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Disruption of Adenylyl Cyclase Type 5 Mimics Exercise Training

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Abstract

Exercise training is key to healthful longevity. Since exercise training compliance is difficult, it would be useful to have a therapeutic substitute that mimicked exercise training. We compared the effects of exercise training in wild type (WT) littermates with adenylyl cyclase type 5 knock out (AC5 KO) mice, a model of enhanced exercise performance. Exercise performance, measured by maximal distance and work to exhaustion, was increased in exercise trained WT to levels already attained in untrained AC5 KO. Exercise training in AC5 KO further enhanced their exercise performance. The key difference in untrained AC5 KO and exercise trained WT was the β-adrenergic receptor signaling, which was decreased in untrained AC5 KO compared to untrained WT but was increased in WT with exercise training. Despite this key difference, untrained AC5 KO and exercise trained WT mice shared similar gene expression, determined by deep sequencing, in their gastrocnemius muscle with 183 genes commonly up or down-regulated, mainly involving muscle contraction, metabolism and mitochondrial function. The SIRT1/PGC-1α pathway partially mediated the enhanced exercise in both AC5 KO and exercise trained WT mice, as reflected in the reduced exercise responses after administering a SIRT1 inhibitor, but did not abolish the enhanced exercise performance in the AC5 KO compared to untrained WT. Increasing oxidative stress with paraquat attenuated exercise performance more in untrained WT than untrained AC5 KO, reflecting the augmented oxidative stress protection in AC5 KO. Blocking nitric oxide actually reduced the enhanced exercise performance in untrained AC5 KO and trained WT to levels below untrained WT, demonstrating the importance of this mechanism. These results suggest that AC5 KO mice, without exercise training, share similar mechanisms responsible for enhanced exercise capacity with chronic exercise training, most importantly increased nitric oxide, and demonstrate more reserve with the addition of exercise training. A novel feature of the enhanced exercise performance in untrained AC5 KO mice is their decreased sympathetic tone, which is also beneficial to patients with cardiovascular disease.

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Keywords

Type 5 adenylyl cyclase; exercise training; SIRT1; β-adrenergic signaling

1. Introduction

Increased sympathetic tone is one of the most important factors regulating acute exercise performance [3, 7, 9], and conversely when there is a decrease in sympathetic tone, exercise performance declines [1, 12, 18]. Exercise training, which also involves increases in sympathetic tone [1, 3, 7, 9, 13, 22], is one of the most widely employed mechanisms to improve exercise performance, with widely recognized health benefits for a number of diseases, most prominently cardiovascular disease and heart failure [37]. Paradoxically, increased sympathetic tone exerts an adverse effect on the ischemic and failing heart [13, 29], and is the rationale for β-adrenergic receptor blocker therapy in these diseases [16, 22]. Accordingly, it would be advantageous to discover a mechanism to increase exercise performance and mimic chronic exercise training without the adverse consequences of increased sympathetic tone. Despite the clear benefits to health, exercise compliance tends to be low and it becomes increasingly difficult to participate in exercise with advancing age, thus making attractive the elucidation of novel mechanisms that offer similar benefits, but can be translated to pharmacological therapy. One potential mechanism is inhibition of adenylyl cyclase type 5 (AC5), since AC5 knockout mice (AC5 KO) exhibit decreased sympathetic tone yet display enhanced exercise capacity [31]. Accordingly it becomes important to compare the enhanced exercise performance in untrained AC5 KO mice with that of exercise training in wild type (WT) mice, and also determine whether the mechanisms mediating both are similar, recognizing the directionally opposite effects on sympathetic tone.

The goal of this investigation was to compare mechanisms mediating the improved exercise performance in untrained AC5 KO mice with that of exercise training in their WT littermates, primarily focusing on mechanisms known to improve exercise performance. To accomplish this, we first compared exercise performance in exercise trained WT mice and untrained AC5 KO mice. We next compared genes up or down regulated in the two models using deep sequencing techniques, and mechanisms that mediate enhanced exercise in WT with exercise training and in untrained AC5 KO. Some of the major mechanisms involved increased SIRT1, mitochondrial biogenesis, protection against oxidative stress and increased nitric oxide.

2. Materials and Methods

Animal experimental procedures

All experiments were performed in 3–6 month old male AC5 KO mice and their corresponding male WT littermates. For exercise studies AC5 KO and WT mice were matched for body weight. Animals were anesthetized using Avertin (290 mg/kg i.p.) and the heart and skeletal muscle were excised immediately. Skeletal muscle was dissected and used for histological and biochemical measurements. The extent of oxidative stress was measured

by 8-hydroxy-deoxyguanosine (8-OHdG) staining in the extensor digitorum longus muscle. Animals used in this study were maintained and all experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Eighth Edition 2011). These studies were approved by the Institutional Animal Care and Use Committee of Rutgers University - New Jersey Medical School. Animals were all placed on standard chow for the length of the study.

Exercise protocol and indices of exercise capacity

Each group of mice was assessed for maximal exercise performance using a mouse treadmill. These same mice were then tested for the contributions of specific related mechanisms to exercise performance using: 1) EX527 (SIRT1 inhibitor); 2) paraquat, which increases superoxide and oxidative stress; 3) L-NAME (NO inhibitor). Upon completion of these exercise performance tests mice were sacrificed and skeletal muscle tissue was removed and used for histological and biochemical experiments. Mice were exercised on a treadmill (Accuscan Instruments Inc. AN5817474) to measure indices defining exercise capacity. All mice were subjected to a practice trial 3 days before the experiment to adapt to the treadmill testing environment. Food was withdrawn 3 hours before the exercise. At the time of the experiment, each mouse was placed on a treadmill with a constant 10% grade. The treadmill was started at 4 m/min and the speed incrementally increased 2 m/min every 2 min until the mice reached exhaustion. After acclimatization and baseline exercise measurements, WT and AC5 KO mice were randomly selected and assigned to training (70% of maximal distance, 1 hour/day, for one month). Exhaustion was defined as spending time (10 seconds) on the electric stimulus platform without attempting to reengage the treadmill belt. Following four weeks of training the animals were retested using the previously described protocols. AC5 KO and WT mice were treated with SIRT1 inhibitor EX527 (6-Chloro-2,3,4,9-tetrahydro-1H-Carbazole-1-carboxamide) (10 mg/kg/day) or Nω-nitro-l-arginine methyl ester (L-NAME; 1.43 μmol (2 μl; i.p.) immediately prior to exercise testing [17]. We have previously demonstrated the specificity and effectiveness of L-NAME [15]. In addition, we measured NOS before and after L-NAME in WT and found decreased NOS by 43%. We have also previously demonstrated that EX527 (10 mg/kg/day) effectively decreases exercise capacity through SIRT1 mediated disruptions in mitochondrial function [31]. In addition, our previous experiments have demonstrated that treatment with L-NAME blocks acetylcholine mediated vasodilator function [15]. We also examined the extent to which 10 days of paraquat treatment (35 mg kg−1, i.p.), which increases oxidative stress [31], affected the response to exercise in the groups studied. Following our preliminary data additional subsets of animals were added to perform the following biochemical experiments leading to unbalanced samples sizes between figures.

Genes identified by RNA sequencing (RNA-seq)

To determine mean genomic expression profiles among WT, WT exercise trained and untrained AC5 KO mice were utilized for deep sequencing techniques. Total RNA was extracted from each tissue sample using RNeasy Fibrous Tissue Kit (QIAGEN) and all groups were processed and analyzed together during a single experiment. Equal amounts of each RNA sample was subjected to sequencing on Illumina Genome Analyzer IIx (1–4 million reads per sample, ~35 nt per read), following the protocol presented by Illumina for

mRNA sequencing (Illumina). The sequencing reads were mapped to target genomic sequences using Bowtie2 [19] and found the genomic position to which each read is uniquely aligned. The number of reads was counted for each transcript in NCBI Reference Sequence (RefSeq) Database (http://www.ncbi.nlm.nih.gov/refseq/). The relative abundance of each transcript was quantified by calculating RPKM (reads per kilobase of exon model per million mapped reads), giving accurate comparison of expression levels among samples and among genes. Calculations were executed by R and Perl script language.

Histological analyses

Histology samples were collected from the gastrocnemius or extensor digitorium longus muscles. Samples were preserved in 10% buffered formalin. Cross-sectional slices were cut and mounted onto glass slides. Succinate dehydrogenase staining was carried out following the protocol as described [29].

Biochemical analyses

Immunoblotting Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes. The membranes were probed with primary antibodies to SIRT1 (Cell Signaling; D739) and MnSOD (Sigma-Aldrich; DD-17) at 4 °C overnight. The bands were visualized using chemiluminescence reagents. The linear range of detection for different proteins and band intensities were determined by densitometry. Blots were re-probed with GAPDH (Sigma-Aldrich; G5262) to equalize sample loading. It has been suggested that increased sympathetic tone induces some variability in the GAPDH measurements [26]. However the AC5 KO is a model of decreased sympathetic tone and as is evident by the data in the figures, there was little variability in the GAPDH measurement.

Immunoprecipitation using Protein A-Sepharose was incubated overnight at 4°C with anti-PGC-1α antibody (Sigma-Aldrich; ST1204). Immunoprecipitation was performed by incubating cellular extract with the antibody conjugated beads, and the complex was washed 3 times in phosphate buffered saline (PBS). Immunoprecipitates were denatured by boiling, resolved on SDS-PAGE gels, and then transferred to membranes following immunoblotting analysis for acetylated lysine.

Citrate synthase activity of the soleus muscle was then measured using a citrate synthase activity kit (Sigma Aldrich; CS 0720). Complex IV activity was measured in the gastrocnemius using a Complex IV rodent assay kit (Abcam; ab109911). Total nitric oxide expression was measured at a wavelength of 540nm in the gastrocnemius using a Total Nitric Oxide Assay (Biosystems).

Cyclic AMP (cAMP; Invitrogen; EMSCAMPL) Animals were sacrificed 2 hours following their final bout of exercise. The gastrocnemius was removed and snap frozen immediately and the tissue was stored at −80 C°. The tissue was than homogenized and a multispecies Cyclic AMP Competitive ELISA Kit was used to assay cAMP activity.

Statistical analysis

All data are expressed as mean ± SD. To compare experiments using three or four independent groups, a one-way ANOVA with a Tukey's post-hoc was employed. A Student's t test was used for comparison of two groups. A 2 × 2 repeated measures ANOVA with "group" as a between subject factor and "treatment" as a within subject factor was run for exercise performance test using treatment of L-NAME, paraquat and EX527. A one-way ANOVA was employed for post-hoc analysis to assess group differences when appropriate. The alpha level was set at $P < 0.05$. The numbers of animals selected were based on past experience with these protocols and power analyses. This investigation tested the null hypotheses that the exercise performance in untrained AC5 KO and WT trained mice were not the same and mechanisms mediating the enhanced exercise performance were not the same.

3. Results

Enhanced exercise capacity in exercise trained WT and untrained AC5 KO mice

Exercise performance was measured using the indices of maximal distance attained and work to exhaustion. Untrained AC5 KO mice ran significantly longer than untrained WT mice in distance (755± 79 vs. 510 ± 58 m; Fig. 1a), and demonstrated 51% increased work to exhaustion compared with untrained WT mice, $p<0.05$. (Fig. 1b). Chronic exercise training increased performance in WT, e.g., distance rose to (707 ± 86 m) (Fig 1a), similar to levels in AC5 KO mice without training, and increased average work to exhaustion. Exercise training in untrained AC5 KO mice enhanced their exercise performance even further to levels exceeding those in WT mice with exercise training (Fig. 1a,b).

Exercise and beta adrenergic signaling

A key goal of the current study was to correlate exercise performance and β-adrenergic signaling. Accordingly we measured cAMP concentration in the gastrocnemius and found that it was significantly reduced in the untrained AC5 KO group by 40% compared to untrained WT mice (Fig. 1c), and that chronic training in WT increased cAMP concentration by 41% confirming the decreased sympathetic tone in AC5 KO mice and increased sympathetic tone with chronic exercise.

Gene comparison in untrained AC5 KO mice and WT with exercise training

There were 78 genes commonly up-regulated and 105 genes commonly down-regulated in WT with exercise training and AC5 KO without training (Fig. 2a). These common genes were mainly involved in the pathways of muscle contraction, metabolism, contractile fibers and mitochondrion. Based on these genomic similarities we studied specific mechanisms mediating the improved exercise performance in AC5 KO and WT with exercise training (Fig. 2b). Genes that are commonly up regulated with improvements in metabolism and contractile function and common to exercise were also increased in untrained AC5 KO mice (Fig. 1d). The expression of Pfkm, an established marker of glucose metabolism, was increased with exercise training. In addition, two other important energy regulating enzymes were upregulated, Idh2 and Ckmt, which encode for isocitrate dehydrogenase and creatine

kinase [5]. Two contractile proteins which are sensitive to exercise were upregulated (Actn2 and Myh1) [14, 17].

The skeletal muscle phenotype of untrained AC5 KO mice resembles that of exercise-trained mice

Mitochondrial Biogenesis—Exercise training is known to induce a skeletal fiber type switch from fast-twitch glycolytic fiber into a more oxidative fiber state, a phenomenon that is dependent upon the mitochondrial content [29, 32]. In addition, citrate synthase activity, a marker of exercise training, was increased similarly in the soleus muscle of untrained AC5 KO and in exercise trained WT mice (Fig. 3a). There were also similar increases in Complex IV activity present in untrained AC5 KO and exercise trained WT compared to sedentary WT (Fig. 3b). Relative mRNA expression of mitochondrial specific genes were upregulated by exercise training and AC5 deletion encoding for both improved mitochondrial function and biogenesis (Fig. 3c). Mitochondrial fusion was only weakly increased (Mfn1 and Mfn2), in both WT trained and untrained AC5 KO groups, indicating that this specific mechanism may not be as important a mediator of the improvements in mitochondrial function seen with exercise training.

SDH Staining—Succinate dehydrogenase (SDH) staining of extensor digitorum longus (EDL) muscles, indicated more oxidative SDH positive muscle fibers both in untrained AC5 KO and exercise-trained mice [29] (Fig. 3d).

Sirt1/PGC1-α Pathway

SIRT1 levels and the SIRT1/PGC-1α pathway were compared in untrained AC5 KO and exercise trained WT and found to be up regulated similarly (Fig. 4a, b). In addition to studying the expression of SIRT1 we administered EX527, a SIRT1 inhibitor. After EX527 exercise capacity was reduced in all groups (Fig. 4d,e), verifying a key role of SIRT1 in mediating enhanced exercise performance, but not eliminating the better exercise performance in AC5 KO untrained and WT trained compared with WT untrained.

Oxidative Stress

A key beneficial component of exercise is the increased resistance to oxidative stress. We measured protein expression of the mitochondrial endogenous antioxidant MnSOD in the gastrocnemius, which was up regulated in untrained AC5 KO and exercise trained mice by 84% and 66% respectively, when compared to sedentary WT (Fig. 5a). Paraquat, which increases oxidative stress, reduced running distance more in untrained WT (−36 ± 21%) and exercise trained WT (−39 ± 16%), more than in AC5 KO without exercise training (−10 ± 8%) (Fig. 5 c,d) supporting the concept that the AC5 KO are already protected against oxidative stress.

Nitric Oxide

Nitric oxide (NO) is also linked to mitochondrial metabolism and oxidative stress. Total NO was increased by 50% in the exercise trained WT, while there was a 75% increase in the untrained AC5 KO group (Fig. 6a). To block the effects of nitric oxide, we administered L-

NAME to mice just prior to exercise testing (Fig 6c,d). While all groups had significant decreases in running distance (WT = − 12 ± 14%; WT-Trained = −63 ± 13%; AC5 KO = − 74 ± 7%), The untrained AC5 KO group had greater losses in exercise capacity, consistent with their greater nitric oxide and consistent with the greatest increases in NOS1, NOS2 and NOS3 genes (Fig 6b). NO blockade actually decreased exercise performance below that of untrained WT in both the untrained AC5 KO and WT trained groups. These data support the concept that increased NOS production is crucial to the improvement in exercise performance in AC5 KO and exercise training.

Further enhanced exercise capacity in exercise trained AC5 KO mice

Exercise training in untrained AC5 KO mice enhanced their exercise performance even further to levels exceeding those in WT mice with exercise training (Fig. 7a,b). SIRT1 inhibition with EX527 reduced exercise performance in exercise trained AC5 KO, but levels remained higher than those in exercise trained WT (Fig 7a, b). Paraquat reduced exercise performance only slightly in exercise trained AC5 KO and not as much as in trained WT (Fig 7c, d). Nitric oxide blockade reduced exercise performance in AC5 KO trained to levels equivalent to those in untrained WT (Fig. 7e, f).

Intra-Individual Changes with Exercise Training and Drug Treatment

Although there were highly significant differences in this study between responses of WT and AC5 KO, it was also important to examine the intra-individual changes. We found that the intra-individual changes with exercise training were similar for WT (197 ± 73 m) and AC5 KO for running distance (191 ± 123 m) and for work to exhaustion (6 ± 2 J vs. 9 ± 5 J). Although there was variability from animal to animal, the mean ± SD values for both running distance and work to exhaustion were significantly greater in AC5 KO than WT. Additionally, we assessed intra-individual changes in WT and AC5 KO before and after treatment with 1) EX527, 2) paraquat, and 3) L-NAME. Similar to chronic exercise training, there was variability from animal to animal. However the mean ± SD shown in each figure reflects highly significant changes between groups.

4. Discussion

The current investigation identified similar mechanisms mediating enhanced exercise performance with exercise training in WT mice compared with enhanced exercise performance in untrained AC5 KO mice. Utilizing gene deep sequencing we found 78 genes commonly up-regulated and 105 genes commonly down-regulated in skeletal muscle of untrained AC5 KO and exercise trained WT animals. Some of the major mechanisms involved increased SIRT1, mitochondrial biogenesis, protection against oxidative stress and increased nitric oxide, all known to be involved in improved exercise performance. All of these mechanisms have been found to mediate enhanced exercise performance with chronic exercise training [2, 4, 8, 10–12, 29, 31, 32, 36].

An increase in either mitochondrial function or biogenesis augments skeletal muscular oxygen extraction and improves exercise performance [32, 33]. We found that mitochondrial function, as assessed by citrate synthase and complex IV activity, was improved similarly in

untrained AC5 KO mice and WT exercise trained mice. To further support this we found a number of mitochondrial specific genes were similarly up regulated in both exercise trained WT mice as well as untrained AC5 KO mice. We previously reported that cardiac specific AC5 KO did not improve exercise performance [31], indicating that skeletal muscle specific increases in mitochondria appear to be the principal mediating factor for the improvements seen in the AC5 KO mice. We also found that the AC5 KO, like exercise training, was characterized by a skeletal muscle fiber type switch to a more oxidative state, a phenomenon that is dependent upon the mitochondrial content [29, 32, 34], and as shown by skeletal muscle SDH staining, which indicated more oxidative SDH positive muscle fibers both in untrained AC5 KO and exercise trained WT mice (Fig. 3d).

A key mechanism mediating mitochondrial oxidative capacity and the enhanced exercise performance in untrained AC5 KO mice is the up regulation of the SIRT1/PGC-1α pathway, which plays an important role in the control of skeletal muscle mitochondrial content and mediating exercise performance [10, 11, 20, 31] and was found to be increased similarly in the untrained AC5 KO and chronic exercise trained WT mice (Fig. 4). Inhibiting this pathway with the SIRT1 blocker, EX 527, diminished exercise capacity in all groups, but did not eliminate the enhanced exercise performance in the untrained AC5 KO or WT trained mice indicating that alternative pathways that were not blocked by EX527 play an important role with enhanced exercise capacity.

Oxidative stress is also known to inhibit exercise performance [31]. The untrained AC5 KO and exercise trained WT were both protected against oxidative stress. Interestingly, when oxidative stress was augmented with paraquat, there was less reduction in exercise performance in AC5 KO than in WT, even with exercise training, demonstrating that deletion of AC5 protects against oxidative stress, even in the absence of exercise training. Another mechanism closely allied to oxidative stress is NO, a key regulator in skeletal muscle and mitochondrial function and the prevention of oxidative stress [6, 25]. Since NO is linked to the SIRT1 pathway, we assessed its role in mediating the enhanced exercise capacity present in untrained AC5 KO mice and trained WT mice [24, 27, 35]. We demonstrated that both exercise training and AC5 deletion increased the total expression of NO in skeletal muscle, and that NO blockade with L-NAME eliminated the increased exercise performance in both the untrained AC5 KO and WT trained mice. Interestingly, blocking NO actually reduced exercise performance in both of these groups below that observed in the untrained WT group, supporting the major role of the NO pathway in mediating the enhanced exercise performance found in both these groups. Although NO is known to be a mediator of exercise performance [17, 21], and is involved in exercise training [28, 30], this is the first demonstration that the increased exercise performance induced by exercise training can not only be completely eliminated by NO blockade, but can be reduced below levels observed in untrained animals. One reason for this novelty is that NO blockade did not reduce exercise performance in untrained WT mice, the model that has been examined most extensively in prior studies.

The potentially most important feature of the current investigation, with respect to its role in identifying a novel therapeutic modality, is that the increased exercise performance in the AC5 KO model is accompanied by decreased β-adrenergic signaling and cAMP, as adenylyl

cyclase is the major enzyme responsible for increased β-adrenergic signaling [7, 9], whereas exercise training is accompanied by increased cAMP [3, 7]. In fact, increased exercise performance is almost universally associated with increased β-adrenergic signaling [9, 13, 23, 33], whereas decreases in β-adrenergic signaling are most always associated with decreased exercise performance, as reflected by reduced exercise tolerance with β–adrenergic blocking drugs [13]. This is important because increased exercise performance is therapeutic for almost all disease states, most significantly for cardiovascular disease and heart failure [37], where it is essential to reduce β-adrenergic signaling, which is the rationale for β-adrenergic blockade therapy.

In conclusion, we found that despite having a decrease in sympathetic tone, untrained AC5 KO mice had a greater level of exercise capacity then their WT littermates, as they were able to perform as well as exercise trained WT mice, and were able to increase their exercise capacity even further with training. AC5 deletion appears to mimic the effects of exercise training by up-regulating the SIRT1/PGC-1α pathway, nitric oxide and oxidative stress protection. These beneficial effects occurred independent of β-adrenergic signaling suggesting that increased sympathetic tone is not necessary for SIRT 1 induction. The nitric oxide mechanism was unique and most important in that after blockade of this mechanism with L-NAME, exercise capacity was not only blocked, but actually fell below that observed in untrained WT. The finding that AC5 KO exercise performance mimics that of exercise training, with many of the same mechanisms mediating the enhanced exercise performance, has important implications for those patients that are obese or diabetic or with heart disease, where increasing exercise is important therapeutically, but cannot or choose not to exercise.

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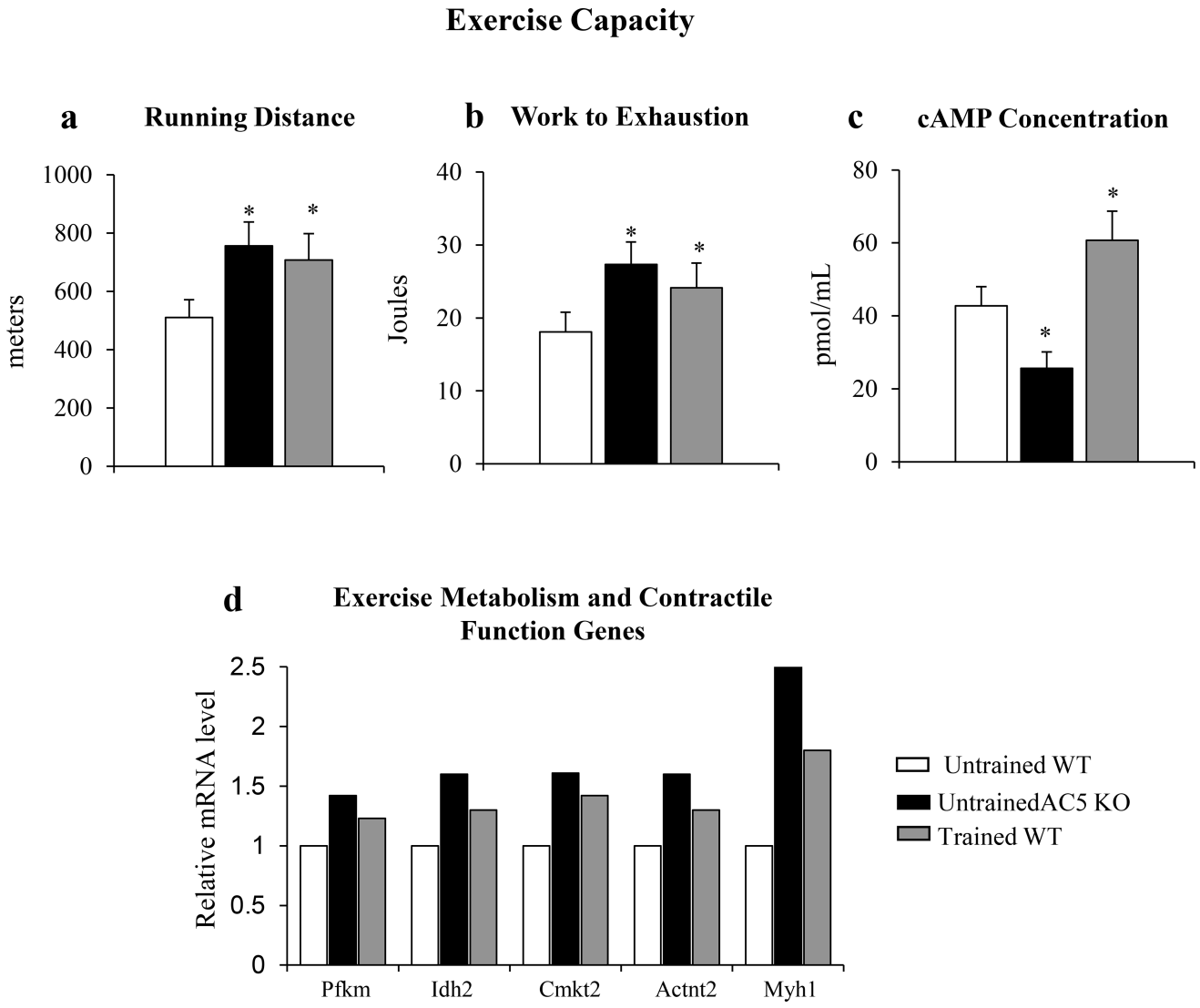

Fig. 1. Similar exercise capacity and gene expression but opposite cyclic AMP expression in untrained AC5 KO and WT trained mice

a) Untrained AC5 KO mice (black bars) ran longer distances and b) had greater maximal work capacity than untrained WT (open bars). One month of exercise training improved performance in WT to the level of AC5 KO without training (n=10/group). These mice were used for subsquent experiments involving SIRT1 (EX527) and NO inhibitor (L-NAME), along with superoxide induction (paraquat; Figs. 4–6). c) Untrained AC5 KO mice had a decrease in cAMP concentration when compared to untrained WT, while exercise training increased cAMP in the WT mice. (n=5–6/group) d) In untrained AC5 KO and WT trained there was similar gene up-regulation related to ATP-PCr system, glycolysis and the Kreb's cycle (Pfkm, Idh2, and Ckmt), and for those reflecting regulation of contractile proteins (Actn2, Myh1) (n=3/group). (* = $p<0.05$ vs. WT). Results are expressed as the mean ± SD.

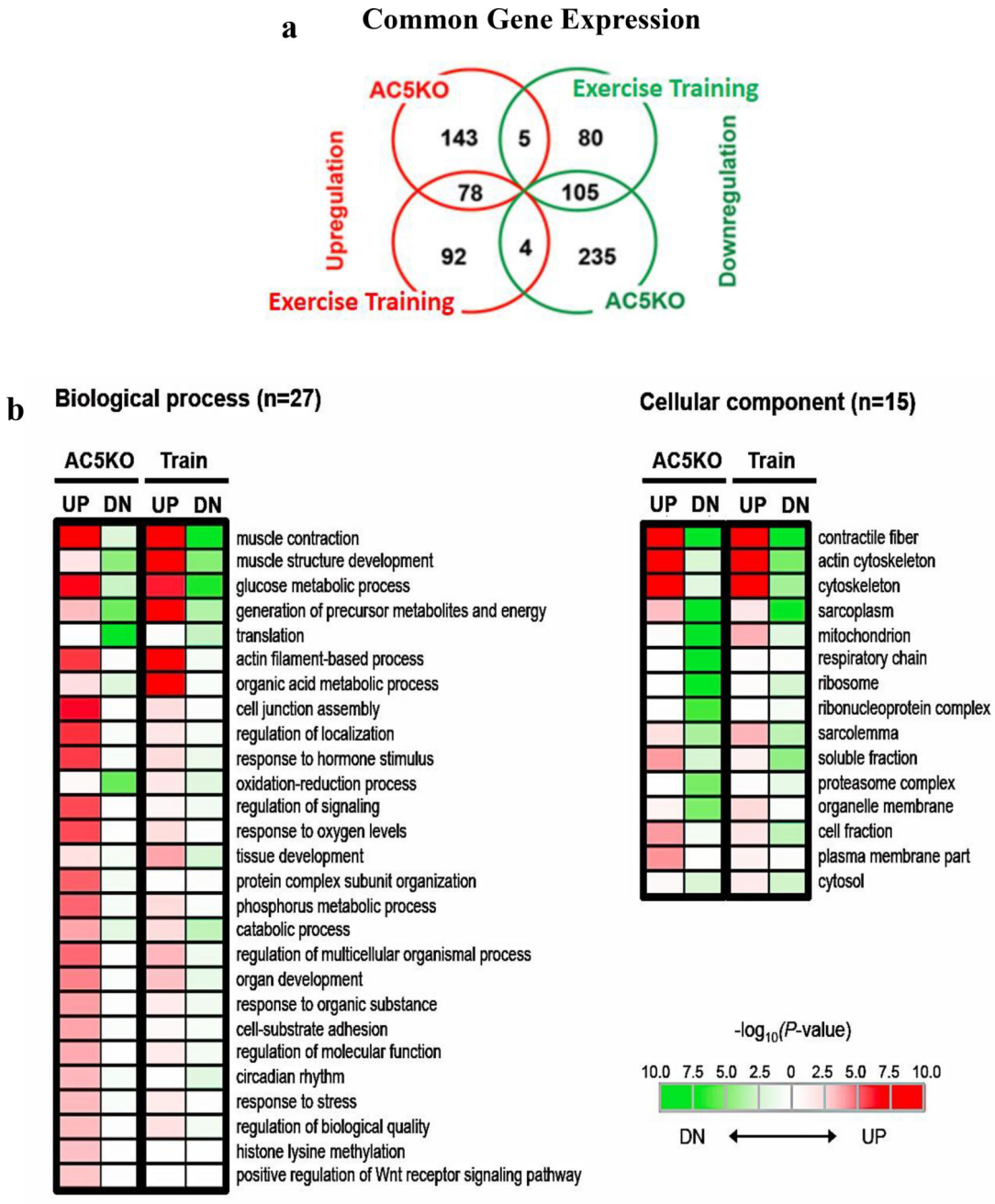

Fig. 2. AC5 KO mice have similar molecular signaling mechanisms to WT mice with chronic exercise training

a) There were 78 genes commonly up-regulated and 105 genes commonly down-regulated in WT with exercise training and AC5 KO without training. b) Heat map showing Gene Ontology (GO) terms associated with regulated genes. Each GO term was tested for bias to up-regulation (UP) and down-regulation (DN) by the Fisher's exact test. Significant GO terms ($P < 0.001$ for either up- or down-regulation) are shown (n=3/group).

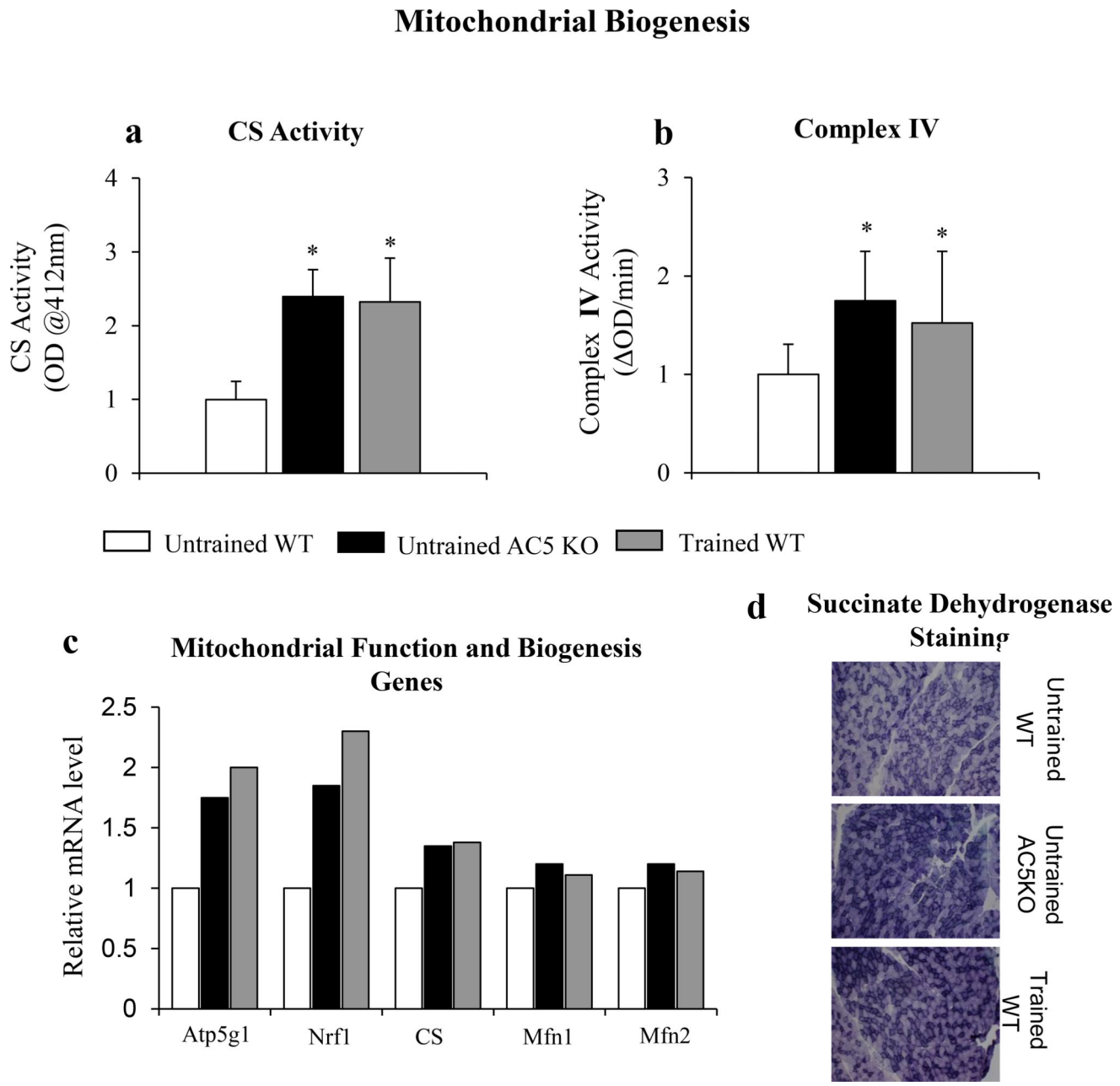

Fig. 3. AC5 KO and chronic exercise training in WT improve mitochondrial biogenesis

a) Citrate synthase (CS) activity and b) mitochondrial complex IV were increased in untrained AC5 KO (black bars), and exercise trained WT (gray bars) mice compared to untrained WT (open bars) mice. (n=5–6/group) c) Genomic analysis of relative mRNA expression of mitochondria-related genes, which were up-regulated similarly in the gastrocnemius muscle in both untrained AC5 KO and exercise trained mice (ATP5G1, Nrf1, CS, Mfn1, Mfn) (n=3/group). d) Succinate dehydrogenase (SDH) staining of extensor digitorum longus (EDL) muscles was increased similarly in untrained AC5 KO and exercise trained WT mice compared to WT without training. Results are expressed as the mean ± SD. (* = $p<0.05$ vs. WT).

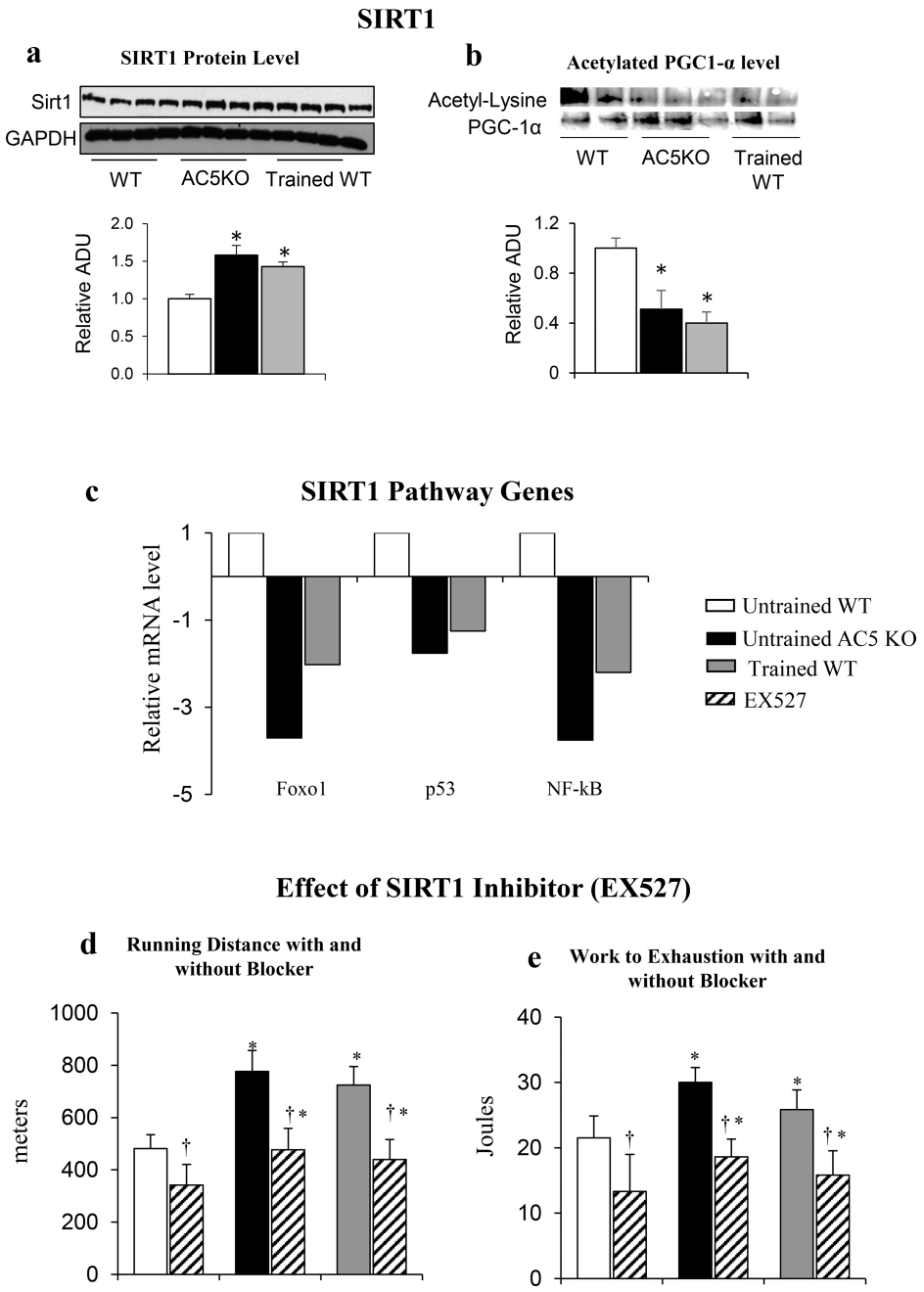

Fig. 4. The enhanced exercise capacity in the WT trained and untrained AC5 KO group is partially mediated through the SIRT1/PGC1-α pathway

AC5 KO untrained and WT trained mice induced mitochondrial biogenesis through the Sirt1/PGC-1α pathway. a) Increased SIRT1 expression in untrained AC5 KO (black bars) and exercise trained WT (gray bars) compared to untrained WT (open bars, (n=4/group). Western blot for the WT untrained and AC5 KO untrained were included in a previous publication [31], but the data from the WT trained was not included in that publication. b) PGC-1α acetylation was decreased similarly in skeletal muscle of untrained AC5 KO and exercise trained WT mice (n=3/group). c) Genomic analysis revealed that genes specific to the SIRT1 pathway (Foxo1, P53, NF-kB) were downregulated similarly in the WT exercise

trained and untrained AC5 KO mice (n=3/group). d) Enhanced exercise capacity in untrained AC5 KO and exercise trained WT mice is compared with and without the SIRT1 blocker (EX 527). Administration of the blocker was given for running distance and for e) work to exhaustion (n=8–9/group). The SIRT1 inhibitor, EX 527 reduced exercise capacity in all groups but did not affect the pattern of greater exercise capacity in untrained AC5 KO compared to untrained WT. Results are expressed as the mean ± SD. (* = $p<0.05$ vs. WT, † = $p<0.05$ vs. w/o treatment).

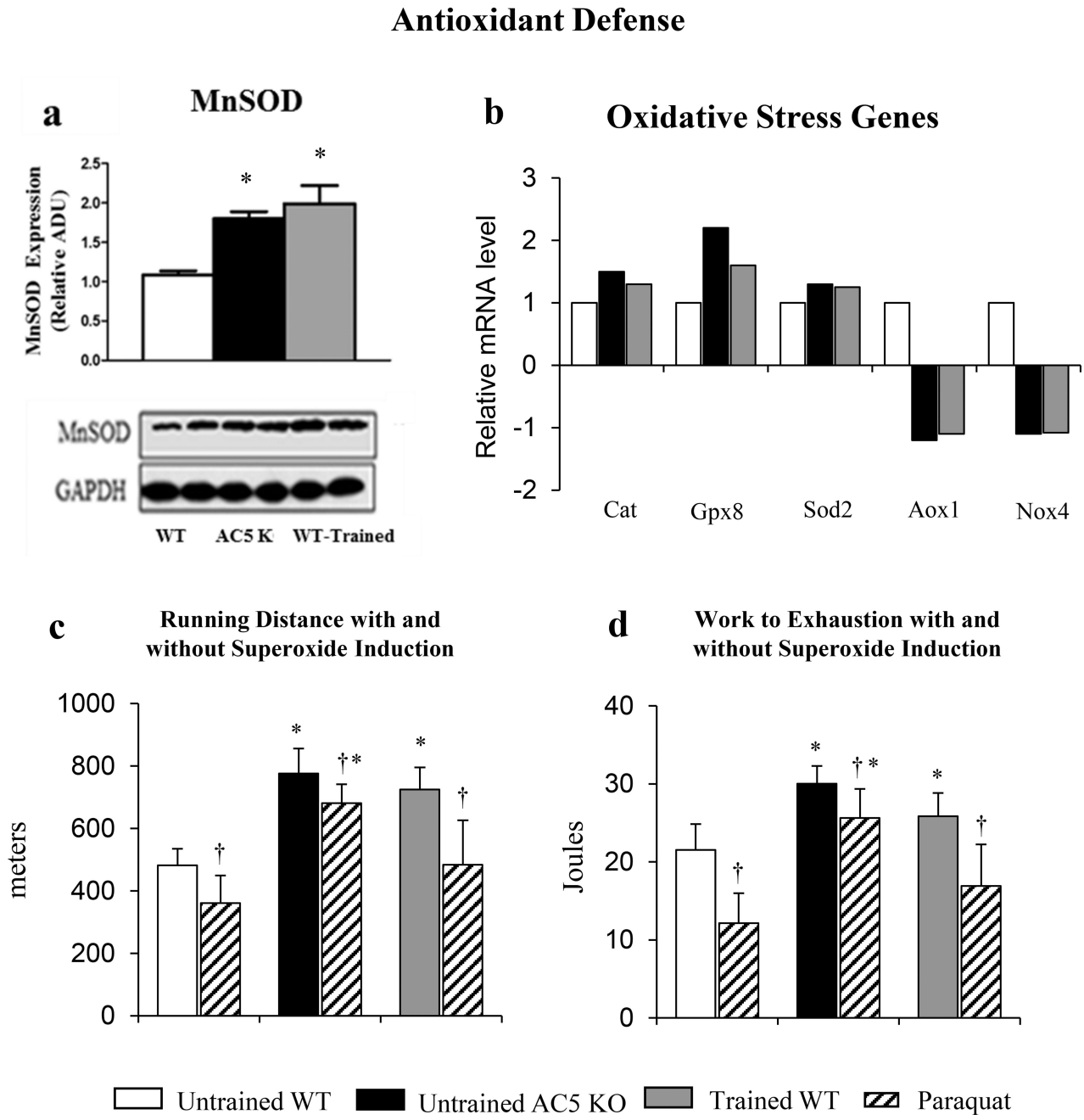

Fig. 5. Untrained AC5 KO and exercise training in WT protect against oxidative stress

a) Greater MnSOD expression was present in the untrained AC5 KO and exercise trained WT mice compared to WT (n=4/group). b) Genes regulating oxidative stress were up-regulated similarly in both untrained AC5 KO and exercise trained WT groups (Cat, Gpx8, Sod2), as were genes encoding for enzymes which catalyze the formation of the free radical superoxide (Aox1, Nox4) (n=3/group). c) Running distance and d) work to exhaustion are shown before and after paraquat adminstration. (n=8–9/group). Treatment with paraquat reduced running distance and work to exhaustion in all groups, but actually less in AC5 KO,

due to their enhanced protection against oxidative stress. (* = $p<0.05$ vs. WT, † = $p<0.05$ vs. w/o treatment).

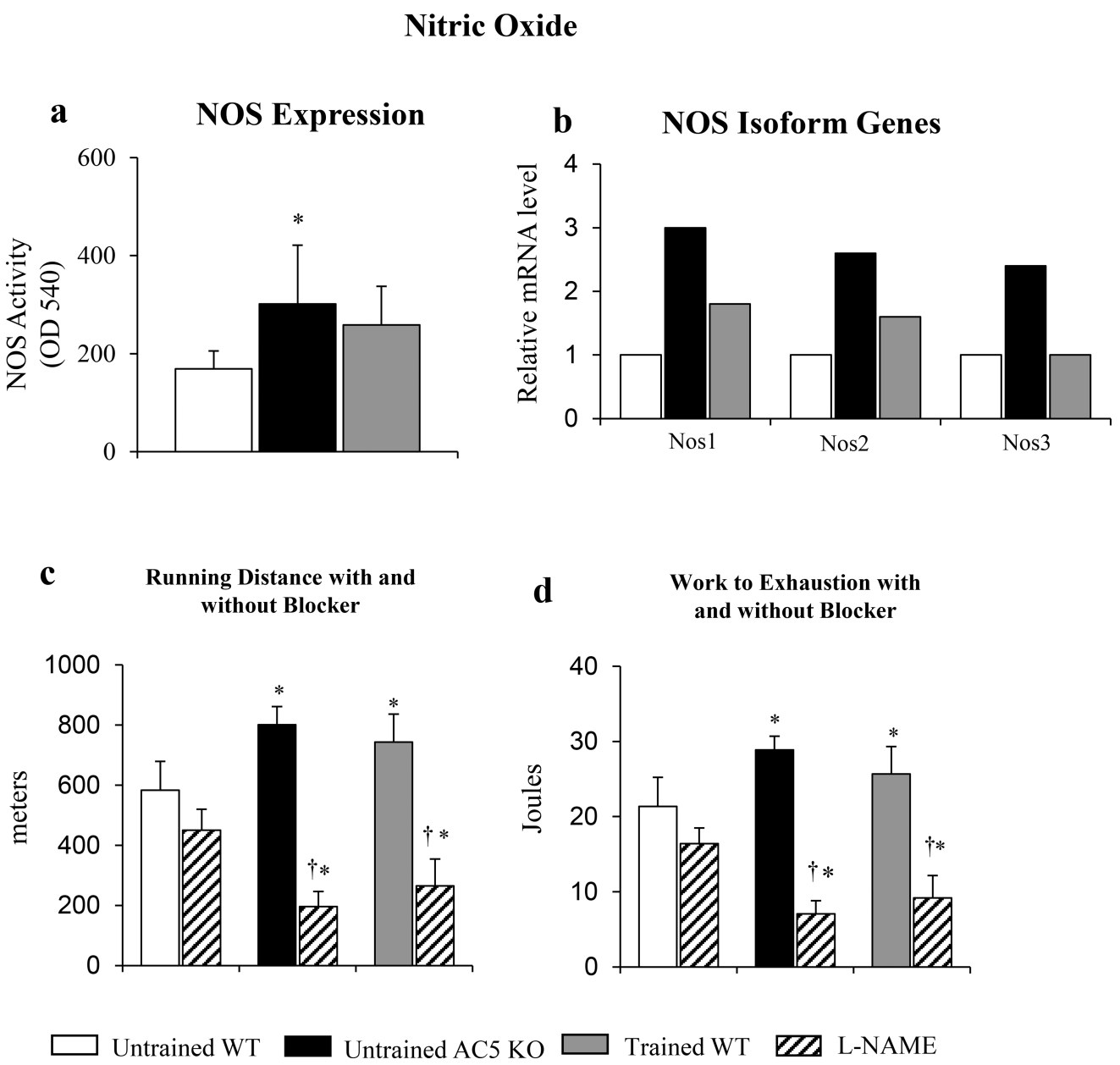

Fig. 6. The enhanced exercise capacity in the AC5 KO group and WT trained group totally mediated through increased NOS protein and gene expression

a) Total NOS expression was increased in the untrained AC5 KO and WT trained groups, but tended to be higher in both untrained AC5 KO mice (n=5-group). b) Genomic analysis demonstrated that the relative mRNA expression of the different isoforms of NOS in the untrained AC5KO was up-regulated more than in the WT trained group (n=3/group). L-NAME abolished enhanced c) running distance and d) work to exhaustion in untrained AC5 KO and WT exercised trained groups but to a greater extent in the AC5 KO groups, where exercise capacity was actually lower than in the WT group (n=5–6/group). Results are expressed as the mean ± SD. (* = $p<0.05$ vs. WT; † = $p<0.05$ vs. w/o treatment).

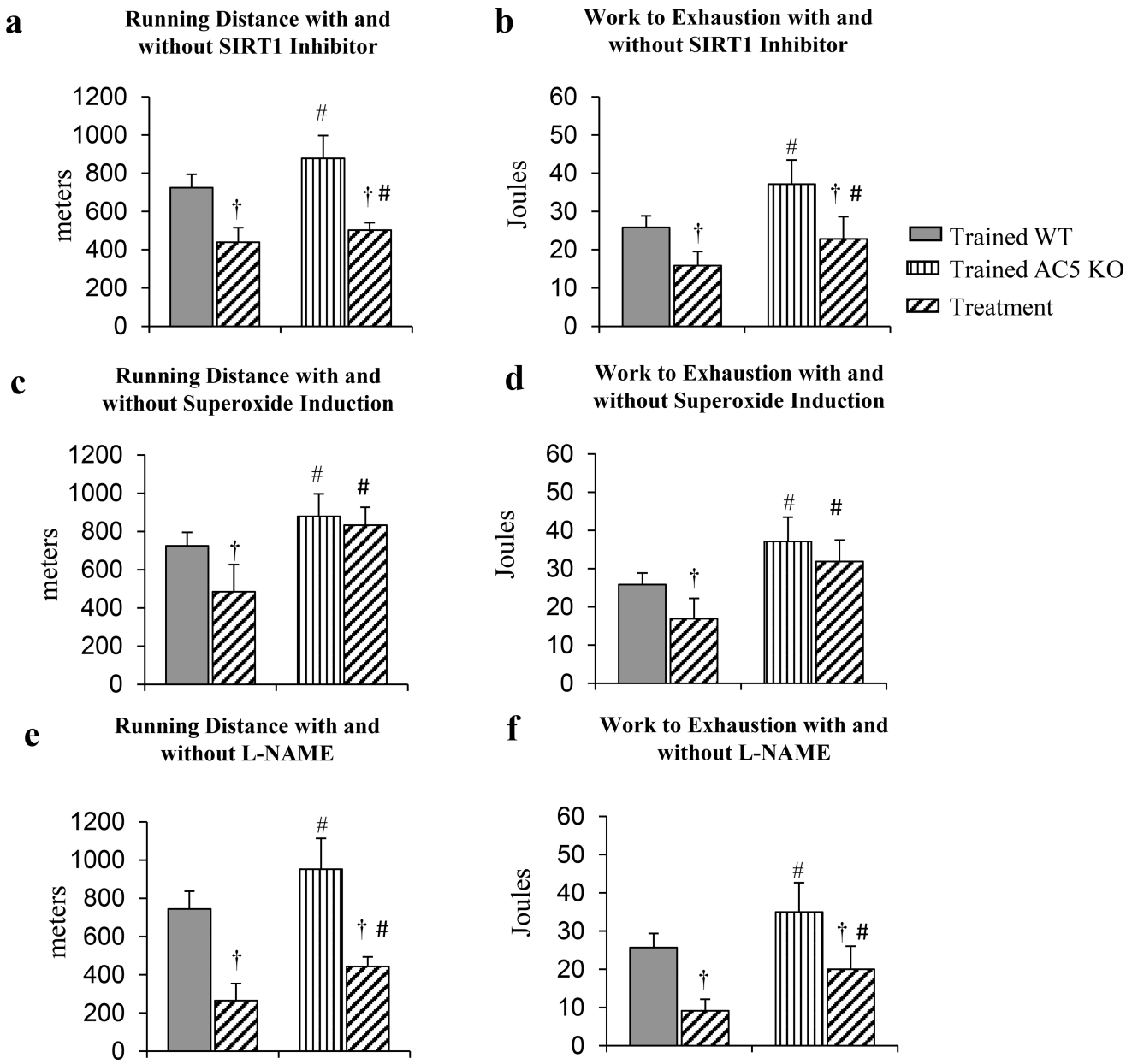

Fig. 7. Exercise capacity between trained WT and trained AC5 KO mice

Enhanced exercise capacity in exercise trained AC5 KO (lined bars) and exercise trained WT mice (gray bars) are compared with and without the SIRT1 blocker (EX 527) (panels a,b), with and without superoxide induction with paraquat (panels c,d) and with and without L-NAME (panels e,f). Exercise performance was enhanced in trained AC5 KO compared with trained WT and persisted after each treatment. There were 6–9 mice in each group, with paired data compared with and without treatment. Treatment with paraquat reduced running distance and work to exhaustion in both groups, but actually less in exercise trained AC5 KO, due to their enhanced protection against oxidative stress. EX 527 and L-NAME reduced running distance and work to exhaustion in both AC5 KO exercise trained and WT exercised

trained groups. Results are expressed as the mean ± SD. (# = $p<0.05$ vs. WT trained, † = $p<0.05$ vs. w/o treatment).