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Perm1 enhances mitochondrial biogenesis, oxidative capacity, and fatigue resistance in adult skeletal muscle

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ABSTRACT Skeletal muscle mitochondrial content and oxidative capacity are important determinants of muscle function and whole-body health. Mitochondrial content and function are enhanced by endurance exercise and impaired in states or diseases where muscle function is compromised, such as myopathies, muscular dystrophies, neuromuscular diseases, and age-related muscle atrophy. Hence, elucidating the mechanisms that control muscle mitochondrial content and oxidative function can provide new insights into states and diseases that affect muscle health. In past studies, we identified Perm1 (PPARGC1- and ESRR-induced regulator, muscle 1) as a gene induced by endurance exercise in skeletal muscle, and regulating mitochondrial oxidative function in cultured myotubes. The capacity of Perm1 to regulate muscle mitochondrial content and function in vivo is not yet known. In this study, we use adeno-associated viral (AAV) vectors to increase Perm1 expression in skeletal muscles of 4-wk-old mice. Compared to control vector, AAV1-Perm1 leads to significant increases in mitochondrial content and oxidative capacity (by 40–80%). Moreover, AAV1-Perm1–transduced muscles show increased capillary density and resistance to fatigue (by 33 and 31%, respectively), without prominent changes in fiber-type composition. These findings suggest that Perm1 selectively regulates mitochondrial biogenesis and oxidative function, and implicate Perm1 in muscle adaptations that also occur in response to endurance exercise.—Cho, Y., Hazen, B. C., Gandra, P. G., Ward, S. R., Schenk, S., Russell, A. P., Kralli, A. Perm1 enhances mitochondrial biogenesis, oxidative capacity, and fatigue resistance in adult skeletal muscle. FASEB J. 30, 000–000 (2016). www.fasebj.org

Key Words: oxidative metabolism • angiogenesis • endurance exercise responses • skeletal muscle plasticity

Adult skeletal muscle shows remarkable plasticity, with many of its features and functional properties being shaped by the type and degree of physical exertion. For example, in response to endurance exercise training, muscle adapts with increases in mitochondrial content, oxidative capacity and vascularization, and changes in fiber-type composition (1). Conversely, physical inactivity, immobilization, aging, and many disease states are associated with decreases in muscle oxidative capacity and distinct changes in fiber-type composition (2–4). The skeletal muscle adaptations to physical activity (or inactivity) affect muscle performance and exercise tolerance, as well as whole-body health and well-being (5). Remarkably, genetic or pharmacologic manipulations that mimic exercise-induced adaptations in rodent skeletal muscle can also counteract the impact of diseases affecting muscle or aging. For example, transgene-mediated enhancement of oxidative capacity ameliorates symptoms of muscular dystrophy, myopathies, and aging in mouse models (6–9). Hence, elucidating the signaling networks that control skeletal muscle mitochondrial biogenesis and oxidative function is important for understanding the mechanisms that underlie muscle plasticity and enable adaptations, and for identifying novel targets for the treatment of diseases associated with muscle mitochondrial defects.

Endurance exercise induces intracellular signaling pathways, via the activation of the p38 MAPK, the AMPK, and Ca2+-dependent kinases and phosphatases, such as CaMK and calcineurin (1, 10–12). These signal transducers control the activity and expression of transcriptional regulators [e.g., ATF2, PGC-1 (peroxisome proliferator-activated receptor γ coactivator 1), NRF1 (nuclear respiratory factor 1), GABP (GA-binding protein), NFAT, MEF2s, PPARs (peroxisome proliferator-activated receptors), and ERRs (estrogen-related receptors)], which subsequently drive the expression of sets of nuclear genes important for the adaptive responses. The induction of mitochondrial biogenesis is generally

Abbreviations: AAV, adeno-associated virus; AAV1, adeno-associated virus, serotype 1; AMPK, AMP-activated protein kinase; CPT1, carnitine palmitoyltransferase 1; CS, citrate synthase; EDL, extensor digitorum longus; ERR, estrogen-related receptor; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; FDB, flexor digitorum brevis; GABP, GA-binding protein; 

(continued on next page)
thought to be under the control of the transcriptional coactivators PGC-1α and PGC-1β, which are recruited via NRF1, GABP, and ERRα to the promoters of nuclear genes encoding regulators of mitochondrial replication and transcription (e.g., Tfam, Tfb1m, and Tfb2m), components of the tricarboxylic acid cycle and oxidative phosphorylation (OxPhos) systems, and many other proteins important for mitochondrial biogenesis and oxidative function (13, 14). The coactivators PGC-1α and PGC-1β and the nuclear receptors ERRα and ERRγ also drive skeletal muscle angiogenesis, at least in part via the induction of Vegfa (15–17). Changes in fiber-type composition are thought to be primarily under the control of NFAT, though they are also controlled by PGC-1α, PGC-1β, PPARγ, and ERRβ/ERRγ (17–24). Notably, many of these transcriptional regulators seem to work as a tightly knit network, crosstalking and regulating each other’s expression. For example, ATF2, MEF2, and PPAR factors regulate PGC-1α expression, whereas PGC-1α and ERRα regulate ERRα, PPARγ, NRF1, and GABP expression (25–31), suggesting the presence of feed-forward regulatory loops that get activated to coordinate appropriate responses to exercise.

All of the regulators implicated so far in exercise-induced responses are present at high levels in skeletal muscle but are also expressed in multiple other tissues. The extent to which muscle may have tissue-specific factors regulating mitochondrial biogenesis in response to exercise has not yet been addressed. In our previous study, we identified Perm1 (PPARGC1- and ESRR-induced regulator, muscle 1) as a protein with a remarkably muscle-specific expression, induced by PGC-1α and ERRα, and required for the enhancement of oxidative capacity by PGC-1α in cultured myotubes (32). Besides skeletal and cardiac muscles, where its expression is very high, Perm1 is detectable only in brown adipose tissue, a depot that shares developmental and expression similarities to muscle (33). Skeletal muscle Perm1 levels are induced by endurance exercise and decreased in disease states with decreased oxidative capacity, as seen in patients with amyotrophic lateral sclerosis (32). Our findings suggested that the induction of Perm1 may be part of the mechanism by which exercise (and PGC-1/ERRs) regulates mitochondrial oxidative function in muscle. Interestingly, Perm1 regulates the expression of only a subset of genes induced by PGC-1α or ERRγ expression in C2C12 myotubes, suggesting that Perm1 selectively functions in specific PGC-1/ERR-driven pathways (32). The ability of Perm1 to control mitochondrial biogenesis or other

PGC-1/ERR pathways in adult skeletal muscle is so far unknown.

To address the function of Perm1 in adult skeletal muscle in vivo, we used adeno-associated viral (AAV) vectors to enhance Perm1 expression in specific muscles and determine the effect of increased Perm1 levels on mitochondrial content, oxidative capacity, and muscle functional properties. Our findings show that Perm1 is a potent positive regulator of mitochondrial biogenesis and oxidative capacity. Perm1 enhanced also muscle capillarity and fatigue resistance, without altering fiber-type composition.

MATERIALS AND METHODS

Generation of adeno-associated virus, serotype 1

The AAV1 (adeno-associated virus, serotype 1)-FLAG-Perm1 vector was produced by the Vector Core of the University of Pennsylvania (Philadelphia, PA, USA), using the plasmid pZac2.1/FLAG-Perm1 (pZac2.1 with an insert coding for the Perm1 protein with an N-terminal FLAG epitope) (32).

AAV injection and animal studies

C57BL/6J mice (TSRI breeding colony) were housed at constant temperature on a 12 h light-dark cycle and with free access to food and water. All experimental procedures were conducted according to protocols approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute (Protocol number 14-0004). For i.m. injections of AAV vectors, mice (4 wk old) were anesthetized with isoflurane and injected with AAV vectors at the following doses: 2.5 × 1010 viral genomes when injecting the frontal area of tibialis anterior (TA)/extensor digitorum longus (EDL) muscles, and 1.0 × 1010 viral genomes for injections into the flexor digitorum brevis (FDB) muscle. Each mouse had 1 leg injected with AAV1-Perm1 and the contralateral leg injected with AAV1-LacZ as a control. Four weeks following the injections, mice were killed (~11 AM, unless indicated otherwise), and muscles were dissected and stored at −80 °C (for processing of protein, DNA, and RNA), frozen in liquid nitrogen-cooled isopentane (for histology and immunohistochemistry), subjected to electrical stimulation (for measurements of force production), or dissociated to single fibers (for oxygen consumption measurements).

Histologic analyses and immunohistochemistry

Skeletal muscle samples were pinned to corkboard and frozen in liquid nitrogen-cooled isopentane (34). Cross-sections (10 μm thick) were cut from the midbelly of the TA muscle on a cryostat at −18°C (Microm HM500; Waldorf, Germany). To assess overall fiber morphology, serial sections were stained with hematoxylin and eosin (H&E), and images of AAV1-LacZ and AAV1-Perm1–transduced TA muscles from 4 mice were taken using a Zeiss Axiovert S100 fluorescence microscope (Carl Zeiss Microscopy, Jena, Germany). For succinate dehydrogenase (SDH) staining, sections were air dried for 30 min and then incubated in 100 mM Tris-HCl buffer containing 50 mM sodium succinate, 0.6 mM nitro blue tetrazolium, and 0.02 mM phenazine methosulfate for 20 min at 37°C. For immunostaining, sections were fixed with PBS containing 4% paraformaldehyde (PFA), followed by blocking in 5% goat serum, and incubation with, first, primary antibodies
buffer and post medium, using standard procedures (35). Briefly, TA muscles from 3 mice perfused with 4% PFA and 1.5% glutaraldehyde in 0.1 M cacodylate buffer were dissected, cut into small pieces (1 mm²), and fixed overnight with 2.5% glutaraldehyde/2% PFA in 0.1 M phosphate buffer (pH 7.4). Samples were then washed in 0.1 M sodium cacodylate buffer and postfixed for 5 h with 1% OsO4 in cacodylate buffer. Samples were dehydrated in a graded ethanol series and embedded in Epon/Araldite resin (Electron Microscopy Sciences, Hatfield, PA, USA) overnight. Ultrathin sections were obtained, contrasted with uranyl acetate/lead citrate, and examined with a Philips CM100 microscope (FEL, Hillsboro, OR, USA).

Transmission electron microscopy

Transmission electron microscopy was performed using standard techniques (35). Briefly, TA muscles from 3 mice perfused with 4% PFA and 1.5% glutaraldehyde in 0.1 M cacodylate buffer were dissected, cut into small pieces (1 mm²), and fixed overnight with 2.5% glutaraldehyde/2% PFA in 0.1 M phosphate buffer (pH 7.4). Samples were then washed in 0.1 M sodium cacodylate buffer and postfixed for 5 h with 1% OsO4 in cacodylate buffer. Samples were dehydrated in a graded ethanol series and embedded in Epon/Araldite resin (Electron Microscopy Sciences, Hatfield, PA, USA) overnight. Ultrathin sections were obtained, contrasted with uranyl acetate/lead citrate, and examined with a Philips CM100 microscope (FEL, Hillsboro, OR, USA).

Reverse transcription and quantitative real-time PCR analysis

Total RNA was extracted from whole muscles using TRIzol reagent (Life Technologies), and cDNA was synthesized using the SuperScript II Reverse Transcription system (Invitrogen, Life Technologies), as published (36). Relative mRNA levels were determined by quantitative PCR using cDNA, gene-specific primers (Table 1), SYBR Green reagent (Affymetrix, Santa Clara, CA, USA), and normalization to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described (37).

DNA isolation and quantification

Total DNA was prepared from whole TA muscle, according to standard procedures (38), and digested with 100 µg/ml RNase A for 30 min at 37°C. The relative copy numbers of mitochondrial and nuclear genomes were determined by quantitative PCR with primers specific to the CoxII (mitochondrial) and Dio3 (nuclear) genes (Table 1).

Western blot and antibodies

Whole muscles were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM Na2VO4, 5 mM

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<th>Gene</th>
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Mitochondrial enzyme activity assays

Whole TA muscles were homogenized in 50 mM Tris-HCl (pH 7.4), 5 μM/ml protease inhibitor cocktail (P8340), and 20 μg/ml PMSF. Homogenates were centrifuged at 1000 g for 10 min (at 4°C), and the resulting supernatants were tested for the enzymatic activities of electron transfer chain complexes I-IV and citrate synthase (CS), as described (39).

Fiber-type composition analysis

TA muscle myosins were extracted and analyzed by electrophoresis and silver staining as described (40). Silver-stained gels were scanned and quantified using ImageJ. TA cross-sections immunostained with antibodies to myosins were also used to determine fiber-type composition (41).

Isolation of single FDB fibers and oxygen consumption assay

Oxygen consumption of single FDB fibers was measured using the XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, Danvers, MA, USA); anti-Rt/Ms Total (anti-C1orf170; Sigma-Aldrich); anti-tubulin (#2184; Cell Signaling Technology, Danvers, MA, USA); and anti-β-actin (clone 4G10; Sigma-Aldrich). Images of all cellular Flux Analyzer and as described (42). Images of all wells (at ×4 magnification) were used to determine the number of fibers in each well. Basal, uncoupled (in the presence of 1 μM oligomycin), and maximal [in the presence of 800 nM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; FCCP] OCRs were calculated by subtracting background rates (in the presence of 1 μM antimycin A and 1 μM rotenone), normalized by the number of fibers in each well, and expressed as OCR per 20 fibers. Data shown are the means ± SD of 6 AAV1-LacZ and 6 AAV1-Perm1-transduced FDB muscles, with each muscle OCR being the average of 6–12 replicates.

Cell culture

Primary myoblasts were isolated from 6- to 8-wk-old mice and differentiated to myotubes as described (43). Differentiated myotubes were infected on d 4 of differentiation with adenoviruses expressing LacZ (control) or Perm1 at multiplicity of infection 50 (32). Cells were harvested 24 h after infection.

Ex vivo muscle contractile function and fatigue resistance

Mice were anesthetized, and the muscle belly of the fifth digit of the EDL was dissected as described (44). Matched muscles from the same animal transduced with AAV1-LacZ or AAV1-Perm1 were analyzed. Muscles were mounted in a specialized muscle chamber continuously perfused with Ringer’s solution (137 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 24 mM NaHCO₃, 1 mM NaH₂PO₄, 11 mM glucose, 1 mM MgSO₄, and 0.01% tubocurarine chloride [pH 7.5]) at 25°C, bubbled with 100% O₂. One of the tendons was tied with 5-0 silk suture to the arm of a muscle lever system (model 300B; Aurora Scientific, Aurora, ON, Canada), and the other tendon was tied to a micromanipulator allowing control of muscle position and length. Muscles were electrically stimulated (Model SRT; AstroMed, West Warwick, RI, USA) via parallel platinum electrodes (~35 V, 300 ms train duration, 0.3 ms pulse duration) with single twitches to set the length for maximal twitch tension. After a 10 min rest, muscles were stimulated at different frequencies (1–120 Hz) with 120 s intervals between contractions to determine the force-frequency relationship. The force-frequency curves were fitted by a nonlinear regression equation, $P = P_{\text{min}} + (P_{0X} / F_{0X}) \times (1 + X_{0X}^{2})$, where $P_{\text{min}}$ is the minimum tension developed, $F_{0X}$ is the midpoint of the curve (in hertz), and $n_{H}$ is the Hill coefficient. After the force-frequency protocol, muscles rested for 10 min, and the fatigue resistance was determined by repeated isometric contractions at the calculated $F_{0X}$ frequency. During the fatigue protocol, rest intervals started at 4 s and progressively decreased every 1 min (to 3 and 2 s). Time to fatigue was defined as the time it took each muscle to reach 40% of the force generated in the first contraction of the fatigue protocol. Force development was normalized to the muscle physiologic cross-sectional area, calculated as described (44). EDL muscles were also analyzed by Western blot analysis for Perm1 and OxPhos protein expression, to confirm efficient transduction and Perm1 function.

EDTA, 1% Triton X-100, 5 μM/ml protease inhibitor cocktail P8340 (Sigma-Aldrich), and 20 μg/ml PMSF. Protein lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Hybond-C Extra; GE Healthcare Life Sciences, Pittsburgh, PA, USA). Western blotting was performed using the following antibodies: anti-FLAG (Clone M2; Sigma-Aldrich); anti-PERMI (anti-C1orf170; Sigma-Aldrich); anti-tubulin (#2184; Cell Signaling Technology, Danvers, MA, USA); and anti-R/Myosin (ab76228; Abcam, Cambridge, MA, USA); anti-PGC-1α (29); anti-Sirt3 (sirtuin 3; #5490; Cell Signaling Technology); anti-Myoglobin (sc-25607; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-p38 MAPK (#9212; Cell Signaling Technology); and anti-Rt/Ms Total (anti-C1orf170; Sigma-Aldrich); anti-tubulin (#2184; Cell Signaling Technology); Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies were purchased from Bio-Rad (Hercules, CA, USA). The blots were developed using the Pierce ECL reagent (Thermo Fisher Scientific, Waltham, MA, USA).
RESULTS

Increasing muscle Perm1 expression does not affect muscle gross morphology or fiber size

Skeletal muscle Perm1 expression is induced in response to an acute endurance exercise bout, elevated in trained compared to untrained individuals and mice, and enriched in red/oxidative fiber-rich compared to white/glycolytic fiber-rich parts of muscle [(32, 45); Supplemental Fig. 1], suggesting that increased Perm1 levels could be driving or contributing to some of the exercise-induced adaptations. To determine the consequences of increasing Perm1 levels in adult skeletal muscle, in the absence of endurance exercise, we used AAV1 vectors to transduce muscle and achieve stable Perm1 transgene expression (46). First, we confirmed the efficiency of transduction by injecting an AAV1-green fluorescent protein vector into the frontal area of TA and EDL muscles. When analyzed 4 wk post-injection, >95% of TA and EDL fibers were green fluorescent protein positive, as reported by others (Supplemental Fig. 2) (46). Next, we injected 4-wk-old mice with AAV1-FLAG-Perm1 into the TA of 1 hind limb and the control AAV1-LacZ into the contralateral TA muscle. Four weeks later, FLAG-Perm1 was efficiently expressed, resulting in significantly increased levels of the slower migrating Perm1 protein isoform (arrow in Fig. 1A). By use of an antibody that detects only the AAV-expressed protein, FLAG-Perm1 was detected in the AAV1-Perm1-transduced muscle but not the control AAV1-LacZ-transduced contralateral muscle, confirming that AAV1 vector expression stays restricted to the injected leg. The increased expression levels of Perm1 did not alter muscle weight or cause any morphologic changes, as judged by H&E staining (Fig. 1B, C). Myofiber cross-sectional area was also similar in LacZ- and Perm1-transduced muscles (Fig. 1D, E). Thus, AAV1-Perm1 leads to efficient increases in Perm1 expression in the TA muscle, and increased levels of Perm1 do not affect muscle gross morphology or fiber size.

Perm1 enhances mitochondrial biogenesis and oxidative activity in adult skeletal muscle

In previous studies, we found that Perm1 was required for the induction of mitochondrial biogenesis by PGC-1α in cultured myotubes (32). Thus, we next assessed the effect of increased Perm1 expression on skeletal muscle mitochondrial content and oxidative activity. As shown in Fig. 2A, Perm1 overexpression resulted in an increase in skeletal muscle mitochondrial DNA (mtDNA) content. The increase in mtDNA copy number was accompanied by higher expression levels of genes encoded by the mitochondrial genome, mt-CoxII.

\[ \text{PERM1 DRIVES SKELETAL MUSCLE OXIDATIVE METABOLISM IN VIVO} \]
Perm1 increases muscle spare respiratory capacity

To assess the extent to which the increased mitochondrial content in Perm1 muscles leads to enhanced mitochondrial function and, in particular, respiratory capacity, we next compared oxygen consumption in Perm1 and control intact muscle fibers. For this, we administered the AAV1 vectors expressing FLAG-Perm1 or LacZ into the FDB muscles of 4-wk-old mice. As seen in the TA muscle, the increased Perm1 expression led to elevated protein levels of OxPhos complexes in FDB muscles (Fig. 3A). Dissociated intact single FDB fibers were placed in matrix-coated Seahorse Bioscience XF96 plates (Fig. 3B), and oxygen consumption was assessed in the presence of drugs that enable assessment of different components of respiration (see Supplemental Fig. 3 for representative traces of OCRs). In the basal state (absence of any drugs), there was no difference in OCRs between control LacZ- or Perm1-over-expressing fibers, suggesting that Perm1 did not influence the basal energetic needs of muscle (Fig. 3C). Additionally, the increased Perm1 expression had no significant effect on oxygen consumption in the presence of oligomycin (inhibitor of ATP synthesis), suggesting that it did not alter the coupling of respiration to ATP synthesis. In contrast, Perm1 overexpression dramatically increased maximal OCRs (measured in the presence of the uncoupler FCCP) (Fig. 3D). These findings show that the increased

Figure 2. Perm1 enhances mitochondrial biogenesis and oxidative activity in skeletal muscle. A) The relative mtDNA content was determined as the ratio of mitochondrial (CoxII) to genomic (Dio3) DNA copy numbers, and expressed relative to the ratio seen in control (LacZ) TA muscles. B) mRNA levels for the indicated mitochondrial genome-encoded OxPhos genes were determined by RT-quantitative PCR, normalized to GAPDH levels, and expressed relative to the ratio seen in control (LacZ) TA muscles. A and B) Data are the means ± s.e. (n = 14). **P < 0.01; ***P < 0.001. C) The levels of OxPhos complex proteins in TA muscles were determined by Western blot analysis, using total protein lysates and the Total OxPhos Complex antibody cocktail (C). The intensity of the bands was quantified using ImageJ software, and the values are expressed relative to the signal intensity in control (LacZ) TA muscles (D). Data are the means ± s.e. **P < 0.01; ***P < 0.001. G) Representative transmission electron micrographs of LacZ and Perm1 TA muscles. Arrowheads indicate mitochondria. Scale bar, 1 μm. H) Representative images of cross-sections of LacZ and Perm1 TA muscles stained for SDH activity. Scale bar, 100 μm.
mitochondrial content in Perm1-over-expressing muscles translates to higher oxidative capacity, with these muscles showing specifically higher spare respiratory capacity.

**Increasing expression of Perm1 does not significantly alter fiber-type composition in TA muscles**

The degree of mitochondrial content and oxidative capacity is characteristic of the different muscle fiber types: highest in type I slow-twitch and type IIA fast-twitch oxidative fibers, intermediate in type IIX fast-twitch mixed glycolytic/oxidative fibers, and lowest in IIB fast-twitch glycolytic fibers. Therefore, we next asked whether Perm1 affects the muscle fiber-type composition. First, we used high-resolution SDS-PAGE and silver staining to quantify the relative protein levels of the MHC isoforms that define fiber-type identity. As shown in Fig. 4A, B, increasing Perm1 expression led only to a minor shift from IIb to IIA and IIX MHC content (2% of all MHCs), which whereas appearing significant in statistical terms ($P = 0.002$), is unlikely to have a significant physiologic impact on muscle function or to explain the marked increase in mitochondrial oxidative function. Next, we used staining of muscle cross-sections with antibodies against type I, IIA, IIX, and IIB fast-twitch glycolytic fibers. Therefore, we next asked whether Perm1 affects the muscle fiber-type composition. Based on this approach, Perm1 had no effect on fiber-type composition (Fig. 4C, D). Both SDS-PAGE/silver staining and immunostaining confirmed the lack of type I fibers in LacZ- and Perm1-expressing TA muscles (Fig. 4A; data not shown). Moreover, we did not detect any significant changes in the mRNA levels of the genes encoding the different MHC isoforms (Fig. 4E), consistent with Perm1 having no prominent effect on fiber-type switching in adult skeletal muscle.

**Increased expression of Perm1 enhances skeletal muscle vascularization**

The signals that increase Perm1 expression (i.e., endurance exercise, PGC-1α and PGC-1β, and ERRs) also increase angiogenesis, via the transcriptional induction of Vegfa (15–17). Thus, we next determined the effect of increased Perm1 expression on skeletal muscle capillary density, by staining TA muscle cross-sections with antibodies against platelet endothelial cell adhesion molecule-1 (CD31). We observed an ~30% increase in the number of CD31-positive endothelial cells in AAV1-FLAG Perm1-transduced muscle (Fig. 5A, B) compared to control LacZ muscle, suggesting that Perm1 enhances vascularization. Consistent with the increase in capillary density, Perm1-over-expressing muscles had an increase in Vegfa mRNA content (Fig. 5C).

**Perm1 remodels muscle p38 signaling and enhances the expression of select regulators of mitochondrial biogenesis and oxidative function**

To gain insights into the pathways by which Perm1 remodels skeletal muscle, we next assessed the expression levels of genes known to be important for mitochondrial biogenesis and other aspects of skeletal muscle oxidative function. First, we assessed the mRNA levels of nuclear transcriptional regulators, including the coactivators of the PGC-1 family, the corepressor Rip140 (Nrip1), members of the ERR and PPAR subfamilies of nuclear receptors, and the DNA-binding transcription factors NRF1 and GABP. As shown in Fig. 6A, Perm1 led to increases in PGC-1α and ERRα mRNA levels. Next, we analyzed the expression of mitochondrial regulators and found that Perm1 led to increased expression of the mtDNA replication/transcription factors Tfam and Tfb2m, mitochondrial endonuclease Endog, and deacetylase Sirt3 (Fig. 6B). The increases in PGC-1α, ERRα, and Sirt3 were confirmed at the protein level (Fig. 6D). In addition to regulators involved in mitochondrial biogenesis and function, Perm1 also led to increases of other genes known to be induced by endurance exercise and/or to be important for nutrient utilization and energy transduction, such as Glut4 (glucose transporter type 4) and Cpt1b (carnitine palmitoyltransferase 1b) (Fig. 6C). For some genes, such as Mb (encoding myoglobin), Perm1 led to increased protein levels, even
though we did not detect an increase at the mRNA level (Fig. 6C, D).

Finally, we tested the extent to which Perm1 affected signaling pathways known to control mitochondrial biogenesis and responses to exercise. Comparison of AAV1-FLAG-Perm1 and control-transduced TA muscle lysates showed that the increase in Perm1 led to changes in the active [phosphorylated (phospho)] form of the MAPK p38. At daytime, when mice are resting, muscles with elevated Perm1 expression showed decreased levels of phospho-p38, whereas at nighttime, when mice are active, Perm1 muscles had significantly increased levels of phospho-p38 kinase (Fig. 7 and Supplemental Fig. 4). To determine the acute effect of Perm1 on p38 in a system independent of diurnal changes, we next expressed Perm1 in primary myotubes. Perm1 led to an increase in the phosphorylated form of the p38 kinase (Fig. 7C). We did not detect differences in phospho- or total AMPK at any time (data not shown). These findings suggest that Perm1 induces a remodeling of skeletal muscle signaling pathways.

Perm1 expression enhances fatigue resistance in skeletal muscle

To gain better insight into the physiologic impact of the Perm1-induced skeletal muscle remodeling, we next compared the contractile and fatigue properties of isolated EDL muscles that had been transduced with either
the AAV1-FLAG-Perm1 or AAV1-LacZ viruses. We found no significant differences in isometric force generation (at submaximal or maximal stimulation) or the rates of force generation and relaxation (Fig. 8A and Table 2), suggesting no changes in contractile properties. However, Perm1 muscles showed delayed fatigue (Fig. 8B, C), suggesting that the increased Perm1 levels confer resistance to fatigue.

DISCUSSION

We previously identified Perm1 as a PGC-1α/β and ERR-induced gene that is important for mitochondrial oxidative function in C2C12 myotubes in vitro (32). Perm1 is also induced in skeletal muscle by endurance exercise, raising the hypothesis that it plays a role in the enhanced oxidative capacity seen in response to exercise in vivo. Consistent with this hypothesis, we show here that increasing the levels of Perm1 in skeletal muscle postnatally is sufficient to enhance mitochondrial content and oxidative capacity. Moreover, Perm1 enhances muscle vascularization and resistance to fatigue, without affecting fiber-type composition. Future studies using loss-of-function approaches will be needed to determine the extent to which Perm1 is required for specific endurance exercise adaptations.

Increasing Perm1 levels in skeletal muscle for ~4 wk led to significant enhancements of mitochondrial content and oxidative function, comparable to those seen in mice in response to endurance training (39) or in transgenic mice overexpressing upstream regulators of Perm1 (e.g., PGC-1α, PGC-1β, or ERRγ) or other regulators of oxidative capacity [e.g., PPARβ or members of the NR4A subfamily of nuclear receptors (20, 21, 23, 24, 41, 50)]. It is noteworthy that the increased oxidative capacity of muscles over-expressing Perm1 did not alter basal levels of respiration in myofibers, suggesting that Perm1 does not affect the ATP demands of resting muscle. Rather, Perm1 selectively enhanced the spare respiratory capacity (i.e., the ability of fibers to supply ATP at times of high energy demand), suggesting that muscles with high Perm1 expression use a lower fraction of their mitochondrial reserve to perform similar work as control muscles (51). The enhanced respiratory capacity is probably due to increases in both mitochondrial content and enzymatic activities. The increase in mitochondrial content may be driven by the enhanced expression of Tfam, Tfb2m, and Endog, which interact with the mitochondrial genome and control mitochondrial mass (52, 53). Enhancements in enzymatic activities may be driven by the increase in mitochondrial Sirt3, which deacetylates and activates SDH and other subunits of OxPhos complexes, and fatty acid oxidation enzymes (54, 55).

Besides enhancing mitochondrial content and oxidative capacity, increased Perm1 levels led to other muscle adaptations typically seen in response to endurance exercise, including enhanced vascularization, myoglobin content, and resistance to fatigue. These changes suggest that Perm1 promotes angiogenesis and oxygen transport, to meet the increased muscle oxidative capacity. The increase in vascularization is reminiscent of changes seen in mice with muscle-specific overexpression of PGC-1α, PGC-1β, or ERRγ, suggesting that PGC-1, ERRs, and Perm1 act coordinately to remodel skeletal muscle (15–17). As in these other models, Perm1 led to increased expression of Vegfa, a gene important for angiogenesis. The increase in ex vivo fatigue resistance in isolated EDL muscles over-expressing Perm1 is likely related to the increased mitochondrial oxidative capacity (56). It is also possible that Perm1 remodels other muscle pathways that contribute to fatigue mechanisms, such as, for example, Ca2+ handling. Future studies will need to address this.

Increased Perm1 levels in muscle led to enhancements in the expression of genes that regulate mitochondrial biogenesis and function (Tfam, Tfb2m, Endog, and Sirt3), angiogenesis (Vegfa), and substrate metabolism (Glut4 and Cpt1b). These genes are known to be under the control of

Figure 5. Increased expression of Perm1 enhances skeletal muscle vascularization. A) Representative images of cross-sections of LacZ and Perm1 TA muscles stained with anti-CD31 and anti-laminin antibodies. Scale bars, 50 μm. B) Capillary density of LacZ and Perm1 TA muscles, quantified from CD31-stained muscles, using ImageJ software. Data are the means ± se (n = 5). *P < 0.05. C) Vegfa mRNA levels were determined by RT-quantitative PCR, normalized to GAPDH levels, and expressed relative to the levels in control (LacZ) muscle. Data are the means ± se (n = 14). **P < 0.01.
Protein levels of PGC-1α intensity of the bands was quantified using ImageJ software, normalized to tubulin, and is indicated below each lane [expressed relative to the mean signal intensity in control (LacZ) muscles]. Note that the anti-PGC-1α antibody detects a protein isoform of ~115 kDa, corresponding to full-length PGC-1α (also called PGC-1α1) (48, 49).

Figure 6. Increased expression of Ptm1 enhances the expression of genes important for mitochondrial biogenesis and oxidative function. Total RNA was harvested from whole LacZ and Perm1 TA muscles. A–C) RNA levels for the indicated genes were determined by RT-quantitative PCR, normalized to GAPDH levels, and expressed relative to the levels of each gene in control (LacZ) muscles. Note that the primers used to quantify PGC-1α expression detect all PGC-1α isoforms, and it is thus unclear whether Perm1 affects differentially the different isoforms (47–49). Data are the means ± se (n = 14). *P < 0.05; **P < 0.01. D) Protein levels of PGC-1α, ERα, Sirt3, and myoglobin (Mb) were determined by Western blot analysis of whole TA muscles from 4 mice, expressing LacZ (L1–L4) or Perm1 (P1–P4, with numbers indicating contralateral muscles from the same mouse). The intensity of the bands was quantified using ImageJ software, normalized to the loading control (tubulin), and is indicated below each lane [expressed relative to the mean signal intensity in control (LacZ) muscles]. Note that the anti-PGC-1α antibody detects a protein isoform of ~115 kDa, corresponding to full-length PGC-1α (also called PGC-1α1) (48, 49).

multiple nuclear transcriptional regulators, including PGC-1α, PGC-1β, NRF1, GABP, PPARα, PPARδ, and ERRs (13–15, 53–55). Perm1 also led to enhanced levels of PGC-1α and ERα, suggesting that the effects of Perm1 on gene expression are at least in part mediated through increases in these transcriptional regulators. Hence, whereas our initial observation that Perm1 is induced by PGC-1 and ERRs suggested that Perm1 acts as a downstream effector of PGC-1/ERR signaling (32), our current study shows that Perm1 can also act upstream of PGC-1/ERRs, by enhancing the expression (and possibly activity) of PGC-1α and ERα. This finding suggests the presence of a feed-forward regulatory loop, reminiscent of what has been shown for PGC-1α and other regulators (e.g., Mef2 factors) in skeletal muscle (13, 26). Based on these reciprocal interactions, we propose that the regulatory system controlling muscle oxidative capacity may be better viewed not as a linear pathway but rather as a network of interacting factors, where PGC-1α and ERRs induce the transcription of Perm1, and Perm1 promotes the expression of PGC-1α and ERα, thereby leading to long-term adaptive changes in skeletal muscle.

The exact molecular mechanism by which Perm1 leads to changes in gene expression is currently unclear. The Perm1 protein is predominantly cytoplasmic, does not harbor a transcriptional function, and does not physically interact with PGC-1α or ERα (32). Hence, we tested the possibility that Perm1 impacts on signaling pathways that control the activity of PGC-1α or ERR proteins. Although we did not see changes in the AMPK pathway, we observed that the increased Perm1 levels led to the activation of p38 MAPK during nighttime, when mice are physically active. We also found that acute expression of Perm1 in primary myotubes activated p38, suggesting that this activation is an early event in Perm1 action. The p38 MAPK gets activated in response to muscle contraction, and p38 activity in skeletal muscle is essential for exercise-induced PGC-1α expression and mitochondrial biogenesis (57, 58). Moreover, p38 phosphorylates and activates the PGC-1α protein and may thus act to drive a feed-forward loop of increased PGC-1α activity leading to higher PGC-1α expression (25, 26, 59, 60). Accordingly, it seems plausible that the Perm1-induced increase in muscle phospho-p38 activates the PGC-1α protein, resulting in enhanced PGC-1α/ERα expression and mitochondrial biogenesis. Interestingly, the increase in p38 activity is not seen during the day, when mice rest. This
PERM1 DRIVES SKELETAL MUSCLE OXIDATIVE METABOLISM IN VIVO

Increased Perm1 expression enhances fatigue resistance in skeletal muscle. A) Specific force generated at different frequency stimulations of EDL muscles injected with AAV1-LacZ (control) or AAV1-Perm1. Other contractile properties of the muscles are shown in Table 2. B) Relative force generation of LacZ and Perm1 EDL muscles during repeated stimulation for 3 min (with rest intervals between stimulations of 4 s for the first minute, 3 s for the second minute, and 2 s for the third minute). Fatigue curves were obtained from control (LacZ) or Perm1 EDL muscles. Force is expressed relative to force generated in the first contraction. C) Time to fatigue is defined here as time taken for each muscle to reach 40% of the force generated in the first contraction. Data are the means ± SE (n = 8). *p < 0.05.
TABLE 2. Contractile properties of LacZ- and Perm1-transduced EDL muscles

<table>
<thead>
<tr>
<th>Muscle weight (mg)</th>
<th>LacZ</th>
<th>Perm1</th>
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<tr>
<td>4.44 ± 0.16</td>
<td>4.23 ± 0.11</td>
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<tr>
<th>$L_0$ (mm)</th>
<th>10.09 ± 0.18</th>
<th>9.95 ± 0.16</th>
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<tbody>
<tr>
<td>$P_0$ (kPa)</td>
<td>244.43 ± 14.00</td>
<td>216.45 ± 10.01</td>
</tr>
<tr>
<td>$P_i$ (kPa)</td>
<td>71.04 ± 3.94</td>
<td>70.37 ± 3.50</td>
</tr>
<tr>
<td>TPT (ms)</td>
<td>26.75 ± 0.90</td>
<td>25.25 ± 0.70</td>
</tr>
<tr>
<td>$RT_{1/2}$ (ms)</td>
<td>63.88 ± 6.11</td>
<td>65.00 ± 4.87</td>
</tr>
<tr>
<td>$F_{50}$ (Hz)</td>
<td>25.21 ± 1.70</td>
<td>23.33 ± 0.98</td>
</tr>
</tbody>
</table>

Data are the means ± s.e. $F_0$, frequency to evoke 50% of maximal tetanic force; $L_0$, length that promotes maximal isometric tension; $P_0$, specific maximal isometric tetanic force; $P_i$, specific twitch peak force; $RT_{1/2}$, twitch half-relaxation time; TPT, time to peak twitch tension. kPa, kilopascal.

contractile twitch properties, the force-frequency relationship, or maximal tetanic force. The lack of impact of Perm1 on muscle fiber-type composition is surprising, given the increase in PGC-1α, which is known to increase type I and IIa fibers (20). Possibly, the increase in PGC-1α expression in our Perm1 model is much more moderate than the one achieved in PGC-1α transgenic mice, thereby leading to the activation of a subset of pathways that can be activated by PGC-1α. In addition, the timing of PGC-1α increase (postnatally in our model versus throughout development in the PGC-1α transgenic mice) and/or posttranslational modifications of PGC-1α (via the altered p38 signaling in Perm1-expressing mice) may restrict PGC-1α action at select target genes. Notably, mice lacking muscle p38α also show defects specifically in mitochondrial biogenesis and not in fiber-type switching in response to training (58). Moreover, mice that express the gain-of-function R225Q mutant of the AMPKγ3 subunit show a selective enhancement of oxidative activity, with no changes in fiber-type composition (64). Thus, it seems likely that Perm1 (like p38α and the R225Q mutant AMPKγ3) regulates a subset of pathways that respond to exercise.

Endurance exercise is an effective way of improving muscle performance and metabolic health, with some of the beneficial effects attributed to enhanced skeletal muscle oxidative capacity (1). Consistent with this notion, muscle-specific expression of PGC-1α or ERRγ transgenes enhances mitochondrial oxidative capacity, ameliorates symptoms of diseases affecting muscle health, and delays age-related loss of muscle function (6–9). Perm1 provides an alternate means for enhancing mitochondrial oxidative capacity, likely having overlapping but not identical actions to those of PGC-1α and ERRγ. Future experiments will determine the capacity of Perm1 to improve muscle function in mouse disease models.

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