Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle

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Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle. Physiol Genomics 37: 58–66, 2009. First published December 23, 2008; doi:10.1152/physiolgenomics.90346.2008.—p53 is a tumor suppressor protein that also plays a role in regulating aerobic metabolism. Since skeletal muscle is a major source of whole body aerobic respiration, it is important to delineate the effects of p53 on muscle metabolism. In p53 knockout (KO) mice, we observed diminished mitochondrial content in mixed muscle and lowered peroxisome proliferator-activated receptor-γ (PPARγ) coactivator (PGC)-1α protein levels in gastrocnemius muscle. In intermyofibrillar (IMF) mitochondria, lack of p53 was associated with reduced respiration and elevated reactive oxygen species production. Permeability transition pore kinetics remained unchanged; however, IMF mitochondrial cytochrome c release was reduced and DNA fragmentation was lowered, illustrating a resistance to mitochrondially driven apoptosis in muscle of KO mice. p53-null animals displayed similar muscle strength but greater fatigability and less locomotor endurance than wild-type (WT) animals. Surprisingly, the adaptive responses in mitochondrial content to running were similar in WT and KO mice. Thus p53 may be important, but not necessary, for exercise-induced mitochondrial biogenesis. In WT animals, acute muscle contractions induced the phosphorylation of p53 in concert with increased activation of upstream kinases AMP-activated protein kinase and p38, indicating a pathway through which p53 may initiate mitochondrial biogenesis in response to contractile activity. These data illustrate a novel role for p53 in maintaining mitochondrial biogenesis, apoptosis, and performance in skeletal muscle.

endurance exercise; fatigue; mitochondrial turnover; signaling cascades

THE ROLE OF P53 as a critical tumor suppressor protein has been well established in the literature (43). During basal nonstressed conditions, p53 levels are low in the cell. On receipt of a genotoxic stress signal, p53 protein concentration increases, leading to a myriad of downstream effects depending on the cellular environment (25, 29, 48). p53 can induce the expression of cell cycle arrest and pro- and antioxidant proteins (34, 36, 39, 43, 48). It can mediate apoptosis by directly manipulating the mitochondrial pathway of cell death (30, 31) or by the transcriptional induction of proapoptotic nuclear DNA-encoded proteins (7, 25, 31, 34, 36, 39, 43, 48). In addition, it can also stimulate the expression of several genes involved in promoting and maintaining optimal mitochondrial function (10, 15).

p53 is involved in regulating mitochondrially mediated cellular metabolism primarily through its transcriptional activity. Recent work has demonstrated that several nuclear genes encoding mitochondrial proteins (NUGEMPs) have p53 response elements in their promoter regions and are therefore regulated, in part, by p53. For example, p53 modulates the expression of nuclear-encoded synthesis of cytochrome-c oxidase 2 [SCO2 (28)], apoptosis-inducing factor [AIF (39)], and the mitochondrial DNA-transcribed gene 16S rRNA (15). SCO2 is crucial for the proper assembly and function of cytochrome-c oxidase (COX) enzyme complex in the electron transport chain. The COX complex plays a crucial role in aerobic respiration by catalyzing the transfer of electrons from reduced cytochrome c to molecular oxygen. Abrogation of a functional p53 protein manifests as impairments in COX activity (27, 28, 49). This decrease in COX activity has been attributed to the associations observed between p53 and the regulation of COX subunits I and II (17, 27, 32, 49). 16S rRNA is pivotal for the translation of mitochondrial DNA-encoded genes, such as the 13 proteins that are part of the electron transport chain (2, 19).

It has also been reported that a small fraction of p53 is present in the mitochondrial matrix during nonstressed basal conditions. p53 directly interacts with and enhances the function of mitochondrial (mt)DNA polymerase γ (pol γ), the lone DNA polymerase in the mitochondria that is responsible for mtDNA replication and repair (1). Additionally, others have identified a putative p53 response element in the mtDNA, indicating the possibility of p53 inducing mtDNA gene transcription (18). p53 has been shown to interact directly with mitochondrial transcription factor A (Tfam), a nuclear DNA-encoded protein that is known to regulate mtDNA copy number and transcriptional activity (21, 47). Thus p53 is clearly involved in regulating mitochondrial biogenesis and cellular metabolism in the cell.

Skeletal muscle is a unique tissue because of its multineurinated and metabolically active phenotype. It houses two different populations of mitochondria, the organelles responsible for oxygen consumption generation of ATP. Mitochondria located underneath the sarcolemmal membrane of the muscle fiber are known as subsarcolemmal (SS) and those interspersed between the myofibrils are called intermyofibrillar (IMF) mitochondria (12). The former make up ~10–20% of the total mitochondrial volume and are mainly responsible for generating energy for membrane-bound functions (13). Conversely, IMF mitochondria account for the remaining ~80–90% of the mitochondrial content and are likely most responsible for generating ATP for contractile purposes (13). SS and IMF respiration rates depend on the availability of ADP. In the absence of ADP, respiration is regulated by proton leak across the inner membrane, which is slower and is known

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as state 4 respiration. With the availability of ADP as a substrate, the respiration rate increases and is referred to as state 3 or active respiration (45).

In skeletal muscle, chronic contractile activity induces a wide array of metabolic, biochemical, and physiological adaptations. It is firmly recognized that imposed long-term contractile activity or 6–8 wk of endurance training elicits a measurable change in mitochondrial content and activity in both humans and animals (2, 19, 20, 23). Peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α) is an important regulator of mitochondrial biogenesis in muscle because of its role in the specific activation of NUGEMPs (20) in response to contractile activity. PGC-1α can bind and activate the transcription of nuclear respiratory factor (NRF)-1 and NRF-2, two transcription factors that are responsible for expressing genes such as cytochrome c, proteins of the electron transport chain, import machinery proteins, transcription factors such as Tfam, and heme biosyntheses proteins (19, 20, 23, 33). While p53 has been illustrated as a positive regulator of mitochondrial biogenesis, to date no study has examined the role of p53 in basal and exercise-induced alterations in mitochondrial synthesis and function in skeletal muscle. Thus our goal was to address this question by investigating the metabolic and oxidative milieu of skeletal muscle from p53 wild-type (WT) and null or knockout (KO) mice. Furthermore, we sought to examine how these WT and KO animals respond to a program of endurance activity imposed via a voluntary wheel running training regimen. We hypothesized that p53 could be activated by exercise and that it could arbitrate, in part, the process of mitochondrial biogenesis in muscle. In contrast, we expected that the absence of p53 would attenuate the exercise-induced improvements in mitochondrial content and function, oxidative metabolism, and apoptotic susceptibility, thereby reducing skeletal muscle health.

**Experimental procedures**

Animal breeding. Transgenic p53 mice (16) were obtained from Taconic Labs (Germantown, NY). All animal care protocols were submitted to the York University Animal Care Committee, and they were approved in accordance with the regulations imposed by the Canadian Council on Animal Care. Each progeny of the breeding pair was genotyped as follows. An ear clipping obtained from each animal was used for a crude DNA extraction. Extracted DNA was added to a PCR tube containing DNA Taq polymerase (Sigma Jumpstart REDTaq Ready Mix PCR Reaction Mix) and forward and reverse primers for the WT p53 gene or the KO p53 gene. Differences in the genome were detected by polymerase chain reaction (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. A deficiency of the p53 protein was also verified at the protein level (data not shown).

Voluntary wheel running protocol. p53 WT and null mice were matched by age and sex and assigned to a control or runner group. The mice were housed individually, allowed access to food and water ad libitum, and kept on a 12:12-h light-dark cycle. Runners had access to a freely rotating wheel attached to a magnetic counter that recorded the number of revolutions. The number of revolutions was noted daily and converted into distance (kilometers) per day based on a conversion formula. The training protocol lasted ~6–8 wk, after which the animals were killed and muscles were extracted. WT and KO mice were roughly 65–85 days of age at the start of the training program.

Muscle extracts and protein content. Animals were anesthetized with a mixture of ketamine-xylazine at 9 mg/100 g body wt. All muscle groups from the forelimb and hindlimb were excised and placed in ice-cold buffer. Freshly isolated muscle was used for mitochondrial isolations, or muscle was frozen in liquid nitrogen and stored at −80°C for further analysis. 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 buffer, sonicated, centrifuged, and stored in liquid nitrogen until further use. The protein content of whole muscle and mitochondrial extracts was determined by the Bradford method as previously described (8).

Cytochrome-c oxidase enzyme activity. COX activity was measured as previously detailed (14). Briefly, protein extracts from mixed hindlimb muscle were added to a test solution containing fully reduced cytochrome c. Enzyme activity was determined as the maximal rate of oxidation of fully reduced cytochrome c measured by the change in absorbance at 550 nm in a Synergy HT microplate reader at 30°C.

Mitochondrial isolation. Freshly isolated mixed forelimb and hindlimb muscle tissue was minced, homogenized, and subjected to differential centrifugation to isolate the SS and IMF mitochondrial subfractions, as described previously (12, 26, 41). Mitochondria were suspended in resuspension medium (100 mM KCl, 10 mM MOPS, and 0.2% BSA). After the isolation procedure, SS and IMF mitochondria were used for analyses of mitochondrial respiration, reactive oxygen species (ROS) production, protein release, and mitochondrial permeability transition pore (mtPTP) kinetics.

Mitochondrial respiration. Isolated SS and IMF mitochondria (50, 100, or 300 µl) were incubated with 250 µl, 200 µl, or 2 ml of VO2 buffer (in mM: 250 sucrose, 50 KCl, 25 Tris, HCl, 10 K2HPO4, pH 7.4) respectively, at 30°C in a water-jacketed respiratory chamber with continuous stirring. Respiration rates (nanoatoms O2·min−1·mg−1) were evaluated in the presence of 10 mM glutamate (state 4 or passive respiration) and 0.44 mM ADP (state 3 or active respiration) with the use of a Clark oxygen electrode as described previously (26, 41).

Mitochondrial reactive oxygen species production. SS and IMF mitochondria (50 µg) were incubated with 50 µM dichlorodihydrofluorescein diacetate (H2DCF-DA) and VO2 buffer at 37°C for 30 min in a black polystyrene 96-well plate. The fluorescence emission (between 485 and 528 nm) is directly proportional to ROS production and was measured with a Synergy HT microplate reader. ROS production was assessed during state 4 and state 3 respiration by the addition of 10 mM glutamate and 0.44 mM ADP, respectively, to isolated mitochondria immediately before the addition of H2DCF-DA as described previously (4).

Protein release assay. Isolated SS and IMF mitochondrial fractions (150 µg) were incubated in resuspension medium for 60–90 min at 30°C as described previously (3). Reaction mixtures were subsequently centrifuged at 14,000 g (4°C) to pellet mitochondria, and the supernate was analyzed for cytochrome c release from the mitochondria by Western blot analysis.

Western blotting. Supernatant aliquots from the release assay and protein extracts (75 µg) were separated by 10–15% SDS-PAGE and subsequently transferred onto a nitrocellulose membrane, washed, blocked, and immunoblotted overnight at 4°C with primary antibody directed against cytochrome c (1:750 dilution), PGC-1α/b (1:100, Cayman Chemicals), AMP-activated protein kinase (AMPK)-phospho (1:1,000, Cell Signaling), AMPK-total (1:1,000, Cell Signaling), p38-phospho (1:1,000, Cell Signaling), and p38-total (1:1,000, Cell Signaling). Membranes were washed three times with Tris-buffered saline-Tween 20 (TBST) solution containing 25 mM Tris·HCl (pH 7.5), 1 mM NaCl, and 0.1% Tween 20. Membranes were then treated with a 1:1,000 dilution of secondary antibody (1:1,000, Cell Signaling). Membranes were washed three times with TBST and exposed to ECL reagents (Amersham) and autoradiograms were developed with X-ray film.
incubated with appropriate secondary antibody coupled to horseradish peroxidase (HRP) at room temperature for 60 min. After incubation, membranes were washed three times in TBST, developed with an enhanced chemiluminescence (ECL) kit, and quantified via densitometric analysis of the intensity of signal with Sigma Scan Pro v.5 software (Jandel Scientific, San Rafael, CA).

Mitochondrial permeability transition pore assay. Isolated SS and IMF mitochondria were resuspended in buffer containing (in mM) 215 mannitol, 71 sucrose, 3 HEPES, and 5 succinate (pH 7.4) to obtain a concentration of 1 mg/ml. SS and IMF mitochondria were treated with 400 μM CaCl₂ and 75 μM t-BuOOH, and the decrease in absorbance was monitored for 10 min with a spectrophotometer as detailed previously (5). mPTP opening was measured by monitoring the decrease in light scattering associated with mitochondrial swelling at 540 nm.

Electron microscopy. Samples from the gastrocnemius muscle were cut into 2-mm-square pieces, fixed in 4% glutaraldehyde buffered with 2X phosphate-buffered saline (PBS), and processed for electron microscopy. Ultrathin sections (60 nm) were cut, collected on copper grids, and stained with uranyl acetate and lead citrate. Electron micrographs were obtained with a Philips EM201 electron microscope.

Acute in situ muscle stimulation. The stimulation protocol was conducted in a manner described previously (40, 42). Briefly, animals were anesthetized and the Achilles tendon of the gastrocnemius muscle was isolated, affixed to a strain gauge, and adjusted to be at resting length. Stimulating electrodes were placed next to the sciatic nerve. A thermistor was employed to monitor muscle temperature, which was maintained at 37°C with heat lamps. The gastrocnemius muscle of the other limb was also exposed and wrapped in plastic to prevent tissue dehydration. Mice were subjected to an in situ contraction protocol in which the gastrocnemius muscle from one leg was stimulated via the sciatic nerve at 1 tetanic contraction per second (TPS) and 3 TPS for 5 min each, intensities that are sufficient to cause mild and more severe muscle fatigue, respectively. At the end of the stimulation period, the gastrocnemius muscles from the electrically stimulated leg and the nonstimulated contralateral leg of the animal were excised, quickly frozen, and weighed. Frozen gastrocnemius tissue was subsequently used to measure p53(Ser15), PGC-1α/β, AMPK, and p38 kinase phosphorylation.

Phosphorylated p53(Ser15) ELISA. Phosphorylated p53(Ser15) in whole muscle protein extracts from WT mice was measured with a colorimetric ELISA kit (Millipore). Extracts from control or stimu-

lated gastrocnemius muscle were resuspended in a buffer with pro-
tase inhibitors (1 mM PMSF, 1 mM DTT, 1 mM Na₃VO₄, 10 μM leupeptin, 5 μM pepstatin A, and 10 mg/ml aprotonin), rotated for 1 h at 4°C, sonicated, centrifuged, and stored in liquid nitrogen until further use. Samples (200 μg) were added to the microwells of the 96-stripwell immunoplate, which was precoated with a specific mouse monoclonal p53 capture antibody, and incubated for 3 h with mild agitation (150 rpm) at room temperature. After incubation, the plate was washed to remove any unbound nonspecific material. The wells were then incubated with a specific rabbit anti-phospho-p53(Ser15) antibody to detect the captured p53 on the plate well that is phos-
phorylated on Ser15. The unbound detection antibody was washed away, followed by incubation with an HRP-conjugated anti-rabbit antibody. After the addition of tetramethylbenzidine (TMB) substrate and stop solution, the absorbance was measured at 450 nm with a Synergy HT microplate reader. A set of standards was run as a positive control and to generate a standard curve for phosphorylated p53(Ser15) measurement.

DNA fragmentation ELISA. Isolated mixed forelimb and hind-
limb muscles from WT and KO mice were minced, homogenized, suspended, and centrifuged at 800 g for 10 min. The supernate was filtered through a layer of cheesecloth, and 200 μl of the filtrate was extracted. The extracted cytosolic subfractions were further spun three times at 3,500 g for 5 min, and the protein concentration was determined by the Bradford assay (8). DNA fragmentation was measured with a DNA fragmentation ELISA kit (Roche). Samples (20 μg) were placed into a streptavidin-coated microplate well and incubated with a mixture of anti-histone-biotin and anti-DNA-POD for 2 h on a shaker (300 rpm) at room temperature. Subsequently, wells were washed and reincubated with 2,2’-azino-bis(3-ethyl- benzthiazoline-6-sulfonic acid) (ABTS) on a plate shaker for 18 min. Absorbance was measured at 405 nm with a Synergy HT microplate reader and adjusted for background values. A positive and a negative control were run with the assay each time.

Statistical analysis. Data were analyzed with Graph Pad 4.0 software, and values are reported as means ± SE. The weekly running distance values and fatigability curves for p53 WT and null mice were analyzed with a repeated-measures two-way ANOVA. Basal COX activity, protein expression, cytochrome c release, mtPTP kinetics, and p53 phosphorylation were analyzed with a Student’s t-test. All other data were analyzed with a two-way ANOVA unless otherwise indicated. Significance levels were set at P < 0.05.

RESULTS

p53 KO mice manifest reduction in markers of mitochondrial content. SS and IMF mitochondrial yield was measured in p53 WT and KO mice as a crude estimate of basal mitochondrial content. The yield was reduced by ~24% for both SS and IMF mitochondria derived from p53 KO ani-

mals (P < 0.05, Fig. 1A). As expected, SS mitochondrial yield was lower than that of IMF mitochondria for both strains of animals (P < 0.05, Fig. 1A). Mixed hindlimb muscle COX activity, an established indicator of mitochon-
drial biogenesis, was reduced by 26% in p53 KO mice compared with WT counterparts (P < 0.05, Fig. 1B). Further-
more, PGC-1α, an important regulator of mitochondrial content, was reduced by 40–45% in the KO mice (P < 0.05, Fig. 1C), whereas PGC-1β expression remained unchanged in the KO gastrocnemius muscle compared with the WT counterparts (Fig. 1C). Representative electron micrographs, prepared from sections of the gastrocnemius muscle from WT and KO animals, illustrate the SS and IMF mitochondrial populations in the two mouse strains (Fig. 1D). A deficit in both SS and IMF mitochondrial content was manifest in the KO mice (Fig. 1D). IMF mitochondria exhibited a greater reticular network formation in the WT animals, and appeared to be larger than in the KO mice (Fig. 1D, right). In addition, SS mitochondria in the KO mice exhibited some morphological deformities, as evident from the irregular cristae formation (Fig. 1D, left).

Impaired mitochondrial function is observed in muscle from p53 KO mice. Respiration rates were measured as indicators of mitochondrial function. As expected, state 4 respiration rates were lower than state 3 respiration rates in both mitochondrial subfractions (P < 0.05, Fig. 2, A and B). Ablation of p53 reduced state 3 respiration within IMF mitochondria by 40% (P < 0.05, Fig. 2B) but did not affect state 4 respiration (Fig. 2B). State 4 and state 3 respiration rates were not altered in SS mitochondria (Fig. 2A) with absence of p53. ROS production was measured in isolated SS and IMF mitochondria and expressed per unit of oxygen consumed. As expected (4), ROS generation was higher during state 4 respiration than during state 3 respiration in
WT mice (P < 0.05, Fig. 2, C and D). In SS mitochondria, ROS production was not different between p53 KO and WT mice during state 4 or state 3 respiration (Fig. 2C). However, in IMF mitochondria, ROS production was elevated by ~1.5- to 3-fold in KO mice during state 4 and state 3, respectively, compared with WT animals (P < 0.05, Fig. 2D).

Altered mitochondrial apoptotic potential is evident in p53 KO skeletal muscle. To assess mitochondrial apoptotic susceptibility, we measured the maximal rate of pore opening (Vmax) and the time to Vmax in SS and IMF mitochondria. In SS mitochondria, Vmax was decreased by ~46% and time to Vmax was elevated by 19% in the KO mice (P < 0.05, Table 1). No changes were observed in IMF mitochondrial pore kinetics between the two strains (Table 1). To further augment our understanding of apoptotic susceptibility in mice lacking p53, we assessed the basal rate of cytochrome c release in isolated SS and IMF mitochondria from WT and KO mice. In the KO mice, SS mitochondria liberated ~2.5-fold higher levels of cytochrome c under basal conditions (P < 0.05, Fig. 3A). Conversely, IMF mitochondria from the KO mice displayed a 69% decrease in cytochrome c release compared with their WT counterparts (P < 0.05, Fig. 3B). Bax protein levels were not different in gastrocnemius muscle extracts from p53 WT and KO animals (data not shown). However, DNA fragmentation, a hallmark measure of apoptosis, was ~5.5-fold lower in KO animals compared with their WT counterparts (P < 0.05, Fig. 3C).

Muscle mass, fatigability, and force production in p53 KO mice. There was no difference in overall body weight between WT and KO mice (Table 2). However, p53 KO mice had higher forelimb and hindlimb muscle weight-to-body weight ratios compared with WT mice (P < 0.05, Table 2). To evaluate the role of p53 in muscle force generation and fatigability, we used an acute in situ model of muscle contraction. Stimulation eliciting 1 and 3 TPS resulted in a reduction of force generation over time (i.e., fatigue) in both WT and KO animals (P < 0.05, Fig. 4A). In the KO mice, the extent of fatigue was 50% greater than the WT animals at both 1 TPS and 3 TPS (P < 0.05, Fig. 4A). KO animals exhibited a higher maximum tetanic force in the gastrocnemius muscle (P < 0.05, Table 2) as expected because of the greater muscle mass; however, there was no difference in muscle strength between WT and KO mice when expressed per unit of muscle mass (Table 2).

Acute contractile activity elicits an increase in p53 Ser15 phosphorylation. We assessed whether p53 phosphorylation on Ser15, as well as activation of upstream kinases, is increased by muscle contractile activity in the gastrocnemius muscle of WT mice. Acute stimulation at 1 and 3 TPS resulted in a 2.2-fold increase in p53 phosphorylation (P < 0.05, Fig. 4B). This oc-
curred in concert with four- and eightfold increases in AMPK and p38 phosphorylation (P<0.05, Fig. 4, C and D), suggesting a possible pathway through which p53 could be activated in response to contractile activity.

**Response of p53 KO mice to voluntary exercise.** To evaluate the effect of p53 on exercise-induced adaptations, both WT and KO animals were subjected to 8 wk of voluntary exercise training. Both sets of animals increased their running distances with time. However, the WT animals ran approximately fivefold greater distances (5.83 km/day wk⁻¹; P<0.05, Fig. 5A) over the 8-wk period compared with the KO animals (1.25 km·day⁻¹·wk⁻¹). After 8 wk of voluntary wheel running, modest ~15–22% increases in forelimb muscle mass were observed for both WT and KO mice (P<0.05, Table 2). This increase in muscle mass was greater in the KO animals. Furthermore, both the WT and KO runners exhibited an increase in heart weight-to-body weight ratio (0.05 < P < 0.1; Table 2), an expected consequence of endurance training.

COX activity was enhanced by ~26% with training in both WT and KO mice (P<0.05, Fig. 5B). The increase in COX activity in the KO animals with training effectively normalized the COX activity to that observed in control animals. Representative electron micrographs prepared from sections of the gastrocnemius tissue from p53 WT and KO runners displayed an increase in mitochondrial content with training (Fig. 5D). Exercise training had no effect on SS (data not shown) or IMF (Fig. 5C) mitochondrial respiration rates in p53 WT or null mice with running. Similarly, ROS production remained unchanged with training in either the SS or IMF mitochondria for all animals (data not shown), and voluntary wheel running had no significant effect on DNA fragmentation in either KO or WT animals (Fig. 3C).

**DISCUSSION**

p53 has a well-established function in preventing cancer progression primarily by its ability to induce apoptosis and halt cell cycle progression. However, it is becoming apparent that p53 has a much broader role in facilitating normal growth and development. Recent studies have indicated emerging roles for p53 in regulating maternal reproduction, aerobic respiration, glycolysis, longevity and aging, DNA repair, and angiogenesis (22, 44). As shown by Matoba et al. (28), lack of p53 induced deficits in aerobic respiration in liver mitochondria and cancer cell lines. Our data highlight the detrimental effects of the absence of p53 on mitochondrial content and respiration in skeletal muscle. The yield of SS and IMF mitochondria was
significantly lower in muscle from KO compared with WT mice. Mixed muscle COX activity, a marker of mitochondrial biogenesis, was also reduced in the KO animals. Additionally, the expression of PGC-1α, a master regulator of mitochondrial biogenesis, was reduced in gastrocnemius muscle extracted from p53 KO animals compared with their WT counterparts. Electron micrographs of skeletal muscle corroborated these findings, because mitochondrial content was visibly diminished in the KO mice. Interestingly, the presence of perturbed mitochondrial cristae structure was also evident in mitochondria from the KO mice.

Given these results on mitochondrial content, we also sought to elucidate the effects of p53 on mitochondrial respiratory function and ROS production. Our data indicate that while SS mitochondrial respiration rates remained relatively constant between the two genotypes, the ablation of p53 impaired state 3 (active) respiration in the IMF mitochondrial subfraction by 40%. This deficit in oxygen consumption in the IMF mitochondria carries significant repercussions for the KO mice, because IMF mitochondria constitute ∼80–85% of the total mitochondrial volume and are surmised to be primarily responsible for providing ATP during muscle contraction (19). Furthermore, our data indicate that the production of ROS was elevated in null animals, specifically in the IMF mitochondrial subfraction. These data clearly specify that the absence of p53 induces a reduction in mitochondrial content and function, which could contribute substantially to detriments in muscle performance.

Since ROS facilitate the opening of the mtPTP, we expected faster kinetics of pore opening and greater apoptotic protein release from muscle mitochondria isolated from the KO mice. Surprisingly, pore kinetics were similar in the predominant IMF mitochondrial subfraction between WT and KO mice, and basal cytochrome c release from IMF mitochondria was markedly decreased. As p53 regulates pathways of apoptotic protein discharge from the mitochondria, such as the mtPTP and Bax:Bax and Bak:Bak dimers (30, 31), the absence of p53 appears to impede the liberation of cytochrome c from the mitochondria. Since IMF mitochondria predominate over SS mitochondria in muscle, we surmise that this will lead to a reduced rate of apoptosis in muscle of KO mice. Indeed, a significant decrease in DNA fragmentation, a hallmark measure of apoptosis, was observed in the KO mice compared with WT control mice. Previous work has shown that experimental models of muscle atrophy such as hindlimb unloading and denervation-induced muscle disuse are associated with an up-regulation of p53 protein content, suggesting a role for p53 in determining muscle mass (37, 38). Similarly, in the absence of p53 we observed a significantly greater muscle mass in the KO mice. This could certainly be due, in part, to the reduced rate of apoptosis that we have found, or to alternative mechanisms of enhanced muscle growth that remain to be established in this animal model.

**Table 1. Mitochondrial permeability transition pore kinetics**

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<tr>
<th>Mitochondrial Fraction</th>
<th>$V_{\text{max}}, \text{AU}$</th>
<th>Time to $V_{\text{max}}, \text{s}$</th>
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<tr>
<td>SS</td>
<td>WT: 10.40±0.38</td>
<td>5.63±0.77*</td>
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<tr>
<td></td>
<td>KO: 9.25±1.65</td>
<td>113.60±48.81*</td>
</tr>
<tr>
<td>IMF</td>
<td>WT: 11.66±1.60</td>
<td>10.03±1.01</td>
</tr>
<tr>
<td></td>
<td>KO: 145.20±22.84</td>
<td>125.10±8.97</td>
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Data are presented as mean ± SE. Subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial maximal rate of pore opening ($V_{\text{max}}$) and time to $V_{\text{max}}$ in p53 wild-type (WT) and knockout (KO) animals; $n = 4–16$ animals. *$P < 0.05$, WT vs. KO. AU, arbitrary units; s, seconds.

Fig. 3. Reduced apoptotic potential in p53 KO skeletal muscle. A and B: Western blots illustrating cytochrome c release from untreated isolated SS mitochondria (A) with representative graph ($n = 4$; *$P < 0.05$, WT vs. KO) and IMF mitochondrial basal cytochrome c release (B) with representative graph, corrected for endogenous mitochondrial cytochrome c content in p53 WT and KO mice ($n = 4$ or 5; *$P < 0.05$, WT vs. KO). C: amount of DNA fragmentation, a hallmark indicator of apoptosis, in whole muscle from p53 WT and KO mice in control and animals allowed to train on a wheel ($n = 4–7$). Data are means ± SE (**$P < 0.05$, main effect of genotype). AU, arbitrary units.
It is well known that the mitochondrial content of the muscle is intimately associated with muscle endurance (2). High mitochondrial content is closely associated with enhanced endurance performance, whereas low mitochondrial content induces rapid fatigability and low exercise tolerance. To evaluate whether deficits in mitochondrial content and function translate into ineffective muscle performance, we investigated the contractile properties of skeletal muscle of p53 WT and KO mice in situ. Our data indicate that while there was no difference in the maximum strength of the muscle (expressed per unit of muscle mass), the endurance capacity of p53 KO mice was markedly impaired. These data suggest that muscular deficits underlie the inefficient exercise performance of the null animals, as observed with the voluntary wheel running program. These data are consistent with those of Matoba et al. (28) using swimming as an exercise model. However, it should be noted that this displayed aversion to running in p53 null animals is likely a collective result of neurobehavioral problems in addition to the impairments in muscle contractility as noted above. Amson et al. (6) illustrated that the lack of p53 expression induces apoptotic brain lesions accompanied by deficits in learning and behavior. Additionally, p53 null mice also display a significant deficit in rapid walking synchronization (9) that may contribute to the poor locomotory activity of the KO mice.

The data summarized thus far clearly reveal the value of p53 in maintaining optimal mitochondrial content and function. If p53 plays a role in regulating exercise-induced

### Table 2. Muscle and heart weight-to-body weight ratios and maximum tetanic force

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<tr>
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<th>p53 WT Mice</th>
<th>p53 KO Mice</th>
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<tr>
<td></td>
<td>Control</td>
<td>Runner</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body wt, g</td>
<td>38.96±4.18</td>
<td>34.97±2.46</td>
</tr>
<tr>
<td>Forelimb wt/body wt</td>
<td>13.09±1.37</td>
<td>15.04±1.37†</td>
</tr>
<tr>
<td>Hindlimb wt/body wt</td>
<td>31.83±4.16</td>
<td>33.91±5.96</td>
</tr>
<tr>
<td>Maximum tetanic force, N</td>
<td>0.68±0.09</td>
<td>ND</td>
</tr>
<tr>
<td>Maximum tetanic force, N/mg tissue</td>
<td>0.0085±0.0001</td>
<td>ND</td>
</tr>
<tr>
<td>Heart wt/body wt, ×1,000</td>
<td>3.54±0.18</td>
<td>3.73±0.18</td>
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</table>

Data are presented as means ± SE. Combined wet muscle mass is corrected for body weight in forelimb and hindlimb muscle fractions in p53 WT and KO control and runner mice. Despite higher forelimb and hindlimb muscle mass in KO mice, body weights were similar between p53 WT and KO control and runner mice (n = 7–9). A trend toward an increase in heart weight-to-body weight ratio was noted with 8 wk of voluntary wheel running (0.05 < P < 0.1) in p53 WT and KO control and runner mice (n = 8 or 9). Maximum tetanic contraction is expressed as absolute force in Newtons (N) and as corrected for gastrocnemius muscle weight for both genotypes (n = 5 or 6). *P < 0.05, WT vs. KO; †P < 0.05, main effect of voluntary wheel running. ND, not determined.
mitochondrial biogenesis, it would be logical to assume that a single bout of exercise should be able to enhance the activity of p53, possibly via phosphorylation. Previous research has indicated that p53 can be phosphorylated on Ser15 (Ser18 in mouse) via AMPK (24) and p38 (11, 35), leading to increased protein stabilization and activity. There is ample evidence to indicate that AMPK and p38 activation precede mitochondrial biogenesis in response to contractile activity (19, 23, 46). Indeed, acute stimulation of muscle induced an approximately twofold increase in p53 phosphorylation at Ser15. This occurred in concert with marked increases in AMPK and p38 phosphorylation, indicating a possible signaling cascade through which p53 may initiate mitochondrial biogenesis in response to exercise. However, it appears that the presence of p53, and/or its activation, is not a necessary component of exercise-induced mitochondrial biogenesis. We observed similar 26% increases in COX activity in WT and KO runners in response to voluntary exercise training. Indeed, the training program attenuated the impairment in COX activity seen in the KO animals, bringing the values up to control levels. Electron micrographs from WT and KO runners also appeared to corroborate the biochemical data, by demonstrating an increase in mitochondrial content.

These data suggest that a lack of p53 induces a deficit in basal mitochondrial function (respiration, ROS production, apoptosis) and content but that the absence of p53 does not hinder the ability of the animal to adapt to exercise. This may be because exercise provokes a wide range of redundant overlapping signals that can ultimately induce mitochondrial biogenesis in response to contractile activity. This exercise-induced adaptation observed in KO animals occurred despite the ability of the KO animals to run only ~20–25% of the distance covered by the WT counterparts. This suggests the necessity of compensatory increases in other regulators of mitochondrial synthesis in the KO mice during exercise. The extent to which p53 coordinates with and/or controls other regulators of mitochondrial synthesis such as PGC-1α and the signaling mechanisms by which it potentiates its effect on mitochondrial biogenesis remain to be elucidated.

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