

# Exercise-induced Mitochondrial Biogenesis Begins before the Increase in Muscle PGC-1 $\alpha$ Expression\*

Received for publication, June 26, 2006, and in revised form, October 4, 2006 Published, JBC Papers in Press, November 12, 2006, DOI 10.1074/jbc.M606116200

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Exercise results in rapid increases in expression of the transcription coactivator peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) and in mitochondrial biogenesis in skeletal muscle. PGC-1 $\alpha$  regulates and coordinates mitochondrial biogenesis, and overexpression of PGC-1 $\alpha$  in muscle cells results in increases in mitochondrial content. In this context, it has been proposed that the increase in PGC-1 $\alpha$  protein expression mediates the exercise-induced increase in mitochondrial biogenesis. However, we found that mitochondrial proteins with a short half-life increase as rapidly as, or more rapidly than, PGC-1 $\alpha$  protein. This finding led us to hypothesize that activation, rather than increased expression, of PGC-1 $\alpha$  mediates the initial phase of the exercise-induced increase in mitochondria. In this study, we found that most of the PGC-1 $\alpha$  in resting skeletal muscle is in the cytosol. Exercise resulted in activation of p38 MAPK and movement of PGC-1 $\alpha$  into the nucleus. In support of our hypothesis, binding of the transcription factor nuclear respiratory factor 1 (NRF-1) to the cytochrome *c* promoter and NRF-2 to the cytochrome oxidase subunit 4 promoter increased in response to exercise prior to an increase in PGC-1 $\alpha$  protein. Furthermore, exercise-induced increases in the mRNAs of cytochrome *c*,  $\delta$ -aminolevulinic synthase, and citrate synthase also occurred before an increase in PGC-1 protein. Thus, it appears that activation of PGC-1 $\alpha$  may mediate the initial phase of the exercise-induced adaptive increase in muscle mitochondria, whereas the subsequent increase in PGC-1 $\alpha$  protein sustains and enhances the increase in mitochondrial biogenesis.

Exercise is a powerful inducer of mitochondrial biogenesis in skeletal muscle (1, 2). Mitochondrial biogenesis involves the orchestrated expression of the mitochondrial genome and the nuclear genes that encode mitochondrial proteins. Discovery of nuclear receptors and transcription factors that activate the genes encoding mitochondrial proteins and of the transcription

coactivator peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ )<sup>4</sup> has provided major new insights regarding how increases in mitochondrial biogenesis are mediated and coordinated (3–5). PGC-1 $\alpha$  docks on and coactivates the transcription factors that regulate expression of nuclear genes encoding mitochondrial proteins and that induce expression of mitochondrial transcription factor A, which regulates mitochondrial DNA transcription, thus activating the coordinated expression of mitochondrial proteins (3–6).

A bout of exercise or stimulation of muscle contractions induces increases in PGC-1 $\alpha$  mRNA and protein in skeletal muscle (7–11). It has been assumed that the exercise-induced increase in PGC-1 $\alpha$  expression mediates the increase in mitochondrial biogenesis (7–12). However, when we examined the time course of this adaptive response, we found that mitochondrial proteins with short half-lives increase in parallel with the increase in PGC-1 $\alpha$  protein. In this context, the purpose of the present study was to evaluate the hypothesis that the initial phase of the exercise-induced adaptive increase in mitochondria is mediated by activation, rather than increased expression, of PGC-1 $\alpha$ . Our findings that increases in the binding of NRF-1 to the cytochrome *c* promoter and NRF-2 to the cytochrome oxidase subunit 4 promoter, as well as the increases in the mRNAs of cytochrome *c*,  $\delta$ -aminolevulinic synthase (ALAS), and citrate synthase, precede the increase in PGC-1 $\alpha$  protein support our hypothesis.

## EXPERIMENTAL PROCEDURES

**Materials**—Reagents for SDS-PAGE were from Bio-Rad. Reagents for ECL were obtained from Amersham Biosciences. TRIzol reagent for isolation of RNA was purchased from Invitrogen. Reagents for the isolation of mRNA were obtained from Ambion (Austin, TX). Nuclear isolation kits were obtained from Pierce. The anti-PGC-1 antibody directed against amino acids 777–797 in the carboxyl terminus was purchased from Calbiochem. Anti-phospho p38 MAPK and ATF-2 (activating transcription factor 2) antibodies were products of Cell Signaling (Beverly, MA). Mouse anti-human monoclonal antibody against succinate-ubiquinone oxidoreductase (SUO) 70-kDa subunit was obtained from Molecular Probes (Eugene, OR). A rabbit polyclonal antibody directed against the 19 carboxyl-terminal amino acids of  $\delta$ -ALAS was generated by Alpha

\* This research was supported by National Institute of Health Grant AG00425. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Initially supported by National Institute on Aging Institutional National Research Service Award AG00078, and subsequently by individual NRSA DK070425.

<sup>2</sup> Supported by National Institute on Aging Institutional National Research Service Award AG00078.

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<sup>4</sup> The abbreviations used are: PGC-1 $\alpha$ , peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$ ; NRF-1, nuclear respiratory factor 1; NRF-2, nuclear respiratory factor 2; COXIV, cytochrome oxidase subunit 4; ALAS,  $\delta$ -aminolevulinic synthase; p38 MAPK, p38 mitogen-activated protein kinase; ATF-2, activating transcription factor 2; SUO, succinate-ubiquinone oxidoreductase; ChIP, chromatin immunoprecipitation.

Diagnostics International (San Antonio, TX). NRF-1 and NRF-2 antibodies were obtained from Rockland (Gilbertsville, PA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The antibody for lactate dehydrogenase was purchased from Sigma. The antibody against histone H2B was obtained from Imgenex (San Diego, CA). Anti-estrogen-related receptor  $\alpha$  antibody (13) was a gift from Daniel Kelly (Washington University, St. Louis, MO). Horseradish peroxidase-conjugated secondary antibodies were from the Jackson Laboratory. All other reagents were purchased from Sigma. ChIP assay kits were purchased from Upstate Biotechnology (Lake Placid, NY).

**Animals and Exercise Program**—Male Wistar rats (150–180 grams) were obtained from Charles River and maintained on a diet of Purina rat chow and water. To evaluate the effects of exercise on mitochondrial biogenesis, some rats were exercised by swimming for 6 h using a modification of the protocol of Plough *et al.* (14) in which the exercise session was divided into two 3-h bouts separated by 45 min of rest during which time the rats were kept warm and given food and water, as described previously (15). Other rats were exercised by swimming for 2 h. Rats were anesthetized with 5 mg/100 g of body weight sodium pentobarbital given intraperitoneally. Triceps and epitrochlearis muscles were dissected out and trimmed of fat and connective tissue. Some triceps muscles were used immediately for isolation of nuclei. The other muscles were clamp-frozen in aluminum tongs cooled to the temperature of liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . This protocol was approved by the Animal Studies Committee of Washington University.

**Preparation of Nuclear Extracts**—Triceps muscles were homogenized in 10 volumes of Pierce cytosolic extraction reagent supplemented with 1% Nonidet P-40; 0.25% sodium deoxycholate; 1 mM each EDTA, phenylmethylsulfonyl fluoride, NaF; 1  $\mu\text{g}/\text{ml}$  each aprotinin, leupeptin, and pepstatin; 0.1 mM bis-peroxovanadium, 1,10-phenanthroline; 25  $\mu\text{M}$  okadaic acid; and 2 mg/ml  $\beta$ -glycerophosphate aprotinin, leupeptin, and phenylmethylsulfonyl fluoride. Nuclear and cytosolic fractions were isolated according to the manufacturer's specifications using a Pierce nuclear isolation kit.

**Western Blot Analysis**—Clamp-frozen epitrochlearis muscles were powdered and then homogenized in a 10:1 volume-to-weight ratio of ice-cold buffer containing: 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM each EDTA, phenylmethylsulfonyl fluoride, NaF, 1  $\mu\text{g}/\text{ml}$  each aprotinin, leupeptin, and pepstatin, 0.1 mM bis-peroxovanadium, 1,10-phenanthroline, 25  $\mu\text{M}$  okadaic acid, and 2 mg/ml  $\beta$ -glycerophosphate. The homogenates were sonicated on ice for 5 s. Samples were centrifuged for 15 min at  $700 \times g$  at  $4^{\circ}\text{C}$ . Nuclear and cytosolic fractions of triceps muscle were prepared as described above. Protein concentration was determined by the method of Lowry *et al.* (16). PGC-1 $\alpha$ , SUO, ALAS, p-P38 MAPK, p-ATF-2, lactate dehydrogenase, and histone H2B were measured by Western analysis using the appropriate primary and secondary antibodies as described previously (13, 17).

**ChIP Assay**—ChIP assays were performed using an EZ-ChIP kit from Upstate Biotechnology. Two epitrochlearis muscles were pooled for each sample and cross-linked in 10 ml of phos-

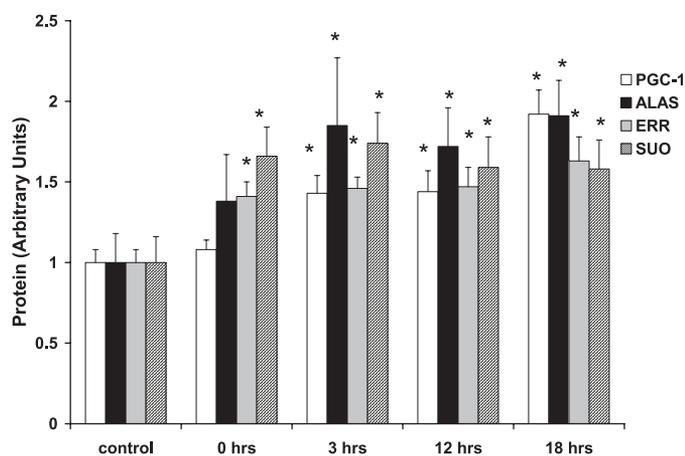
phate-buffered saline containing 1% formaldehyde for 10 min at room temperature. 1 ml of  $10\times$  glycine (Upstate Biotechnology) was added to stop fixation. Muscles were homogenized in 1 ml of SDS lysis buffer containing 5  $\mu\text{l}$  of Protease Inhibitor Mixture II (Upstate Biotechnology) in glass Kontes grinding tubes. Chromatin was sheared by sonicating each sample for  $4 \times 10$  s on ice. Following centrifugation at  $10,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min, supernatant containing 1 mg of protein was diluted to 1 ml with dilution buffer (Upstate Biotechnology). 5  $\mu\text{g}$  of anti-NRF-1, NRF-2, or anti-IgG antibody was added per sample and incubated overnight at  $4^{\circ}\text{C}$ . Anti-IgG antibodies were used as a nonspecific control. The following morning, 60  $\mu\text{l}$  of protein G-agarose was added, and the sample was mixed for 1 h at  $4^{\circ}\text{C}$  with rotation. Precipitated complexes were eluted in 100  $\mu\text{l}$  of elution buffer (Upstate Biotechnology), and cross-linking was reversed by the addition of 8  $\mu\text{l}$  of 5 M NaCl per sample followed by incubation at  $65^{\circ}\text{C}$  overnight. Coimmunoprecipitated DNA was purified according to the manufacturer's directions. Primers were designed to amplify the NRF-1 binding region of the cytochrome *c* promoter and the NRF-2 binding region of the cytochrome oxidase subunit IV (COXIV) promoter. The following specific primers were used: cytochrome *c*, forward, 5'-TGT CGT AAG TGT CGG GCA AAC-3', and reverse, 5'-TCA TTG GCT CCA GGA TTG GG-3'; COXIV, forward, 5'-ACC GCG ARC CTG AAG CTG AT-3', and reverse, 5'-CTG TCA AAG GCC GTC ACC-3'.

PCR conditions were as follows:  $94^{\circ}\text{C}$  for 3 min and then 36 20-s cycles at  $94^{\circ}\text{C}$ , 36 30-s cycles at  $59^{\circ}\text{C}$ , and 36 20-s cycles at  $72^{\circ}\text{C}$ . 50  $\mu\text{l}$  of PCR product was loaded onto a 2% agarose gel and electrophoretically separated, and the densities of the bands were quantified. Purified DNA from the input sample that did not undergo immunoprecipitation was PCR-amplified and used to normalize signals from ChIP assays.

**Determination of mRNA**—RNA was extracted from triceps muscles from sedentary and exercised rats using TRIzol reagent as described previously (18, 19). Exercise-induced changes in ALAS, citrate synthase, and cytochrome *c* mRNAs were determined using semiquantitative reverse transcription-PCR as described in detail previously (20). Universal 18s (Ambion, Austin, TX) was used as an internal control for citrate synthase and ALAS, whereas 18s Classic (Ambion, Austin, TX) was used for cytochrome *c*. PCR products were run on a 2% agarose gel and visualized with Sybr Green (Molecular Probes, Eugene, OR). Transcript intensity was expressed relative to 18s. The following primers were used:  $\delta$  ALAS, forward, 5'-GCTCTTG-TTCTCTTCCTGCTTCG-3', and reverse, 5'-CAAACCTCGT-GCTGGCAATGTATC-3'; citrate synthase, forward, 5'-CAC-AGCCCCCTCAACAGTGAAAGC-3', and reverse, 5'-GTA-GTCTCGTAACTTCTCATCTGACACG-3'; cytochrome *c*, forward, 5'-GGAGGCAAGCATAAGACTGG-3', and reverse, 5'-GTCTGCCCTTTCTCCCTTCT-3'.

**Statistical Analysis**—Data are presented as means  $\pm$  S.E. Analysis of differences between groups was performed with one-way analysis of variance ( $p < 0.05$  was considered to be significant) followed by Fisher's least significant differences post hoc test when appropriate.

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**FIGURE 1. Time course of exercise-induced increases in PGC-1 $\alpha$  and of proteins whose expression is regulated by PGC-1 $\alpha$ .** Rats were sacrificed immediately 3, 12, or 18 h after exercise. Values are means  $\pm$  S.E. for 5–10 muscles per group. \*,  $p < 0.05$  when compared with the corresponding sedentary control value. ERR, estrogen-related receptor.

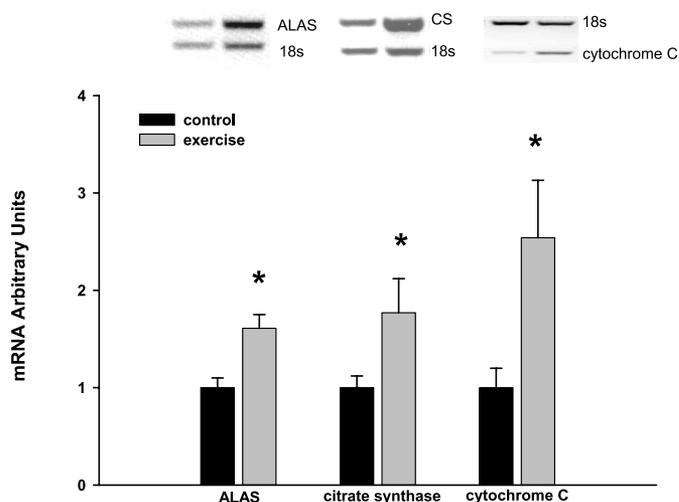
## RESULTS

**Time Course of Exercise-induced Increases in PGC-1 $\alpha$  and Proteins, the Expression of Which It Regulates**—Immediately following a bout of swimming ALAS, SUO and estrogen-related receptor  $\alpha$  proteins were increased in epitrochlearis muscle (Fig. 1). The expression of these proteins, which is regulated by PGC-1 $\alpha$  (4, 21, 22), occurred before there was an increase in PGC-1 $\alpha$  protein. PGC-1 $\alpha$  protein content was significantly increased 3 h following exercise, with a further increase over the next 15 h (Fig. 1). The finding of increases in proteins that are regulated by PGC-1 $\alpha$  prior to an increase in PGC-1 protein provides evidence that the initial adaptive increase in mitochondrial biogenesis is not mediated by the exercise-induced increase in PGC-1 $\alpha$  protein expression.

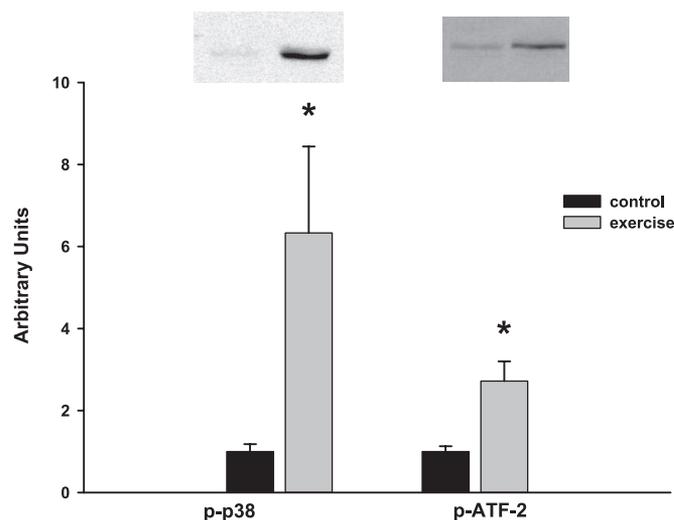
**Exercise-induced Increases in mRNAs of Mitochondrial Markers**—To obtain further evidence regarding the role of PGC-1 $\alpha$  in the early phase of the adaptation to exercise, we measured ALAS, citrate synthase, and cytochrome *c* mRNAs immediately after a bout of swimming. As shown in Fig. 2, the mRNAs for ALAS, citrate synthase, and cytochrome *c* were significantly increased at this time point, prior to the increase in PGC-1 $\alpha$  protein.

**Exercise Results in Increased Phosphorylation of p38 MAPK and ATF-2**—It has been shown that p38 MAPK phosphorylates and activates PGC-1 $\alpha$  (23, 24). As shown in Fig. 3, 2 h of swimming resulted in a 4-fold increase in p38 MAPK phosphorylation. There was also an increase in phosphorylation of the p38 MAPK substrate ATF-2. The transcription factor ATF-2 binds to the cAMP-response element-binding protein site on the PGC-1 $\alpha$  promoter and induces PGC-1 $\alpha$  transcription (12).

**PGC-1 $\alpha$  Moves from the Cytosol into the Nuclei**—It has been shown that in COS cells, PGC-1 $\alpha$  is located in the nuclei (25). However, as shown in Fig. 4, in resting skeletal muscle, most of the PGC-1 $\alpha$  is present in the cytosol. Lactate dehydrogenase protein was used as a marker of the degree of contamination of the nuclear fraction with cytosolic proteins (Fig. 4). Histone H2B protein was measured to evaluate the effect of exercise on the yield of nuclei. As shown in Fig. 4, there was no detectable



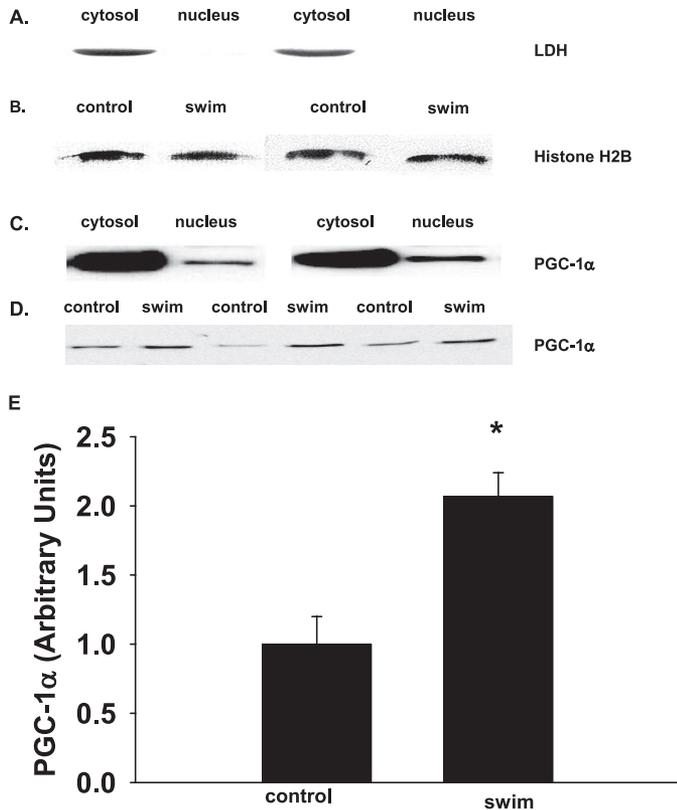
**FIGURE 2.  $\delta$ -Aminolevulinic acid synthase, citrate synthase (CS), and cytochrome *c* mRNAs are increased in triceps muscles immediately following exercise.** Values are means  $\pm$  S.E. for eight muscles per group. \*,  $p < 0.05$  when compared with sedentary control values.



**FIGURE 3. Exercise increases p38 MAPK and ATF-2 phosphorylation.** Triceps muscles were obtained immediately following 2 h of exercise. Values are means  $\pm$  S.E. for 7–11 muscles per group. \*,  $p < 0.05$  when compared with sedentary control.

contamination of the nuclear fraction by the cytosolic protein, lactate dehydrogenase. There was also no difference in the quantity of nuclei extracted from resting and exercised muscles as reflected in the nuclear protein histone H2B (Fig. 4). 2 h of swimming resulted in a large increase in the amount of PGC-1 $\alpha$  located in the nuclei.

**NRF-1 and NRF-2 DNA Binding**—PGC-1 $\alpha$  coactivates both NRF-1 and NRF-2, transcription factors that play major roles in mitochondrial biogenesis (4). To further evaluate the possibility that activation rather than increased expression of PGC-1 $\alpha$  mediates the initial phase of exercise-induced mitochondrial biogenesis, we measured NRF-1 and NRF-2 DNA binding. We used the ChIP assay to quantify binding of NRF-1 to the NRF-1 binding site on the cytochrome *c* promoter and binding of NRF-2 to an NRF-2 binding sequence on the cytochrome oxidase subunit IV (COXIV) promoter. As shown in Fig. 5, NRF-1 and NRF-2 DNA binding was increased in response to a bout of



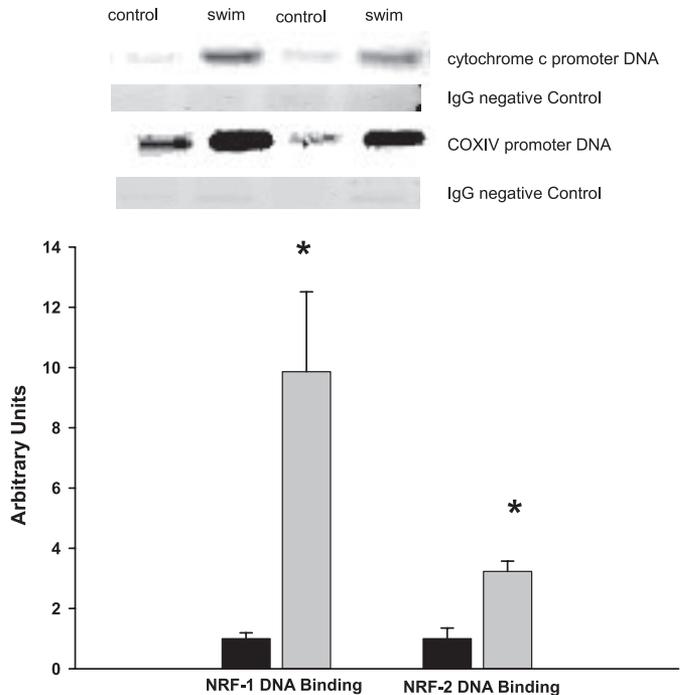
**FIGURE 4. Exercise causes an increase in PGC-1 $\alpha$  protein content in the nucleus.** Rats were sacrificed immediately following 2 h of swimming, and PGC-1 $\alpha$  content was determined in the nuclear fraction of epitrochlearis muscles. *A*, lactate dehydrogenase (LDH) protein content was measured in the cytosolic and nuclear fractions as an indicator of the degree of contamination of the nuclear fraction with cytosolic protein. *B*, the yield of nuclei from resting and exercised muscles was similar, as reflected in equal amounts of the nuclear protein histone H2B in the nuclear extracts. *C*, most of the PGC-1 $\alpha$  protein in skeletal muscle is present in the cytosol. *D*, representative blot showing exercise-induced increases in PGC-1 $\alpha$  in the nucleus. *E*, increase in PGC-1 $\alpha$  protein in the nucleus in response to exercise. Values are means  $\pm$  S.E. for seven muscles per group, \* $p$  < 0.05 versus sedentary control.

exercise, before there was an increase in PGC-1 $\alpha$  protein expression.

## DISCUSSION

The transcription coactivator PGC-1 $\alpha$  regulates the coordinated expression of the mitochondrial proteins that mediate substrate oxidation and ATP synthesis (3, 4, 26–29). PGC-1 $\alpha$  mediates this effect by docking on, and coactivating, transcription factors that control expression of genes encoding mitochondrial proteins (6, 22, 26). Overexpression of PGC-1 $\alpha$  in muscle cells results in increases in mitochondria, with enhancement of the capacity for respiration and oxidative phosphorylation (26, 27). Similarly, transgenic overexpression of PGC-1 $\alpha$  increases the mitochondrial content of mouse skeletal muscle and conversion of low oxidative white muscle fibers to high oxidative red muscle fibers (30).

Exercise training induces an adaptation in skeletal muscle that is similar to that induced by PGC-1 $\alpha$  overexpression, with increases in the levels of the mitochondrial enzymes of the citrate cycle (31) mitochondrial respiratory chain (13, 32) and the fatty acid oxidation pathway (13, 33) and proportional increases in the capacity to oxidize pyruvate and fatty acids (32–34) and



**FIGURE 5. NRF-1 binding to the cytochrome *c* promoter and NRF-2 binding to the COXIV promoter is increased in rat epitrochlearis muscles immediately following 2 h of swimming.** Values are means  $\pm$  S.E. for 8–9 muscles per group. \* $p$  < 0.05 when compared with the corresponding sedentary control value.

generate ATP via oxidative phosphorylation (35). Advances in understanding how mitochondrial biogenesis is regulated, the discovery of the transcription factors that regulate the genes encoding mitochondrial proteins, and most importantly, the discovery of PGC-1 $\alpha$ , (3, 4, 25, 26) has made it possible to examine the mechanisms by which exercise stimulates mitochondrial biogenesis.

A number of studies have shown that exercise/muscle contraction results in rapid increases in PGC-1 $\alpha$  mRNA (7–9) and PGC-1 protein (10, 11) in skeletal muscle. In light of the finding that PGC-1 $\alpha$  overexpression in cells (26, 27) and in muscles of transgenic mice (30) increases mitochondrial biogenesis, it was generally assumed that the exercise-induced stimulation of mitochondrial biogenesis is mediated by the increase in PGC-1 $\alpha$  protein (7, 8, 10–12). However, during an examination of the time course of the adaptive response, we were impressed by its rapidity. Some of the mitochondrial proteins with very short half-lives that are regulated by PGC-1 $\alpha$  appeared to increase roughly in parallel with PGC-1 $\alpha$  protein.

This finding led to the hypothesis that the initial phase of the adaptive increase in mitochondrial biogenesis induced by exercise is mediated by activation of PGC-1 $\alpha$  rather than by the increase in PGC-1 $\alpha$  protein expression. The results of the present study support this hypothesis. One piece of evidence is that the binding of the transcription factor NRF-1 to its response element on the cytochrome *c* promoter and of NRF-2 to its response element on the COXIV promoter was markedly increased in response to exercise 7 h prior to an increase in PGC-1 $\alpha$  protein. NRF-1 and NRF-2 binding activities are regulated by coactivation by PGC-1 $\alpha$  (22, 26). A second piece of evidence that PGC-1 $\alpha$  activation, rather than the increase in

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PGC-1 $\alpha$  protein, mediates the initial stimulation of mitochondrial biogenesis induced by exercise is that the mRNA and protein levels of a number of mitochondrial constituents were increased prior to the increase in PGC-1 $\alpha$  protein.

To our knowledge, this is the first evidence suggesting that mitochondrial biogenesis can be increased by activation of PGC-1 $\alpha$ . The evidence that PGC-1 $\alpha$  regulates mitochondrial biogenesis has come from studies in which PGC-1 $\alpha$  content of cells or tissues was increased by transgenic overexpression or transfection or in response to biological stimuli that increase mitochondrial biogenesis such as cold or exercise (3, 4). However, it has also been shown that the transcriptional activity of PGC-1 $\alpha$  can be augmented by phosphorylation by p38 MAPK (3, 23, 24). This effect is apparently mediated by release of an inhibitory binding protein, termed p160 myb-binding protein by Spiegelman's group (36). Although it has not yet been possible to demonstrate phosphorylation of PGC-1 $\alpha$  in skeletal muscle *in vivo*, it seems reasonable that this mechanism is involved in the biological regulation of PGC-1 $\alpha$ .

Exercise/muscle contraction results in activation of p38 MAPK (11, 12, 37, 38). Activation of p38 MAPK increased ATF-2 phosphorylation and PGC-1 $\alpha$  promoter activity in C<sub>2</sub>C<sub>12</sub> myotubes (12). Furthermore, muscle-specific overexpression of the p38 MAPK-activating kinase MKK6E in transgenic mice resulted in increased expression of PGC-1 $\alpha$  (12). Akimoto *et al.* (12) concluded from these findings that activation of p38 is linked to the adaptive increase in mitochondrial biogenesis through phosphorylation of ATF-2, which binds to and stimulates the cAMP-response element-binding protein site on the PGC-1 $\alpha$  promoter and induces increased PGC-1 $\alpha$  expression (12, 39). Our findings that exercise results in activation of p38 and phosphorylation of ATF-2 agree with and support this interpretation. However, we hypothesize that the increase in PGC-1 $\alpha$  protein expression represents the second phase of this adaptive response and that the initial phase involves increased induction of mitochondrial proteins mediated by activation of pre-existing PGC-1 $\alpha$ .

In this study, we found that most of the PGC-1 $\alpha$  in resting skeletal muscle is in the cytosol and that exercise causes movement of PGC-1 $\alpha$  into the nucleus. This is in contrast to COS cells in which PGC-1 $\alpha$  is localized to the nuclei (25). Based on our findings, we propose the following sequence of events. Exercise results in activation of p38 MAPK, which phosphorylates and activates PGC-1. The activated PGC-1 $\alpha$  moves into the nucleus and coactivates the transcription factors and nuclear receptors that regulate expression of mitochondrial proteins, and thus, mediates the first phase of the adaptive response. Activation of the PGC-1 $\alpha$  promoter by ATF-2 and by transcription factors that are coactivated by PGC-1 $\alpha$ , such as MEF2 (40, 41), subsequently results in an increase in PGC-1 $\alpha$  expression. The increase in PGC-1 $\alpha$  protein then mediates the second phase of the adaptation by sustaining and enhancing the increase in mitochondrial biogenesis initiated by activation of PGC-1 $\alpha$ .

*Acknowledgment*—We are grateful to Victoria Reckamp for expert assistance with preparation of the manuscript.

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