Modest PGC-1α Overexpression in Muscle in Vivo Is Sufficient to Increase Insulin Sensitivity and Palmitate Oxidation in Subsarcolemmal, Not Intermyofibrillar, Mitochondria*

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PGC-1α overexpression in skeletal muscle, in vivo, has yielded disputed and unexpected effects, including disrupted cellular integrity and insulin resistance. These unanticipated results may stem from an excessive PGC-1α overexpression in transgenic animals. Therefore, we examined the effects of a modest PGC-1α overexpression in a single rat muscle, in vivo, on fuel-handling proteins and insulin sensitivity. We also examined whether modest PGC-1α overexpression selectively targeted subsarcolemmal (SS) mitochondrial proteins and fatty acid oxidation, because SS mitochondria are metabolically more plastic than intermyofibrillar (IMF) mitochondria. Among metabolically heterogeneous rat hindlimb muscles, PGC-1α was highly correlated with their oxidative fiber content and with substrate transport proteins (GLUT4, FABPpm, and FAT/CD36) and mitochondrial proteins (COXIV and mTFA) but not with insulin-signaling proteins (phosphatidylinositol 3-kinase, IRS-1, and Akt2), nor with 5′-AMP-activated protein kinase, α2 subunit, and HSL. Transfection of PGC-1α into the red (RTA) and white tibialis anterior (WTA) compartments of the tibialis anterior muscle increased PGC-1α protein by 23–25%. This also induced the up-regulation of transport proteins (FAT/CD36, 35–195%; GLUT4, 20–32%) and 5′-AMP-activated protein kinase, α2 subunit (37–48%), but not other proteins (FABPpm, IRS-1, phosphatidylinositol 3-kinase, Akt2, and HSL). SS and IMF mitochondrial proteins were also up-regulated, including COXIV (15–75%), FAT/CD36 (17–30%), and mTFA (15–85%). PGC-1α overexpression also increased palmitate oxidation in SS (RTA, +116%; WTA, +40%) but not in IMF mitochondria, and increased insulin-stimulated phosphorylation of Akt2 (28–43%) and rates of glucose transport (RTA, +20%; WTA, +38%). Thus, in skeletal muscle in vivo, a modest PGC-1α overexpression up-regulated selected plasmalemmal and mitochondrial fuel-handling proteins, increased SS (not IMF) mitochondrial fatty acid oxidation, and improved insulin sensitivity.

The peroxisome proliferator-activated receptor γ (PPARγ) co-activator 1α (PGC-1α) is a nuclearly encoded transcriptional co-activator that has been implicated in the regulation of mitochondrial biogenesis and oxidative metabolism in muscle tissues (cf. Ref. 1). In C57BL/12 and L6 muscle cells, PGC-1α overexpression up-regulates PPAR-target genes involved in fatty acid catabolism and β-oxidation, as well as genes involved in glucose transport, fatty acid trafficking, and oxidative phosphorylation (2–4). These observations in muscle cells (2–4) have been extrapolated to suggest that PGC-1α is likely also a key factor regulating fatty acid oxidation and insulin sensitivity in mammalian skeletal muscle (1, 2). However, in vivo, PGC-1α has failed to improve insulin sensitivity (5, 6). Thus, although there is no doubt that PGC-1α expression is reduced in obesity (7) and diabetes (8–10), or after a high fat diet (11), it is not certain that the metabolic changes in skeletal muscle in these conditions are necessarily attributable to reductions in PGC-1α.

In mammalian muscle tissues, the effects of PGC-1α on genes involved in fatty acid metabolism have been examined in animal models in which PGC-1α has been overexpressed (12) or ablated (13, 14). In these studies, changes in the mRNA abundance of selected mitochondrial genes have been interpreted as leading to concurrent changes in fatty acid oxidation (12–15). However, this may not occur because the mRNA abundance of carnitine palmitoyltransferase-I (CPTI), a key regulator of mitochondrial fatty acid oxidation, was not altered either in

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PGC-1α-overexpressing or in PGC-1α null mice (12–14), except for one report (15). Moreover, in these studies (12–15) the effects of PGC-1α on CPTI protein expression and CPTI activity were not determined. Thus, it remains to be demonstrated whether PGC-1α up-regulates fatty acid oxidation in mammalian skeletal muscle mitochondria in vivo. For these purposes it may be important to determine whether different types of mitochondria in muscle are selectively targeted by PGC-1α, because subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria are functionally and biochemically distinct (16, 17), and SS mitochondria are much more responsive to perturbations that alter cellular energy states (cf. Ref. 17).

Animal models in which PGC-1α has been ablated (14, 18) or overexpressed (5, 6), have also been used to examine PGC-1α-mediated changes in insulin sensitivity. Results from these studies have been very perplexing. Specifically, it is unknown why PGC-1α null mice are not prone to becoming insulin-resistant (14, 18) and why PGC-1α-overexpressing mice are insulin-resistant (5, 6). These observations are completely contrary to the PGC-1α-induced insulin-sensitizing effects observed in C2C12 and L6 cells (3). Thus, based on the available evidence, in vivo, it is unclear whether PGC-1α has an insulin-sensitizing effect in mammalian muscle.

There is now some question as to whether PGC-1α-overexpressing or null animals are appropriate models (19), given the unexpected phenotypes that occur. For example, some PGC-1α null mice are hyperactive (18), whereas cardiac PGC-1α overexpression causes severe abnormalities in muscle function and metabolism, either when PGC-1α is massively overexpressed or when it is ablated. Indeed, because the effects of very large PGC-1α alterations in vivo have been disappointing, with respect to cardiac performance, insulin sensitivity, and diet-induced insulin resistance (5, 6, 12, 14, 18, 20), Handschin and Spiegelman (19) have recently speculated that only a moderate overexpression of PGC-1α may be required to observe its potential therapeutic benefits. This remains to be determined.

Transfecting PGC-1α into a single rodent muscle in vivo, such as we have done for other genes (21, 22), would allow an examination of a modest overexpression of this co-activator while also avoiding the phenotypic anomalies that occur in PGC-1α-overexpressing animals (12, 20). Therefore, in this study, we have examined the following: the relationship between the endogenous PGC-1α protein expression and the oxidative capacity and selected metabolic proteins in six metabolically heterogeneous skeletal muscles (extensor digitorum longus (EDL), soleus, red gastrocnemius (RG), and white gastrocnemius (WG); plantaris (PL), extensor digitorum longus (EDL), soleus, red gastrocnemius (RG), and white gastrocnemius (WG)) were excised, freeze-clamped in liquid nitrogen, and stored at −80 °C for future analysis.

**Comparison of PGC-1α with Oxidative Capacity and Selected Proteins**

**Tissue Harvesting**—Rats were anesthetized with somnotol administered intraperitoneally (6 mg/100 g body weight). Metabolically heterogeneous skeletal muscles (extensor digitorum longus (EDL), RTA, white tibialis anterior (WTA), plantaris (PL), soleus, red gastrocnemius (RG), and white gastrocnemius (WG)) were excised, freeze-clamped in liquid nitrogen, and stored at −80 °C for future analysis.

**Protein Isolation and Western Blotting**—Muscles were homogenized and proteins separated using SDS-PAGE as we have described previously (24, 25). Proteins from isolated mitochondria were similarly separated using SDS-PAGE. Western blotting was performed as we have reported elsewhere (24, 25). Signals were detected using enhanced chemiluminescence (PerkinElmer Life Sciences) and were subsequently quantified by densitometry by Gene Tool as per the manufacturer's instructions (SynGene, ChemiGenius2, PerkinElmer Life Sciences). All membranes were stained with Ponceau S to confirm equal loading and to enable normalization for purposes of densitometry.

**Muscle Fiber Composition**—The muscle fiber compositions of metabolically heterogeneous rat hindlimb muscles (EDL, IMF, RTA, WTA, PL, RG, and WG), as determined by histochemical analysis using a modification of the method of Ehmke et al. (30), were as follows: (i) SS but not IMF mitochondrial fatty acid oxidation, and (ii) in insulin sensitivity in mammalian skeletal muscle.

**EXPERIMENTAL PROCEDURES**

**Materials**

[1-14C]Palmitate and [3H]3-O-methyl glucose were purchased from Amersham Biosciences. Insulin (Humulin-R) was purchased from Lilly. Proteins were determined with commercially available antibodies from the following sources: PGC-1α from Calbiochem; anti-Akt2, anti-IRS-1, anti-AMPKα2, and anti-PI 3-kinase from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-Akt2 (phosphoserine 474) from Abcam (Cambridge MA); anti-GLUT4 from Chemicon International (Temecula, CA); anti-CoxIV from Molecular Probes (Eugene, OR); goat anti-rabbit secondary antibodies from Chemicon International (Temecula, CA); and donkey anti-rabbit secondary antibody from Amersham Biosciences. Anti-HSL was a gift from Dr. F. Kraemer (Stanford University School of Medicine). The monoclonal antibody MO25 (23) was used to detect FAT/CD36, and a polyclonal antibody (gift from Dr. G. Woldegiorgis (Oregon Graduate Institute of Science and Technology, Beaverton, Oregon)) was used to detect CPTI, as we (24) have done previously. Temgesic was obtained from Reckitt and Benckiser Healthcare Ltd. (Hull, UK). Isoflurane was obtained from Baxter Corp. (Mississauga, Ontario, Canada). All other reagents were obtained from Sigma.

**Animals**

Male Sprague-Dawley rats (~350 g) were used in all experiments. All animals were bred on site and housed in a temperature-controlled room with a 12:12 h reversed light/dark cycle. Rats were fed water and standard rat chow ad libitum. All experimental procedures were approved by the Animal Care Committee at the University of Guelph.
PGC-1α-induced Metabolic Alterations in Skeletal Muscle

RTA, WTA, soleus, PL, RG, and W(G) were also determined in a separate group of animals. For all muscles, serial cross-sections (10 μm) were stained for myofibrillar ATPase and succinate dehydrogenase from which we determined their physiologic and metabolic characteristics (26).

Transfecting Muscle with PGC-1α

The PGC-1α expression construct (gift from Dr. B. Spiegelman, Harvard University, Boston) was produced by sub-cloning the PGC-1α coding sequence into a mammalian expression vector (pcDNA 1.0) (Invitrogen). The pcDNA3.0 vector was used for control experiments (Invitrogen). PGC-1α-pcDNA and pcDNA3.0 plasmid stocks for electroporation were produced by large scale plasmid isolation from transformed Escherichia coli cells (One-Shot® Invitrogen) using commercially available kits (GIGA-prep kits, Invitrogen).

Electrotransfection experiments were performed as described by us (21, 22) and others (27, 28), with some modifications. Briefly, animals were anesthetized with isoflurane. Once sedated, the lower hindlimb was shaved and sterilized (iodine and 70% ethanol), whereas the contralateral limb served as the control. To increase transfection efficiency (28–30), hyaluronidase (100 μl (0.15 units/μl in 50% v/v saline)) was injected through the skin and into the tibialis anterior (TA) muscle. Thereafter, rats were allowed to recover for 2 h (28–30). The TA muscles of rats were then either electrotransfected with PGC-1α-pcDNA plasmid (500 μg of PGC-1α in 50% v/v saline) or electrotransfected with empty pcDNA3.0 plasmid (500 μg of pcDNA in 50% v/v saline). Plasmid DNA and hyaluronidase were both injected using a short (1.25 cm) 27-gauge needle inserted through the skin, into the TA muscle, parallel to the orientation of the muscle fibers. The needle was initially inserted into the distal aspect of the belly of the muscle and was then directed proximally and parallel to the orientation of the muscle fibers. During injection, the area around the needle tip would swell with fluid, then the needle was inserted further proximally in the muscle for the remaining injection.

Immediately following the final injections, a pair of 0.8-cm diameter plate electrodes, attached to a set of ruled calipers (BTX, San Diego), were applied onto the skin that overlies the TA muscle. Electroporation of the intact TA muscle was performed as described previously (21, 31, 32), with slight modifications. Briefly, nine electric pulses were administered with the distance between the electrodes determined from the calipers so that the voltage produced an electric field of 180 V/cm (1 Hz, 20 ms in duration). The anode and cathode electrodes were then alternated between the lateral and medial aspects of the hindlimb while also traversing along the TA muscle from the proximal to its distal insertion, after each set of three pulses (ECM 830 Square Wave Electroporator; BTX, San Diego, CA). After the pulse delivery, the rats were provided with an analgesic (Temgesic) and allowed to recover for 2 weeks after transfection, as recommended elsewhere (33). This protocol results in the transfection of 30% of the muscle fibers (data not shown). Transfection with higher voltages can improve transfection efficiency but causes muscle damage (28).

Two weeks after transfection, the muscle tissue was harvested. Separation of the red and white TA compartments was based on their distinct anatomical locations and the distinct differences in coloration (control muscle). Within each animal the sectioning of the control muscle was used as a guide to separate the red and white compartments in the contra-lateral PGC-1α transfected TA muscle, in which coloration differences were less pronounced. Red and white TA sections from both hindlimbs were frozen for later analysis, for selected proteins using Western blotting. In additional transfection experiments, SS and IMF mitochondria were isolated to determine rates of fatty acid oxidation, and rates of basal and insulin-stimulated glucose transport were determined in hindlimb perfused muscles.

Mitochondrial DNA, PGC-1α mRNA, and Enzyme Activities

Mitochondrial DNA—Mitochondrial DNA content was determined using real time PCR. Total DNA was isolated using DNeasy blood and tissue kit (Qiagen). Real time PCR was performed on a 7500 real time PCR system (Applied Biosystems) using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). Reactions were prepared as per the manufacturer’s recommendations in a total volume of 25 μl and 10 ng of template DNA. mtDNA primers were designed using the rat mitochondrial genome sequence (GenBank™ accession number NC_001665) within the NADH dehydrogenase subunit 5 gene as follows: forward, 5′-GCAGCCACAGGAAAATCCG-3′; reverse, 5′-GTAGGGCAGAGACGGGAGTTG-3′. Primers measuring genomic content were designed within the solute carrier family 16, member 1 gene sequence on chromosome 2 (GenBank™ accession number NC_005101): forward, 5′-TAGCTGGATCTCCCTGATGCGA-3′; reverse, 5′-GCATCAGACTTCCCCAGCTTCC-3′. mtDNA content was calculated by the ΔΔCT method using genomic DNA content as an internal standard with 7500 System SDS software version 1.2.1.22 (Applied Biosystems).

PGC-1α mRNA—PGC-1α mRNA was determined in the same manner as described by Handschin et al. (34). Briefly, RNA was isolated using a combination of TRizol reagent (Invitrogen) and RNeasy mini kit (Qiagen). Total RNA was treated with RNase-free DNase (Qiagen) during the column purification as outlined by the manufacturer. Reverse transcription was carried out using First Strand cDNA synthesis kit for reverse transcription-PCR (avian myeloblastosis virus) (Roche Applied Science) using random primers. Real time PCR was performed using a 7500 real time PCR system (Applied Biosystems) using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). Relative PGC-1α RNA levels were calculated using the ΔΔCT method using 7500 System SDS software version 1.2.1.22 (Applied Biosystems). 18S ribosomal RNA was used as an endogenous control. The following primer sets were used: PGC-1α forward, 5′-CGATGACCTTCTCCTACCCA-3′, and PGC-1α reverse, 5′-TTGGCTTGAAGTGTGTCG-3′; 18S forward, 5′-GGTGGTTTTTGCGAACTGAGG-3′, and 18S reverse, 5′-GTCGGGACTTGTATGTCG-3′.

Enzyme Activities—Citrate synthase activity was measured in muscle homogenates and in isolated SS and IMF mitochondria using the method of Srere (35). CPTI activities were measured

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*A. Bonen, unpublished observations.*
in isolated SS and IMF mitochondria with the method of McGarry et al. (36), using 75 μM of palmitoyl-CoA, which approximates the concentration at which the \( K_m \) of CPTI activity is observed (37).

**Muscle Triacylglycerol Content, Mitochondrial Isolation, and Palmitate Oxidation**

**Muscle Triacylglycerol**—Concentrations of intramuscular triacylglycerol were determined using thin layer chromatography, as outlined elsewhere (38). Briefly, muscle (50 mg) was homogenized (Polytron, Kinematica AG, Brinkmann, Littau-Lucerne, Switzerland) in 2 ml of 1:1 chloroform/methanol on ice. Solvent solution was recovered by centrifugation (6000 × g for 10 min at 4 °C). This solution was washed, and the lipid-containing chloroform phase was separated from the aqueous phase with another centrifugation step. Thereafter, the chloroform phase (500 μl) was dried under nitrogen, and samples were reconstituted with 2.1 chloroform/methanol (v/v, 100 μl). Samples were then spotted onto 250-mm silica gel plates and resolved (60:40:3 heptane/isopropyl ether/acetic acid). Afterward, the air-dried plate was sprayed with chlorofluorescein dye (0.02% w/v in ethanol). The triacylglycerol lipid band was visualized under long wave UV light and quantified against known standards.

**Mitochondrial Isolation**—To obtain sufficient mitochondria for fatty acid oxidation and Western blotting, muscles from three rats were pooled for each of five independent experiments. For mitochondrial isolations, fresh RTA and WTA muscles were excised and placed in ice-cold Buffer 1 (100 mM KCl, 50 mM Tris-HCl, 5 mM MgSO4, 5 mM EDTA, pH 7.4). Thereafter, SS and IMF mitochondria were obtained as we have described previously (24, 39). To analyze protein content in the mitochondria with Western blotting, the samples were purified further to remove possible sarcolemmal membrane debris, as we have done previously (24).

**Palmitate Oxidation by Isolated Mitochondria**—Oxidation of palmitate was measured in isolated mitochondria as we have described previously (24, 37), using a modified Krebs-Ringer buffer (MKR: 115 mM NaCl, 2.6 mM KCl, 1.2 mM KH2PO4, 10 mM NaHCO3, 10 mM HEPES, pH 7.4) that was gassed for 15 min (with 5% CO2, 95% O2) and then supplemented with 5.0 mM ATP, 1.0 mM NAD+, 0.5 mM DL-carnitine, 0.1 mM coenzyme A, 25 μM cytochrome c, and 0.5 mM malate. Palmitate (75 μM, [1-14C]palmitate (0.5 μCi)), complexed to BSA (6:1 (24, 40)) was used in all experiments. The reactions were initiated by the addition of the palmitate-BSA complex (24, 40) and were allowed to proceed for 30 min at 37 °C. Palmitate oxidation was determined as we have described previously (24).

**Basal and Insulin-stimulated Glucose Transport and Insulin-stimulated Akt2 Phosphorylation**

PGC-1α-induced changes in basal and insulin-stimulated glucose transport were examined using a hindlimb perfusion procedure (41), in which muscles were clamped at a constant level of glucose while flow-controlled perfusions (18 ml/min) were performed in the absence (basal) or presence of insulin (20 milliunits/ml). Briefly, the animals were surgically prepared under anesthesia (65 mg of pentobarbital sodium/100 g body weight) as outlined in detail by Ruderman et al. (42). The cell-free perfusate consisted of a Krebs-Henseleit buffer, 2 mM pyruvate, 4% BSA under constant gassing (95% O2, 5% CO2). Initially (5 min) the venous outflow was discarded, and the perfusion flow was adjusted to 18 ml/min. Thereafter, muscles were pre-perfused, either without (basal) or with insulin for 20 min. At that point 3-O-methylglucose, corresponding to concentrations at which the maximal rate of glucose transport occurs (43) (40 mM, 10 μCi of [3H]-3-O-methylglucose), was added to the perfusion reservoir. Because 3-O-methylglucose transport increases linearly for up to 30 and 15 min for basal and insulin-stimulated conditions, respectively,8 perfusions for the basal conditions were performed for 20 min to accumulate sufficient counts in the muscles, whereas perfusion with insulin was performed for 9 min (43). In each animal the PGC-1α-transfected and control muscles were perfused simultaneously. Upon completion of the perfusion, the RTA and WTA from both hindlimbs were immediately excised, blotted for excess liquid on paper, and frozen in liquid N2. In all experiments, mannitol (2 mM, 10 μCi of [14C]mannitol) was used as an extracellular space marker. Muscle samples were boiled with 1 N NaOH for 15 min and chilled on ice. Thereafter, 200 μl of aqueous solution as well as perfusate sample were counted in a liquid scintillation counter. Determinations of 3-O-methylglucose transport rate were performed using standard calculations.

**Insulin-stimulated Phosphorylation of Akt2**—Phosphorylation of Akt2 was determined in control and PGC-1α-transfected muscles under basal conditions (saline injection, intraperitoneal) and after injecting insulin (20 units/rat intraperitoneal, 10 min), which reduced blood glucose by 50–60% after 10 min. Red and white control and PGC-1α-transfected muscles were removed 10 min after saline or insulin injection, rapidly frozen in liquid N2, and stored until analyzed for phosphorylation of Akt2.

**Statistics**

Comparisons between muscle fiber composition and PGC-1α protein expression, as well as between PGC-1α protein and other proteins, were made using least squares linear regression. For these purposes the means of the data were used. Repeated measures analyses of variance were used to compare the effects of transfection on protein expression in red and white skeletal muscle, in SS and IMF mitochondria, and on

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**TABLE 1**

**Muscle fibers composition (%) of rat hindlimb muscles**

Mean ± S.E., n = 9, except RG where n = 4, and RTA and WTA where n = 5–6. FOG means fast-twitch oxidative glycogen; SO means slow-twitch oxidative; FG means fast-twitch glycolytic; and SOL means soleus.

| Muscles | Skeletal muscle |
|---------|-----------------|
| EDL | RTA | WTA | SOL | PL | RG | WG |
| Oxidative | 42.6 | 76.8 | 19.9 | 100 | 43.9 | 89.4 | 16.1 |
| (FOG + SO) | ±2.8 | ±2.3 | ±4.0 | ±2.0 | ±2.0 | ±1.0 |
| FOG | 38.7 | 68.7 | 19.9 | 31.1 | 37.1 | 68.4 | 16.1 |
| SO | ±0.8 | ±2.0 | ±1.9 | ±2.4 | ±3.7 | ±3.9 | ±1.0 |
| FG | 57.4 | 23.2 | 80.1 | 0 | 56.0 | 10.6 | 83.9 |
| ±2.9 | ±1.9 | ±1.9 | 0 | ±4.2 | ±1.1 | ±1.0 |

*The oxidative muscle fiber composition of the differing muscles was based on summing the FOG and SO muscle fiber content of each muscle.*
palmitate oxidation by SS and IMF mitochondria, as well as on basal and insulin-stimulated rates of glucose transport. Post hoc analyses, when warranted, were performed using Fisher’s least squares difference test. All data are reported as mean ± S.E.

RESULTS

We first examined the relationship between endogenous PGC-1α and the oxidative capacity of rat skeletal muscles and selected proteins in these muscles. Thereafter, we examined the effects of PGC-1α overexpression on selected proteins, on palmitate oxidation in SS and IMF mitochondria, and on basal and insulin-stimulated rates of glucose transport.

Relationship between Endogenous PGC-1α with Muscle Oxidative Capacity and Selected Metabolic Proteins

PGC-1α Protein Expression and Skeletal Muscle Oxidative Fiber Type—The rat hindlimb muscles included in this study exhibited a wide range of oxidative fibers (Table 1) as well as a wide range of selected proteins (Fig. 1). PGC-1α protein expression was positively related to the proportion of oxidative fibers in metabolically heterogeneous rat hindlimb muscles (Fig. 2).

PGC-1α and Metabolic Marker Proteins—To gain insight as to whether PGC-1α might be acting as a metabolic regulator of diverse metabolic processes, we examined the relationship between the expression of PGC-1α protein and that of the following: (a) transport proteins for fatty acids and glucose, (b) mitochondrial transcription and oxidative phosphorylation, and (c) insulin signaling. Among muscles, PGC-1α was positively related to GLUT4 (r = 0.97; Fig. 3A), the fatty acid transporters FAT/CD36 (r = 0.93; Fig. 3B) and FABPpm (r = 0.97; Fig. 3C), the mitochondrial proteins CPT1 (r = 0.9; Fig. 3D) and COXIV (r = 0.93; Fig. 3E), and mitochondrial transcription factor A (r = 0.89; Fig. 3F). In contrast, no relationship was observed between PGC-1α and AMPKα2 (r = −0.39), HSL (r = 0.25), and selected insulin-signaling proteins (Akt2 (r = 0.49), PI3K (r = −0.48), IRS-1 (r = −0.43)).

Effects of PGC-1α Overexpression on the Expression and Activities of Selected Proteins

To examine directly the effects of PGC-1α on protein expression, this co-activator was transfected into the red and white compartments of the TA muscle in one hindlimb. In control experiments, transfection of empty pcDNA vector did not alter the protein expression of PGC-1α (Fig. 4A) and several other proteins (p > 0.05; data not shown). Transfection with PGC-1α-pcDNA increased the PGC-1α mRNA (+28%; data not shown). PGC-1α protein expression was increased to the same extent in transfected RTA and WTA muscles (24%, p < 0.05; Fig. 4B). This was accompanied by a marked increase in the red coloration of both the red and white compartments of the transfected TA muscle (Fig. 5).

Citrate synthase activities, a widely used index of mitochondrial biogenesis (15), were increased similarly in the transfected red and white muscles (+13%, p < 0.05; Fig. 6A). This increase was also observed in mitochondrial DNA (+13%, p < 0.05; data not shown). Citrate synthase activity was altered within IMF but not SS mitochondria (Fig. 6, B and C).

Effects of PGC-1α Overexpression on Selected Substrate Transport Proteins, Insulin-signaling Proteins, HSL, and AMPKα2 Proteins, and Intramuscular Triacylglycerol Concentrations—PGC-1α overexpression increased GLUT4 protein in RTA (+20%) and WTA (+32%) muscles (p < 0.05; Fig. 7A). In contrast,
the insulin-signaling proteins IRS-1, PI3K, and Akt2 were unchanged following PGC-1α overexpression (p > 0.05; Fig. 7, B–D). However, the insulin-stimulated phosphorylation of Akt2 was more pronounced in PGC-1α transfected red (+43%) and white muscles (+28%) muscles than in the respective control muscles (Fig. 7E; p < 0.05).

The fatty acid transport protein FAT/CD36 was also increased following PGC-1α overexpression in both RTA (+35%) and WTA (+195%) muscles (p < 0.05; Fig. 8A), whereas another fatty acid transport protein, FABPpm, was not altered in either muscle (p > 0.05; Fig. 8B). PGC-1α induced no change in the expression of HSL protein (Fig. 8C; p > 0.05), but PGC-1α did induce an increase AMPKα2 protein in both RTA (25%) and WTA (38%) muscles (p < 0.05; Fig. 8D). Despite the up-regulation of FAT/CD36, triacylglycerol concentrations were not altered in PGC-1α transfected muscles (Fig. 8E; p > 0.05).

**Effect of PGC-1α Overexpression on Mitochondrial COXIV, CPTI, FAT/CD36, and mTFA Proteins**—Because of the importance of PGC-1α as a regulator of mitochondrial genes, the expression of several key mitochondrial proteins was also
examined. In mitochondria of the PGC-1α transfected muscles, COXIV protein was increased (RTA, SS +15%, IMF +33%; WTA, SS +75%, IMF +28%) \((p < 0.05, \text{Fig. 9, A and B})\), whereas CPTI protein was increased in RTA SS mitochondria (+35%, \(p < 0.05\); Fig. 9C), and decreased in WTA IMF mitochondria (−20%, \(p < 0.05\)) (Fig. 9D). Yet, CPTI activity was not altered (\(p > 0.05\); Fig. 6, D and E).

PGC-1α transfection induced an increase in FAT/CD36 protein in SS mitochondria (RTA, SS +17%; WTA, SS +15%) (\(p < 0.05\); Fig. 9E), but no changes were observed in IMF mitochondrial FAT/CD36 protein (Fig. 9F). Finally, mTFA protein was also increased following PGC-1α overexpression, in RTA (SS, IMF, +37%) and WTA (SS, +85%; IMF, +25%) (\(p < 0.05\); Fig. 9, G and H).

**Effects of PGC-1α Overexpression on Palmitate Oxidation in SS and IMF Mitochondria**—To determine whether PGC-1α up-regulated oxidative metabolism, in conjunction with its ability to drive cells toward a more oxidative state, mitochondrial palmitate oxidation was measured in SS and IMF mitochondria of control and PGC-1α-transfected muscles. Transfection with an empty plasmid did not alter mitochondrial palmitate oxidation (\(p > 0.05\); data not shown). In contrast, in the PGC-1α-transfected muscles, palmitate oxidation was increased. However, this was only observed in the SS mitochondria in both the RTA (+116%) and WTA (+40%) muscles (\(p < 0.05\); Fig. 10A). In contrast, no PGC-1α-mediated changes in
PGC-1α-induced Metabolic Alterations in Skeletal Muscle

Effects of PGC-1α Overexpression on Basal and Insulin-stimulated Glucose Transport—Because GLUT4 and insulin-stimulated Akt2 phosphorylation (Ser-474) in red and white TA muscle (p < 0.05, PGC-1α transfected muscle versus control muscle; **, p < 0.05, white versus red muscle; ***, p < 0.05, insulin-stimulated versus basal).

Discussion

Our studies have revealed that PGC-1α protein expression in metabolically heterogeneous rat hindlimb muscles was positively related to the following: (i) the oxidative capacity of these muscles, and (ii) the expression of selected substrate transport proteins (FAT/CD36, FABPpm, and GLUT4) and mitochondrial proteins (mTFA, COXIV, and CPTI), but not HSL or AMPKα2 proteins or insulin-signaling proteins (IRS-1, PI3 kinase, Akt2). In addition, these studies are the first to demonstrate that a modest overexpression of PGC-1α in skeletal muscle, in vivo, (iii) up-regulated substrate transport proteins (GLUT4 and FAT/CD36), selected mitochondrial proteins, and AMPKα2 protein but not insulin-signaling proteins, although the insulin-stimulated phosphorylation of Akt2 was increased. As well, PGC-1α (iv) increased the rate of palmitate oxidation by SS mitochondria, but not IMF mitochondria, and (v) increased the rate of insulin-stimulated glucose transport. Thus, a modest up-regulation of PGC-1α in skeletal muscle is sufficient to up-regulate selected fuel-handling proteins, fatty acid oxidation, and insulin sensitivity.

Endogenous PGC-1α and the Metabolic Capacities of Rodent Skeletal Muscles—A comparative approach was used initially to gain insight about the relationship between PGC-1α and the metabolic profiles of metabolically heterogeneous skeletal muscles. In agreement with previous findings (44), this indicated that there is a broad, positive association between oxidative capacities of muscle tissues and PGC-1α protein expression. However, this study appears to be the first to show that this relationship is preserved across the range of metabolically heterogeneous rat hindlimb muscles. Remarkably, a 2.3-fold range in endogenous PGC-1α protein expression across all muscles is highly related to the oxidative capacities of the muscles, and to the selected substrate transport proteins and mitochondrial enzymes of the muscles. This suggests that even small changes in PGC-1α, such as can occur with physiologic stimuli (2, 11, 44–48), may have pronounced effects on muscle metabolism. These observations support the view that PGC-1α appears to induce a coordinated metabolic program that up-regulates a number of genes simultaneously to produce an oxidative muscle fiber phenotype (1, 49), as well as increasing the transport rates of glucose and fatty acids and the oxidation rates of fatty acids. We recognize that positive associations between PGC-1α and selected proteins do not necessarily infer a causative relationship but could reflect simply a set of independently coordinated metabolic programs that produce different metabolic profiles in skeletal muscles. For these reasons we also examined the effects of PGC-1α overexpression on metabolic proteins, fatty acid oxidation, and glucose transport.

Overexpression of PGC-1α in Red and White Skeletal Muscle—We used an electroporation procedure to up-regulate PGC-1α in the red and white compartments of a single muscle, as we have done previously (21, 22). The transfection-induced changes in PGC-1α protein are comparable with physiologically induced changes in PGC-1α protein that have been observed in rodent (44) and human muscle (50). The advantage of transfecting a single small muscle with PGC-1α is that (a) whole body fuel homeostasis is unlikely to be altered and (b) an unusual phenotype does not result, as has occurred in transgenic mice that overexpress PGC-1α massively (5, 6). In addition, the animal serves as its own control.

FIGURE 7. Effects of PGC-1α overexpression on protein expression of GLUT4 (A), IRS-1 (B), PI3K (C), and Akt2 (D) proteins and on insulin-stimulated Akt2 phosphorylation (Ser-474) in red and white TA muscle (E) (mean ± S.E., n = 3–4 in control and transfected muscles. Equal protein concentrations were loaded for each muscle, *, p < 0.05, PGC-1α transfected muscle versus control muscle; **, p < 0.05, white versus red muscle; ***, p < 0.05, insulin-stimulated versus basal.

palmitate oxidation were observed in the IMF mitochondria, in either the RTA or WTA muscles (p > 0.05; Fig. 10B).

Effects of PGC-1α Overexpression on Basal and Insulin-stimulated Glucose Transport—Because GLUT4 and insulin-stimulated Akt2 phosphorylation were increased, when PGC-1α was transfected into rat muscle, we also examined whether 3-O-methylglucose transport was concomitantly altered. In the absence of insulin, the basal rates of 3-O-methylglucose transport were not altered (p > 0.05; Fig. 10C). However, with insulin stimulation the rates of 3-O-methylglucose transport were increased in both RTA (+20%) and WTA (+38%) muscles (p < 0.05; Fig. 9C).
Whenever muscle fibers are transfected in live animals, either in our laboratory or others (27, 28), transfection of every muscle fiber is never achieved. In our studies we transfected 30% of the fibers. This is purposeful. A greater transfection efficiency requires higher voltages during the electroporation phase that will induce muscle damage (28). The fact that not all muscle fibers are affected in our work also mirrors other experimental interventions in skeletal muscle, e.g. exercise (i.e. the motor unit size recruitment principle). These exercise studies (45–47, 51–53), and our present transfection studies, in which PGC-1α is up-regulated in only some muscle fibers, do not invalidate the observations that metabolic changes are induced on a per g or per whole muscle basis. In exercise studies (53), as well as in the present studies, there will be a considerable heterogeneity of responses in PGC-1α, and in other parameters, among muscle fibers, ranging from no change to very large changes in some muscle fibers. As a consequence, the subpopulation of affected muscle fibers must be sufficiently large so as to observe a change per g of muscle, i.e. an average of all muscle fibers. This was certainly accomplished in our present work, as the average increase in PGC-1α was 23–25%. That only a portion of the muscle fibers “drive” the changes observed within the whole muscle, in some experiments, is well known in the skeletal muscle literature. Indeed, many of our understandings of the adaptive capacities in muscle (e.g. enzyme activities, glucose transport, gene expression, and transcription rates, etc.) are based on studies in which only some of the muscle fibers have been affected. Yet, this is sufficient to observe meaningful metabolic adaptations within the whole muscle.

**PGC-1α Overexpression and Fatty Acid Transporters, FAT/CD36 and FABPpm**—PGC-1α overexpression increased FAT/CD36 protein in muscle, but this was not observed for another fatty acid transport protein, FABPpm. Others have observed an increase in FAT/CD36 mRNA in PGC-1α-overexpressing mice (15). Increases in PGC-1α (44) and in FAT/CD36 and FABPpm protein (54, 55) have been observed when chronic muscle stimulation is used to induce a more aerobic muscle phenotype. However, this study indicates that unlike FAT/CD36, FABPpm is not regulated by the transcription factors that are co-regulated by PGC-1α.

Although the PPAR family of transcription factors are known targets of PGC-1α, and the FAT/CD36 promoter contains a PPRE sequence (56), no such sequence has yet been identified in FABPpm. It is still unknown whether the PPARs, and subsequently PGC-1α, directly regulate FABPpm expression, particularly because there is some conflict in the literature as to
whether the PPARγ activator rosiglitazone induces an up-regulation of FABPpm (55, 57). A further complication with interpreting data for FABPpm is its apparent dual function depending on its subcellular location. At the plasma membrane this protein contributes to facilitating fatty acid uptake (21, 22), whereas at the mitochondrion this same protein is known as mitochondrial aspartate aminotransferase (22, 58–60) and catalyzes the following reversible reaction: glutamate + oxaloacetate ↔ aspartate + 2-oxoglutarate (22). Thus, it is difficult to ascribe a specific metabolic role for mAspAT/FABPpm based solely on its protein expression.

PGC-1α Overexpression and HSL—Langfort et al. (61) had suggested that HSL protein was more prevalent in the highly oxidative soleus muscle than in the more glycolytic EDL muscle. However, our data show that PGC-1α does not correlate with HSL or the oxidative capacity of muscle (data not shown) nor is HSL induced by PGC-1α.

PGC-1α Overexpression and AMPKα2—The absence of a relationship between AMPKα2 and PGC-1α proteins among metabolically heterogeneous rat skeletal muscles is in agreement with studies in which there was no relationship between oxidative capacities of skeletal muscle and the content of AMPKα2 protein (62). However, the increase in the expression of AMPKα2 following PGC-1α overexpression is interesting because AMPK is thought to serve as a fuel gauge to protect against energy deprivation, as it is activated when cellular energy is depleted (increased AMP/ATP and creatine/phosphocreatine ratio). However, it is now known that AMPK is not merely regulated by the AMP/ATP milieu within muscle, but it also responds to hormonal and nutrient signals without any change in the energy status of the cell (cf. Ref. 63). In PGC-1α transgenic mice (PGC-1α mRNA increased 10–13-fold), whole body oxygen consumption

Transfected JOURNAL OF BIOLOGICAL CHEMISTRY

FEBRUARY 15, 2008•VOLUME 283•NUMBER 7

FIGURE 9. Effects of PGC-1α overexpression on COXIV (A and B), CPTI (C and D), FAT/CD36 (E and F), and mTFA (G and H) proteins in SS and IMF mitochondria in red and white TA muscle (mean ± S.E., n = 4–5 independent experiments; each independent experiment was based on pooling respective control and transfected muscles from three animals. Equal protein concentrations were loaded for each mitochondrial fraction. *, p < 0.05, PGC-1α transfected versus control muscle; **, p < 0.05, white versus red muscle.
was increased and AMP concentrations were increased ~10-fold (64). It would seem unlikely that the modest PGC-1α overexpression in a single muscle in the present study would have elicited such effects on AMP concentrations. Nevertheless, the PGC-1α-induced increase in AMPKα2 protein would allow for a greater activation of this kinase, which presumably contributes to stimulating the increase in fatty acid oxidation observed in this study.

**PGC-1α Overexpression and Mitochondrial Proteins and Palmitate Oxidation**—Because of the well established role of PGC-1α in mitochondrial biogenesis (65), we examined the effect of PGC-1α overexpression on selected mitochondrial proteins. Because previous reports have shown that SS mitochondria exhibit greater metabolic plasticity than IMF mitochondria (16, 17, 66), we examined proteins in SS and IMF mitochondria. In agreement with previous studies showing that PGC-1α induced the expression of COXIV and mTFA in C2C12 skeletal muscle cells (44, 65), PGC-1α also induced the expression of COXIV and mTFA proteins in skeletal muscle SS and IMF mitochondria.

**PGC-1α-induced Fatty Acid Oxidation**—The reason for the PGC-1α-induced increase in SS, but not IMF, mitochondrial fatty acid oxidation is unclear at present. However, selectively greater increases in palmitate oxidation in SS mitochondria (+100%) compared with IMF mitochondria (+46%) have been observed previously in muscles of endurance trained rats (66), in which PGC-1α was undoubtedly also increased. All other reports (12–14), except one (15), have found that the mRNA abundance of skeletal muscle CPTI, whose protein product is a key regulator of fatty acid oxidation, was not altered by PGC-1α overexpression. Our results concur, as PGC-1α overexpression did not increase CPTI activity in either SS or IMF mitochondria. Therefore, this lack of change in CPTI activity cannot account for the up-regulation in fatty acid oxidation in SS mitochondria of PGC-1α-transfected muscles. Although it is possible that an altered GPAT activity could have been associated with the increase in fatty acid oxidation (67, 68), this seems unlikely, as the intramuscular triacylglycerol concentrations in our study were not altered in PGC-1α-transfected muscles.

In one study from our laboratory (69) and in several others by Muoio and co-workers (2, 66, 70), the rates of fatty acid oxidation have been shown to be greater in mitochondria obtained from red muscle than in mitochondria obtained from white muscle. These studies suggest that there are intrinsic differences in the capacities to oxidize fatty acids by red and white muscle mitochondria. However, such intrinsic differences between muscles were, for unknown reasons, not observed in this study. Hence, the mitochondrial fatty acid oxidation did not correlate directly with differences in red white muscle mitochondrial FAT/CD36 content, as we have observed in other studies (36, 71). Thus, the role of FAT/CD36 in contributing to the regulation of mitochondrial fatty acid oxidation, as well of the concept of intrinsic differences in red and white muscle mitochondria, will require further examination.

**PGC-1α Overexpression GLUT4 and Insulin-signaling Proteins**—Our study demonstrates that PGC-1α is positively related to GLUT4 protein expression among mammalian muscles and that PGC-1α overexpression in vivo up-regulated skeletal muscle GLUT4 protein. This confirms a previous study in L6 myotubes (3).

In this study, there was no relationship between PGC-1α and selected post-receptor insulin-signaling proteins (IRS-1, PI 3-kinase, and Akt2), nor were these proteins up-regulated when PGC-1α was overexpressed in RTA and WTA muscles. Others had already shown that there was not a strong relationship between insulin-signaling proteins and the metabolic characteristics of rodent skeletal muscle (72), that PGC-1α did not induce insulin-signaling proteins in L6 myotubes (3), and that exercise training, which increases PGC-1α (46), also failed to up-regulate insulin-signaling proteins (73). However, unlike studies in PGC-1α-transfected L6 myotubes (3), we did observe a greater insulin-stimulated phosphorylation of Akt2 in PGC-1α-transfected muscles. The mechanisms of this PGC-1α mediated increased in Akt2 phosphorylation remain to be determined.

**PGC-1α and Glucose Transport**—The modest PGC-1α overexpression did not alter basal glucose transport, as has been observed in L6 myotubes (3) and in skeletal muscle (15), when
PGC-1α has been massively overexpressed (3, 15). The PGC-1α-mediated increase in insulin-stimulated glucose transport (+20% to +38%) is well within the increase observed for insulin-stimulated glucose transport after acute exercise (+12 to +129%) or after 6–8 weeks of exercise training (+10 to +100%) (74, 75). Thus, ours is the first study to demonstrate that a modest PGC-1α overexpression in skeletal muscle increased insulin sensitivity in this tissue. This is likely related to the increased availability of GLUT4 and the increased phosphorylation of Akt2.

Speculation as to Why PGC-1α Transgenic Mice Are Insulin-resistant—In contrast to the findings in this study, transgenic mice that massively overexpress PGC-1α appear to increase basal rates of glucose transport (15), but unexpectedly these animals are prone to becoming insulin-resistant (5, 6), for unknown reasons. In one study, skeletal muscle GLUT4 mRNA was slightly up-regulated (+10%) when the PGC-1α mRNA increase was small (+10%), whereas a 10–13-fold increase in PGC-1α mRNA reduced skeletal muscle GLUT4 mRNA by up to 70%, while concomitantly reducing whole body insulin sensitivity (5). Similarly, in transgenic mice with muscle-specific overexpression of PGC-1α, diet-induced insulin resistance was unexpectedly increased at the whole body level and within skeletal muscle (6). This appeared to be linked to an excessive intramuscular lipid accumulation (6). Although there were no explanations for these unexpected PGC-1α-mediated reductions in insulin-stimulated glucose disposal (5, 6), our observation that PGC-1α increased the fatty acid transporter FAT/CD36 begins to suggest a possible reason. Specifically, when PGC-1α was slightly up-regulated (10%) when the PGC-1α increase was small (10%), whereas a 10–13-fold increase in PGC-1α overexpression in skeletal muscle

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