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p53 is necessary for the adaptive changes in cellular milieu subsequent to an acute bout of endurance exercise

Ayesha Saleem,[1,2] Heather N. Carter,[1,2] and David A. Hood[1,2,3]

[1]*School of Kinesiology and Health Science, York University, Toronto, Ontario, Canada;* [2]*Muscle Health Research Centre, York University, Toronto, Ontario, Canada; and* [3]*Department of Biology, York University, Toronto, Ontario, Canada*

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Saleem A, Carter HN, Hood DA. p53 is necessary for the adaptive changes in cellular milieu subsequent to an acute bout of endurance exercise. *Am J Physiol Cell Physiol* 306: C241–C249, 2014. First published November 27, 2013; doi:10.1152/ajpcell.00270.2013.—An acute bout of exercise activates downstream signaling cascades that ultimately result in mitochondrial biogenesis. In addition to inducing mitochondrial synthesis, exercise triggers the removal of damaged cellular material via autophagy and of dysfunctional mitochondria through mitophagy. Here, we investigated the necessity of p53 to the changes that transpire within the muscle upon an imposed metabolic and physiological challenge, such as a bout of endurance exercise. We randomly assigned wild-type (WT) and p53 knockout (KO) mice to control, acute exercise (AE; 90 min at 15 m/min), and AE + 3 h recovery (AER) groups and measured downstream alterations in markers of mitochondrial biogenesis, autophagy, and mitophagy. In the absence of p53, activation of p38 MAPK upon exercise was abolished, whereas CaMKII and AMP-activated protein kinase only displayed an attenuated enhancement in the AER group compared with WT mice. The translocation of peroxisome proliferator-activated receptor-γ coactivator-1 α to the nucleus was diminished and only observed in the AER group, and the subsequent increase in messenger RNA transcripts related to mitochondrial biogenesis with exercise and recovery was absent in the p53 KO animals. Whole-muscle autophagic and lysosomal markers did not respond to exercise, irrespective of the genotype of the exercised mice, with the exception of increased ubiquitination observed in KO mice with exercise. Markers of mitophagy were elevated in response to AE and AER conditions in both WT and p53 KO runners. The data suggest that p53 is important for the exercise-induced activation of mitochondrial synthesis and is integral in regulating autophagy during control conditions but not in response to exercise.

acute endurance exercise; p53; signaling; PGC-1α; mRNA; mitophagy; autophagy

SKELETAL MUSCLE IS A HIGHLY adaptable tissue that undergoes numerous metabolic and morphological adaptations in response to regular physical activity. In particular, endurance exercise training has been illustrated to induce beneficial physiological alterations that help extend life expectancy and reduce morbidity in disease states, such as obesity, cardiovascular disease, type 2 diabetes, metabolic syndrome, cancer, as well as physical disability in later life (34). Whereas an extensive amount of information is available on the benefits of exercise, complete insight into the molecular mechanisms underlying these changes is lacking. The elucidation of the causes and molecular signaling events behind the adaptations in response to endurance exercise carries great significance for the treatment of physical inactivity-related diseases.

Muscle contraction results in rapid cellular changes that subsequently activate downstream signaling kinases. Upon each bout of muscle contraction, there are variations in the AMP:ATP ratio, increases in intracellular calcium, and generation of reactive oxygen species (ROS) that can activate AMP-activated protein kinase (AMPK) (37), CaMKII (23), and p38 MAPK (1), respectively. These bona fide exercise-responsive signaling kinases then initiate more widespread changes in the muscle by recruiting regulators of mitochondrial biogenesis, such as peroxisome proliferator-activated receptor-γ coactivator-1 α (PGC-1α), which is a transcriptional coactivator and a critical regulator of the transcription of nuclear genes encoding mitochondrial proteins and has been implicated as a master mediator of the adaptive response to exercise in skeletal muscle (18, 22). Both acute exercise (AE) and chronic exercise can activate PGC-1α and cause it to localize to the nucleus (36), where it can control, directly or indirectly, the transcription of its target genes, including nuclear respiratory factor 1 (NRF-1), cytochrome-*c* oxidase subunit IV (COX-IV), and mitochondrial transcription factor (Tfam), among others (33), and thus enhance mitochondrial biogenesis.

In addition to triggering the synthesis of mitochondria, exercise has been recognized recently to play a part in the removal of damaged or dysfunctional mitochondria, thereby maintaining mitochondrial homeostasis (10, 11, 15). Autophagy refers to the process where damaged cellular materials are marked, encapsulated, and delivered to the lysosomes for degradation. Mitophagy is the selective degradation of dysfunctional mitochondria often tagged by enhanced ubiquitination of mitochondrial proteins, a consequence of elevated ROS accumulation, or dissipation of the mitochondrial membrane potential (9). A multitude of proteins has been identified to be a part of this process, including Beclin1, autophagy-related protein 7 (Atg7), p62, and light chain 3 II (LC3II), which participate at the various stages in the process of autophagy (8, 9, 12). Beclin1 and Atg7 are involved in vesicle nucleation and LC3 maturation, p62 and LC3II recognize ubiquitinated proteins, and LC3II is now commonly used as a marker of autophagy, as is necessary for the construction of the autophagosome (3).

The tumor-suppressor protein p53 has an established role in modulating mitochondrial content and subsequently, oxidative capacity (20, 26, 27). Its transcriptional control over many vital factors involved in mitochondrial biogenesis, such as PGC-1α, Tfam, and synthesis of cytochrome-*c* oxidase 2 (SCO2), an important assembly factor in mitochondrial electron transport chain complexes, renders the expression of p53 to be of significance with respect to mitochondrial adaptations in response to exercise training (27). However, it is unknown whether p53 is necessary for the physiological changes that

Address for reprint requests and other correspondence: D. A. Hood, School of Kinesiology and Health Science, Muscle Health Research Centre, 302 Farquharson Bldg., 4700 Keele St., York Univ., Toronto, Ontario, M3J 1P3, Canada (e-mail: dhood@yorku.ca).

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Table 1. *Primer sequences used for RT-PCR analysis*

Gene	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)
PGC-1α	TTCCACCAAGAGCAAGTAT	CGCTGTCCCATGAGGTATT
Tfam	GAAGGGAATGGGAAAGGTAGA	AACAGGACATGGAAAGCAGAT
NRF-1	ATCCGAAAGAGACAGCAGACA	TGGAGGGTGAGATGCAGAGTA
COX-IV	CTCCAACGAATGGAAGACAG	TGACAACCTTCTTAGGGAAC
SCO2	TCCCTTCACCCTTCGCTGAAC	CAGTAGCATCGTGGACCTGAA
β-2 Microglobulin	GGTCTTTCTGGTGCTTGTCT	TATGTTCGGCTTCCCATTCT
TBP	CCCTATCACTCCTGCCACACCAGC	GTGCAATGGTCTTTAGGTCAAGTTTACAGCC
HPRT	GTGCAATGGTCTTTAGGTCAAGTTTACAGCC	AGGGCATATCCAACAACAAACTT
36B4	GGAGCCAGCGAGGCCACACTGCTG	CTGGCCACGTTGCGGACACCCTCC

PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1 α; Tfam, mitochondrial transcription factor; NRF-1, nuclear respiratory factor 1; COX-IV, cytochrome-*c* oxidase subunit IV; SCO2, synthesis of cytochrome-*c* oxidase 2; TBP, TATA-binding protein; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

occur subsequent to an acute bout of exercise. Incidentally, p53 also serves as a dual regulator of autophagy, a positive enforcer via transcriptional regulation of genes that induce autophagy (19), and a negative moderator when it is present in the cytoplasm through a hitherto uncharacterized mechanism (32). With the consideration of the role of p53 in mediating oxidative capacity, autophagy, and its recognition as a target of AMPK and p38 MAPK (16, 30), we hypothesized that the absence of p53 will result in a diminished adaptive cellular response to exercise.

METHODS

Animal breeding. Transgenic p53 mice (5) were obtained from Taconic (Germantown, NY). Heterozygous p53 mice were bred to produce homozygous p53 knockout (KO) and littermate wild-type (WT) mice and were treated experimentally, as outlined in protocols approved by the York Animal Care Committee in accordance with the Canadian Council on Animal Care. Each progeny of the breeding pair was genotyped as described. An ear clipping obtained from each animal was used to produce a crude DNA extract. Extracted DNA was added to a PCR tube containing DNA Taq polymerase (JumpStart REDtaq ReadyMix PCR reaction mix; Sigma-Aldrich, St. Louis, MO) and forward and reverse primers for the WT or the mutated p53 gene. Differences in the genome were detected using PCR amplification. The reaction products were separated on a 2% agarose gel at 90 V for 2–2.5 h and visualized with the use of ethidium bromide.

Exercise performance test. WT and p53 KO mice were subjected to a graded treadmill exercise test to determine maximum exercise capacity. Mice were acclimatized to the treadmill 1 wk before the test. Animals commenced running at 5 m/min on a 0% incline for 5 min, followed by 10 m/min for 10 min. Running speed was increased by 1 m/min every minute until mice reached exhaustion, defined as the point whereby mice remained at the back of the treadmill on an electric shock pad for 5 s. The work performed was calculated by the formula: work (J) = force [body weight (kg) × 9.8 m/s^2] × vertical distance [speed (m/min) × time (min)] (21).

Experimental design. As no differences were observed in the endurance capacity of the two genetic strains of mice, p53 WT and KO mice (n = 6/exercise condition; 18/genotype)—at ~2 mo of age—were matched for sex and body weight and assigned randomly to control (CON), AE, or AE followed by 3 h of recovery (AER) groups. All mice were acclimatized to the treadmill 2 wk before the beginning of the experiment. The animals in both the AE and AER groups were then selected and subjected to an acute bout of treadmill running at 15 m/min for 90 min. All of the mice subjected to treadmill exercise were visibly exhausted at the end of the exercise, as determined by their ability to withstand air and shock stimuli for >5 s. The AE group was killed immediately following exercise. The animals in the CON group were euthanized by cervical dislocation at the same time as the AER group. Both quadriceps femoris muscle groups were extracted from all mice, and a portion was immediately snap frozen and stored at −80°C for subsequent messenger RNA (mRNA) expression and whole-muscle Western blotting purposes. The remaining ~200 mg fresh quadriceps femoris was used for nuclear and mitochondrial fractionation. Frozen muscle samples were pulverized into a powder with a stainless-steel mortar, cooled to the temperature of liquid nitrogen. Powdered tissue was resuspended in assay buffer, sonicated, centrifuged, and stored in liquid nitrogen until further use. The protein content of extracts was determined using the Bradford method (4).

RNA isolation. Total RNA was isolated from ~70 mg frozen muscle using TRI Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RNA concentration and quality were measured using NanoDrop 2000 (Thermo Scientific, Wilmington, DE) and verified further with RNA gels.

mRNA expression analyses. The mRNA expression of PGC-1α, Tfam, NRF-1, COX-IV, and SCO2 was quantified using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green chemistry (PerfeCTa SYBR Green SuperMix, ROX; Quanta BioSciences, Gaithersburg, MD). First-strand cDNA synthesis from 2 μg total RNA was performed with primers using SuperScript III transcriptase (Invitrogen), according to the manufacturer's directions. Forward and reverse primers (Table 1) for the aforementioned genes were designed based on sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank), using the Primer 3 designer software (http:// bioinfo.ut.ee/primer3-0.4.0/primer3/), and were confirmed for specificity using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih. gov/BLAST/). β-2 Microglobulin was used as a control housekeeping gene, the expression of which did not change with exercise. An average of hypoxanthine-guanine phosphoribosyltransferase, 36B4, TATA-binding protein, and β-2 microglobulin was

Table 2. *Body weight and treadmill exercise performance*

Genotype	Body Weight, g	Speed, m/min	Distance, km	Work, kJ
WT (n = 4)	17.6 ± 1.52	42.2 ± 1.44	0.99 ± 0.06	172.4 ± 24.04
KO (n = 3)	19.6 ± 0.17	44.0 ± 1.73	1.06 ± 0.08	204.1 ± 13.0

p53 Wild-type (WT) and knockout (KO) mice were subjected to a graded treadmill exercise challenge until exhaustion. No difference was observed in maximum speed attained, distance covered, or work performed between the WT and KO mice. Values are means ± SE (n = 3–4).

used as the housekeeping "gene" to normalize the mRNA transcripts measured in nonexercised WT and KO mice. All samples were run in duplicate simultaneously with negative controls that contained no cDNA. Melting-point dissociation curves, generated by the instrument, were used to confirm the specificity of the amplified product. Primer efficiency curves were generated for each set to ensure 100 ± 2% efficiency.

Mitochondrial fractionation. Briefly, ~150 mg fresh skeletal muscle was minced, homogenized, and subjected to differential centrifugation, as documented previously, to yield the subsarcolemmal mitochondrial fraction (26). The mitochondria were resuspended in a small volume of resuspension buffer [100 mM KCl, 10 mM MOPS, and 0.2% BSA, pH 7.4, supplemented with Complete EDTA-free protease inhibitor cocktail (Roche Applied Science, Manheim, Germany)]. All centrifugation steps were carried out at 4°C. Mitochondrial homogenates were analyzed for protein content using the Bradford assay and subsequently frozen at −80°C for further biochemical analysis.

Nuclear fractionation. Nuclear fractions were prepared from freshly isolated skeletal muscle using a commercially available nuclear extraction kit (NE-PER; Pierce, Rockford, IL). Briefly, 50–75 mg skeletal muscle was minced and homogenized in Cytoplasmic Extraction Reagent I buffer containing Complete EDTA-free protease inhibitor cocktail (Roche Applied Science). After a series of wash steps, nuclear proteins were extracted in high-salt Nuclear Extraction Reagent buffer, supplemented with protease inhibitors.

Immunoblotting. Proteins were resolved on 8% or 12% SDS-PAGE gels, depending on the molecular weight of the protein of interest. The gels were transferred onto enhanced chemiluminescence (ECL) nitrocellulose membranes, followed by blocking with 1–3% milk in Tris-buffered saline Tween-20 (TBST) overnight at 4°C. Immunoblotting was carried out using rabbit PGC-1α (AB3242, 100 kDa; Millipore, Billerica, MA), phosphorylated (P)-AMPK (2535, 62 kDa; Cell Signaling Technology, Danvers, MA), total (T)-AMPK (2532, 62 kDa; Cell Signaling Technology), P-p38 MAPK (9211, 38 kDa; Cell Signaling Technology), T-p38 MAPK (9212, 38 kDa; Cell Signaling Technology), P-CaMKII (3361, 50 kDa; Cell Signaling Technology), and T-CaMKII (3362, 50 kDa; Cell Signaling Technology); lysosomal markers: cathepsin-D (Cts-D; sc6486, 33 kDa; Santa Cruz Biotechnology, Santa Cruz, CA) and lysosomal-associated membrane protein 2 (lamp2; Ab13524, 105 kDa; Abcam, Cambridge, UK); and autophagy markers: ubiquitin (Ub; SPA-302; Enzo/Stressgen, Enzo Life Sciences, Farmingdale, NY), p62 (P0067, 62 kDa; Sigma-Aldrich), LC3II (2775, 14 kDa; Cell Signaling Technology), Atg7 (A2856, 75 kDa; Sigma-Aldrich), and Beclin1 (3738, 60 kDa; Cell Signaling Technology). Membranes were then incubated with the appropriate secondary antibody coupled to horseradish peroxidase at room temperature for 2 h. After incubation, membranes were washed three times in TBST, developed with an ECL kit, and quantified via densitometric analysis of the intensity of signal with SigmaScan Pro v.5 software (Jandel Scientific, San Rafael, CA). Aciculin, voltage-dependent anion channel (MitoSciences, Eugene, OR), and histone 2 B (Cell Signaling Technology) were used as loading controls for whole-muscle, mitochondrial, and nuclear fractions, respectively.

Statistical analysis. Data were analyzed using Graph Pad 4.0 software, and values are reported as means ± SE, unless indicated otherwise. Data were analyzed using a two-way ANOVA and Bonferroni post-tests. All other data were analyzed using Student's *t*-test. Significance levels were set at $P < 0.05$.

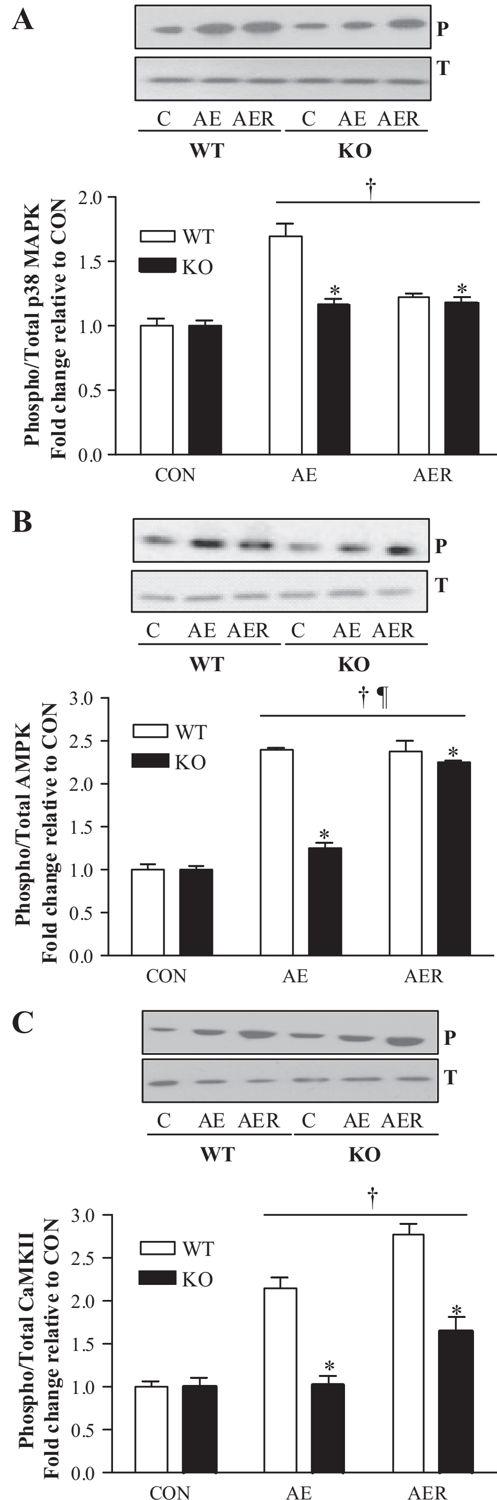

Fig. 1. Exercise-induced activation of signaling kinases. *A*: p38 MAPK phosphorylation (P; Phospho) increased significantly with exercise and recovery in the wild-type (WT) mice and not in the knockout (KO) animals ($P < 0.05$). T, total; C, control (CON); AE, acute exercise; AER, AE + 3 h recovery. *B*: AMP-activated protein kinase phosphorylation (AMPK) levels were enhanced by approximately 2- to 2.5-fold in AE and AER conditions in WT mice, respectively ($P < 0.05$). The p53 KO group displayed a delayed ~2-fold elevation in AMPK phosphorylation in the AER group ($P < 0.05$). *C*: CaMKII phosphorylation content was higher by ~2.5-fold in WT mice in AE and AER and by ~1.5-fold in the AER condition in KO counterparts ($P < 0.05$). †$P < 0.05$ main effect of AE and AER vs. CON; *$P < 0.05$ main effect of WT vs. KO; and ¶$P < 0.05$ main interaction effect. Data are presented as a fold increase over CON values. Error bars represent SE values; n = 6/exercise condition.

RESULTS

Maximum treadmill exercise capacity is similar in WT and p53 KO mice. To estimate the maximum aerobic running potential of p53 KO mice, we subjected p53 WT and KO mice to an acute bout of graded treadmill exercise to exhaustion. p53 KO animals attained a maximum speed comparable with that of their WT counterparts (Table 2), as reported previously by Park et al. (21). Interestingly, p53 KO mice, although demonstrated previously to have impaired respiration (20, 21, 26) and exercise performance in wheel running (26) and swimming tests (20, 21), displayed no deficits in maximum distance run or work performed (Table 2). Thus both p53 WT and KO mice were subjected to the same AE protocol for 90 min at 15 m/min, as no basal differences in exercise capacity were apparent.

Exercise-induced kinase activation is suppressed in p53 KO animals. Whereas a plethora of signals is triggered upon exercise, the transient activation of p38 MAPK, AMPK, and CaMKII in skeletal muscle is a known modification in response to contractile activity. As expected, there was a large increase in P-p38 MAPK in the AE group, which returned to control levels with recovery in WT mice (Fig. 1*A*). In contrast, P-p38 MAPK, upon exercise, was abolished in p53 KO mice (Fig. 1*A*). Similarly, P-AMPK (Fig. 1*B*) and P-CaMKII (Fig. 1*C*) were enhanced by ~2.5-fold in AE and AER groups in WT mice. As opposed to this, p53 KO mice displayed a delayed activation of P-AMPK and P-CaMKII subsequent to endurance exercise. P-AMPK increased by approximately twofold (Fig. 1*B*), and P-CaMKII activation was elevated by ~1.5-fold (Fig. 1*C*), but this only occurred in the AER group in p53 KO mice. Interestingly, whereas no differences were documented between the basal status of P-p38 MAPK and P-AMPK in nonexercised muscle, levels of P-CaMKII tended to be higher in the KO mice compared with their WT counterparts in the CON condition ($P = 0.09$; data not shown).

Nuclear translocation of PGC-1α with AE is attenuated in p53 KO mice. PGC-1α, considered to be an important transcriptional cofactor, has been illustrated previously to translocate rapidly to the nucleus upon an exercise bout (36). We assessed the accumulation of PGC-1α in the nuclear fractions isolated from sedentary CON, AE, and AER groups from p53 WT and KO mice (Fig. 2*A*). PGC-1α increased by 1.5- to twofold in WT animals with AE and AER (Fig. 2*A*). In contrast, PGC-1α nuclear content was not elevated in p53 KO mice in the AE group and displayed only a slight increase in the AER group. The level of nuclear PGC-1α content in KO mice under nonexercised, basal conditions tended to be lower than that of WT, but this did not reach statistical significance.

mRNA levels of genes related to energy metabolism increase following AE in p53 WT but not KO mice. The mRNA levels of PGC-1α and COX-IV were not different in the KO muscle compared with WT counterparts during control, nonexercised conditions (Fig. 3*A*). However, the KO mice displayed a reduction in the basal mRNA expression of NRF-1 and Tfam (Fig. 3*A*). The transcript levels of genes involved in mitochondrial biogenesis, such as PGC-1α, Tfam, NRF-1, and COX-IV, increased immediately following an acute bout of exercise in the WT mice (Figs. 2*B* and 3, *B–D*). The enhancement in mRNA expression of these transcripts was maintained or increased further after 3 h of recovery following the exercise in

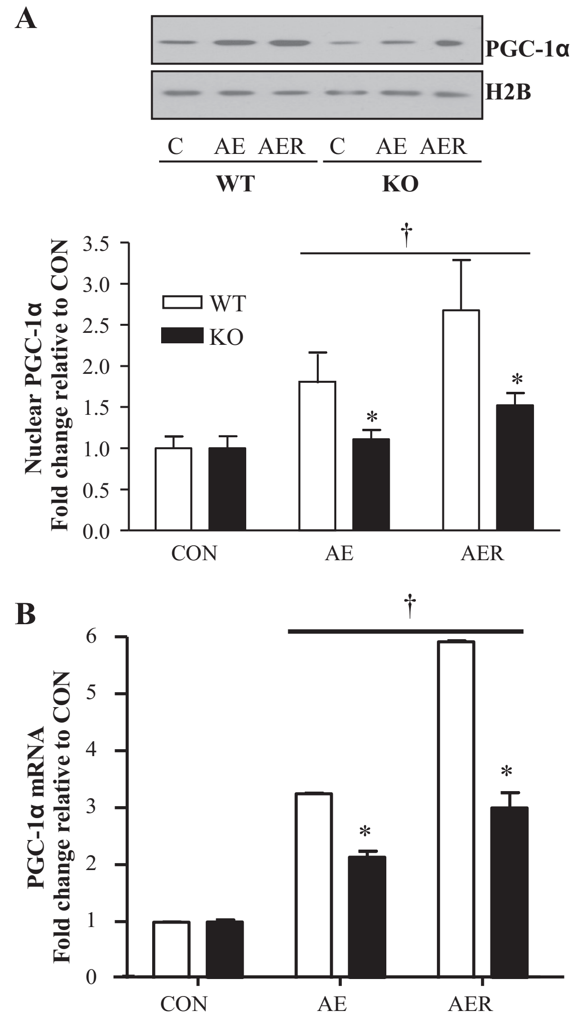

Fig. 2. Alterations in expression of peroxisome proliferator-activated receptor-γ coactivator-1 α (PGC-1α) protein and messenger RNA (mRNA). *A*: PGC-1α nuclear content increased by 1.5- to 2-fold in WT mice with AE and AER ($P < 0.05$), and this effect was nearly absent in the KO mice. Histone 2 B (H2B) was used as a nuclear-loading control. *B*: as expected, a large upregulation of PGC-1α mRNA was evident in WT mice with exercise and recovery. On the other hand, p53 KO-exercised mice displayed an attenuated increase in PGC-1α mRNA content. †$P < 0.05$ main effect of AE and AER vs. CON; and *$P < 0.05$ main effect of WT vs. KO. Data are presented as a fold increase over CON values. Error bars represent SE values; $n = 6$/exercise condition.

WT animals (Figs. 2*B* and 3, *B–D*). Surprisingly, the only transcript that increased as a result of exercise or recovery in p53 KO mice was PGC-1α (Fig. 2*B*). The response of other transcripts to exercise and exercise with recovery remained relatively unchanged in the KO animals (Fig. 3, *B–D*). The mRNA levels of SCO2, a well-known target of p53 that was illustrated initially to be pivotal for p53-mediated mitochondrial biogenesis in cells in culture, did not respond to the AE stimulus in WT or KO animals (Fig. 3*E*).

Muscle autophagic and lysosomal markers are unchanged in response to AE in p53 WT and KO mice. We immunoblotted for lysosomal and autophagic proteins, intimately associated with the induction of the autophagic process. Basal levels of Cts-D and lamp2 in nonexercised muscle were lower in KO animals compared with their WT littermate controls (Fig. 4*B*),

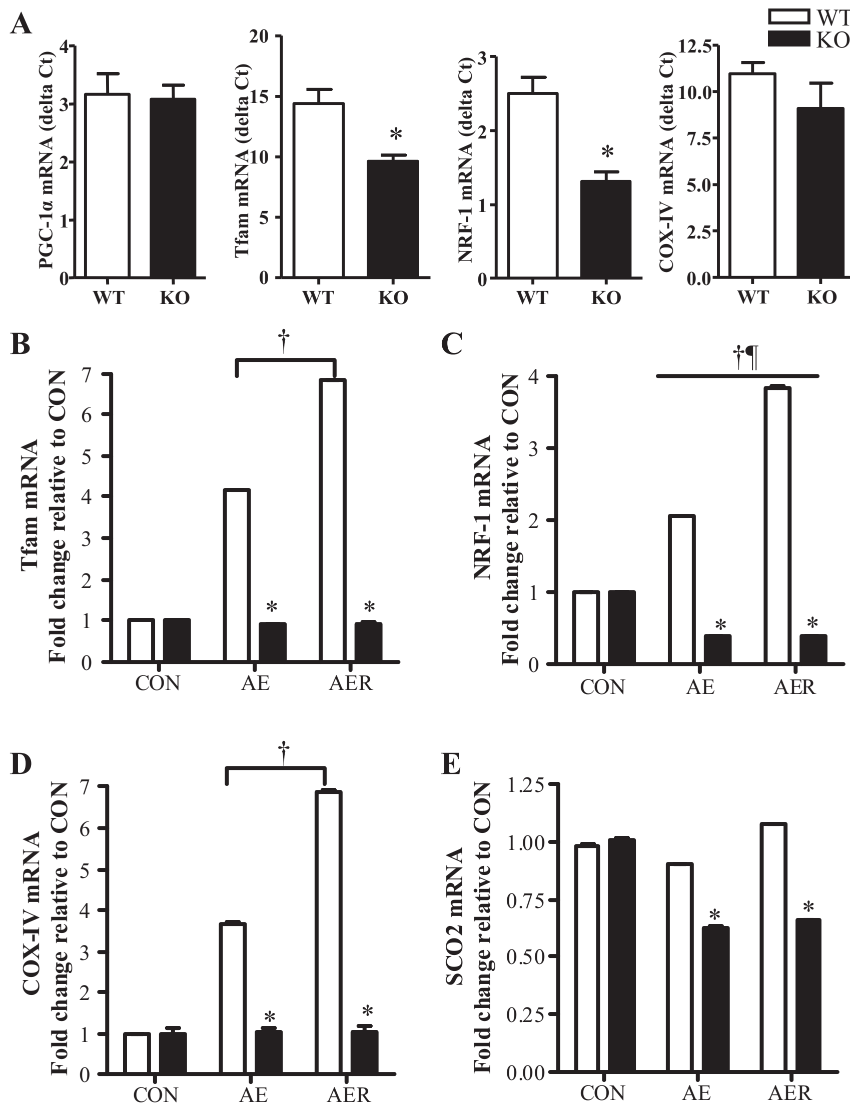

Fig. 3. Changes in mRNA transcripts related to mitochondrial biogenesis. *A*: mRNA levels of PGC-1α, mitochondrial transcription factor (Tfam), nuclear respiratory factor 1 (NRF-1), and cytochrome-*c* oxidase subunit IV (COX-IV) during nonexercised conditions. Whereas PGC-1α and COX-IV transcript levels did not differ between WT and KO, Tfam and NRF-1 mRNA content was decreased significantly in the p53 KO muscle. Ct, comparative threshold. *$P < 0.05$ WT vs. KO. Error bars represent SE values; $n = 5–6$/exercise condition. The transcript levels of (*B*) Tfam, (*C*) NRF-1, and (*D*) COX-IV increased immediately following AE and were elevated further in recovery in the WT mice ($P < 0.05$). This increase in mRNA levels was abolished in the KO mice and decreased in the case of NRF-1 transcript levels in the KO mice with exercise and recovery. *E*: synthesis of cytochrome-*c* oxidase 2 (SCO2), a transcriptional target of p53 involved in maintaining mitochondrial function, did not respond to exercise in the WT mice, and its levels decreased in the absence of p53 ($P < 0.05$). β-2 Microglobulin was used as an internal housekeeping gene. †$P < 0.05$ main effect of AE and AER vs. CON; *$P < 0.05$ main effect of WT vs. KO; and ¶$P < 0.05$ main interaction effect. Data are presented as a fold increase over CON values. Error bars represent SE values; $n = 4$/exercise condition.

suggesting a lower lysosomal capacity in p53-deficient animals. In contrast, LC3II content was similar, and p62 expression was enhanced in the KO animals compared with WT controls. Thus p53 exerts specific effects on the expression of autophagy and lysosomal proteins in muscle. Exercise and recovery had no effect on the level of any of these proteins in muscle. Beclin1 and Atg7 levels were comparable in WT and KO mice at rest and also did not respond to the exercise stimulus (Fig. 4*A*). Ubiquitination, a post-translational modification that most commonly earmarks cellular material for proteosomal or autophagic degradation, was also measured in whole-muscle homogenates (Fig. 4, *B* and *C*). Steady-state levels of ubiquitination were reduced in p53 KO mice (Fig. 4*B*), and whereas WT ubiquitination levels remained unchanged with exercise, the KO animals displayed a significant increase in ubiquitinated proteins in the AE and AER groups compared with WT controls (Fig. 4, *C* and D). This effectively raised the level of ubiquitination in KO tissue back to the level observed in WT animals.

Mitophagy signaling is activated by AE in p53 WT and KO mice. We measured the amount of lipidated LC3, p62, and ubiquitination in isolated mitochondrial fractions from WT and KO mice as indicators of mitophagy (Fig. 5). Levels of mitochondrial LC3II (Fig. 5*B*) were increased ($P < 0.05$), as well as organelle ubiquitination ($P = 0.06$; Fig. 5*D*) in the KO mice compared with their WT counterparts in control, nonexercised conditions, suggesting a heightened drive for mitophagy in the KO muscle. Basal p62 levels remained unchanged in mitochondria from KO compared with WT animals (data not shown). A significant, main effect of exercise and recovery on all three mitophagy indicators was evident in both WT and KO animals (Fig. 5).

DISCUSSION

Steady-state mitochondrial content is determined by the interplay between mitochondrial biogenesis and mitophagy. We and others have extensively characterized the synthesis arm of mitochondrial turnover, but there are only a few studies that have investigated exercise and its effects on mitophagy. Thus the purposes of this study were to explore how mitochondrial biogenesis and mitophagy are regulated in response to acute endurance exercise in skeletal muscle and the role executed by p53 in this context.

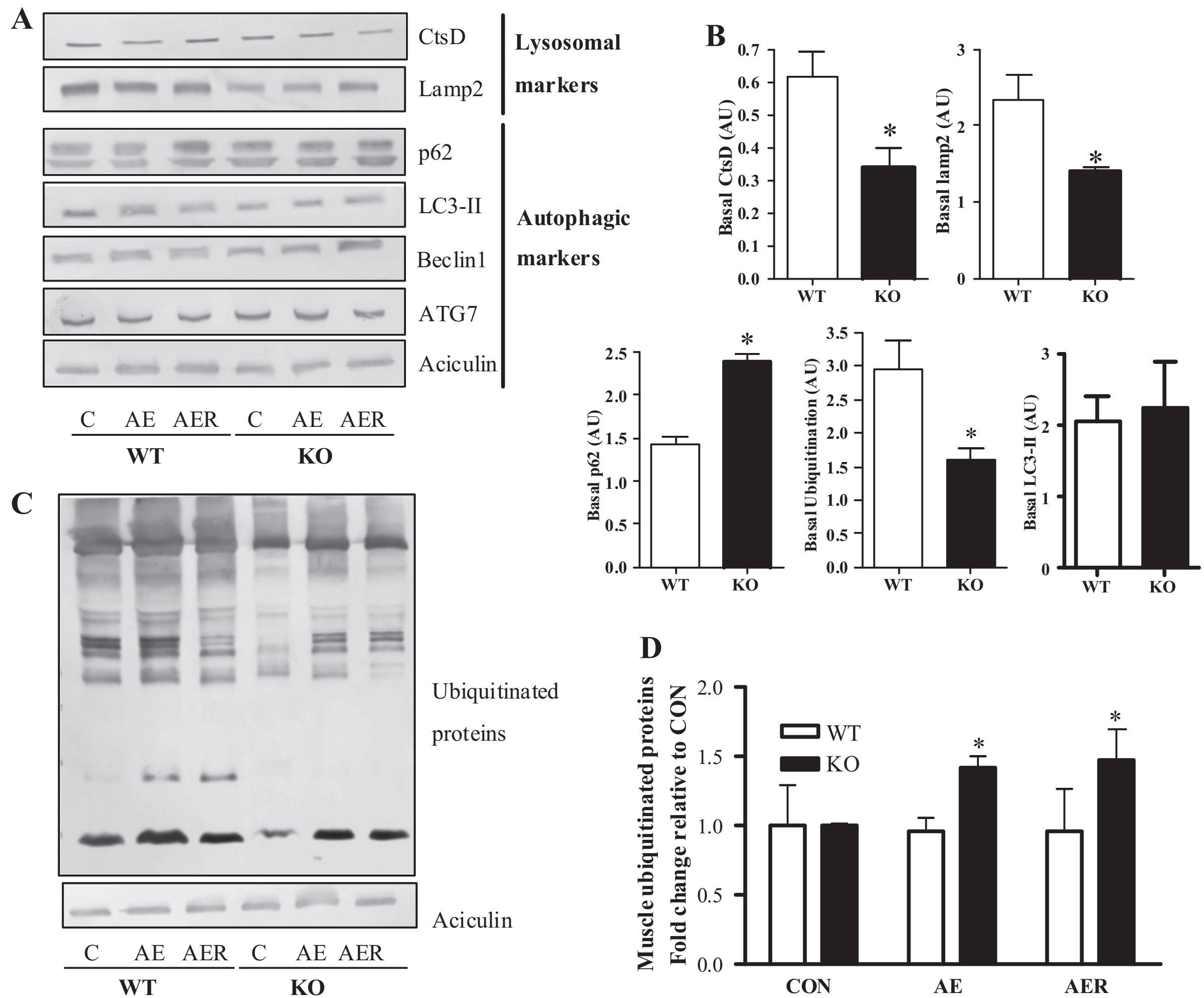

Fig. 4. Response of whole-muscle autophagic and lysosomal markers to AE. *A*: immunoblots depicting the expression of lysosomal [cathepsin-D (CtsD) and lysosomal-associated membrane protein 2 (Lamp2)] and autophagic [p62, light chain 3 II (LC3-II), Beclin1, autophagy-related protein 7 (ATG7)] markers in WT and KO samples in CON, AE, and AER conditions. No changes in protein content were observed in either genotype with exercise. *B*: nonexercised, control levels of LC3-II were unchanged; CtsD, Lamp2, and ubiquitin levels were lower; and p62 content was higher in p53 KO mice compared with littermate WT controls ($P < 0.05$). AU, arbitrary units. *$P < 0.05$ WT vs. KO. Data are presented as mean ± SE values. *C*: representative Western blot for ubiquitinated proteins and (*D*) its graphical illustration depict an exercise-induced elevation of ubiquitin content in p53 KO mice only ($P < 0.05$). Aciculin was used as a loading control. *$P < 0.05$ main effect of WT vs. KO. Data are presented as a fold increase over CON values. Error bars represent SE values; $n = 6$/exercise condition.

PGC-1α is intimately involved in regulating mitochondrial biogenesis, due to its ability to translocate rapidly to the nucleus and coactivate the transcription of numerous downstream mediators of mitochondrial biogenesis upon an exercise signal (18). Interestingly, p53 exerts a significant degree of control over the transcription of PGC-1α. We had earlier identified a putative p53 response element in the PGC-1α promoter (13), which was subsequently demonstrated to both suppress (25) and activate transcription of PGC-1α (2). Clearly, the interaction is dependent on the cellular milieu and the experimental model in which it is investigated. We here report that whereas there is no effect on basal nuclear PGC-1α localization between the two genotypes, the kinetics of its movement into the nucleus upon exercise are impaired in the absence of p53. This is important, as the delayed increase in nuclear PGC-1α may suppress the onset of mitochondrial biogenesis. Indeed, the expected augmentation in the transcript levels of PGC-1α, Tfam, NRF-1, and COX-IV, which occurs with exercise and recovery in WT animals, was abolished completely in the KO mice. It is quite possible that there is an eventual increase in the mRNA content of the different transcripts in the p53 KO animals, requiring either a longer recovery period (>3 h) or multiple bouts of exercise to gain the same adaptation as WT mice. It is important to note that the p53-transcribed gene SCO2, involved in the assembly of complex IV of the electron transport chain, was unresponsive to exercise in either WT or KO mice. Also, whereas there was no difference in the basal amounts of nuclear PGC-1α protein content and mRNA expression, NRF-1 and Tfam mRNA content was lower in the KO mice. The decrease in Tfam mRNA expression could likely be due to the direct transcriptional regulation of the gene by p53.

To understand the mechanisms of reduced PGC-1α translocation to the nucleus, we measured the activation of upstream kinases. p38 MAPK directly phosphorylates PGC-1α at three sites that release it from its repressor-binding protein, known as

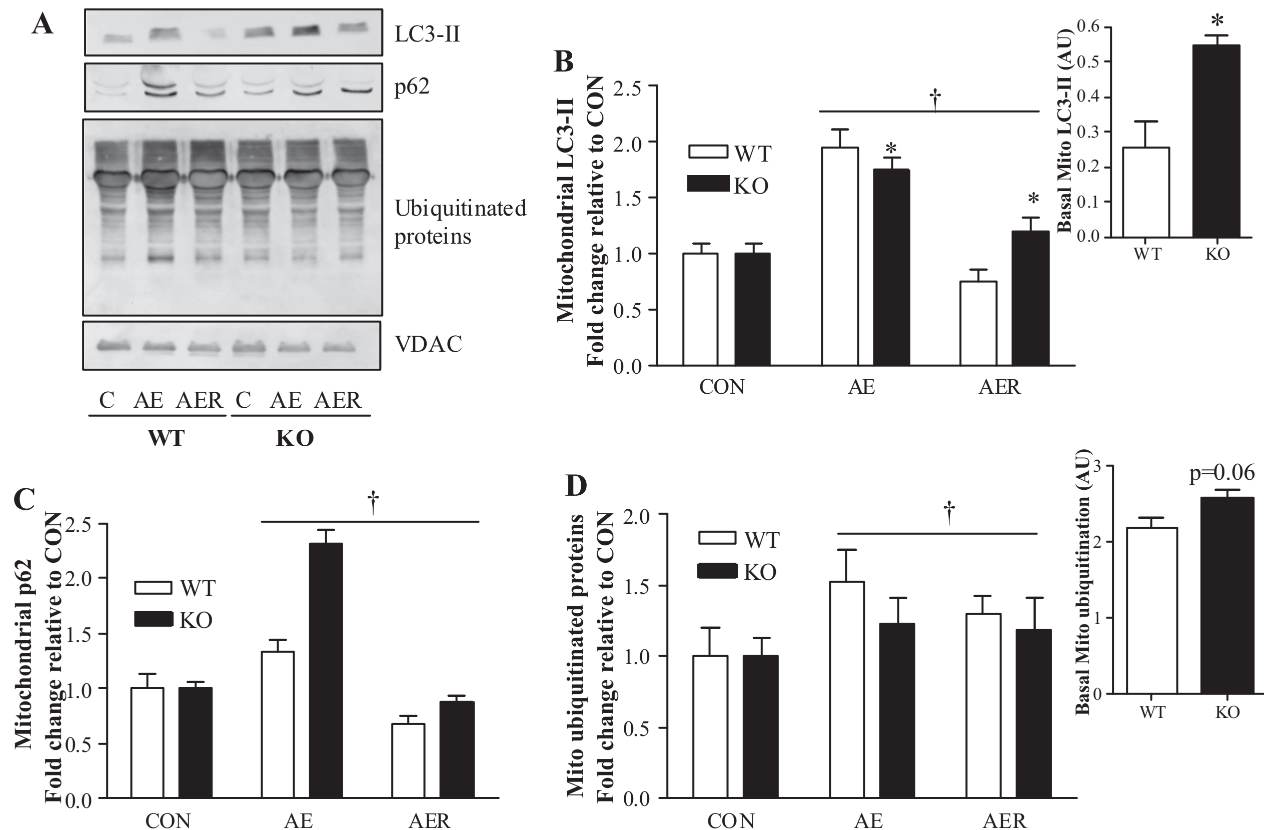

Fig. 5. Activation of mitophagy signaling postacute bout of exercise. *A*: lipidated LC3, p62, and ubiquitination in isolated mitochondrial fractions from WT and KO mice. Voltage-dependent anion channel (VDAC) was used as a loading control. Graphical illustration depicting an increase in (*B*) LC3-II, (*C*) p62, and (*D*) ubiquitinated protein expression with exercise in mitochondria (Mito) isolated from both WT and p53 KO mice. *B*, *inset*: increased amounts of LC3-II ($P < 0.05$), concomitantly with (*D*, *inset*) higher ubiquitination ($P = 0.06$), were documented in the mitochondria from mice with p53 ablation. †$P < 0.05$ main effect of AE and AER vs. CON; and *$P < 0.05$ main effect of WT vs. KO. Data are presented as a fold increase over CON values. Error bars represent SE values; n = 6/exercise condition.

p160, and leads to its stabilization (6). AMPK activates PGC-1α by phosphorylating it on the threonine-177 and serine-538 residues (14). Our data indicate that the absence of p53 results in a lack of, or delay in, P-p38 MAPK and P-AMPK, respectively, in KO mice subjected to AE. This may be the underlying reason behind the sluggish response of PGC-1α translocation into the nucleus upon exercise in p53 KO animals.

The diminished AMPK activity in the KO mice is not likely attributed to reduced levels of the allosteric activator AMP in the muscle of p53 KO animals during exercise. This is because muscle of p53 KO animals has a reduced oxidative capacity and thus is more likely to increase cellular AMP levels, as well as the activation of AMPK (17). However, p53 KO cells have been shown to maintain comparable cellular ATP levels basally and higher ATP levels during conditions of metabolic stress, such as glucose deprivation, compared with WT cells (20, 32). It is possible that the lower AMPK activity may be due to the transcriptional control exerted by p53 over the β1 accessory subunit of the AMPK complex (7). AMPK exists as a heterotrimeric complex, with one catalytic α subunit and two regulatory β and γ subunits. The β subunit functions as a scaffold for the other two and has been shown to modulate AMPK localization and activity (35). Thus it is likely that in the absence of p53, reduced β-subunit expression somehow manifests as a delayed increase in AMPK activity upon AE.

p38 MAPK can be activated by a plethora of different oxidative, DNA damage, and inflammatory stress stimuli and is a bona fide activator of p53, as well as subject to negative regulatory-feedback regulation by p53-inducible genes (31). P-p38 MAPK, in response to AE, was abrogated completely in the KO mice, although basal levels were unchanged. We expected higher basal p38 MAPK activity in the KO mice, having previously reported elevated ROS levels in these animals (26) and also because of the negative correlation that exists between oxidative capacity and activation of p38 kinase activity (17). We do not know why p38 activation is attenuated in control, nonexercised muscle from p53 KO mice, but given that p38 MAPK is an integral component of many cellular processes, further research is warranted to investigate this observation more closely.

The effect of p53 on autophagy is potentially ambiguous, depending on its cellular location. Cytoplasmic p53 can inhibit autophagy during nonstressful cellular conditions, whereas the accumulation of nuclear p53 upon a cellular insult results in the p53-mediated transcriptional upregulation of autophagy genes, including AMPK β1, death-associated protein kinase 1, damage-regulated autophagy modulator, proapoptotic B cell lymphoma 2 proteins (e.g., Bad, Bax, Bnip3, and Puma), sestrin 2, and tuberous sclerosis protein 2 (19, 27). In this study, we have totally abrogated p53 activity and expression and examined the consequence on the expression of autophagy and autophagy-

related proteins. Our results revealed attenuated lysosomal capacity, as illustrated by lower Cts-D and lamp2 protein expression in the KO animals at rest. Concomitantly, there was decreased ubiquitination and an accumulation of p62, indicative of a lower autophagy flux in the resting muscle of KO mice. LC3II, Beclin1, and Atg7 expression remained unchanged in the absence of p53. Interestingly, the mitochondrial localization of LC3II and Ub content was higher in the KO compared with the WT animals, indicative of an underlying basal drive to remove dysfunctional mitochondria during nonexercised conditions but limited by a lower lysosomal capacity for degradation. Consistent with this, it has been shown that liver, kidney, and pancreas from p53 KO mice had higher basal levels of LC3 puncta and autophagosomal accumulation in whole-cell homogenates (32), characteristic of an impaired autophagic process in the absence of p53.

Since both autophagy and mitophagy have been implicated recently to be activated in response to acute endurance exercise in muscle (10, 11, 15), we next investigated whether this avenue of mitochondrial turnover is dependent on the presence of p53. He et al. (11) previously reported an increase in the autophagosomal, membrane-associated, lipidated form of LC3II and degradation of autophagy substrate p62 with AE in WT mice. We investigated a number of autophagy and lysosomal markers and did not observe any changes in the WT mice with the exercise protocol used, despite the considerable induction of gene expression. It is likely that our exercise protocol, although commonly used to invoke mitochondrial synthesis successfully (24, 36) and physiologically equivalent to running at ~75% maximum oxygen consumption (29), is not as intense or long enough as that used by others (11) to induce macroautophagy in WT mice. Indeed, a recent study showed the upregulation of autophagy proteins in humans that could be observed after an ultraendurance exercise bout lasting 24 h (15). Clearly, exercise dosage is of paramount importance, as it dictates the magnitude of the downstream activation of autophagic signaling. The increase in whole-muscle ubiquitination, evident only in p53 KO mice, may play a therapeutic role in raising the attenuated level of this process up to healthy control levels in the absence of p53. Exercise-induced ubiquitination is commonly used to tag cellular debris for removal by the proteosome or for selective autophagy; therefore, it is possible that this change in Ub content is upstream of an activation of the autophagic process in the KO mice. With respect to mitophagic signaling initiation, both WT and KO animals displayed an equal propensity to enhance mitochondrial LC3II, p62, and Ub content with exercise. Thus AE promotes mitophagy by enhanced targeting of LC3II and p62 to the mitochondria and by ubiquitinating mitochondrial proteins, even when p53 is absent.

This study documents a comprehensive analysis of the necessity of p53 for exercise-induced changes in cellular signaling and gene expression in skeletal muscle. Exercise has been touted as a viable means to decrease the incidence and progression of cancer, as well as many inactivity-related pathologies, such as obesity, type 2 diabetes, and cardiovascular disease. We have previously identified that endurance exercise can directly activate and relocalize p53 to the mitochondria, where it affects mitochondrial DNA transcription (28). Here, we demonstrate that the presence of p53 is requisite for the activation and subsequent initiation of mitochondrial biogenesis after an acute bout of exercise. In addition, lack of p53 seems to be important for the basal expression and recruitment of autophagic proteins but has no effect on the induction of mitophagic signaling after an acute bout of exercise. Further research is warranted to fully appreciate the interplay between autophagy and mitophagy and the dependence of these cellular processes on p53 expression in skeletal muscle after exercise. These findings add considerably to the literature on muscle adaptations to exercise, and they suggest that individuals with decreased or ablated p53 expression may require a greater exercise dose to benefit from the mitochondrial adaptations that normally accompany the pursuit of a regular program of physical activity.

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DISCLOSURES

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Author contributions: A.S. and D.A.H. conception and design of research, A.S. and H.N.C. performed experiments, A.S. analyzed data, A.S. and D.A.H. interpreted results of experiments, A.S. prepared figures, A.S. drafted manuscript, A.S. and D.A.H. edited and revised manuscript, A.S., H.N.C., and D.A.H. approved final version of manuscript.

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