Endurance exercise is known to induce metabolic adaptations in skeletal muscle via activation of the transcriptional co-activator peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α). PGC-1α regulates mitochondrial biogenesis via regulating transcription of nuclear-encoded mitochondrial genes. Recently, PGC-1α has been shown to reside in mitochondria; however, the physiological consequences of mitochondrial PGC-1α remain unknown. We sought to delineate if an acute bout of endurance exercise can mediate an increase in mitochondrial PGC-1α content where it may co-activate mitochondrial transcription factor A to promote mtDNA transcription.

Endurance exercise increases mitochondrial PGC-1α content and promotes nuclear-mitochondrial cross-talk to coordinate mitochondrial biogenesis.

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Physical inactivity is a major threat to public health worldwide. It is a primary modifiable risk factor for sarcopenia, cardiovascular diseases, type 2 diabetes, obesity, stroke, hypertension, and other chronic diseases including colon and breast cancer, end-stage renal disease, osteoporosis, osteoarthritis, and neuromuscular and neurometabolic disorders (1). Given the dire consequences associated with sedentary living, public health initiatives and therapeutic strategies that improve independent living and promote a physically active lifestyle are an international priority. Indeed, there is incontrovertible evidence from epidemiological studies and randomized trials that illustrate that regular physical activity and endurance exercise reduces the risk of chronic diseases and physical disability in later life and even extends lifespan (1, 2). The therapeutic effects of endurance exercise are associated with the maintenance of homeostatic energy metabolism via mitochondrial biogenesis in various tissues including skeletal muscle, heart, brain, adipose tissue, and liver (3–4). Endurance exercise-mediated enhancement of skeletal muscle mitochondrial content and oxidative capacity is a well established phenomenon in physiology (3), yet the molecular mechanisms promoting this process have only recently begun to be unraveled (5).

Mitochondrial biogenesis is a complex process requiring the coordinated expression and assembly of ~1500 proteins encoded by both the nuclear and mitochondrial genomes (6, 7). Peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α)4 is regarded as a crucial regulator of mitochondrial biogenesis by virtue of its ability to co-activate and augment the expression and activity of several transcription factors that in turn bind to the promoters of distinct sets of nuclear-encoded mitochondrial genes (6, 8). PGC-1α is also indirectly involved in regulating the expression of mitochondrial DNA (mtDNA) transcription via increased expression of mitochondrial transcription factor A (Tfam) as executed by its co-activation of nuclear respiratory factor 1 (NRF-1) (9–11). In this manner, PGC-1α may act as a central regulator of the adaptive response to exercise by coordinating metabolic gene expression in both the nuclear and mitochondrial genomes. We and others have
shown that an acute bout of exercise induces an increase in PGC-1α mRNA and protein content in skeletal muscle (12–14). Both human and murine studies have shown that endurance exercise induces mitochondrial biogenesis by activating PGC-1α, yet the molecular mechanisms promoting this process have only recently begun to unravel (15). It is likely that exercise triggers a fine-tuned metabolic response that results in several post-translational modifications to PGC-1α by various metabolic sensors, including phosphorylation by 5′-AMP-activated protein kinase and deacetylation by sirtuin 1 (SIRT1) (16). An additional mechanism to acutely modulate PGC-1α activity involves a potential shift in its subcellular localization (13, 17, 18). Wright et al. (13) provide evidence that acute endurance exercise caused PGC-1α protein to translocate from the cytosol to the nucleus. The increase in nuclear PGC-1α abundance coincided with increased binding of NRF-1/2 to mitochondrial promoter sequences, the aim of the present study was to comprehensively examine the subcellular localization of PGC-1α in response to an acute bout of endurance exercise, a potent stimulus for the nuclear and mitochondrial DNA-encoded transcription of mitochondrial genes. Specifically, we investigated whether PGC-1α resides in skeletal muscle mitochondria in vivo and if acute exercise can cause a coordinated increase in the abundance of nuclear and mitochondrial PGC-1α. In addition, we also evaluated the sub mitochondrial localization of PGC-1α and interrogated whether PGC-1α is complexed with Tfam at the mtDNA transcription start sites. Our findings indicate that acute exercise mediates an increase in mRNA expression of nuclear and mitochondrial DNA-encoded mitochondrial genes, with no alterations in mtDNA replication. This up-regulation of mitochondrial genes in response to exercise coincides with the increase in abundance of PGC-1α in nuclear and mitochondrial fractions prepared from murine skeletal muscle. Within mitochondria, PGC-1α is not bound directly to mtDNA but is found in a complex with Tfam in the D-loop region of mtDNA. Overall, this suggests that an acute increase in nuclear and mitochondrial PGC-1α may facilitate a coordinated nuclear-mitochondrial cross-talk to promote net mitochondrial biogenesis in response to physiological stressors such as exercise.

**Experimental Procedures**

**Animal Housing and Exercise Protocol**—C57BL/6J mice, bred in an institutional central animal facility (McMaster University), were housed in micro-isolator cages in a temperature- and humidity-controlled room and maintained on a 12-h light-dark cycle with food and water ad libitum (20). At 3 months of age, mice (n = 12/group; ♀ = ♂) were matched for body weight and randomly assigned to either sedentary (SED), forced-endurance (END) exercise, or forced endurance followed by 3 h of recovery (END+3h) group. None of the mice had been previously exposed to a structured exercise regime. Both the END and END+3h groups were subjected to an acute bout of treadmill (Eco3/6 treadmill; Columbus Instruments, Columbus, OH) running at 15 m/min for 90 min (20). All of the mice in the END and END+3h exercise groups completed the 90-min trial and were visibly exhausted (i.e. mouse will sit at the lower end of the treadmill, on the shock bar, for 5 s) (20). The END exercise group was sacrificed immediately after exercise. The SED group served as a control group for the analyses and was euthanized by cervical dislocation at the same time as the END+3h group. Quadriceps femoris muscle (~70 mg) was extracted from all mice into RNase-free cryovials, immediately snap-frozen, and stored at −80°C for subsequent mRNA expression, mtDNA copy number, and whole muscle protein analyses. ~200 mg of fresh quadriceps femoris was utilized for nuclear and mitochondrial fractionation, and ~20 mg of fresh tibialis anterior was used for ChIP assay. The study was approved by the McMaster University Animal Research and Ethics Board (Animal Utilization Protocol 05-03-11), and the experimental protocol strictly followed guidelines put forth by Canadian Council of Animal Care.

**RNA Isolation**—Total RNA was isolated from ~25 mg of quadriceps femoris using RNeasy® Mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions (20). RNA samples were treated with TURBO DNA-freeTM (Ambion Inc., Austin, TX) to remove DNA contamination. RNA integrity and concentration was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA) (20). The average RIN (RNA integrity number) value for all samples was 9.5 ± 0.2 (scale 1–10), indicating high quality of isolated RNA.

**mRNA Expression Analyses**—The mRNA expression of PGC-1α, Tfam, pyruvate dehydrogenase kinase 4 (PDK4), cytochrome c, 5-aminolevulinate synthase, citrate synthase, NADH dehydrogenase subunit 1 (ND1), NADH dehydrogenase subunit 4 (ND4), and cytochrome c oxidase subunits I and IV (COX-I and COX-IV) were quantified using 7300 Real-time PCR System (Applied Biosystems Inc., Foster City, CA) and SYBR® Green chemistry (PerFeCTa SYBR® Green Supermix, ROX, Quanta BioSciences, Gaithersburg, MD) as previously described (20). First-strand cDNA synthesis from 1 μg of total RNA was performed with random primers using a high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s directions (20). Forward and reverse primers (supplemental Table S1) for the aforementioned genes were designed based on sequences available in GenBankTM using the MIT Primer 3 designer software and were confirmed for specificity using the basic local alignment search tool. β2-microglobulin was used as a control housekeeping gene, the expression of which did not alter between groups. All samples were run in duplicate simultaneously with negative control that contained no cDNA. Melting point dissociation curves generated by the instrument was used to confirm the specificity of the amplified product.
**Total DNA Isolation**—Total DNA (genomic and mtDNA) was isolated from ~15 mg of quadriceps femoris using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions. DNA samples were treated with RNase (Fermentas, Mississauga, ON, Canada) to remove RNA contamination. DNA concentration and quality was assessed using Nanodrop 2000 (Thermo Scientific, Wilmington, DE).

**mtDNA Copy Number Analysis**—mtDNA copy number, relative to the diploid chromosomal DNA content, was quantitatively analyzed in quadriceps femoris using mtDNA copy number, relative to the diploid chromosomal DNA content, was quantitatively analyzed in quadriceps femoris using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions. DNA samples were treated with RNase (Fermentas, Mississauga, ON, Canada) to remove RNA contamination. DNA concentration and quality was assessed using Nanodrop 2000 (Thermo Scientific, Wilmington, DE).

**Whole Muscle Homogenization**—Total protein was extracted from frozen skeletal muscle samples as detailed previously (20). Briefly, ~30 mg of quadriceps muscle was homogenized on ice in a 2-ml Wheaton glass homogenizer (Fisher) with 25 volumes of radiomunoprecipitation assay homogenization buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS supplemented with protease inhibitor mixture (Complete Mini, EDTA-free, Roche Applied Science) and phosphatase inhibitor mixture (PhosSTOP, Roche Applied Science)). The lysate was mixed by end-over-end inversion for 30 min at 4 °C followed by centrifugation at 14,000 × g for 25 min at 4 °C to separate cellular debris. The supernatant was separated into aliquots, snap-frozen in liquid nitrogen, and stored at −80 °C for further analysis. The BCA protein assay (Pierce) was used to quantify the total protein content of samples.

**Nuclear and Cytosolic Fractionation**—Nuclear and cytosolic fractions were prepared from freshly obtained skeletal muscle (quadriceps femoris) using a commercially available nuclear extraction kit (Pierce NE-PER®) as previously described (17, 18). Briefly, ~50 mg of fresh skeletal muscle was homogenized in CER-I buffer containing protease inhibitor mixture Complete, EDTA-free (Roche Applied Science) using an electronic homogenizer (Pro 250, Pro Scientific, Oxford, CT). Pellets containing nuclei were isolated by centrifugation at 16,000 × g for 10 min at 4 °C and were subsequently washed 4 times in PBS to remove cytosolic contaminating proteins. Nuclear proteins were extracted in high salt NER buffer supplemented with protease inhibitors according to the manufacturer’s instructions. To obtain pure cytosolic fraction (S100 cytosolic extract), the cytosolic fraction was spun at 100,000 × g (Optima™ TLX Benchtop Ultracentrifuge, Beckman Coulter, Mississauga, ON, Canada) for 10 min at 4 °C, and the supernatant was collected (S100 fraction).

**Chromatin Immunoprecipitation Assay—**ChIP assay was performed using an EZ-ChIP™ kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. A 20-mg piece of tibialis anterior muscle was cross-linked in 5 ml of phosphate-buffered saline containing 1% formaldehyde for 10 min at room temperature. 1 ml of 10× glycine was added to stop fixation. Muscles were homogenized in 1 ml of SDS lysis buffer supplemented with protease inhibitor mixture Complete, EDTA-free (Roche Applied Science). Chromatin was sheared by sonicating each sample on ice using a Branson Digital Sonifer® S-450D (output 20%, 4 times for 20 s, with a 20 s pause each time; Branson Ultrasonics Corp., Danbury, CT). After centrifugation at 10,000 × g at 4 °C for 10 min, the supernatant containing 1 mg of protein was diluted to 1 ml with dilution buffer. 10 μg of anti-NRF-1 or anti-IgG antibody was added per sample and incubated overnight at 4 °C. Anti-IgG antibodies were used as a nonspecific control. 60 μl of protein G-agarose was added, and the sample was mixed for 1 h at 4 °C with rotation. Precipitated complexes were eluted in 100 μl of elution buffer, and cross-linking was reversed by the addition of 8 μl of 5 M NaCl per sample followed by incubation at 65 °C for 10 h. Co-immunoprecipitated DNA was purified according to the manufacturer’s instructions. Primers were designed to amplify the NRF-1 binding region of the cytochrome c promoter (supplemental Table S1). The amount of cytochrome c promoter immunoprecipitated with NRF-1 was quantified using 7300 Real-time PCR System (Applied Biosystems) and SYBR® Green chemistry (PerfeCTa SYBR® Green Supermix, ROX, Quanta BioSciences). Purified DNA from the input sample that did not undergo immunoprecipitation was PCR-amplified using β-globin primers and used to normalize signals from ChIP assays.

**Mitochondrial Fractionation**—Mitochondrial fractions were isolated using conventional differential centrifugation protocol. Briefly, ~150 mg of skeletal muscle (quadriceps femoris) was minced and homogenized on ice in 1:10 (w/v) ice-cold isolation buffer A (10 mM sucrose, 10 mM Tris/HCl, 50 mM KCl, and 1 mM EDTA, and 0.2% fatty acid-free BSA, pH 7.4, supplemented with protease inhibitor mixture Complete, EDTA-free (Roche Applied Science)) using a Potter-Elvehjem glass homogenizer. The resulting homogenates were centrifuged for 15 min at 700 × g, and the resulting supernatants were centrifuged for 20 min at 12,000 × g. The mitochondrial pellets from the 12,000 × g spin were washed twice and then re-suspended in a small volume of ice-cold isolation buffer B (10 mM sucrose, 0.1 mM EGTA/Tris, and 10 mM Tris/HCl, pH 7.4, supplemented with protease inhibitor mixture Complete, ETDA-free (Roche Applied Science)). All centrifugation steps were carried out at 4 °C. Enriched mitochondrial fraction was further purified on Percoll® (Sigma) gradient as previously described (21). To eliminate the possible presence of nuclear DNA contaminants, Percoll®-purified mitochondria were incubated for 1 h at 4 °C in the presence of DNase I (Pierce NE-PER®), and reaction was then stopped by the addition of 5 mM EDTA, pH 8.0. In addition, to prevent contamination of isolated mitochondria with cytosolic proteins weakly bound to the cytosolic-face of the mitochondrial outer membrane, Percoll®-purified mitochondria were mildly treated with 0.05 μg/ml proteinase K (Fermentas Life Science, Burlington, ON, Canada) for 10 min at 25 °C, and the reaction was then stopped by adding 2 ml phenylmethylsulfonyl fluoride (Sigma) (22). The mitochondrial pellets from a subset of animals were washed and immediately frozen at −80 °C for further biochemical analysis. The rest of the mitochondrial pellets were immediately cross-linked with 1% formaldehyde for 10 min at 4 °C followed by 10× glycine treatment to stop the cross-linking reaction and shearing on ice using a Branson Digital Sonifer® S-450D (output 20%, 10 times for 25 s with a 20-s pause each time). After sonication, the mitochondria...
Mitochondrial Pellets were immediately frozen at −80 °C for further mtDNA co-immunoprecipitation analyses.

**Mitochondrial Subfractionation**—Mitochondria were subfractionated using a phosphate swelling-shrinking procedure (23, 24) with minor modifications. Briefly, Percoll®-purified mitochondria were subjected to swelling by the addition of swelling buffer (10 mM KH₂PO₄, pH 7.4, supplemented with protease inhibitor mixture Complete, ETDA-free) at a concentration of 200 μg of mitochondrial protein/ml and incubated for 20 min at 4 °C with gentle mixing. Subsequently, an equal volume of shrinking buffer (10 mM KH₂PO₄, pH 7.4, 32% sucrose, 30% glycerol, 10 mM MgCl₂, supplemented with protease inhibitor mixture Complete, ETDA-free) was added, and samples were incubated for an additional 20 min at 4 °C. The suspension was centrifuged at 10,000 × g for 10 min, yielding a supernatant-containing outer membrane and the intermembrane-space fractions (mixture 1) and a pellet containing mitoplasts. The mitoplasts were washed three times in the 1:1 mixture of swelling and shrinking buffer, then re-suspended in 500 μl of swelling buffer and sonicated using a Branson Digital Sonifier® S-450D (output 20%, 4 times for 15 s, with a 30-s pause each time). The resulting suspension contained the inner membrane and matrix-enriched fractions (mixture 2). Mixtures 1 and 2 were centrifuged at 150,000 × g (Optima™ TLX Ultracentrifuge) for 60 min. The supernatant from mixture 1 was extracted as the intermembrane space fraction, and the supernatant from mixture 2 was extracted as the matrix-enriched fraction. These supernatant fractions were concentrated using protein filter concentrators (9000 molecular weight cut-off, Pierce) and diluted in gel sample buffer. The outer membrane pellet in mixture 1 and the inner membrane pellet in mixture 2 were washed once in swelling buffer and centrifuged at 150,000 × g for 60 min. The outer and inner membrane pellets were solubilized in radioimmune precipitation assay homogenization buffer.

**Mitochondrial Trypsin Digestion**—For trypsin digestion, 100 μg of Percoll®-purified mitochondria were treated with 200 μg/ml trypsin in 200 μl of digestion buffer (10 mM sucrose, 0.1 mM EGTA/Tris, and 10 mM Tris/HCl, pH 7.4) for 20 min at 4 °C. The reaction was stopped by adding protease inhibitor mixture Complete Mini, ETDA-free (Roche Applied Science) along with 500 μg/ml soybean trypsin inhibitor (Sigma) to the reaction mixture. The mitochondria were centrifuged at 20,800 × g for 5 min, and mitochondrial extracts were obtained by incubating mitochondria for 30 min at 4 °C in radioimmune precipitation assay homogenization buffer.

**Mitochondrial Respiration**—Samples of isolated mitochondria were incubated with 200 μl of respiration buffer (250 mM sucrose, 50 mM KCl, 25 mM Tris-HCl, 10 mM K₂HPO₄, pH 7.4) at 30 °C in a water-jacketed respiratory chamber with continuous stirring. Respiration rates (nmol of O₂ min⁻¹·mg of protein⁻¹) were evaluated in the presence of exogenous 10 mM glutamate (state 4 respiration) and 0.44 mM ADP (state 3 respiration) with the use of a Clark oxygen electrode (Model 782 1-2 Channel Oxygen Meter, Harvard Apparatus, Holliston, MA) as previously described (25). To assess the integrity of mitochondrial membrane, 3 mM of NADH was added, and change in respiration rate was assessed.

**Mitochondrial Co-immunoprecipitation Assay**—Mitochondrial co-immunoprecipitation assay was performed on isolated mitochondrial fractions using the Pierce co-immunoprecipitation kit according to the manufacturer’s instructions. Briefly, mitochondrial fractions were homogenized in lysis buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% Nonidet P-40, 5% glycerol, pH 7.4) supplemented with protease inhibitor mixture Complete, ETDA-free (Roche Applied Science). 2 mg of protein from mitochondrial fraction was precleared by incubation with 100 μl of control agarose resin to minimize nonspecific binding. 40 μg of anti-PGC-1α (H-300) or anti-Tfam (A-17) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were covalently coupled onto an amine-reactive resin. The precleared lysates were subsequently incubated with antibody-coupled beads overnight at 4 °C. Co-immunoprecipitates were collected by centrifugation, boiled in 50 μl of Laemmli sample buffer, and used for immunoblot analysis for Tfam or PGC-1α. Anti-IgG antibodies were used as a nonspecific control.

**mtDNA Immunoprecipitation Assay**—mtDNA immunoprecipitation was performed on skeletal muscle mitochondrial fraction that were cross-linked and sonicated as previously described (26). 1 mg of mitochondrial proteins were precleared in 25% v/v pre-clearing matrix F (Santa Cruz Biotechnology) overnight at 4 °C. The supernatant was then incubated with 20 μg of anti-PGC-1α (H-300) or anti-Tfam (A-17) antibody (Santa Cruz Biotechnology) and ExactaCruz™ F matrix (Santa Cruz Biotechnology) (27) with mixing by end-over-end inversion overnight at 4 °C in the presence of 5 μg of shared salmon sperm DNA (Sigma) to reduce nonspecific DNA-bead interactions. Anti-IgG antibodies were used as a nonspecific control. The matrix was centrifuged at 16,000 × g for 30 s, and the pellet matrix-immune complex precipitate was washed 4 times under stringent conditions (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 2 mM EDTA) and incubated overnight at 65 °C in the presence of 1% SDS for cross-linking reversion. DNA was extracted from supernatants using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions. The mtDNA D-loop region (supplemental Table S1) and non-D-loop region (COX-II) were quantified using 7300 Real-time PCR System (Applied Biosystems) and SYBR® Green chemistry (PerfeCta SYBR® Green Supermix, ROX, Quanta BioSciences) as previously described.

**Oligonucleotide Pulldown Assay**—Mitochondrial fractions were lysed in cold lysis buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100) supplemented with protease inhibitor mixture Complete, ETDA-free (Roche Applied Science). 500 μg of mitochondrial protein extracts were incubated with in silico-synthesized mtDNA D-loop biotinylated at 5’ (Integrated DNA Technologies®, San Diego, CA), and proteins were allowed to bind the oligonucleotide for 60 min at room temperature (28). The oligonucleotides were precipitated with UltraLink streptavidin beads (Pierce) for 4 h at 4 °C. Bound fractions were washed 3 times with wash buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100), eluted with Laemmli sample buffer, and used for immunoblot analysis for PGC-1α.

**Cytosolic Co-immunoprecipitation Assay**—Cytosolic co-immunoprecipitation assay was performed on isolated S100 cyto-
solic fractions using the Pierce co-immunoprecipitation kit according to the manufacturer’s instructions. Briefly, 2 mg of S100 cytosolic fraction was preclared with incubation with 100 μl of control agarose resin to minimize nonspecific binding. 40 μg of anti-PGC-1α (H-300) (Santa Cruz Biotechnology) were covalently coupled onto an amine-reactive resin. The pre- cleared lysates were subsequently incubated with antibody-coupled beads overnight at 4 °C. Co-immunoprecipitates were collected by centrifugation, boiled in 50 μl of Laemmli sample buffer, and used for immunoblot analysis for Tfam. Anti-IgG antibodies were used as a nonspecific control.

**Immunoblotting**—Proteins were resolved on 10 or 12.5% SDS-PAGE gels depending on the molecular weight of the protein of interest (20, 27, 29). The gels were transferred onto Hybond ECL nitrocellulose membranes (Amersham Biosciences) followed by blocking with 5% milk in TBS-Tween overnight at 4 °C. Immunoblotting was carried out using rabbit monoclonal PGC-1α, cytochrome c, and Bcl-2 antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA), goat polyclonal Tfam (1-17) antibody (1:800 dilution; Santa Cruz Biotechnology), rabbit polyclonal superoxide dismutase 2 and TOMM22 antibodies (1:6000 and 1:1000 dilution, respectively; Abcam®, Cambridge, MA), mouse monoclonal COX-IV antibody (1:1000 dilution, MitoSciences, Eugene, OR), and rabbit polyclonal citrate synthase antibody (1:1000 dilution, a kind gift of Dr. Brian H. Robinson, The Hospital for Sick Children, Toronto, ON, Canada). Membranes were then incubated with anti-rabbit or anti-goat horseradish peroxidase-linked secondary antibody (1:5000 dilution, Bio-Rad) and were visualized by enhanced chemiluminescence detection reagent (Amersham Biosciences). Relative intensities of the protein bands were digitally quantified by using NIH ImageJ, Version 1.37, analysis software (Scion Image, NIH). Actin (1:5000 dilution; BD Biosciences), histone H2B (1:1000 dilution; Abcam®), and voltage-dependent anion channel (VDAC) and lactate dehydrogenase A (1:1000 dilution; Cell Signaling Technology) were used as loading controls.

**Cytochrome c Oxidase Activity**—Mitochondrial electron transport chain COX (EC 1.9.3.1) activity (indicative of mitochondrial oxidative capacity) was determined by measuring the rate of oxidation of reduced cytochrome c, as previously described by our group (27). Briefly, stock cytochrome c (oxidized) was reduced by sodium ascorbate in 0.05 mM potassium phosphate buffer (KH2PO4, pH 7.4). Fifteen microliters of mitochondrial or nuclear fractions were added to 955 μl of 0.05 mM potassium phosphate buffer and 15 μl of reduced cytochrome c. Absorbance was recorded at 550 nm every 30 s for 3 min at 37 °C. All mitochondrial and nuclear fractions were analyzed in duplicate on a spectrophotometer (Cary Bio-300, Varion, Inc., Palo Alto, CA).

**In Silico Analyses**—The presence of mitochondrial targeting sequences was examined using MitoProt II (30) and TargetP (31).

**Statistical Analyses**—Data were analyzed using one-way analysis of variance using Statistica 5.0 software (Statsoft, Tulsa, OK). For all analyses, a two-tailed test was employed. We used Tukey’s Honestly Significant Difference test post-hoc to identify individual differences when statistical significance was observed. Statistical significance was established at a p ≤ 0.05. Data are presented as the means ± S.E.

**RESULTS**

**Purity of the Nuclear and Mitochondrial Fractions**—The purity of nuclear and mitochondrial fractions is of extreme importance for the subsequent subcellular localization analyses of PGC-1α carried out in this study. We rigorously confirmed the enrichment and purity of nuclear and mitochondrial fractions utilizing three independent analyses. First, a real-time PCR analysis of the abundance of β-globin and ND1 genes was carried out that confirmed the exclusive presence of β-globin and ND1 genes in nuclear and mitochondrial fractions, respectively (supplemental Fig. S1). Second, we assessed the protein content of histone H2B and VDAC in our nuclear and mitochondrial fractions. H2B and VDAC were only detected in nuclear and mitochondrial fractions, respectively, further confirming the purity of our fractions. There was no superoxide dismutase 2 detected in both the nuclear and cytosolic fractions.

**Exercise Increases Mitochondrial PGC-1α Content**

![Figure 1. Assessment of the purity of nuclear and mitochondrial fractions using Western blotting methodology.](https://example.com/fig1.png)
isolated mitochondria maintained the native structural environment and consequently retain mitochondrial integrity and function. These control experiments clearly document that our nuclear and mitochondrial fractions are sufficiently pure and can, therefore, be confidently utilized to study the effects of exercise on the subcellular localization of PGC-1α.

Validation of the PGC-1α Antibody—In this study we have utilized two different antibodies raised against PGC-1α: rabbit polyclonal PGC-1α (H-300) antibody (Santa Cruz Biotechnology) for immunoprecipitation assays and rabbit monoclonal PGC-1α antibody for immunoblotting analyses (Cell Signaling Technology). The specificity of anti-PGC-1α (H-300) antibody has been recently confirmed (19). Because we used monoclonal PGC-1α antibody for immunoblotting analyses, we further characterized its specificity using wild-type C57Bl/6J mice skeletal muscle homogenates. We observed a prominent band at ~94 kDa and increased abundance of PGC-1α in slow oxidative muscle than mixed and fast glycolytic, which further confirms that the band we observe in our immunoblots indeed corresponds to PGC-1α protein (supplemental Fig. S2). We have previously tested and published the specificity of this antibody in human vastus lateralis whole muscle homogenates and have utilized it to study the effects of exercise on nuclear PGC-1α abundance in humans (17, 18).

Acute Endurance Exercise Increases mRNA Expression of Nuclear and Mitochondrial DNA-encoded Mitochondrial Transcripts without Altering mtDNA Copy Number—We observed an increase in the nuclear DNA (PGC-1α, Tfam, PDK4, COX-IV, citrate synthase, 5-aminolevulinate synthase, cytochrome c- and mtDNA- (ND1, ND4, and COX-I)-encoded transcripts immediately after an acute bout of endurance exercise (p < 0.05, Fig. 3A). The increase in mRNA expression of these transcripts was maintained or further enhanced 3 h after acute endurance exercise (END+3h) versus both the SED and END groups (p < 0.05, Fig. 3A). PDK4 mRNA expression is extremely sensitive to acute endurance exercise (14, 20). A robust up-regulation of PDK4 mRNA expression in both the END and END+3h groups confirms the acute bout mRNA effect in END and END+3h mice established in previous acute exercise studies (14, 20). We observed no net change in mtDNA copy number in SED, END, and END+3h groups (Fig. 3B). Together, the above results illustrate that altered energy demands, as a result of acute endurance exercise, positively modulate the expression of mitochondrial transcripts without altering mtDNA replication (which may occur as a latent phenomenon requiring multiple bouts of endurance exercise). We speculate that the immediate increase in the global mitochondrial transcriptome promotes homeostatic recovery and the adaptive response to physiological stressors such as exercise.

Time Course of Acute Endurance Exercise-induced Increase in Total PGC-1α Content—There is no immediate increase in total PGC-1α protein content in skeletal muscle from END versus SED groups (Fig. 4). However, PGC-1α protein content was significantly increased (2.4-fold, p < 0.05) 3 h after acute endurance exercise (END+3h) versus both the SED and END groups (Fig. 4). This result provides evidence that the initial adaptive increase in mitochondrial transcripts encoded by nuclear and mitochondrial genomes in END mice is not mediated by an endurance exercise-induced increase in total PGC-1α protein content.

An Acute Bout of Exercise Increases PGC-1α Nuclear and Mitochondrial Abundance—PGC-1α has been widely studied as a nuclear protein co-activating the transcription of nuclear DNA-encoded mitochondrial genes in response to endurance exercise (13, 17, 18, 34). Aquilano et al. (19) recently reported mitochondrial localization of PGC-1α in cells and in vivo in mouse tissues. We carried out Western blot analyses of PGC-1α in purified nuclear and mitochondrial fractions from mouse skeletal muscle and confirmed the presence of PGC-1α protein in both subcellular fractions (Fig. 5). To ensure that PGC-1α is contained within the mitochondria and to identify the sub mitochondrial localization of PGC-1α, we also subjected isolated mitochondria further subfractionation and trypsin digestion. The results clearly indicate that the mitochondrial PGC-1α is primarily localized in the matrix (Fig. 6). Both nuclear and mitochondrial content of PGC-1α were signifi-
Exercise Increases Mitochondrial PGC-1α Content

FIGURE 3. Acute exercise increases mRNA expression of nuclear and mitochondrial DNA-encoded mitochondrial transcripts without altering mtDNA copy number. A, shown are gene expression analyses of PGC-1α, Tfam, PDK4, nuclear DNA-encoded mitochondrial genes (CS, 5-aminolevulinate synthase (ALAS), cytochrome c, and COX-IV), and mtDNA-encoded mitochondrial genes (ND1, ND4, and COX-I) expression levels in skeletal muscle (quadriceps femoris) of END and END + 3h groups compared with SED mice (n = 10/group) at three months of age. B, mtDNA copy number in skeletal muscle (quadriceps femoris) from SED, END, and END + 3h mice (n = 10/group) is shown. mtDNA copy number is normalized to the diploid nuclear genome marker (β-globin). The asterisk (versus SED): *, p < 0.05; **, p < 0.01; †, p < 0.05 (versus both END and SED). Error bars represent S.E.

FIGURE 4. There is no increase in total PGC-1α content immediately after acute exercise. PGC-1α protein content in whole muscle homogenate (quadriceps femoris) of END and END + 3h compared with SED mice (n = 9/group) is shown. †, p < 0.05, versus both END and SED). Error bars represent S.E.

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Significantly increased (2.2- and 1.8-fold, respectively, p < 0.05) in the END versus SED group (Fig. 5). This subcellular increase was further exacerbated 3 h after acute endurance exercise, as depicted by the significantly higher nuclear and mitochondrial abundance of PGC-1α protein in END + 3h group when compared with both the SED and END groups (p < 0.05, Fig. 5). The increase in nuclear and mitochondrial abundance of PGC-1α immediately after exercise coincides with the initial adaptive augmentation of mitochondrial transcripts observed in END mice. H2B and VDAC protein content served as loading controls and were also used to evaluate the effect of exercise on the yield of nuclei and mitochondria. There were no differences in the quantity of nuclei or mitochondria extracted from resting and exercised muscles (Fig. 5).

Mitochondrial PGC-1α Is in a Complex with Tfam, an Association That Is Positively Modulated by Acute Exercise—Given that PGC-1α is a transcriptional co-activator and that no bona fide transcriptional co-activators of mtDNA transcription factors have been reported to date, we posited a possible association of mitochondrial PGC-1α as a transcriptional co-activator of the indispensable mitochondrial transcription factor Tfam. We performed co-immunoprecipitation assays in mitochondrial fractions from SED, END, and END + 3h groups using both anti-PGC-1α and anti-Tram antibodies to test this hypothesis. Mitochondrial PGC-1α was found to be in a complex with Tfam (Fig. 7, A and B). This observation is in congruence with the fact that both Tfam and PGC-1α share the same mitochondrial compartment (Fig. 6). The content of PGC-1α-Tfam complex is significantly higher in both the END and END + 3h groups versus SED (Fig. 7, A and B). To ensure specificity of PGC-1α-Tfam complex in mitochondria, we carried out similar co-immunoprecipitation in cytosolic S100 fractions from SED, END, and END + 3h groups. We did not observe any interaction between cytosolic PGC-1α and Tfam (Fig. 7C).
Together these results indicate that mitochondrial PGC-1α may act as a transcriptional co-activator and augment Tfam activity, an association that is highly dependent on altered energy demands (such as exercise stimulus) and is associated with the observed increase in the expression of mtDNA-encoded gene transcripts (Fig. 3A).

**Mitochondrial PGC-1α and Tfam Form a Complex at mtDNA D-loop Region That Is Positively Modulated by Acute Exercise**—PGC-1α does not have any known DNA binding domains and can only participate in protein-protein interactions with transcription factors and transcriptional machinery at the site of transcription initiation (35). mtDNA D-loop region is the site of transcription initiation for both mtDNA heavy and light strands and is the only domain of mtDNA that contains a Tfam binding consensus sequence (36). We hypothesized that if mitochondrial PGC-1α acts as a transcriptional co-activator, then it would complex with Tfam at the D-loop region only. We employed an mtDNA co-immunoprecipitation assay to assess whether PGC-1α-Tfam complex is bound to mtDNA and if this association is mtDNA D-loop-specific or nonspecific. PGC-1α forms a complex with Tfam specifically at the mtDNA D-loop region (Fig. 8A). We were unable to PCR-amplify any other regions of mtDNA (except mtDNA D-loop) after mtDNA immunoprecipitation that boosted a PGC-1α-Tfam complex (Fig. 8A). This association of PGC-1α-Tfam complex to mtDNA D-loop region was further confirmed using an oligonucleotide pulldown assay where PGC-1α-Tfam complex was precipitated with in silico-constructed mtDNA D-loop region (Fig. 8B). Furthermore, PGC-1α-Tfam complex at the D-loop region is positively modulated in response to acute exercise (END and END + 3 h groups) versus SED group (p < 0.05, Fig. 8, A and B). Because the mtDNA D-loop region contains mtDNA transcription start sites, these observations further strengthen the putative role of mitochondrial PGC-1α as a mitochondrial transcriptional co-activator for Tfam.

PGC-1α co-activates both NRF-1 and NRF-2, transcription factors that play a major role in skeletal muscle mitochondrial biogenesis in response to endurance exercise (6). To further study the dynamics of PGC-1α-mediated activation of nuclear DNA-encoded mitochondrial transcripts, we used a ChIP assay to quantify binding of NRF-1 to the NRF-1 response element in the cytochrome c promoter. NRF-1 DNA binding was increased immediately after acute exercise (4-fold, p < 0.01) and was further enhanced 3 h post-recovery from acute exercise bout (6.7-fold, p < 0.01) versus SED group (Fig. 8C). Together these results indicate that acute exercise induces an increase in the abundance of PGC-1α in nuclear and mitochondrial fractions that coincides with the early adaptive response to acute exercise in skeletal muscle, i.e. the increase in global mitochondrial gene transcription.

**DISCUSSION**

In this study we confirmed that PGC-1α, which has long been considered a nuclear specific transcription co-activator regulating transcription of nuclear DNA-encoded mitochondrial genes (6), also localizes in mitochondria purified from C67Bl/6 mouse skeletal muscle. To our knowledge we are the first to identify a novel process wherein there is an acute increase in the mitochondrial abundance of PGC-1α protein in response to a physiological stressor, that is, acute endurance exercise. Mitochondrial PGC-1α is primarily localized in the mitochondrial matrix where it forms a complex with Tfam only when Tfam is bound to the mtDNA D-loop region. This region of mtDNA possesses the light and heavy strand transcription start sites, thus, creating the tantalizing possibility of PGC-1α acting as a transcriptional co-activator of Tfam. We also observed enhanced binding of both the nuclear transcription factor (NRF-1) and mitochondrial transcription factor (Tfam) to their respective transcription initiation sites immediately after acute exercise, which coincides with the increase in both nuclear and mitochondrial DNA-encoded mitochondrial gene transcription. Taken together, we speculate that in response to acute endurance exercise and altered energy demands, cytosolic PGC-1α shuttles to nuclei and mitochondria where it acts as a transcriptional co-activator to transcription factors that coordinate mitochondrial biogenesis. We believe that this increase in the mitochondrial transcriptome is crucial for skeletal muscle homeostatic recovery and adaptations in response to endurance exercise. We propose that PGC-1α may act as a central regulator of mitochondrial biogenesis that orchestrates nuclear-mitochondrial cross-talk to co-ordinate mitochondrial biogenesis in skeletal muscle.
PGC-1α regulates the coordinated expression of the mitochondrial proteins that mediate substrate oxidation and ATP synthesis (6, 37–39). This is achieved via PGC-1α physically docking on and co-activating transcription factors that modulate the expression of genes encoding mitochondrial proteins (40, 41). Overexpression of PGC-1α increases mitochondrial content and oxidative capacity in muscle cells (37, 42). Similarly, transgenic overexpression of PGC-1α increases the mitochondrial content of skeletal muscle and mediates the conversion of low oxidative white muscle fibers to high oxidative red muscle fibers (42). Results from PGC-1α transgenic and knockout mice support a critical role for this protein in the regulation of exercise capacity and training adaptation (43). Muscle-specific PGC-1α overexpression in mice results in many of the same adaptations seen after exercise training, such as an increase in mitochondrial content, enhanced fat oxidation, reduced muscle glycogen utilization during exercise, and increased endurance capacity (44–46). In contrast, mice deficient in PGC-1α have reduced exercise capacity and are prone to fatigue (45).

Although the role of post-translational modifications in the modulation of PGC-1α activity with acute exercise has been documented (16), mechanistic studies of how endurance exercise affects the subcellular redistribution of activated PGC-1α are lacking. Exercise and/or muscle contraction results in rapid increases in PGC-1α mRNA and total PGC-1α protein in skeletal muscle (12–14, 17, 18, 27). Because PGC-1α overexpression in cells in vitro and in muscles of transgenic mice increases mitochondrial biogenesis (8), it was generally assumed that the exercise-induced stimulation of mitochondrial biogenesis is mediated by the increase in total cellular PGC-1α protein content. Studies in skeletal muscle (13, 34), adipose tissue (47), and brain (48) indicate that PGC-1α may reside in both the cytosol and nucleus, suggesting that dynamic regulation of subcellular localization may play a role in PGC-1α activity. Wright et al. (13) elegantly demonstrate that two 3-h sessions of acute endurance swimming in rodents led to elevated mRNA and protein expression of several mitochondrial genes before any increase in whole muscle PGC-1α protein. The authors reported that in resting rat skeletal muscle the majority of PGC-1α resided in the cytosol, and in response to acute endurance exercise, PGC-1α translocated from the cytoplasm to the nucleus (13). This coincided with increased DNA binding of transcription factors to the cytochrome c and COX IV promoter and enhanced mRNA expression of several mitochondrial genes (13). Therefore, it appears that the redistribution of PGC-1α from the cytosol to the nucleus is an important facet of the molecular mechanism underlying the adaptation to acute exercise in rodent skeletal muscle. Similarly, Little et al. (17) show an increase in the nuclear abundance and activation of PGC-1α in human skeletal muscle immediately after an acute bout of endurance exercise. Here, we demonstrate that in addi-
tion to the apparent nuclear shuttling, an acute bout of endurance exercise also results in increased abundance of PGC-1α in mitochondria. The presence of PGC-1α within the mitochondria suggests that it could exert similar functions in this cellular compartment as it does in the nucleus.

Aquilano et al. (19) recently reported binding of PGC-1α with Tfam in vitro. Here, we also observed that mitochondrial PGC-1α forms a complex with Tfam in skeletal muscle but only at the mtDNA D-loop region. The absence of PGC-1α binding directly to mtDNA is in agreement with its lack of DNA binding domains (35). In addition to its role in mtDNA transcription, Tfam has also been implicated in mtDNA maintenance and integrity by acting as a histone-like protein for the otherwise naked mtDNA (36). However, the association of Tfam to mtDNA is nonspecific, except for the mtDNA D-loop region, which contains consensus sequence sites for Tfam binding that are crucial for mtDNA transcription (36). We postulate that the binding of Tfam to its consensus sequence in the D-loop region mediates allosteric changes that may promote binding of PGC-1α to Tfam. Such allosteric alterations have been reported for other transcription factors when bound to their sequence recognition site in DNA (49, 50). We hypothesize that these conformational changes may not occur when Tfam is non-specifically bound to mtDNA. This allosteric modulation of Tfam structure may regulate the functional consequence of Tfam bound to mtDNA, i.e. Tfam as a transcription factor or a histone-like protein important to maintaining mtDNA integrity (36). The presence of PGC-1α-Tfam complex only at D-loop region, which contains mtDNA heavy and light strand promoter sequences, further strengthens the concept of mitochondrial PGC-1α as a mtDNA transcriptional co-activator.

Proteomic data suggest that nearly half of the nuclear-encoded proteins associated with mitochondria lack canonical N-terminal mitochondrial targeting signal but instead have cryptic internal signals (51–53). These cryptic signals typically lack consistent patterns, and the nature of these signals remains largely elusive (53). Bioinformatic in silico analyses using currently available software (MitoProt II and TargetP) reveal that PGC-1α does not contain a canonical mitochondrial targeting signal but instead have cryptic internal signals (51–53). These cryptic signals typically lack consistent patterns, and the nature of these signals remains largely elusive (53). Bioinformatic in silico analyses using currently available software (MitoProt II and TargetP) reveal that PGC-1α does not contain a canonical mitochondrial targeting signal but instead have cryptic internal signals (51–53). 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ing events that lead to PGC-1α mitochondrial import and the underlying mitochondrial targeting mechanism will be of great interest for future studies. Studies from Wright et al. (13) and Cantó et al. (16) illustrate that phosphorylation of PGC-1α by p38 MAPK and 5′-AMP-activated protein kinase and deacetylation of PGC-1α by SIRT1 are crucial events for the full activation of PGC-1α in response to acute exercise. We speculate that nuclear and mitochondrial shuttling of PGC-1α may be regulated by similar post-translational modifications shown to be necessary for its activation. Recent studies have shown that mitochondria have resident kinases (MAPK, Akt, PKA, and PKC) and metabolic regulators (SIRT1, SIRT3, and SIRT5) that play a vital role in regulating mitochondrial metabolism (56, 57), and it is conceivable that they may also modulate PGC-1α activity via post-translational modifications. In addition, acute endurance exercise results in the production of reactive oxygen species that activate stress kinases, most notably p38 MAPK and ERK-1/2, which promote an increase in both mitochondrial and antioxidant enzymes in skeletal muscle (58). Indeed, in vitro and in vivo studies have shown that p38 MAPK can modulate both the activity and mRNA expression of PGC-1α (59). Reactive oxygen species can also directly modulate the activity of PGC-1α (60). Mitochondria are the primary site of reactive oxygen species generation in times of stress and increased metabolism, some of which can get released into the cytosol (61). Thus, we speculate that changes in cellular redox status due to reactive oxygen species release immediately upon acute exercise may also act as a chemo-attractant to target PGC-1α to the mitochondrial pool. Last, bioinformatics analyses indicate the presence of classic RNA binding domain in the C terminus of PGC-1α (62). Studies have shown that proteins containing RNA binding domains are involved in most post-transcriptional gene expression processes such as mRNA and rRNA processing, RNA export, and stability (63, 64). Furthermore, this domain is not only involved in RNA/DNA recognition but also in protein-protein interactions (64). Hence, the possibility exists that PGC-1α interacts with proteins that have mitochondrial targeting sequences and subsequently gets imported into the mitochondria along with them. PGC-1α may also interact with the TOM-TIM complex (integral for mitochondrial import) via its RNA binding domain and, thus, get imported.

Our study strengthens the role of PGC-1α as a central regulator of mitochondrial biogenesis and energy metabolism in skeletal muscle in response to acute endurance exercise and provides new insight into the regulatory mechanisms govern-
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FIGURE 9. Prediction of the probability of mitochondrial targeting of known mitochondrial proteins using MitoProt II and TargetP. Note that well-known mitochondrial proteins such as COX-IV, mitochondrial Tafm, superoxide dismutase 2 (SOD2), citrate synthase (CS), and SIRT3 possess canonical mitochondrial targeting signal and, thus, have a higher probability of mitochondrial translocation. In contrast, newly discovered mitochondrial-located proteins such as PGC-1α, SIRT1, superoxide dismutase 1 (SOD1), and protein kinase B (Akt) possess cryptic mitochondrial targeting signal and, thus, show a lower probability of mitochondrial localization using the above in silico prediction tools.

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