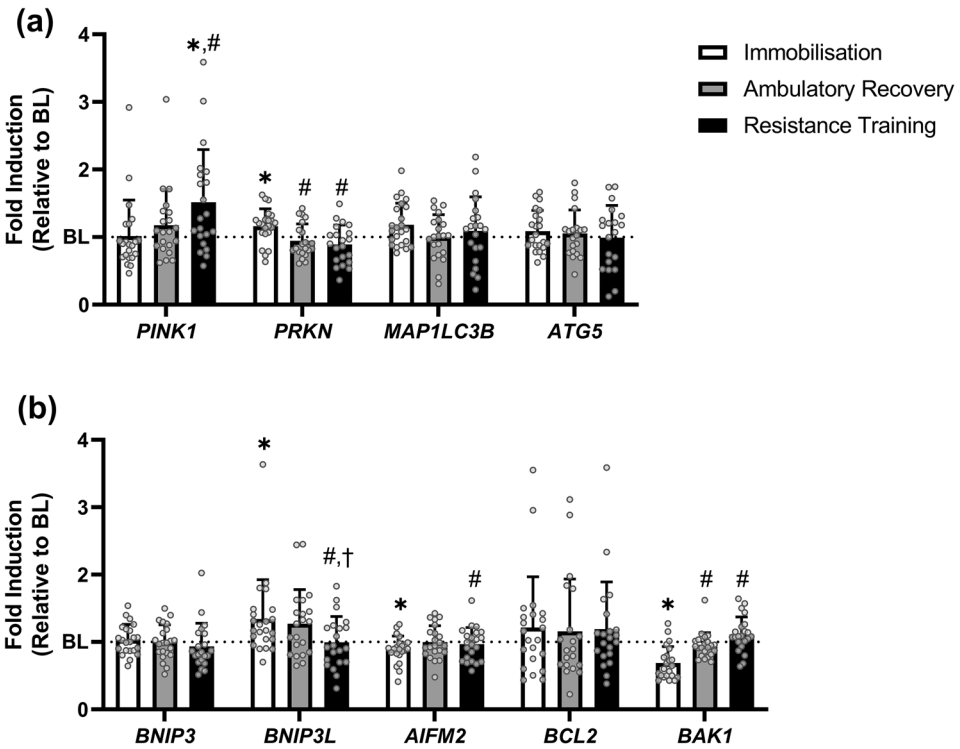

Fig. 4 Gene expression of mitophagic and apoptotic factors are minimally altered with immobilisation and retraining. **a** *PRKN* gene expression modestly increased with immobilisation, but *ATG5* and *MAP1LC3B* expression did not differ across interventions. *PINK1* gene expression increased following 2 weeks of supervised resistance training, ($n = 20$–23). **b** *BNIP3* gene expression was unchanged across interventions. *BNIP3L* and *AIFM2* gene expression increased following immobilisation, whereas *BAK1* expression decreased. Ambulatory recovery restored *BAK1* expression, whereas resistance training recovered *BNIP3L* and *AIFM2* to baseline levels, ($n = 20$–23). Data are expressed mean ± SD relative to baseline for each individual. $p < 0.05$ * vs. Baseline, # vs. Immobilisation, † vs. Ambulatory recovery

Table 2 Summary of changes of gene and protein expression across the immobilisation and retraining intervention periods

Intervention	Biogenesis	Fusion	Fission	Mitophagy and apoptosis
Immobilisation	Gene: ↓ *PPARGC1A*, *TFAM* Protein: ↓NRF1	Protein: ↓MFN1	Gene: ↓*DNM1L*, *MTFP1*	Gene: ↑*PRKN*, *BNIP3L*, ↓*AIFM2*, *BAK1*
Ambulatory recovery	Gene: ↑ *PPARGC1A*, *TFAM* Protein: ↑NRF1	Protein: ↑MFN1	Gene: ↑ *MTFP1*	Gene: ↑*BAK1*, ↓*PRKN*
Resistance training		Gene: ↑*OPA1*, *MFN2* Protein: ↑OPA1	Gene: ↑*Fis1, DNM1L, MTFP1* Protein: ↑FIS1	Gene: ↑*PINK1*, *AIFM2*, ↓ *BNIP3L*

Changes from RT-qPCR and immunoblot analyses of vastus lateralis muscle at baseline, after 2 weeks of immobilisation, 2 weeks post-ambulatory recovery, and 2 weeks following supervised resistance training

(Smith et al. 2019) and inhibition of DRP1 by phosphorylation (Cereghetti et al. 2008). Thus, while the expression of fission proteins did not differ greatly following immobilisation, the small decrease in *DNM1L* gene expression may reflect a compensatory mechanism to preserve bioenergetic function following the immobilisation period.

The resumption of physical activity following a period of immobilisation resulted in enhanced expression of OPA1 and MFN1 fusion machinery, and FIS1 protein expression. An interconnected fused mitochondrial network is more efficient at ATP production than smaller fragmented mitochondria (Romanello and Sandri 2013), which permits greater mitochondrial oxidative capacity (Pich et al. 2005). Therefore, increased expression of OPA1 and MFN1 following resumption of physical activity likely occurs to meet the increased demand for energy production. Moreover, our findings of an increase in FIS1 protein expression are in line with findings from Perry et al. (Perry et al. 2010) that demonstrated continuously robust increases in FIS1 after repeated training sessions. Mitochondrial H_2O_2 emission is elevated following the resumption of physical activity (Pileggi et al. 2018), and is thought to act as a signalling molecule to induce muscle hypertrophy (Merry and Ristow 2015). Therefore, we hypothesise that increased mitochondrial networking following the resumption of physical activity may occur as an adaptive response to increased ROS production, and to enhance mitochondrial respiration.

Damaged mitochondria can undergo engulfment by autophagosomes for lysosomal degradation to prevent activation of apoptosis and cell death. We have recently reported that the mammalian target of rapamycin complex I (mTORC1) activity is decreased following unilateral limb immobilisation in middle-aged men, as evident by decreased phosphorylation of downstream targets (Zeng et al. 2021). mTORC1 is a major regulator of autophagy activation by sequestering the Unc-51 like autophagy activating kinase 1 (ULK1) complex as inactive and prohibiting phagophore formation (an immature autophagosome). In response to mitochondrial damage, PINK1 is sequestered on the mitochondrial membrane and recruits Parkin to ubiquitinate target proteins (Sarraf et al. 2013). Sequestosome-1 (SQSTM1)/p62 recognizes the ubiquitinated proteins and tethers the targeted mitochondria to LC3B-II. However, despite our previous observations of increased p62 protein expression (Zeng et al. 2021), we did not observe an increase in gene expression of PINK1/Parkin-mediated mitophagy markers following the immobilisation period. Instead, we observed an increase in markers of receptor-mediated mitophagy. Bcl-2 and adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and Bnip3-like (NIX) are receptor proteins that contain LC3 interacting regions (LIRs) on the outer mitochondrial membrane which can facilitate the encapsulation of mitochondria into autophagosomes through direct interactions with LC3B-II (Novak et al. 2010; Hanna et al. 2012). The expressions of BNIP3 and NIX are transcriptionally regulated by FoxO3 (Mammucari et al. 2007), which we have previously shown was elevated following immobilisation (Zeng et al. 2021). BNIP3 and NIX are can also activate apoptosis through the opening and release of cytochrome C from the mitochondrial permeability transition pore (mPTP). Similarly, the loss of MTFP1 function can initiate cytochrome c release (Tondera et al. 2004) and activate AIFM2 translocation to the nucleus, which promotes nuclear DNA fragmentation and can induce cellular apoptosis (Yang et al. 1997; Tryon et al. 2014). mTORC1 regulates MTFP1 translation which subsequently phosphorylates and recruits DRP1 to the mitochondria to promote apoptosis (Morita et al. 2017). As mTORC1 signalling is blunted following muscle disuse (Zeng et al. 2021), and the gene expressions of *BNIP3L* and *AIFM2* were increased following the immobilisation period, with a concurrent decline in *MTFP1* expression, it is likely that disruptions in the mitochondrial network are acting as signals for cellular apoptosis and may contribute to the loss of skeletal muscle mass following immobilisation. Consistent with previous findings (Memme et al. 2021), the resistance training periods increased *PINK1* and *MTFP1* gene expression, suggesting that mitophagy increases with exercise training to remove damaged mitochondria and promote mitochondrial network recovery. In support of this, expressions of *BNIP3L* and *AIFM2* were restored to baseline levels following the resumption of physical activity.

A notable limitation of this study is that mitochondrial fission, fusion, and mitophagy are dynamic processes and can be difficult to capture changes to the mitochondrial network when only analysing steady-state levels. As such, future experiments should assess GTPase activity of fission and fusion proteins and incorporate microscopy to allow for visualisation of the mitochondrial network.

Contrary to our hypothesis, expression of factors regulating mitochondrial dynamics did not differ greatly with immobilisation. Instead, factors controlling both fusion and fission were elevated following ambulatory recovery, supporting a role for the dynamic capacity of the mitochondrial network to adjust to the resumed physical activity demand for ATP. Following the immobilisation period, receptor-mediated mitophagy may act as a bridge to activate of apoptosis, whereas the resistance training period may promote mitophagy as a quality control mechanism via the mTORC1/MTFP1 axis (Morita et al. 2017). Collectively, these data indicate subtle regulation of gene expression with 2-week limb immobilisation, which is consistent with the lack of widespread gene changes reported in skeletal muscle of cachexia/sarcopenia (Byrne et al. 2019).

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Author contributions Conceived and designed the experiments: CAP, CJM, and DC-S; performed the experiments: CAP, NZ, BRD, RFD, VCF, and CJM; analysed data: CAP and CJM; drafted the manuscript: CAP; wrote the paper: CAP. Critically evaluated the paper: CAP, CPH, AJR, VCF, CJM, and DC-S. All authors edited and revised manuscript and approved the final version of the manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no competing interests to declare.

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