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Regulation of PGC-1α and PGC-1α-responsive genes with forskolin-induced Schwann cell differentiation

Rita M. Cowell[a,b], Kathryn R. Blake[b], Tatsuya Inoue[c], James W. Russell[c,*]

[a] Department of Psychiatry, University of Alabama, Birmingham, AL 35294, USA
[b] Department of Neurology, University of Michigan, Ann Arbor, MI 48109, USA
[c] Department of Neurology, University of Maryland and VA Maryland Health care system, Baltimore, MD 21201, USA

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ABSTRACT

Recent evidence indicates that mitochondrial homeostasis is critical for myelination and maintenance of peripheral nerve function. Mice lacking the metabolic transcriptional coactivator peroxisome proliferator activated receptor γ coactivator 1α (PGC-1α) show reductions in expression of myelin-related proteins and exhibit myelin-associated lesions, so we identified PGC-1α target genes in Schwann cells (SCs) in vitro to determine potential roles for PGC-1α in glia and tested whether PGC-1α was sufficient for SC differentiation and myelination. Forskolin-induced differentiation was associated with an upregulation of PGC-1α mRNA and protein, and while overexpression of PGC-1α upregulated genes such as manganese superoxide dismutase and estrogen-related receptor α, it was not sufficient for induction of differentiation. Both PGC-1α overexpression and forskolin exposure caused an increase in the mitochondrial fusion-related protein mitofusin 1. These studies suggest that PGC-1α might be a potential target to promote mitochondrial stability during differentiation and myelination.

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Recent evidence suggests that the maintenance of mitochondrial stability is critical for normal peripheral nerve function and neuronal–glial interactions. Charcot-Marie Tooth (CMT) disease, a devastating illness characterized by progressive peripheral nerve fiber deterioration, paralysis, and muscular atrophy, is frequently associated with mutations in genes controlling mitochondrial homeostasis (especially CMT2a, reviewed in Ref. [29]). Mitochondrial homeostasis requires a delicate balance between fission and fusion, the disturbance of which can lead to decreased cellular respiration, decreased cell growth, and cell death [2,7,8,28]. Mitochondrial fusion is orchestrated by several groups of proteins, including the transcription factor estrogen-related receptor α (ERRα; [21]) and the mitofusins (Mfn1, Mfn2; [5,18]), which regulate mitochondrial network formation, glucose and oxygen consumption, and mitochondrial membrane potential [1]. The process of mitochondrial proliferation (biogenesis), on the other hand, is associated with increases in the transcription factors nuclear respiratory 1 (NRF-1) and nuclear respiratory factor 2 (GA-binding protein α and β) and the expression of proteins involved in mitochondrial DNA transcription and oxidative phosphorylation [20,26]. The ERRα and NRF-1/NRF-2-dependent pathways function in parallel to influence mitochondrial gene expression and function [25].

A series of studies have demonstrated that the transcriptional coactivator peroxisome proliferator activated receptor γ coactivator 1α (PGC-1α) can regulate mitochondrial function, ERRα and NRF-1 activity, and mitofusin expression [4,14,21]. Interestingly, myelin abnormalities have been observed in brains from mice lacking PGC-1α [16], but to date, there is no information about the roles of PGC-1α in glia. In the liver, PGC-1α expression is induced by glucocorticoids and agents that activate the cAMP/protein kinase A pathway, leading to the stimulation of gluconeogenesis via activation of the transcription factor CREB (cyclic AMP responsive element binding protein [10,27]). In the peripheral nerve, the protein kinase A/CREB pathway is crucial for the differentiation of Schwann cells (SCs) [12], so it is possible that PGC-1α is involved in SC differentiation.

In this study, we hypothesized that differentiation of SCs by activation of the protein kinase A pathway involves the upregulation of PGC-1α and PGC-1α-target genes and sought to determine whether differentiation and/or mitochondrial pathways are regulated by PGC-1α in SCs.

SCs were isolated from postnatal day 2 rat sciatic nerve and cultured as described [3] with modifications. Briefly, sciatic nerves were digested with Trypsin and collagenase and plated in DMEM with 10% fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA). Cells

* Corresponding author at: Department of Neurology, University of Maryland School of Medicine, 22 South Greene Street, Baltimore, MD 21201, USA.
Tel.: +1 410 706 6689; fax: +1 410 706 4949.
E-mail address: JRussell@som.umaryland.edu (J.W. Russell).

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were fed for 3 days with 10 μM cytosine arabinofurosine and fibroblasts were eliminated using mouse IgM anti-Thy 1.1 hybridoma supernatant (clone T11/D7/e2 from Bob Hyman, the Salk Institute) and rabbit complement (Research Diagnostics, Inc., Concord, MA). Cells were maintained and passaged in low glucose DMEM, 10% fetal bovine serum, 1% penicillin–streptomycin, forskolin (2 μM), and pituitary extract (20 μg/ml). Only passages 4–7 were used for experiments. For all experiments involving forskolin-induced differentiation, cells were harvested, plated at ~80% confluency, and forskolin-starved for 48–72 h before addition of media containing forskolin. Dideoxyforskolin (10 μM) was used as a negative control in differentiation experiments. All animal procedures were approved by the University of Michigan and Ann Arbor VA Medical Center committees for use of laboratory animals. Reagents are from Sigma–Aldrich (St. Louis, MO) unless otherwise noted.

PGC-1α adenovirus was provided by Bruce M. Spiegelman (Dana Farber Cancer Research Center, Harvard University [13,15]), and was purified and amplified at the University of Michigan Cancer Center Vector Core (director, Thomas Lanigan). Cells were exposed for 48 h to adenovirus containing the gene for GFP (5.1×10^{10} pfu/ml) or GFP and PGC-1α (1.4×10^{11} pfu/ml; the gene for GFP was in tandem with the PGC-1α gene [13]). The optimal multiplicity of infection (MOI) was determined to be 200:1 based on expression analysis and evidence of cell death at higher concentrations.

RNA was isolated from cells using the Trizol method, according to manufacturers instructions (Invitrogen Corporation, Carlsbad, CA), and RT-PCR was performed as previously described using Taqman gene expression assays [9]. Primer/probe sets (Applied Biosystems) included β-actin (Rn00667869), myelin protein zero (MPZ; Rn00566746), peripheral myelin protein 22 (PMP22; Rn00566835), Laminin β2 (Rn00564264), PGC-1α (rat: Rn00580241; mouse: Mn00447183), cyclin D1 (Rn00432359), estrogen-related receptor (ERRα; Rn01479215), mitochondrial transcription factor A (TFAM; Rn00580051), mitofusin 1 (Mfn1; Rn00594496), mitofusin 2 (Mfn2; Rn00500120), superoxide dismutase 2 (MnSOD; Rn00566942), glutathione peroxidase (GPx; Rn00577994), and NRF-1 (Forward: TCTATCCGAAAGAGACAGCAGACA; Reverse: TCCCACTCGTGTCGTATATTCATCT; Probe: 6FAM-ttgcttcggaaactca-TAMRA). As a negative control for the RT reaction, reverse transcriptase was omitted in the reaction mix. For negative controls for the PCR reaction, either the primer sets or the cDNA were omitted from the reactions. Relative concentrations of cDNA were calculated with comparison to a standard curve made with dilutions (1:5, 1:10, 1:20) of cDNA from the sample with the highest dose in that particular experiment (calibrator method). Values were normalized to actin-β and expressed as arbitrary units or fold control, ±standard error.

Cells were treated for 3 days with forskolin (0–10 μM), rinsed in cold PBS, and collected in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in 50 mM Tris, pH 8.0) and protease inhibitors (Complete Mini-tablets; Roche Diagnostics Corporation, Indianapolis, IN). Cell membranes were disrupted with a sonicator, and samples were spun at 14,000 × *g* to remove cell debris. Supernatants were immediately mixed in SDS sample buffer and boiled. Protein content was determined using bovine serum albumin as a standard (Pierce Biotechnology Incorporated, Rockford, IL). Proteins were separated with SDS-PAGE electrophoresis (10 μg/lane; Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and Western blotting was performed as described by Cell Signaling Technology, Inc. (Danvers, MA), with slight modifications. Briefly, blocking was performed for 1 h with 5% dry milk and 0.1% Tween in Tris-buffered saline (TBS), the membrane was washed 15 min with TBS, incubated with the primary antibody in 5% IgG-free BSA (Jackson Immunoresearch; West Grove, PA) overnight at 4 °C, and incubated with the peroxidase-conjugated secondary antibody (Jackson Immunoresearch) in 5% milk for 1 h. Signal was detected with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and exposure to Biomax Kodak film (Eastman Kodak Company, Rochester, NY). Primary antibodies included mouse anti-myelin protein zero (Juan Archelos, Medical University, Graz, Austria), mouse anti-cyclin D1 (Millipore, Billerica, MA; cat#: 05-815), rabbit anti-PGC-1α (Daniel P. Kelly, Center for Cardiovascular Research, Washington University, St. Louis; [9,13]), and mouse anti-β actin (Millipore; cat#: MAB1501R).

SCs (1×10^5) were plated on poly-L-lysine-coated coverslips in 24-well plates in forskolin-free media, and then treated 48 h later with forskolin for 24 h. Cells were fixed in warmed paraformaldehyde (4% in PBS, pH 7.4) and washed with PBS before storing at 4 °C overnight. For visualization of cytoskeletal structure, cells were stained with alexa fluor 647-phalloidin for 2 h at room temperature (0.1 μM; Molecular Probes, Eugene, OR) and mounted with Pro-long anti-fade aqueous mounting media (Molecular Probes). For immunostaining, cells were blocked in PBS with 10% donkey serum (Jackson Immunoresearch, West Grove, PA) for 1 h and then treated with 10 mM citrate buffer for 30 min at 37 °C before incubation overnight with rabbit anti-mouse PGC-1α (1:10,000) and 1 h at room temperature with Alexa fluor 568 donkey anti-rabbit IgG (1:2000; Molecular Probes). To rule out non-specific binding of the secondary antibody and autofluorescence, primary antibodies were replaced with species-matched non-specific IgG (Vector Labs, Burlingame, MA). In some cases, cells were counterstained with DAPI (300 nM; Molecular Probes) for 10 min after incubation with the secondary antibody.

Western blotting and quantitative RT-PCR data were expressed as mean ± standard error. Significant differences among groups were determined using *T*-tests. A Bonferroni correction for multiple comparisons was used for data regarding changes in gene and protein expression with increasing doses of forskolin (Fig. 1). Statistical significance was set at $p = 0.05$.

To study gene regulation in an in vitro model of SC differentiation, we isolated and purified primary SCs from postnatal day 2 rat sciatic nerve. Initial experiments determined that forskolin-induced proliferation when cells were less confluent, so for all differentiation experiments, cells were plated at high density (80–90% confluency). Forskolin-induced differentiation was confirmed by several methods. Based on preliminary experiments indicating that 2–3 days of forskolin treatment reduced proliferation of SCs as measured by the MTT assay (not shown), for mRNA expression analysis, we exposed cells to different doses of forskolin for 3 days (Fig. 1). There was a dose-dependent increase in the expression of the myelin-related gene products myelin protein zero (MPZ) and peripheral myelin protein 22 (PMP22) (Fig. 1A), with no effect on β2-laminin expression (Fig. 1A).

Based on reports in the literature indicating that PGC-1α expression is stimulated by adenyl cyclase activators [10,27] and that PGC-1α is involved in the differentiation of adipocytes [23], we hypothesized that SC differentiation also involves PGC-1α upregulation. PGC-1α mRNA expression was robustly stimulated by forskolin in a dose-dependent manner, with almost an 8-fold increase in expression noted with 10 μM forskolin treatment (Fig. 1B). Forskolin was also able to dose-dependently upregulate MPZ and PGC-1α protein expression and downregulate cyclin D1 protein expression (Fig. 1C and D), and forskolin-stimulated changes in protein expression were accompanied by a change in SC morphology as detected by CY-5-labeled phalloidin. Forskolin-starved SCs and their associated processes were trapezoidal in shape (Fig. 1E) whereas forskolin-treated SCs showed elongated cell bodies and long, filamentous processes (Fig. 1F). Dideoxyforskolin, a biologically inactive analog of