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ORIGINAL CONTRIBUTION

Mitochondrial biogenesis and PGC-1α deacetylation by chronic treadmill exercise: differential response in cardiac and skeletal muscle

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Abstract Posttranslational modifications of the transcriptional coactivator PGC-1α by the deacetylase SIRT1 and the kinase AMPK are involved in exercise-induced mitochondrial biogenesis in skeletal muscle. However, similar investigations have not been performed in the left ventricle (LV). Here, we tested whether treadmill training (12 weeks) modifies PGC-1α and mitochondrial biogenesis in gastrocnemius muscle and LV of C57BL/6 J wild-type mice and IL-6-deficient mice with a reported impairment in muscular AMPK activation similarly. Physical activity lowered the plasma insulin and glucose in both mouse strains, suggesting improved insulin sensitivity. The gastrocnemius muscle of IL-6-deficient mice showed reduced mitochondrial respiration and enzyme activity, which was partially normalized after training. Chronic exercise enhanced the mitochondrial biogenesis in gastrocnemius muscle as indicated by increased mRNA or protein expression of primary mitochondrial transcripts, higher mtDNA content and increased citrate synthase activity. Parallel to these changes, we observed AMPK activation, SIRT1 induction and PGC-1α deacetylation. Chronic treadmill training resulted in a mild cardiac hypertrophy in both mouse strains. However, none of these changes observed in skeletal muscle were detected in the LV (both mouse strains) with the exception of AMPK activation and a mildly increased succinate-dependent respiration. Thus, chronic endurance training induces a sustained mitochondrial biogenic response in mouse gastrocnemius muscle but not in the LV. Although AMPK activation occurs in both muscular organs, the absence of SIRT1-dependent PGC-1α deacetylation may be responsible for this significant difference. AMPK activation by IL-6 appears to be dispensable for the mitochondrial biogenic responses to chronic treadmill exercise.

Keywords Exercise · Hypertrophy · Mitochondrial biogenesis · Heart

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Introduction

Mitochondrial biogenesis, the complex process involving various mechanisms responsible for the synthesis and assembly of mitochondrial components, controls mitochondrial number and determines the cellular energy production. Due to the high energy demand of the heart, a decline in mitochondrial function can result in a deterioration of cardiac performance. A reduced activity of complex I of the respiratory chain is a common feature in heart failure [50] or cardiac aging [41].

Exercise training [24] and caloric restriction [37] enhance mitochondrial biogenesis and performance and improve insulin sensitivity in skeletal muscle. They may thus represent an important lifestyle interventions in preventing and managing diabetes. The mitochondrial transcriptional regulator peroxisome-proliferators activated receptor gamma coactivator-1α (PGC-1α) plays a key role in coordinating metabolic flux and is a major regulator of mitochondrial biogenesis [47]. PGC-1α is enriched in tissues with high oxidative capacity such as the heart. It is rapidly induced under conditions with increased energy demand such as exercise or fasting to promote mitochondrial biogenesis. Transcription factors involved in mitochondrial biogenesis include PGC-1α, the estrogen-related receptors alpha and gamma (ERR), the nuclear respiratory factors (NRF-1 and NRF-2) and the mitochondrial transcription factor A (Tfam). PGC-1α activity is influenced by various posttranslational modifications (review in [46]). AMP-dependent protein kinase (AMPK) was suggested to phosphorylate PGC-1α at Thr177 and Ser536, which is required for the PGC-1α-dependent induction of the PGC-1α promoter and the mitochondrial biogenic response [26]. Besides these phosphorylation sites, PGC-1α also contains multiple distinct acetylation sites. PGC-1α deacetylation has been demonstrated to occur via SIRT1 in vitro as well as in vivo during fasting [20, 47] and can be mimicked by resveratrol [31]. Recently, a reduced PGC-1α acetylation in skeletal muscle was described following a single bout of exercise [8] as well as chronic exercise [34]. Furthermore, an exercise-induced increase in SIRT1 activity has been described in both heart and adipose tissue [14].

Although a significant amount of experimental data on mechanisms involved in exercise-induced mitochondrial biogenesis has been obtained in skeletal muscle, less information is available about the heart. Regular exercise has been confirmed as a countermeasure to protect against cardiac injury. The proposed mechanisms to explain the cardioprotective effects of exercise include alterations in coronary circulation as well as functional changes at the mitochondrial level. Long-term voluntary wheel running results in significantly reduced H_2O_2 production from both the subsarcolemmal and interfibrillar cardiac mitochondria [27]. It was also shown to result in increased complex IV activity and reduced oxidative damage in the heart [7]. Even under conditions such as aging when other cardioprotective mechanisms like ischemic pre and postconditioning fail [5, 6], exercise may act cardioprotective by limiting oxidative damage.

The present study was performed in IL-6 KO mice in comparison to the respective wild-type mice. IL-6 is synthesized and released by contracting muscles during exercise. IL-6 plasma concentrations have been shown to rise dramatically during and after exercise in animal models as well as in human studies. AMPK activation by the released IL-6 was suggested to play an important role in promoting some of the beneficial metabolic effects of exercise including increased insulin sensitivity. Indeed, IL-6 was shown to directly activate AMPK in muscular cells and isolated mouse muscles in vitro [18, 29]. The IL-6 KO mouse may thus demonstrate lower AMPK activation due to the total absence of IL-6. Changes in AMPK activity in response to exercise have not been investigated in cardiac tissue of IL-6 KO mice so far. The purpose of the present study was to compare the endurance training-mediated effects on mitochondrial biogenesis and mitochondrial function in the skeletal muscle and the LV of IL-6-deficient mice and wild-type mice. Furthermore, we tried to elucidate the questions whether IL-6-mediated AMPK activation in response to chronic exercise contributes to changes in mitochondrial function and biogenesis.

Research design and methods

Animals and training protocol

Mice homozygous for the targeted mutation of IL-6 ($IL6^{tm1Kopf}$) on a C57BL/6 J background and C57BL/6 J wild-type mice as described previously [16] were used in the present study. Animals were housed in an environmentally controlled laboratory with a 12 h/12 h light–dark cycle with 2–4 mice in each cage. Age-matched, male IL-$6^{-/-}$ (IL-6 KO) mice and the according littermates (wild-type mice on a C57BL/6 J background) were randomly assigned to the sedentary control group (C, $n = 6$–12 per group) or the training group (T, $n = 6$–12 per group). Treadmill exercise training started at age 10–12 week. Mice were trained 5 days weekly for 12 weeks on an Exer 3/6 treadmill equipped with a stimulus assembly (Columbus Instruments). Two weeks before the experimental period, the treadmill speed was gradually increased from 5 to 15 m/min and 10% incline. According to the results from the pilot experiment, mice performed 60 min treadmill training at a speed of 15 m/min for 12 weeks plus a warm-up and cool-down phase on each day. Concurrently, matched untrained mice maintained familiarity with the treadmill (5 m/min, 0% incline, 15 min duration, and three times/week). In order to rule out that a more intense exercise protocol may affect cardiac muscle differently, we also performed treadmill exercise at a speed of 23 m/min for 12 weeks in a subset of animals of both genotypes. For measurement of fasting serum parameters, food was withdrawn 12 h before the death by cervical dislocation. LV and gastrocnemius muscle were removed 24 h after the last training session. Gastrocnemius muscle was carefully dissected prior to analyses by microrespirometry or prior to

freezing in small aliquots in liquid nitrogen. Only the deep red portion of gastrocnemius muscle was used. Blood was drawn by aortic puncture at that time. The protocol was approved by the Animal Care and Use Committee of the Martin Luther University Halle-Wittenberg.

RNA and DNA extraction

Total RNA was isolated from frozen tissue (deep-red portion of the gastrocnemius muscle or left ventricle) by guanidine thiocyanate/cesium chloride centrifugation. Integrity and quality of the RNA was confirmed by agarose gel electrophoresis and the concentration determined by measuring UV-absorption. DNA isolation was performed with the Puregene DNA isolation kit (Biozym).

Real-time PCR

Reverse transcription of RNA samples (500 ng total RNA) was carried out for 30 min at 42°C using the SuperScript™ III First-Strand cDNA Synthesis Kit (Invitrogen). Real-time PCR (Primer sequences: Table. 1) and data analysis were performed using the Mx3000P Multiplex Quantitative PCR System (Stratagene) as described previously [48]. All data of mRNA are given as relative units of 18S rRNA (18S rRNA Control kit, Yakima Yellow®-Eclipse® Dark Quencher, Eurogentec) concentrations. The relative copy number of mtDNA per diploid nuclear genome was measured as described previously [34] using a fragment of mtDNA and a fragment of β-globin.

Western blotting and immunoprecipitation

Protein samples from frozen tissue (deep-red portion of the gastrocnemius muscle and left ventricle) were prepared as described previously [34, 48]. 50 μg of protein were separated by SDS-PAGE gel and immunoblotted with antibodies directed against SIRT1 (1:2,000, Upstate), SIRT3 (1:1,000, BioCat), PGC-1α (1:1,000, Santa Cruz) and phospho-AMPK (Thr172, 1:1,000, Cell Signaling Technology), MitoProfile® Total OXPHOS Rodent WB Antibody Cocktail (1:1,000, MitoSciences) and GAPDH (1:3,000, Abcam). After incubation with peroxidase-conjugated secondary antibody, blots were subjected to the enhanced chemiluminescent detection method with the Fusion FX7 imaging system (Peqlab). For acetylation analyses, PGC-1α or Complex II was immunoprecipitated with the respective antibody (1 μg antibody/250 μg total protein, PGC-1α, Santa Cruz; Complex II Immunocapture antibody, MitoSciences). The immunoprecipitates were then separated by SDS-PAGE and immunoblotted using an acetyl-lysine antibody (1:1,000, Cell Signaling Technology).

Plasma analyses

Serum adiponectin, leptin and insulin were measured using commercial Enzyme Linked-Immunosorbent Assays (Mouse/Rat Adiponectin ELISA-kit, B-Bridge International Inc.; Mouse/Rat Leptin ELISA-Kit, BioVendor; Mouse Insulin ELISA, Mercodia AB) and serum values for glucose were determined with a Glucose Assay Kit (BioCat GmbH). Triglycerides were measured using a commercial kit (GPO Trinder, Sigma). Blood lactate concentration was determined three times in all mice: at the beginning of the chronic exercise, after 6 weeks of exercise and at the end of the last session. Blood was collected from the tip of the tail for this purpose immediately after the end of the session and lactate was measured with an enzymatic Lactate Assay Kit (BioCat GmbH).

Preparation of skinned fibers and respirometric measurements

Immediately before oxygraphic measurements the muscular fibers (deep-red portion of the gastrocnemius muscle or left ventricle) were permeabilized with saponin for 30 min. After removal of saponin and adenine nucleotides, the measurements were performed on an OROBOROS® oxygraph at 30°C in incubation medium (75 mM mannitol,

Table 1 Primer sequences

	GenBank accession number	Forward primer	Reverse primer
Cytochrome b	AY999076	CCCTAGCAATCGTTCACCTC	TCTGGGTCTCCTAGTATGTCTGG
β-globin	NM_008220	TGGGTAATCCCAAGGTGAAG	TTCTCAGGATCCACATGCAG
ND5	AY999076	ACCAGCATTCCAGTCCTCAC	ATGGGTGTAATGCGGTGAAT
NRF-1	BC005410	GCACCTTTGGAGAATGTGGT	GGGTCATTTTGTCCACAGAGA
NRF-2	U20532	CCAGCTACTCCCAGGTTGC	CCTGATGAGGGGCAGTGA
PGC-1α	NM_00894	AAACTTGCTAGCGGTCCTCA	TGGCTGGTGCCAGTAAGAG
Tfam	NM_009360	CCTTCGATTTTCCACAGAACA	GCTCACAGCTTCTTTGTATGCTT
BNP	NM_008726	CCGGGTCCAGCAGAGACCTCAA	AACAACTTCAGTGCGTTACAGCCC

25 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 0.5 mM EDTA, 5 mM $MgCl_2$, 20 mM Tris–HCl, 1 mg/ml BSA) using different substrates: 10 mM pyruvate + 2 mM malate and 10 mM succinate + 5 μM rotenone. The weight specific oxygen consumption was calculated as the time derivative of the oxygen concentration (DATGRAPH Analysis software, OROBOROS®).

Determination of enzyme activities

Small pieces of frozen tissue were homogenized in a solution containing 50 mM Tris buffer (pH 7.5), 100 mM potassium chloride, 5 mM $MgCl_2$, and 1 mM EDTA using a glass/glass homogenizer. Enzyme activities were referenced to the activity of the mitochondrial marker enzyme citrate synthase (CS). The activity of complex I, complex I+III, complex II+III, complex III, complex II and complex IV were performed as described previously [19] using a Cary 50 spectrophotometer (Varian Instruments, Australia).

Electron microscopy

Hearts were fixed by vascular perfusion with 1.5% glutaraldehyde, 1.5% paraformaldehyde in 0.15 M Hepes buffer and stored in the fixative for at least 24 h at 4°C. The LV including the interventricular septum was separated from the rest of the heart, carefully weighed and sampled according to an established systematic uniform random sampling protocol [12]. The volume of the LV was calculated by dividing the mass by density of muscle tissue, 1.06 g/cm^3. The tissue pieces sampled for embedding were subsequently washed in Hepes buffer, postfixed in 1% osmium tetroxide in H_2O, washed with H_2O, stained en bloc in half-saturated aqueous uranyl acetate, washed again, dehydrated in an ascending ethanol series and finally embedded in epoxy resin.

From the tissue blocks, semi- and ultra-thin sections were generated and stained with toluidine blue or lead citrate and uranyl acetate, respectively. The semi thin sections were investigated using an Olympus BX51 microscope (Olympus) equipped with a digital camera and the newCAST stereology software (Visiopharm). Transmission electron microscopy was performed using a LEO 902 electron microscope (Zeiss). At light microscopic level (objective lens magnification: 40×) fields of view sampled in a systematic uniform random way were overlaid with a point grid. Points hitting cardiomyocytes or the rest of the heart were counted to estimate the volume fraction of cardiomyocytes. At electron microscopic level (primary magnification: 4,400×), randomly sampled fields of view were overlaid with a point grid. Points hitting mitochondria or the rest of the cardiomyocytes were counted to estimate the volume fraction of mitochondria. The total volume of mitochondria within the LV was calculated by multiplying the volume fraction of mitochondria with the volume fraction of cardiomyocytes and the volume of the LV [40].

Statistical analysis

All values are expressed as mean ± SEM. Statistical analysis of differences observed between numeric parameters of all groups was performed by one-way ANOVA using an all pairwise multiple comparison procedure (Turkey's test). Statistical significance was accepted at the level of $p < 0.05$.

Results

Systemic effects of treadmill training

At the beginning of the study (age 10–12 week), body weight was comparable in IL-6 KO mice and C57BL/6 J wild-type mice (not shown). During the study the IL-6 KO mice had gained significantly more weight than the wild-type mice (C57BL/6: 28.1 g ± 0.7; IL-6 KO: 32.8 g ± 0.8, $p < 0.05$). The epididymal fat pads at the end of the study were heavier in the untrained IL-6 KO mice compared to untrained wild-type mice (C57BL/6: 320 mg ± 26; IL-6 KO: 460 mg ± 49, $p < 0.05$ vs. WT). Endurance training did not result in significant changes in body weight or fat pads. Glucose, insulin and leptin plasma levels demonstrated a significant reduction together with a strong induction of plasma adiponectin in wild-type and in IL-6 KO mice in response to the training (Table. 2), indicating a change in insulin sensitivity in these animals. Plasma triglycerides were significantly higher in IL-6 KO than in wild-type, but did not change in response to three months of treadmill exercise (Table. 2).

Chronic exercise induced a mild LV hypertrophy in both mouse strains (LV weight/tibia length: C57BL/6 J: 5.15 mg/mm ± 0.13 vs. 5.81 ± 0.14, $p < 0.01$; IL-6 KO: 5.12 mg/mm ± 0.21 vs. 5.76 ± 0.19, $p < 0.05$). IL-6 KO mice did not demonstrate cardiac abnormalities and no major difference in left ventricular BNP mRNA expression was observed between the four groups (C57BL/6 J: 1.19 ± 0.18 vs. 1.21 ± 0.14, control vs. trained; IL-6 KO: 1.25 ± 0.21 vs. 1.24 ± 0.19, control vs. trained). After the first exercise session, blood lactate (mmol/l) in both strains showed a similar increase compared to the respective control mice (C57BL/6 J: 3.69 mmol/l ± 0.48 vs. 5.15 ± 0.43, $p < 0.05$; IL-6 KO: 3.95 ± 0.46 vs. 5.52 ± 0.39, $p < 0.05$). After 6 weeks (not shown) and 12 weeks (Table. 2) of chronic exercise, blood lactate concentrations in the trained C57BL/6 J and trained IL-6 KO mice were no longer different from the respective control mice.

Table 2 Plasma/blood parameters at the end of the study

	C57BL/6 J		IL-6$^{-/-}$	
	Control	Training	Control	Training
Glucose (mmol/l)	6.72 ± 0.54	4.52 ± 0.62*	7.29 ± 0.42	5.13 ± 0.48*
Insulin (ng/ml)	1.55 ± 0.39	0.51 ± 0.15*	1.44 ± 0.26	0.78 ± 0.12*
Leptin (pg/ml)	1427.6 ± 242.1	328.8 ± 52.5*	1081.9 ± 275.6	348.3 ± 101.9*
Adiponectin (μg/ml)	17.8 ± 0.8	34.4 ± 1.7*	15.2 ± 1.3	30.6 ± 1.8*
Triglycerides (mg/dl)	79.7 ± 14.4	61.2 ± 9.5	105.4 ± 21.9**	95.8 ± 16.0
Lactate (mmol/l)	3.79 ± 0.41	4.52 ± 0.30	3.92 ± 0.32	4.37 ± 0.35

* $p < 0.05$ versus. respective control

** $p < 0.05$ versus. C57BL/6 J controls

Influence of treadmill training on mitochondrial biogenesis

The mRNA expression of ND5 (complex I) and cytochrome b (complex III) was higher in gastrocnemius muscle of wild-type and IL-6 KO mice after 3 months of training (Fig. 1a). Furthermore, it increased citrate synthase activity in wild-type and IL-6 KO mice (Fig. 1a) in skeletal muscle, suggesting a higher mitochondrial density. An increased expression of primary mitochondrial transcripts or a higher citrate synthase activity was not observed in the LV of wild-type and IL-6 KO mice after 3 months of training (Fig. 1b). Accordingly, no significant change in mtDNA content was observed in the LV (Fig. 1b), while an approximately 40% increase occurred in the gastrocnemius muscle of both mouse strains (Fig. 1a). Western Blot analyses of the relative levels of the five respiratory chain complexes revealed an increased expression of complex I and complex IV following treadmill exercise in the gastrocnemius muscle of both mouse strains while no significant changes were detectable in cardiac samples (Fig. 1c, d). Finally, we conducted electron microscopy studies for quantification of mitochondrial adaptations. These analyses showed a minor increase in mitochondrial volume density in the skeletal muscle of trained mice (Suppl. Fig. 1), while mitochondrial volume and mitochondrial volume density were not increased in the LV (Fig. 2). Furthermore, no major differences in mitochondrial morphology were detectable. Similarly, we observed a mild induction of NRF-1 and Tfam mRNA (Fig. 3a) in the gastrocnemius muscle of wild-type and IL-6 KO mice but not in the LV (Fig. 3b) after 3 months of treadmill exercise. NRF2 mRNA (Fig. 3a, b) was not altered in any tissue. A significant increase of PGC-1α mRNA was observed in the LV and in the gastrocnemius muscle of both mouse strains following treadmill exercise (Fig. 4a, b), although the effect appeared to be more pronounced in the skeletal muscle. PGC-1α protein was not significantly altered in any group or tissue (Fig. 4a, b). Besides total amount of PGC-1α protein, acetylation status of PGC-1α has a major impact on PGC-1α activity/stability and was shown to be altered in response to low nutrient conditions and exercise [8, 20, 34, 47]. As shown in Fig. 4a, PGC-1α acetylation was significantly reduced in the gastrocnemius muscle of trained wild-type and IL-6 KO mice, while the LV showed no difference between the two treatment groups (Fig. 4b). Accordingly, the protein expression of SIRT1, which specifically deacetylates PGC-1α in vivo and in vitro, was induced in the gastrocnemius muscle of both mouse strains but not in the LV (Fig. 5a, b) following endurance training. The mitochondrial sirtuin SIRT3 on the other hand showed an exercise-induced increased protein expression in both muscular organs, which was even more pronounced in the LV (Fig. 5a, b). However, besides acetylation also PGC-1α phosphorylation by AMPK may contribute to training-induced changes in mitochondrial biogenesis and function. Indeed, training resulted in increased phosphorylation of AMPK which was as strong in the LV of wild-type and IL-6 KO mice as in the gastrocnemius muscle (Fig. 5a, b). Although AMPK activation at rest was significantly lower in the red gastrocnemius muscle of IL-6 KO mice compared with wild-type mice, the percentage increase in response to treadmill exercise was comparable in both mouse strains (Fig. 5a). No such difference in p-AMPK at rest between IL-6 KO and wild-type mice was seen in the LV (Fig. 5b).

Influence of treadmill training on mitochondrial function

Pyruvate- and succinate-dependent respiration, measured in permeabilized muscle fibers of the deep-red portion of the gastrocnemius muscle, was lower in mildly obese IL-6 KO mice compared with C57BL/6 J mice (Fig. 6a). Physical activity resulted in increased succinate-dependent respiration in these mice (Fig. 6a). Left ventricular succinate-dependent respiration was comparable in C57BL/6 J and mice IL-6 KO but increased by approximately 20%

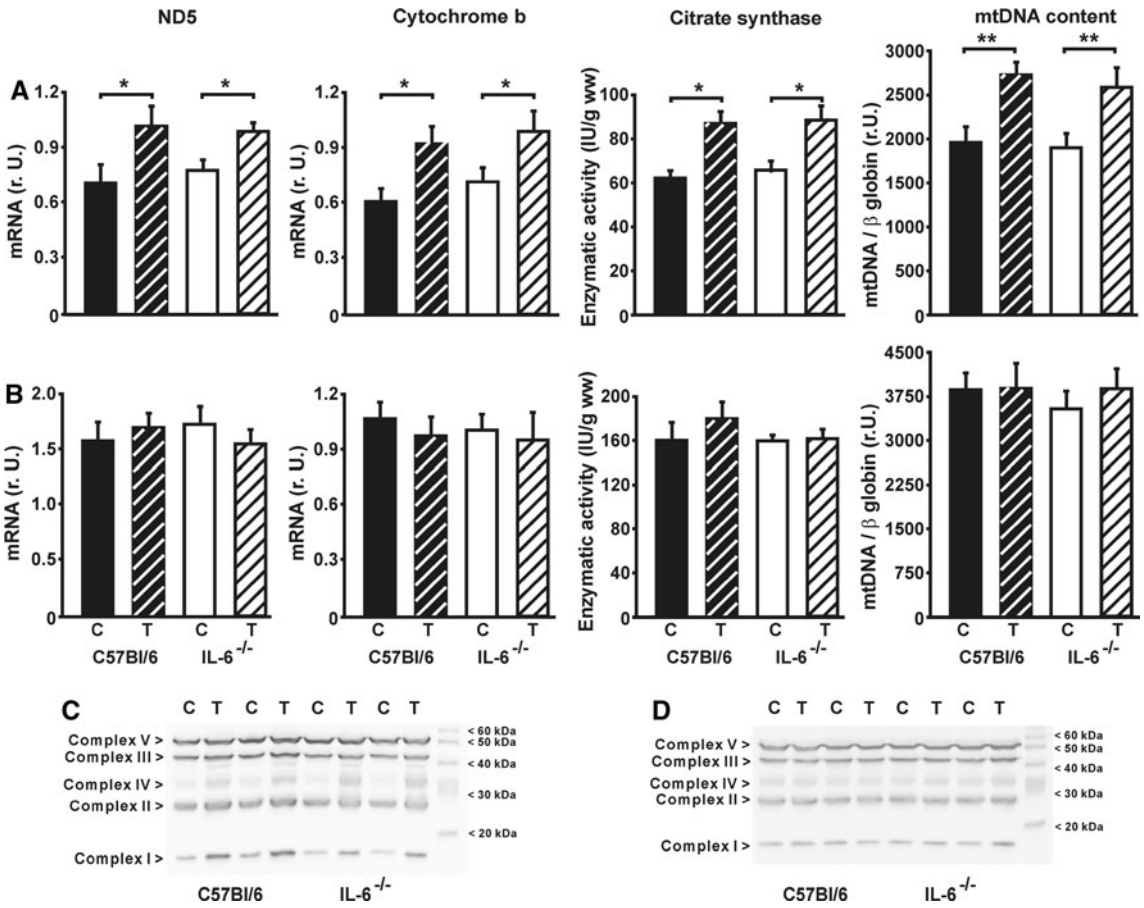

Fig. 1 Markers of mitochondrial biogenesis **a** Real-time PCR analyses of primary mitochondrial transcripts ND5 (complex I) and cytochrome b (complex III) in gastrocnemius muscle. Data are normalized per 18S rRNA. Citrate synthase enzyme activity (IU/g wet weight) and relative copy number of mtDNA per diploid nuclear genome in the deep-red portion of the gastrocnemius muscle. **b** Results from analyses described in **a** obtained in the LV. *Black columns* C57BL/6 J wild-type mice (12 controls, 12 trained animals), *white columns* IL-6 KO mice (6 controls, 6 trained animals). Results obtained from the trained mice are depicted with hatched columns. Data are mean ± SEM. $*p < 0.05$, $*p < 0.01$. *C* control, *T* trained mice. **c** Representative blots from Western Blot analyses of the relative levels of the five respiratory chain complexes in homogenates of mouse gastrocnemius muscle. **d** Representative blots from Western blot analyses of the five respiratory chain complexes in LV tissue

after 12 weeks of treadmill exercise in both mouse strains (Fig. 6b). The mitochondrial protein SIRT3 has been shown to deacetylate a number of mitochondrial proteins including the SDHA subunit of complex II, resulting in increased SDH activity [9]. Our analyses suggest that treadmill exercise resulted in a small decrease in SDHA acetylation in the LV (C57BL/6 J: 0.17 ± 0.04 vs. 0.07 ± 0.01, $p < 0.05$; IL-6 KO: 0.16 ± 0.01 vs. 0.08 ± 0.01, $p = 0.051$, Fig. 6d) as well as in gastrocnemius muscle (C57BL/6 J: 0.31 ± 0.06 vs. 0.19 ± 0.04, $p = 0.062$; IL-6 KO: 0.28 ± 0.02 vs. 0.20 ± 0.01, $p < 0.05$, Fig. 6c), possibly mediated via SIRT3 upregulation (Fig. 5a, b).

A typical feature of an impaired respiratory chain function in diabetes or heart failure is the reduction in the activity of complex I and IV [7], both containing proteins transcribed from the mitochondrial DNA directly. No change in the enzyme activity of any of the complexes of the respiratory chain in response to chronic exercise was observed in the LV of C57BL/6 J and IL-6 KO mice (Fig. 6b). However, the skeletal muscle of IL-6 KO animals demonstrated a significant reduction in complex I activity compared to wild-type mice (Fig. 6a), which was not detectable in the LV of IL-6 KO animals (Fig. 6b). Physical activity resulted in a higher activity of complex I and complex IV in the skeletal muscle of both mouse strains (Fig. 6a), which was accompanied by a higher complex I and complex IV protein expression (Fig. 1c). The other complexes (not shown) of the respiratory chain demonstrated only minor changes related to obesity or physical activity in the gastrocnemius muscle.

Finally, we conducted 12 weeks of treadmill exercise using a higher maximal speed (23 m/min) in a subset of mice from both strains to rule out that the intensity of

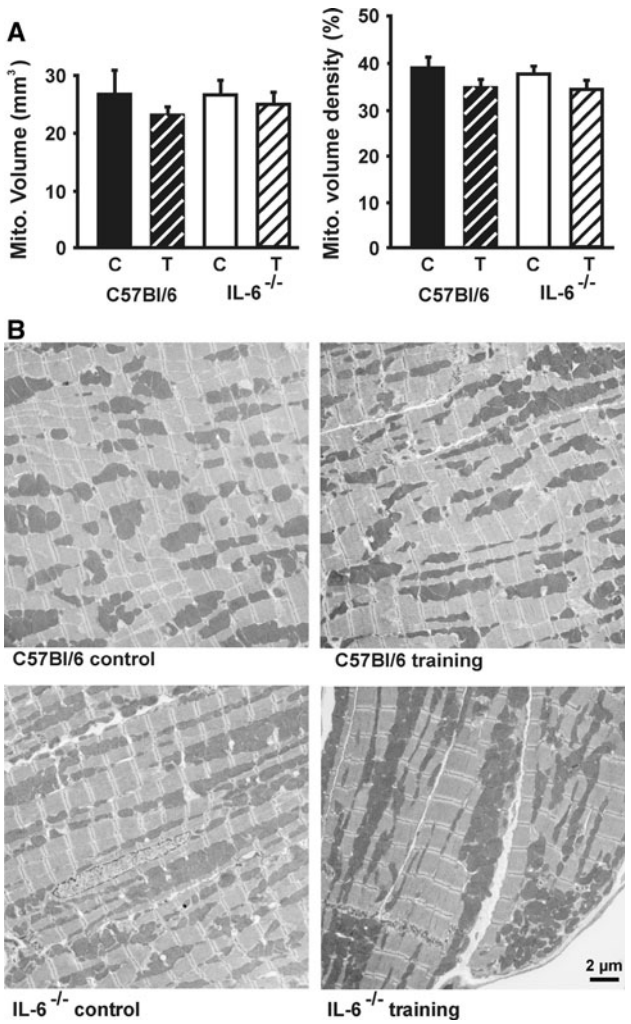

Fig. 2 Electron microscopy **a** Mitochondrial volume and mitochondrial volume density in LV tissue. Data are mean ± SEM. *C* control, *T* trained mice ($n = 4$ per group). **b** Electron micrographs of representative mouse LV sections from different experimental groups. *Scale bar* represents 2 µm

treadmill exercise may have been to low to achieve significant, chronic changes in the LV. Similar to the results obtained from mice running at a maximal speed of 15 m/min for 12 weeks, we did not observe significant changes in the expression of the primary mitochondrial transcript cytochrome b, in citrate synthase activity, PGC-1α expression or acetylation in LV samples from these mice (Suppl. Fig. 2a, b), although gastrocnemius muscle showed a robust response in all of these parameters (not shown). Similarly, electron microscopy did not reveal significant changes in LV mitochondrial volume or mitochondrial volume density (not shown). SIRT1 protein expression was not altered, while SIRT3 protein and phospho-AMPK were strongly increased in the LV of wild-type and IL-6 KO mice following endurance training at 23 m/min (Suppl. Fig. 2c).

Discussion

The present study demonstrates that 3 months of endurance exercise resulted in enhanced mitochondrial biogenesis in the gastrocnemius muscle but not in the LV of wild-type and IL-6 KO mice as indicated by the higher expression of primary mitochondrial transcripts, mtDNA content, citrate synthase activity and mitochondrial volume density. The significant higher expression of complex I and complex IV components following treadmill exercise in the gastrocnemius muscle may have resulted in a higher enzyme activity of these complexes. Parallel to this, we observed PGC-1α deacetylation, AMPK phosphorylation and SIRT1 protein induction in the skeletal muscle, which suggests that these may play a role for the mitochondrial biogenic response. On the other hand, the increased phosphorylation of AMPK in the LV was not sufficient to induce sustained mitochondrial biogenesis or comparable changes in mitochondrial respiratory chain activity as observed in gastrocnemius muscle. IL-6 loss did not alter the exercise-induced adaptations observed in skeletal muscle or heart.

PGC-1α plays a key role in regulating genes involved in myocardial fuel metabolism and cardiac function. A growing literature indicates that PGC-1α expression is altered in heart failure (review in [15]). The hearts of PGC-1α KO mice [1, 33] demonstrate reduced oxidative capacity and mitochondrial gene expression but normal mitochondrial volume density, suggesting additional mechanisms controlling cardiac mitochondrial biogenesis. Cardiac dysfunction under basal conditions is moderate in PGC-1α KO mice [1, 33]. PGC-1α overexpression on the other hand causes uncontrolled mitochondrial proliferation and loss of sarcomeric structure, finally leading to dilated cardiomyopathy and premature death [32]. Thus, a well-balanced and tightly controlled change in the expression of PGC-1α appears to be necessary to maintain optimal cardiac performance. PGC-1α mRNA was increased in the skeletal muscle and LV of wild-type and IL-6 KO in response to chronic treadmill exercise. However, we observed only minor changes in PGC-1α protein, although PGC-1α has been shown to control mitochondrial protein content and mitochondrial mass and determine cardiac capacity [32]. Similar to our results, a study performed in mice (8 weeks voluntary wheel running) has demonstrated changes in mitochondrial gene expression despite unchanged PGC-1α protein in skeletal muscle [23].

SIRT1-mediated deacetylation of PGC-1α is involved in the activation of oxphos genes in skeletal muscle [20]. SIRT1 senses modifications of the muscular NADH/NAD^+ ratio and responds by adjusting skeletal muscle gene expression depending on the metabolic demands [17]. The NADH/NAD^+ ratio is subjected to dynamic fluctuations in skeletal muscle in response to exercise, food intake or

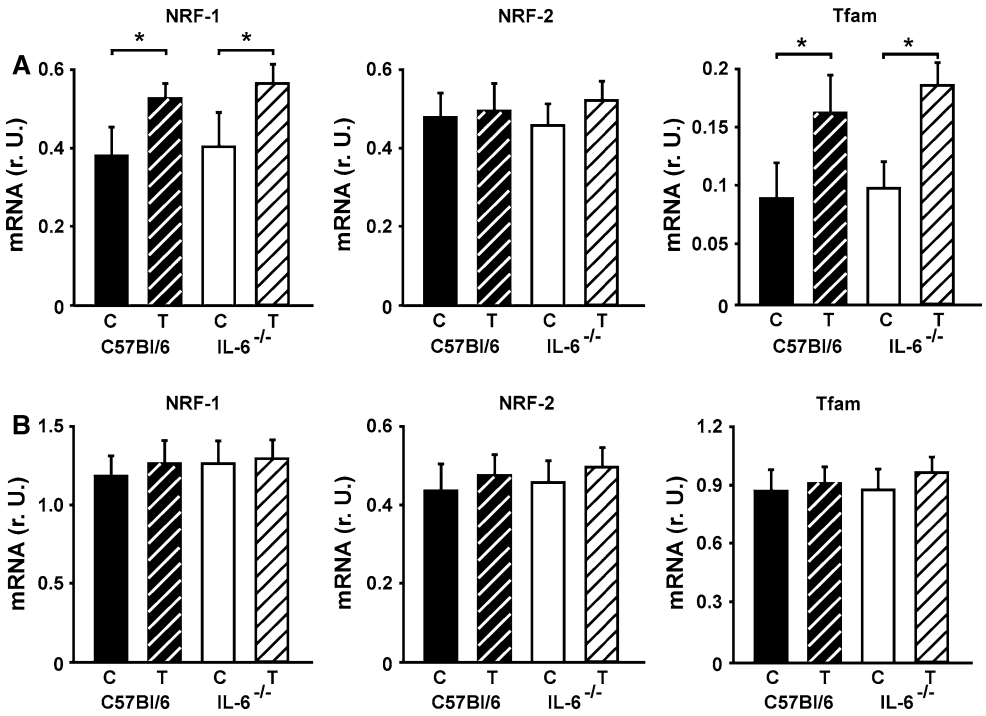

Fig. 3 mRNA expression of transcriptional activators and coactivators of mitochondrial biogenesis **a** Results from real-time PCR analyses of nuclear respiratory factors 1 (NRF-1), nuclear respiratory factors 2 (NRF-2) and the mitochondrial transcription factor A (Tfam) in skeletal muscle samples. **b** Results from analyses described in **a** obtained in the LV. All data are normalized per 18S rRNA. Numbers of animals as described in Fig. 1. Data are mean ± SEM. *$p < 0.05$. *C* control, *T* trained mice

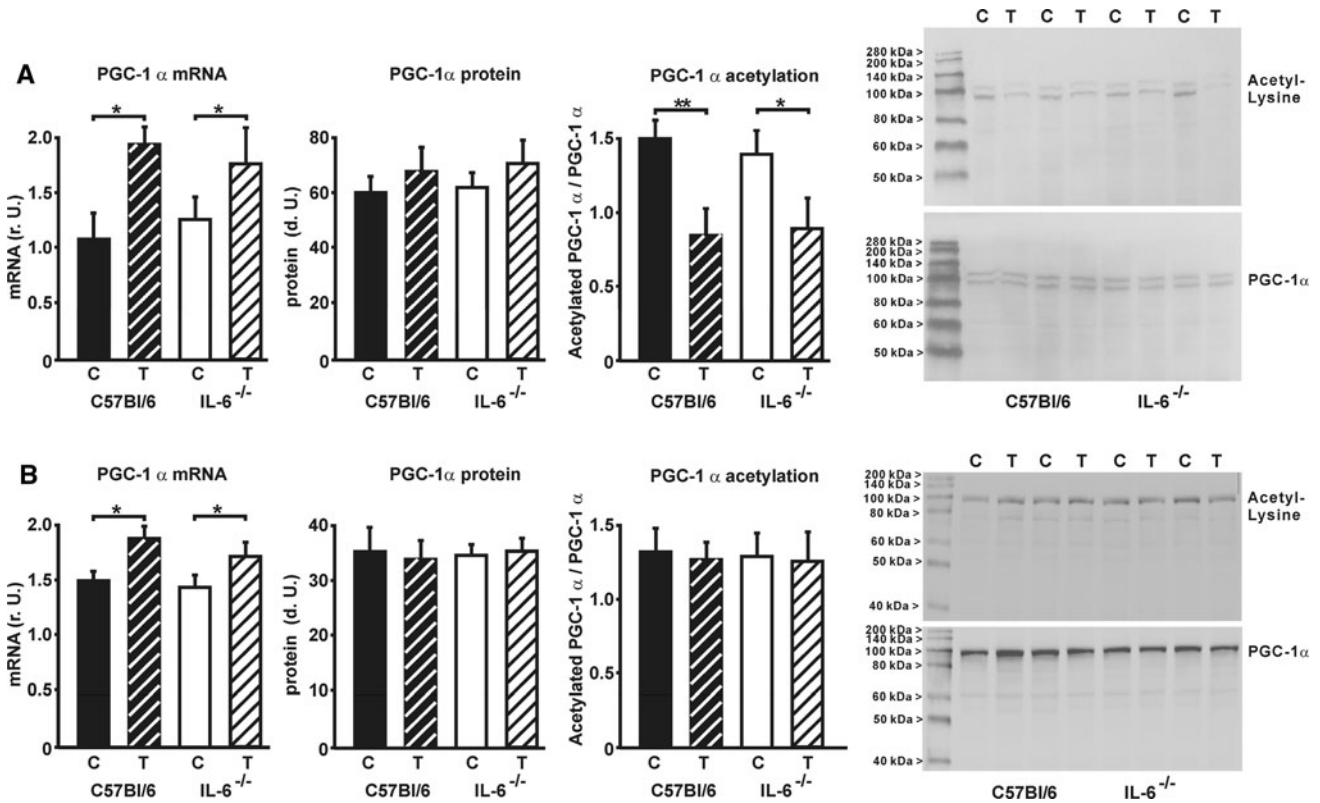

Fig. 4 Changes in PGC-1α expression and acetylation **a** Real-time PCR analyses of PGC-1α mRNA expression in the gastrocnemius muscle of C57BL/6 J wild-type mice and IL-6 KO mice. Data are normalized per 18S rRNA. Densitometry of PGC-1α protein data. Homogenates of skeletal muscle probed with an antibody detecting PGC-1α. Blots were also probed with GAPDH as a loading control. Densitometry and representative, full-size blots of immunoprecipitation experiments performed on skeletal muscle lysates, using PGC-1α for precipitation and an antibody directed against acetyl-lysine or PGC-1α for detection. Data are given as acetylated PGC-1α per total PGC-1α in these samples. **b** Results from analyses described in **a** obtained in the LV. All data are mean ± SEM. *$p < 0.05$; **$p < 0.01$. *C* control, *T* trained mice

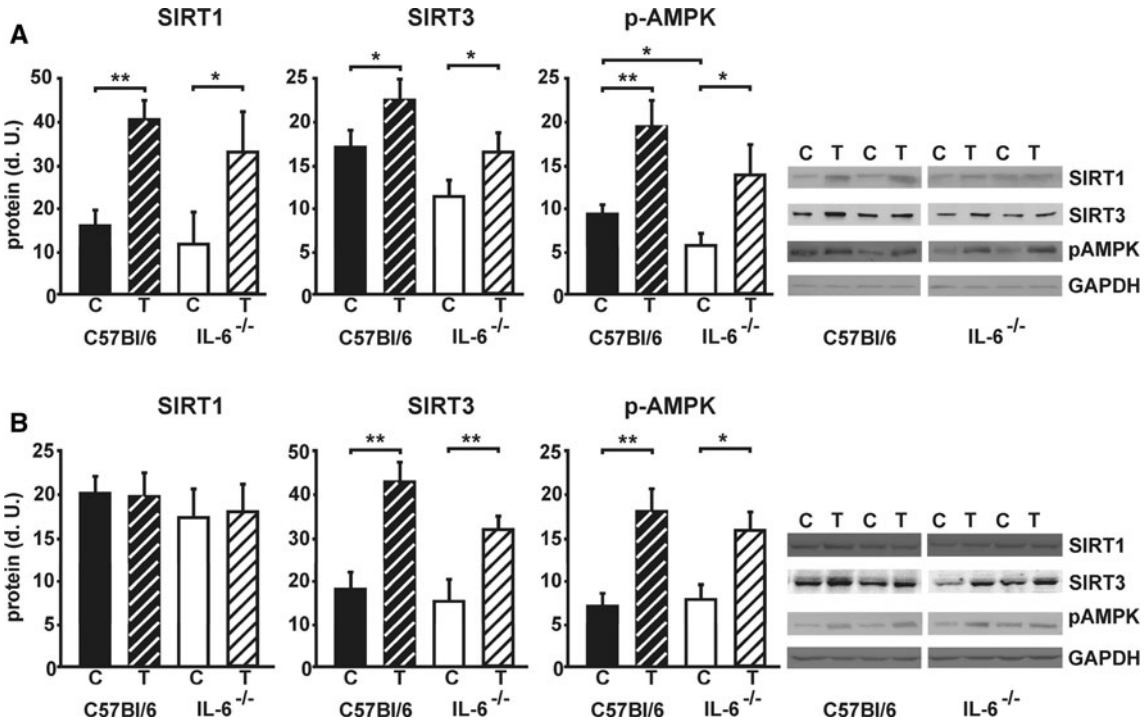

Fig. 5 SIRT1, SIRT3 and phospho-AMPK protein expression **a** Densitometry of protein data and representative blots from gastrocnemius muscle. Homogenates of skeletal muscle were probed with antibodies detecting SIRT1, SIRT3, phospho-AMPK (Thr172) and GAPDH as a loading control. **b** Results from analyses described in **a** obtained in the LV. All data are mean±SEM. $*p < 0.05$; $**p < 0.01$. *C* control animals, *T* trained animals

starvation. An accurate and direct measure of free levels of NAD^+ in the cytosol or other compartments is technically difficult. No such measurements exist from living hearts, but an exercise-induced increase in SIRT1 activity has been described in the rat heart [14]. The SIRT1-mediated effects are enhanced in murine gastrocnemius muscle by resveratrol resulting in higher expression of oxphos genes together with decreased PGC-1α acetylation [31]. These changes were, however, not evident in the hearts of resveratrol-treated mice [31]. This indicates a tissue specific response to resveratrol which can also be envisioned for exercise-induced effects on PGC-1α. Activation of the mammalian mitochondrial sirtuin SIRT3 has been shown to occur in the heart [53] and skeletal muscle [42] in response to various hypertrophic stimuli including chronic exercise. Furthermore, NAD^+-mediated inhibition of cardiac hypertrophy is mediated via SIRT3 but independent of SIRT1 [44]. A depletion of cellular NAD^+ levels, possibly through an increased consumption of NAD by poly(ADP-ribose) polymerase-1 [43], has been reported to occur during pathological cardiac hypertrophy but not during physiological hypertrophy [43, 44]. SIRT1 and SIRT3 both promote mitochondrial biogenesis via PGC-1α but may utilize different ways. Although the deacetylase activity of SIRT3 was shown to be required for its regulation of PGC-1α expression [52], a direct deacetylation of PGC-1α has only been shown to occur by SIRT1 [20] so far. Mice lacking SIRT3 demonstrate mitochondrial protein hyperacetylation [36] and SIRT3 has been shown to deacetylate the SDHA subunit of succinate dehydrogenase, resulting in increased complex II activity [9]. Our investigations show that acetylation of the SDHA subunit of complex II is decreased in response to treadmill exercise in both tissues and mouse strains. Thus, changes in complex II acetylation may be responsible for the small increase in succinate-dependent respiration in trained animals. However, other posttranslational modifications such as phosphorylation of SDHA at Tyr residues by Fgr tyrosine kinase [49] may be involved as well. Interestingly, it has been shown recently, that SIRT3 is a transcriptional target of PGC-1α [30]. Thus, SIRT1 may indirectly modulate SIRT3 expression via PGC-1α deacetylation.

The SIRT1-mediated deacetylation of PGC-1α requires activation of AMPK [8]. AMPK activation at rest was lower in the red gastrocnemius muscle of IL-6 KO mice, but a comparable increase was observed in response to treadmill exercise. No major difference in AMPK activation at rest and a similar increase of p-AMPK after exercise was observed in the LV of wild-type and IL-6 KO mice. The low AMPK activation in gastrocnemius muscle of IL-6 KO mice observed by others [29] after an acute bout of exercise may be related to differences in

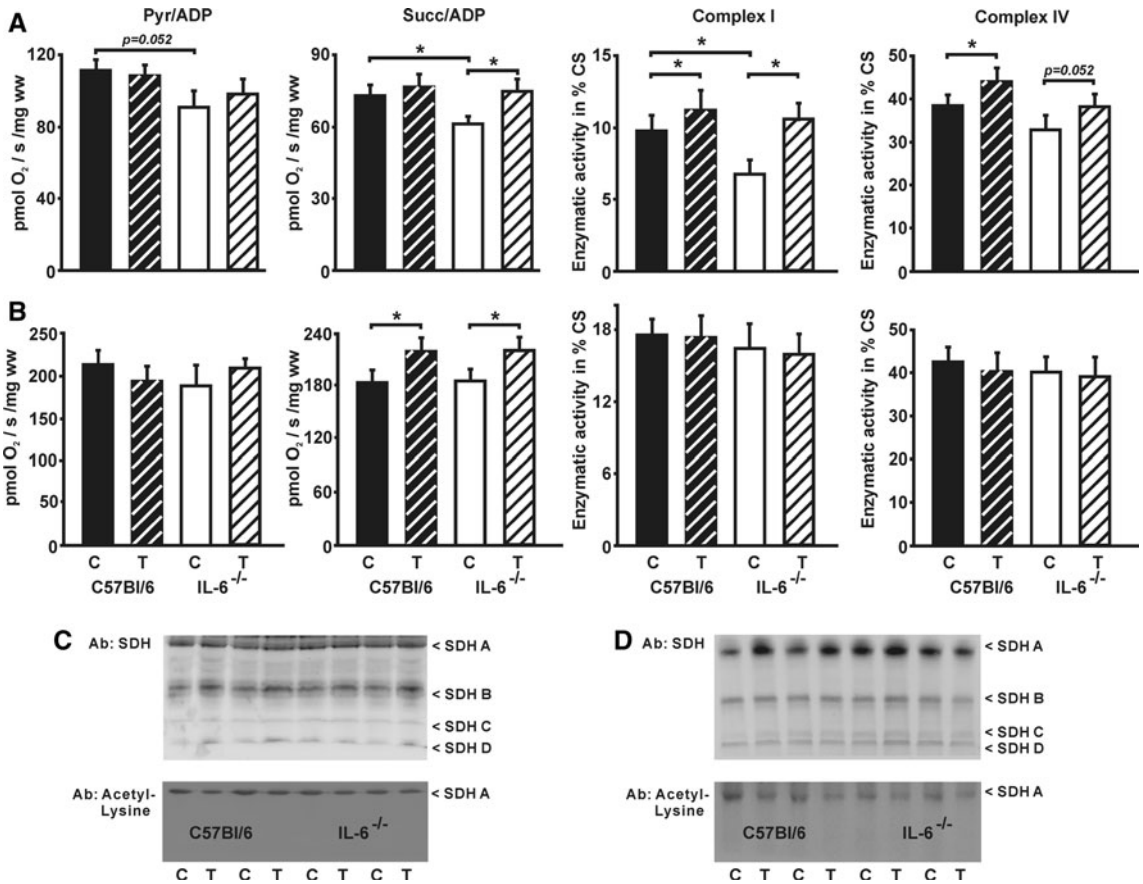

Fig. 6 Mitochondrial function **a** Active rates of respiration (state 3) were measured in saponin-skinned fibers of gastrocnemius muscle in the presence of 5 mM ADP and either 10 mM pyruvate + 2 mM malate or 10 mM succinate in the presence of 5 μM rotenone. Citrate synthase (CS) normalized complex I activity (rotenone-sensitive NADH:CoQ_1 oxidoreductase) and complex IV were measured in gastrocnemius muscle lysates. **b** Results from analyses described in **a** obtained in the LV. All data are mean ± SEM. *$p < 0.05$. **c** The succinate dehydrogenase complex was immunoprecipitated in skeletal muscle lysates and blots were then incubated with an antibody directed against SDH or acetyl-lysine for detection. Representative blots of immunoprecipitation experiments are shown. **d** Results from analyses described in **c** obtained in the LV. *C* control, *T* trained mice

exercise protocol. However, the same authors also reported that AMPK activation in liver tissue was not different and the percentage increase in muscle was similar [29]. Furthermore, the fact that the red and white portions of gastrocnemius muscle were not distinguished may have influenced the results as well [29]. Mouse gastrocnemius muscle is a very heterogeneous tissue and differences between the deep-red and the superficial-white portion have been described before [38]. This is further supported by the fact that resveratrol-induced mitochondrial changes showed significant differences between oxidative and non-oxidative fibers [31]. AMPK was suggested to serve as an initial sensor for exercise-induced adaptations in skeletal muscle [8]. Although AMPK was activated in both muscular tissues in the present study, additional mechanisms appear to be necessary to activate SIRT1 and induce PGC-1α deacetylation. These putative additional coactivators could either be inactive in the heart or specific only for skeletal muscle.

Exercise results in an acute increase in plasma IL-6 and this rise correlates with the activation of AMPK. Thus, it was suggested that IL-6 participates in the exercise-induced increase in muscle insulin sensitivity. Although IL-6 has been described to directly activate AMPK in muscular cells and muscles in vitro [18, 29], some of these studies used rather high concentrations of IL-6 which are unlikely to occur in vivo. Corresponding to these data, no serious deficit was observed in the gastrocnemius muscle of IL-6 KO mice in the present study arguing against a major impact of IL-6 on the mitochondrial biogenic response in skeletal muscle following chronic exercise. IL-6 is also an important activator of the JAK/STAT pathway. In particular activation of STAT3 in the heart may regulate the expression of target genes involved in inflammation, apoptosis and oxidative defence (review in [4]). However, so far only little is known about the role of this pathway in the cardioprotective effects of chronic exercise or its influence on cardiac mitochondrial biogenesis under

exercise conditions. Changes in the release of fat-cell derived hormones have been implicated in AMPK activation during chronic exercise as well. The adipocytokine adiponectin stimulates AMPK [54] and may thus influence mitochondrial biogenesis via increased phosphorylation of PGC-1α [26]. Indeed, endurance training resulted in a significant increase in plasma adiponectin levels in both mouse strains. Recently it was shown that induction of mitochondrial biogenesis after endurance training requires an intact adipocytokine signalling [34]. Furthermore, mice with muscle-specific disruption of AdipoR1 show reduced adiponectin-mediated activation of AMPK and SIRT1 together with reduced mitochondrial content [25]. However, the question remains why circulating acrp30 seems to be sufficient to activate mitochondrial biogenesis via AMPK/SIRT1/PGC-1α in the skeletal muscle but not in the heart.

There has been quite some controversy concerning the obesity in IL-6 KO mice. In 2002 Wallenius et al. [55] reported that IL-6 KO mice develop mature-onset obesity, while others did not find any change in body weight or body composition [11]. At the end of our study the IL-6 KO mice presented higher body weight and epididymal fat pads, moderately increased plasma triglycerides together with normal plasma glucose and insulin. This was associated with only minor functional changes in the heart or in cardiac mitochondria. Others have recently shown that obesity can result in cardiac energetic abnormalities even in the absence of overt diabetes and prior to impairment in cardiac function [10]. On the other hand, obesity and systemic insulin resistance are not directly linked to cardiac insulin resistance or changes in cardiac substrate oxidation [51].

The IL-6 KO mouse has been widely used in the cardiac field for the investigation of potential mechanisms underlying the poor prognosis of patients with congestive heart failure and myocardial infarction with elevated serum IL-6 levels. Recently it has been shown that 3 months old IL-6 KO mice develop severe cardiac dysfunction [2]. Opposite to these data, IL-6 KO mice on a BALB/c genetic background did not show any impairment in LV function or upregulation of ANP [39]. Fuchs et al. also reported no major differences in sham-operated IL-6 KO mice compared to wild-type mice in LV diameter, septum thickness or LV function at the age of 4-5 months [16]. Similarly, we did not observe gross cardiac abnormalities microscopically or by echocardiography (data not shown). Furthermore, our mice did not show a difference in left ventricular BNP mRNA expression. Interestingly, acute exercise in a fasted state compared to exercise in a glucose-fed state or compared to reference conditions was shown to improve systolic function despite increased cardiac lipid content in humans [3].

IL-6 KO mice were also described to demonstrate reduced exercise endurance in a study applying a single exercise bout with increasing workloads [13]. These mice ran at a maximal speed of 18 m/min for 17–35 min depending on the genotype (IL-$6^{-/-}$< IL-$6^{+/+}$). Using our study design, we did not observe any differences between the two mouse strains. Similarly, the time-dependent changes in plasma lactate suggest that the adaptation to the treadmill training was comparable. However, the experimental protocols differ in some aspects. The training in our mice lasted 1 h per day and was continued for 12 weeks. Thus, IL-6 KO mice may have reduced endurance during acute exercise bouts [13] but adapt well to chronic exercise training. Accordingly, other groups did also not report a significantly reduced exercise endurance during 6 weeks of swimming [28] or voluntary wheel running for 2 weeks [22].

The moderate treadmill exercise for 12 weeks was sufficient to result in a mild cardiac hypertrophy in both mouse strains. It has been demonstrated by others that the hypertrophic response of the LV to norepinephrine is blunted in IL-6 KO mice [39]. However, norepinephrine leads to pathological hypertrophy, while physiological hypertrophy occurs in response to exercise. The type of stress leading to cardiac hypertrophy has important consequences in terms of activated signalling cascades, cardiac remodelling and contractile function of the myocyte. Similar to our results, IL-6 KO mice subjected to 6 weeks of swimming did not present a blunted hypertrophic response [28]. Compensated hypertrophy leads to a proportional increase in myofilaments and mitochondrial mass to keep pace with the rising energy demand of the heart in response to increased workload. Any decline in mitochondrial function could otherwise result in a deterioration of cardiac contractile function. Our study suggests that cardiac hypertrophy does not imply a chronically increased mitochondrial biogenesis in the LV. Similarly, it has been shown in a comparative study in rats that physiological hypertrophy induced by treadmill exercise or pregnancy does not lead to changes in mitochondrial biogenesis [45]. On the other hand others have shown that voluntary exercise with a mean running distance of approximately 7 km per day, a distance almost five times longer than in our model, results in a higher total number and total volume of cardiac mitochondria in mice [12]. Interestingly, the mean number-weighed mitochondrial volume was smaller [12], suggesting the biogenesis of new mitochondria rather than enlargement of existing mitochondria. Another interesting aspect of the regulation of mitochondrial biogenesis in long-lived cells such as myocytes may be the link to autophagy. The removal of damaged mitochondria by autophagy (mitophagy) is essential in the heart [21]. The mechanisms of mitochondrial quality control and removal

by mitophagy will most likely impact on the control of mitochondrial biogenesis. However, their precise role has not been investigated so far and comparative analyses in cardiac and skeletal muscle are missing as well. We can not exclude the possibility that during the initial acute response to treadmill exercise mitochondrial biogenesis may have been stimulated in the LV as well. However, opposite to the skeletal muscle, this response is not sustained even when a more intense exercise protocol (23 m/min, 12 weeks) is applied. Mechanisms independent of a chronically increased mitochondrial biogenesis such as enhanced oxidative defence [14] may be involved in the cardioprotective effects of exercise. While PGC-1α overexpression in the heart caused a severe pathology [32], no such changes have been described in skeletal muscle [35], suggesting a tighter range of tolerance in the heart than in the skeletal muscle.

In summary, endurance training induces mitochondrial biogenesis in mouse gastrocnemius muscle but not in the LV. Although AMPK activation occurs in both muscular organs, the absence of SIRT1-dependent PGC-1α deacetylation may be responsible for this significant difference.

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