

# Peroxisome Proliferator Activator Receptor $\gamma$ Coactivator-1 Expression Is Reduced in Obesity

## POTENTIAL PATHOGENIC ROLE OF SATURATED FATTY ACIDS AND p38 MITOGEN-ACTIVATED PROTEIN KINASE ACTIVATION<sup>\*[5]</sup>

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Peroxisome proliferator activator receptor- $\gamma$  coactivator 1 (PGC-1) is a major candidate gene for diabetes-related metabolic phenotypes, contributing to decreased expression of nuclear-encoded mitochondrial genes in muscle and adipose tissue. We have demonstrated that muscle expression of PGC-1 $\alpha$  and - $\beta$  is reduced in both genetic (*Lep<sup>ob</sup>/Lep<sup>ob</sup>*) and acquired obesity (high fat diet). In C57BL6 mice, muscle PGC-1 $\alpha$  expression decreased by 43% ( $p < 0.02$ ) after 1 week of a high fat diet and persisted more than 11 weeks. In contrast, PGC-1 $\alpha$  reductions were not sustained in obesity-resistant A/J mice. To identify mediators of obesity-linked reductions in PGC-1, we tested the effects of cellular nutrients in C2C12 myotubes. Although overnight exposure to high insulin, glucose, glucosamine, or amino acids had no effect, saturated fatty acids potently reduced PGC-1 $\alpha$  and - $\beta$  mRNA expression. Palmitate decreased PGC-1 $\alpha$  and - $\beta$  expression by 38% ( $p = 0.01$ ) and 53% ( $p = 0.006$ ); stearate similarly decreased expression of PGC-1 $\alpha$  and - $\beta$  by 22% ( $p = 0.02$ ) and 39% ( $p = 0.02$ ). These effects were mediated at a transcriptional level, as indicated by an 11-fold reduction of PGC-1 $\alpha$  promoter activity by palmitate and reversal of effects by histone deacetylase inhibition. Palmitate also (*a*) reduced expression of tricarboxylic acid cycle and oxidative phosphorylation mitochondrial genes and (*b*) reduced oxygen consumption. These effects were reversed by overexpression of PGC-1 $\alpha$  or - $\beta$ , indicating PGC-1 dependence. Palmitate effects also required p38 MAPK, as demonstrated by 1) palmitate-induced increase in p38 MAPK phosphorylation, 2) reversal of palmitate effects on PGC-1 and mitochondrial gene expression by p38 MAPK inhibitors, and 3) reversal of palmitate effects by small interfering RNA-mediated decreases in p38 $\alpha$  MAPK. These data indicate that obesity and saturated fatty acids decrease PGC-1 and mitochondrial gene expression and function via p38 MAPK-dependent transcriptional pathways.

The earliest detectable abnormalities in subjects at risk for developing type 2 diabetes (DM)<sup>2</sup> are insulin resistance and accumulation of lipid in skeletal muscle (1–4). Although this metabolic phenotype is increasingly well characterized, the precise molecular basis of diabetes risk remains unknown. Recent genomic and functional studies of muscle from humans with diabetes or at high risk for diabetes have demonstrated that impairments in nuclear-encoded mitochondrial gene expression and function are a key signature of diabetes (5–9). Given the worldwide increase in DM, it is critical to understand the upstream genetic and transcriptional mechanisms mediating mitochondrial dysfunction.

PGC-1 $\alpha$  (PPAR $\gamma$  coactivator 1 $\alpha$ ) and related coactivators are recognized as key regulators of mitochondrial biogenesis and function via interactions with nuclear respiratory factor and other nuclear receptors and transcription factors (10). Because expression of both PGC-1 $\alpha$  and - $\beta$  is reduced by about 50% in skeletal muscle from prediabetic and diabetic humans (5), PGC-1 has emerged as a potential candidate gene mediating diabetes-related metabolic phenotypes.

In turn, both genetic and environmental factors may contribute to reduced expression of PGC-1 $\alpha$  and - $\beta$  in humans. Polymorphisms in PGC-1 have been associated with obesity and diabetes (11–13). We hypothesize that PGC-1 may also mediate the effects of environmental risk factors, including caloric excess, inactivity, and the development of obesity, in the pathogenesis of insulin resistance and diabetes. Indeed, severe caloric restriction, which improves insulin sensitivity, increases PGC-1 $\alpha$  expression in skeletal muscle of obese subjects (14). Likewise, exercise training increases muscle expression of PGC-1 $\alpha$  in both humans and rodents (15, 16). Conversely, infusion of lipids decreases expression of PGC-1 $\alpha$  and nuclear-encoded mitochondrial genes (17). Experimental high fat feeding in healthy humans also results in decreased PGC-1 $\alpha$  and mitochondrial gene expression in skeletal muscle after just 3 days (18).

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<sup>[5]</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–3 and Tables 1 and 2.

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<sup>2</sup> The abbreviations used are: DM, type 2 diabetes; BSA, bovine serum albumin; PPAR, peroxisome proliferator activator receptor; PGC-1, PPAR- $\gamma$  coactivator-1; ACC, acetyl-coA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside; mTOR, mammalian target of rapamycin; MEF2, MADS Box transcription enhancer factor 2; NF- $\kappa$ B, nuclear factor  $\kappa$ B; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; FA, fatty acid(s); FFA, free fatty acid(s).

## Role of Obesity and FFA in PGC-1 Expression

Although such links between obesity, over-nutrition, and PGC-1 expression have been identified, the precise mechanisms underlying these effects remain poorly defined. We now demonstrate a prominent role for saturated fatty acids to inhibit both PGC-1 $\alpha$  and PGC-1 $\beta$  gene expression and downstream mitochondrial gene expression and oxidative function at a transcriptional level, mediated via p38 MAPK-dependent pathways.

### EXPERIMENTAL PROCEDURES

**Reagents and Plasmids**—Fatty acids were obtained from Alltech Associates (Deerfield, IL). SB203580 was obtained from Upstate Cell Signaling Solutions (Chicago, IL), PD98059 and PD169316 were from Calbiochem, and AICAR was from Biomol International (Plymouth Meeting, PA). Rosiglitazone was provided by GlaxoSmithKline. For amino acid treatment, cell media was supplemented with modified Eagle's medium amino acids (Invitrogen). All other chemicals were purchased from Sigma. All antibodies were obtained from Cell Signaling Technology. The pSV- $\beta$ -galactosidase expression vector (Promega, Madison, WI), the pGL3basic luciferase reporter gene vector (Promega) subcloned with a fragment of the 5'-flanking sequence of the PGC-1 $\alpha$  gene (2-kilobase promoter, -2533 to +78 relative to transcriptional start site), and adenoviral vectors containing coding sequences for GFP, GFP-murine PGC-1 $\alpha$ , and murine PGC-1 $\beta$  (Ad-GFP, Ad-PGC-1 $\alpha$ , and Ad-PGC-1 $\beta$ , respectively) were kindly provided by Dr. Bruce Spiegelman (Dana-Farber Cancer Institute, Boston, MA) (19).

**Animal Care and Treatment**—Mice were housed 4 per cage in an Office of Laboratory Animal Welfare-certified animal facility, with a 12-h light cycle. All experimental plans were approved by the Joslin Institutional Animal Care and Use Committee. For basal comparisons, male C57BL6, ob/+, and ob/ob mice (age 10–12 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME). Two cohorts of 6-week-old male mice were placed on chow (17% calories from fat) or high fat diet (42% calories from milk fat) (Harlan Teklad, Madison, WI) and followed from 1 to 11 weeks (C57BL6 and A/J strains,  $n = 5$  per group) or from 1 to 14 days (C57BL6 only,  $n = 8$  per group), respectively. 2-Month-old male *Lep<sup>ob</sup>/Lep<sup>ob</sup>* (ob/ob) (C57BL6 background) or *Lep<sup>ob</sup>/+* (ob/+) mice ( $n = 8$  per group) were treated with rosiglitazone (3 mg/kg/day) or metformin (350 mg/kg/day, mixed with chow) for 3 months. In all mice insulin sensitivity was assessed by insulin tolerance testing (0.75 units/kg humulin R, intraperitoneal), with glucose sampling at time 0, 15, 30, and 60 min. For tissue analysis, randomized mice were anesthetized with pentobarbital (50 mg/kg); quadriceps muscle was removed and snap-frozen in liquid nitrogen.

**RNA Isolation and Expression Analysis**—Total RNA was isolated from mouse tissue or C2C12 cells using RNeasy columns with DNase I treatment (Qiagen, Valencia, CA). cDNA was synthesized using random hexamers (Advantage, Clontech, Mountain View, CA) for subsequent real-time quantitative PCR analysis (ABI Prism 7700 Sequence detection system, Applied Biosystems, Inc., Foster City, CA). PCR products were detected using Sybr Green and normalized to TATA-binding protein expression. Primers were designed using Primer

Express (Applied Biosystems, Inc.). Sequences are provided in supplemental Table 1.

**Cell Culture**—Mouse C2C12 myoblasts (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (Invitrogen) at a confluency of 60–70%. To initiate differentiation, cells were allowed to reach 100% confluency, and medium was changed to Dulbecco's modified Eagle's medium containing 2% horse serum (Invitrogen) and changed every 2 days. Full differentiation, with myotube fusion and spontaneous twitching was observed at 5 days.

**Protein Isolation and Expression Analysis**—Protein was harvested from cells in lysis buffer containing protease inhibitor mixture (Sigma), sodium orthovanadate, and Triton X-100. Protein concentrations were determined using a BCA assay kit (Pierce). Proteins were separated by SDS-PAGE for subsequent Western blotting. Membranes were blocked for 1 h in 3% BSA in phosphate-buffered saline/Tween-20, incubated with primary antibodies (1:1000) overnight, incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000) at room temperature for 1 h, and visualized using chemiluminescence (Western Lightning kit, PerkinElmer Life Sciences).

**Fatty Acid Stock Preparation**—Fatty acids were dissolved in 0.1 N sodium hydroxide (final concentration 100 mM) at 65 °C for 2 h and then complexed with 10% fatty acid-free BSA, yielding a final stock of 5 mM (20).

**Transfection and Reporter Analysis**—C2C12 cells were cotransfected with  $\beta$ -galactosidase and reporter gene expression vectors using Lipofectamine 2000 (Invitrogen) on day 0 of differentiation. Cells were treated with 500  $\mu$ M palmitate or 1% BSA on day 3 and lysed (Galacto-Star System, Applied Biosystems), and luciferase and  $\beta$ -galactosidase activity was determined using the enhanced luciferase assay kit (BD Biosciences) and Galacto-Star Systems (Applied Biosystems), respectively. Luciferase activity was normalized to  $\beta$ -galactosidase.

**Microarray Analysis**—RNA was isolated as described from C2C12 myotubes treated overnight with 500  $\mu$ M palmitate or 1% BSA, and cRNA was synthesized ( $n = 3$  per condition). 10  $\mu$ g of cRNA was hybridized to Affymetrix mouse 430A 2.0 arrays. Intensity values were quantified using MAS 5.0 software. MAPPFinder was used to integrate expression data with known pathways.

**Adenoviral-mediated Overexpression of PGC-1 $\alpha$  and - $\beta$** —Viral vectors were amplified in Ad-293 cells (Stratagene, La Jolla, CA) maintained in Dulbecco's modified Eagle's medium with 10% FBS. C2C12 myotubes were infected at day 2 of differentiation with  $4.3 \times 10^4$  plaque-forming units/well of 6-well plates, allowed to differentiate for 2 more days, treated as indicated, and harvested on day 5.

**Real-time PCR for Mitochondrial Copy Number**—Mitochondrial copy number was measured using primers specific for rRNA, COXII, and ND1 genes and normalized to the  $\beta$ -globin copy number (sequences are provided in supplemental Table 1).

**Oxygen Consumption**—C2C12 myoblasts were seeded in 24-well microplates (Seahorse Labware, Chicopee, MA). After 4 days of differentiation, cells were treated with 250  $\mu$ M palmitate or 1% BSA for 24 or 48 h. Oxygen consumption rates were

measured using a nondestructive, time-resolved method (Sea-horse Bioscience XF24) (21). Three successive 4-min measurements were performed simultaneously at 2-min intervals. Data were normalized to protein content (assayed after completion of measurements).

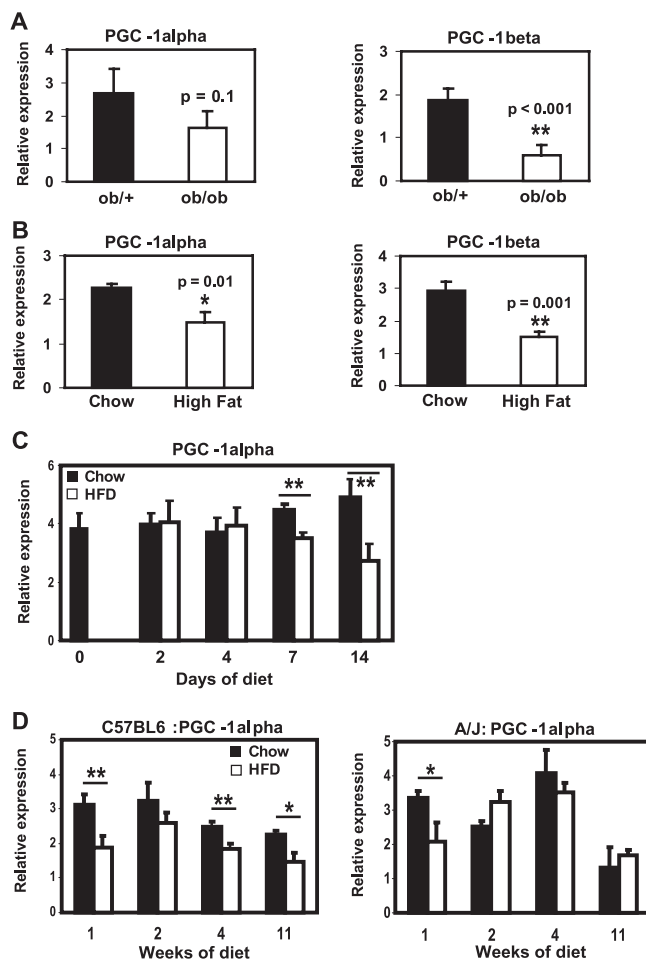
**siRNA**—C2C12 myotubes were transfected at day 3 of differentiation with 100 nM p38 $\alpha$  MAPK, PGC-1 $\alpha$ , or PGC-1 $\beta$  siRNA SMARTpools (Dharmacon, Lafayette, Colorado) or siCONTROL non-targeting siRNA using Dharmafect 3 transfection reagent. Cells were treated overnight with 500  $\mu$ M palmitate or 1% BSA on day 4 of differentiation, and RNA and protein were harvested on day 5. Efficiency of transfection was determined using siGLO-RISC-free non-targeting siRNA and estimation of siRNA uptake by fluorescence detection (absorbance/emission 557/570 nm).

## RESULTS

**Expression of PGC-1 Is Decreased in Skeletal Muscle in Mouse Models of Obesity**—We first assessed whether genetic or experimental diet-induced obesity might contribute to decreased expression of PGC-1 and mitochondrial gene expression. In the leptin-deficient *Lep<sup>ob</sup>/Lep<sup>ob</sup>* (ob/ob) mouse, PGC-1 $\beta$  expression was decreased by 68% ( $p < 0.001$ ) in quadriceps muscle as compared with *Lep<sup>ob</sup>/+* (ob/+) (Fig. 1A); similar trends were observed for PGC-1 $\alpha$  (39% decrease,  $p = 0.1$ ) (Fig. 1A). These differences were also apparent in ob/ob muscle compared with wild-type C57BL6 (supplemental Fig. 1). Likewise, in C57BL6 mice exhibiting diet-induced obesity after 2 months of a high fat diet, both PGC-1 $\alpha$  and PGC-1 $\beta$  expression was decreased in quadriceps by 35% ( $p = 0.01$ ) and 48% ( $p = 0.001$ ), respectively, as compared with chow-fed controls (Fig. 1B). Strikingly, reduced PGC-1 $\alpha$  expression in C57BL6 mice occurred as early as 1 week after initiation of a high fat diet and was maintained over an 11-week time course (Fig. 1, C and D), with expression correlating inversely with weight ( $r = -0.41$ ;  $p = 0.03$ ), plasma leptin levels ( $r = -0.53$ ;  $p = 0.02$ ), and plasma insulin levels ( $r = -0.45$ ;  $p = 0.02$ ) at 11 weeks. In contrast, muscle from obesity-resistant A/J mice exhibited an early reduction in PGC-1 $\alpha$  expression (38% reduction at 1 week,  $p = 0.04$ ), but this was not sustained over the 11-week time course (Fig. 1D). Expression of PGC-1 $\beta$  was also significantly decreased in C57BL6 mice (49% reduction at 11 weeks,  $p = 0.03$ , not shown).

Reductions in PGC-1 $\alpha$  and PGC-1 $\beta$  expression in both genetic and experimental obesity models suggest that obesity and/or related metabolic effects, including hyperinsulinemia, insulin resistance, and cellular over-nutrition, may contribute to patterns observed in humans with insulin resistance and type 2 diabetes (5, 6, 18).

**Sustained Insulin Exposure and Insulin Resistance Have No Effect on PGC-1 Expression**—We next assessed the direct cellular effects of sustained exposure to high insulin concentrations in the absence of complicating systemic metabolic changes. Treatment of fully differentiated C2C12 myotubes overnight (16 h) with insulin (1 to 100 nM) in the absence of serum produced no significant change in mRNA expression of PGC-1 $\alpha$  or - $\beta$  (Fig. 2A). Furthermore, pathway-specific pharmacologic induction of insulin resistance induced by overnight incubation with the MAPK-extracellular signal-related kinase inhibitor



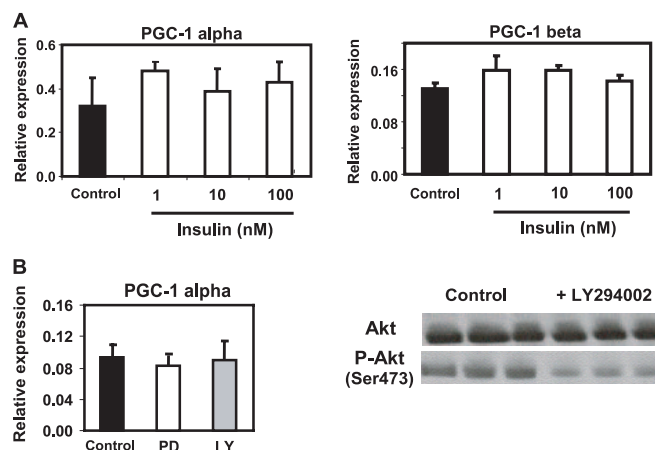
**FIGURE 1. PGC-1 $\alpha$  and - $\beta$  expression are decreased in mouse models of obesity.** Expression of PGC-1 $\alpha$  (left) and - $\beta$  (right) was assessed by real-time PCR, as described under "Experimental Procedures," in quadriceps muscle from 5-month-old *Lep<sup>ob</sup>/Lep<sup>ob</sup>* (ob/ob) mice as compared with *Lep<sup>ob</sup>/+* (ob/+) ( $n = 8$  per group) (A) and 5-month-old C57BL6 mice fed a high fat diet (HFD) as compared with chow for 3 months ( $n = 5$  per group) (B). C, PGC-1 $\alpha$  expression was assessed serially over 14 days in C57BL6 mice fed chow or high fat diet ( $n = 8$  per group) (C) and in C57BL6 (left) and A/J mice (right) fed high fat diet from 1 to 11 weeks ( $n = 5$  per group) (D). Data are the mean  $\pm$  S.E.; \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ .

PD98059 (20  $\mu$ M) had no effect on expression of PGC-1 $\alpha$  (Fig. 2B). Likewise, the phosphatidylinositol 3-kinase inhibitor LY294002 (10  $\mu$ M) was without effect despite induction of insulin resistance as demonstrated by decreased insulin-stimulated Akt Ser-473 phosphorylation (Fig. 2B, right). Similarly, treatment with 1 mM dexamethasone for 24 h or with 1 nM tumor necrosis factor  $\alpha$  for 24–96 h, both potent inducers of insulin resistance in myotubes, did not affect PGC-1 (not shown).

Because leptin deficiency and resistance are key features of genetic and diet-induced obesity, we also tested the effects of leptin on PGC-1 expression in myotubes. Overnight leptin (1 ng/ml) had a modest effect of increasing PGC-1 expression in C2C12 myotubes (29% increase,  $p < 0.01$ ).

**Neither High Glucose Nor Activation of the Hexosamine Biosynthetic Pathway Affects Expression of PGC-1**—In both humans and mice, hyperglycemia is associated with reduced expression of multiple mitochondrial genes (22). To test whether elevations in glucose might mediate reductions in PGC-1 expression, C2C12 myotubes were incubated overnight

## Role of Obesity and FFA in PGC-1 Expression



**FIGURE 2. Overnight insulin exposure or pharmacological induction of insulin resistance has no effect on PGC-1 expression in C2C12 myotubes.** mRNA expression of PGC-1 $\alpha$  (left) and PGC-1 $\beta$  (right) was assessed in C2C12 myotubes incubated for 16 h with 1–100 nM insulin (A) and 20  $\mu$ M PD98059 (PD) or 10  $\mu$ M LY294002 (LY) for 16 h (B). The right panel shows anti-phospho-Akt and total Akt Western blot of cell lysates treated with LY before insulin stimulation 100 nM for 10 min and demonstrates impaired insulin-stimulated Akt phosphorylation (P-Akt) in LY-treated cells. Data are the means  $\pm$  S.E.

with either low (5 mM) or high (25 mM) glucose in the presence or absence of 10 nM insulin to ensure glucose uptake. Expression of PGC-1 $\alpha$  or - $\beta$  did not differ as a function of glucose concentration (supplemental Fig. 2A). Likewise, prolonged exposure (48 h) of myotubes to high glucose had no effect.

The hexosamine biosynthetic pathway is a nutrient-sensing pathway implicated in the development of both hyperglycemia and lipid-mediated insulin resistance. Robust experimental activation of this pathway by glucosamine infusion decreases expression of nuclear-encoded mitochondrial genes in rat skeletal muscle (23). We, therefore, tested whether direct activation of the hexosamine biosynthetic pathway might modulate PGC-1 expression. Overnight incubation with 5 mM glucosamine did not affect PGC-1 $\alpha$  or - $\beta$  expression in either the presence or absence of insulin (supplemental Fig. 2B).

**Excess Amino Acids Have No Effect on PGC-1 Expression—**Nutrient excess, particularly high concentrations of amino acids, can induce insulin resistance via activation of the mTOR/S6kinase nutrient-sensing pathway (24). Mice with ablation of S6 kinase exhibit increased PGC-1 expression and are resistant to high fat induction of insulin resistance and obesity (25). In C2C12s, however, there was no effect of increasing amino acids (to levels 2–4-fold above that in standard media) on expression of PGC-1 (supplemental Fig. 2C).

**Long Chain Saturated Fatty Acids Decrease PGC-1—**Fatty acids (FA) have long been implicated as particularly potent nutrient modulators of insulin resistance (26, 27). Therefore, we incubated C2C12 myotubes overnight with representative saturated and unsaturated BSA-conjugated FA, including caprylic acid (C8:0), lauric acid (C12:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), and oleic acid (C18:1), and the omega-3 fatty acid, eicosapentanoic acid (EPA) or with BSA control. Although short chain, unsaturated, and omega-3 fatty acids in concentrations up to 500  $\mu$ M had no effect on PGC-1 (Fig. 3, A and B), long chain saturated fatty acids potently decreased expression of both PGC-1 $\alpha$  and

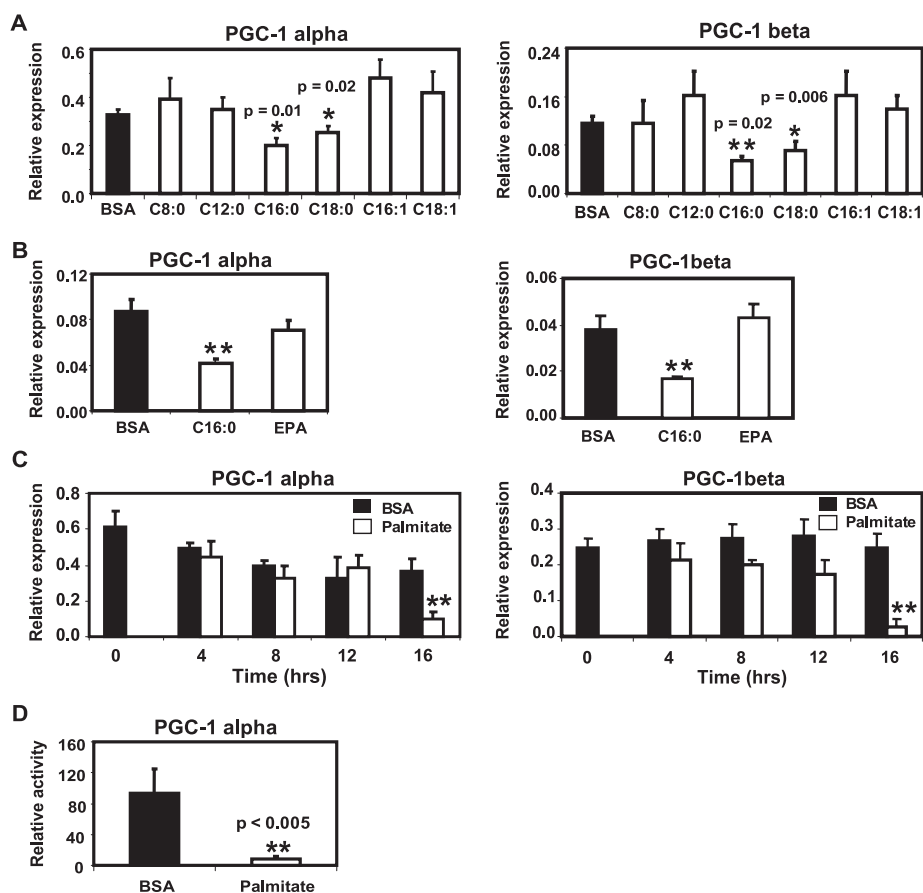
PGC-1 $\beta$  (Fig. 3A). Palmitate decreased expression of both PGC-1 $\alpha$  and - $\beta$  by 38% ( $p = 0.01$ ) and 53% ( $p = 0.006$ ) respectively; stearate similarly decreased expression of both PGC-1 $\alpha$  (22% decrease,  $p = 0.02$ ) and PGC-1 $\beta$  (39% decrease,  $p = 0.02$ ) (Fig. 3A). As expected, this dose of palmitate induced insulin resistance as demonstrated by decreased insulin-stimulated Akt Ser-473 phosphorylation (see Fig. 5C). Interestingly, when cells were incubated in the presence of palmitate and either oleate or EPA, no decrease in PGC-1 expression was observed (not shown).

Plasma levels of fatty acids are increased in individuals with obesity and diabetes, with nocturnal values exceeding 500  $\mu$ M (28). Although our fatty acid experimental dose is within this range, we recognize that plasma contains a mixture of fatty acid subtypes (29). However, lower, more clearly physiological doses of palmitate also decreased PGC-1 $\alpha$  expression (250  $\mu$ M, 40% decrease,  $p = 0.01$ ; 125  $\mu$ M, 36% decrease,  $p = 0.11$ ; not shown) and during a time course ranging from 16 (Fig. 3C) to 72 h of exposure (not shown). Palmitate was chosen as a representative saturated FA for future experiments.

Incubation of cells with 500  $\mu$ M methyl palmitate (a palmitate derivative that cannot form a coenzyme A ester) had no effect on PGC-1 mRNA expression, suggesting that formation of a fatty acyl-CoA moiety is necessary for palmitate-mediated inhibition. Furthermore, pretreatment of cells with etomoxir (an inhibitor of carnitine palmitoyltransferase 1, required for transport of long chain acyl-CoAs from the cytoplasm to the mitochondria) inhibited the effects of palmitate to decrease PGC-1 expression (data not shown).

**Palmitate Decreases PGC-1 $\alpha$  Promoter Activity—**Changes in PGC-1 mRNA expression may reflect altered transcription or transcript stability. To test the impact of palmitate on PGC-1 promoter activity, we cotransfected C2C12 myoblasts with the pGL3basic reporter gene vector (Promega) subcloned with a 2-kilobase fragment of the 5'-flanking sequence of the PGC-1 $\alpha$  gene and a pSV- $\beta$ -galactosidase expression vector. Cells were treated at day 3 of differentiation with 500  $\mu$ M palmitate or BSA. Under these conditions, palmitate decreased PGC-1 $\alpha$  mRNA expression by 33% ( $p = 0.002$ ) (not shown) and decreased PGC-1 $\alpha$  promoter-regulated luciferase activity by 11-fold ( $p < 0.005$ ) (Fig. 3D).

**Palmitate Decreases Mitochondrial Gene Expression and Oxygen Consumption—**Pathway analysis of microarray data from C2C12 myotubes treated overnight with palmitate revealed decreased expression of many mitochondrial genes, including members of the tricarboxylic acid cycle (MAPPFinder  $Z = 3.3$ ,  $p < 0.004$ ) and electron transport chain (MAPPFinder  $Z = 5.1$ ,  $p < 0.001$ ) (supplemental Table 2) as compared with BSA-treated control cells. We confirmed differential expression of selected tricarboxylic acid cycle and electron transport chain genes by PCR, including isocitrate dehydrogenase 2 (67% decrease,  $p = 0.001$ ), citrate synthase (14% decrease,  $p = 0.03$ ) (Fig. 4A), NADH-ubiquinone oxidoreductase 1 $\alpha$  subcomplex 5 (45% decrease,  $p = 0.04$ ), mitochondrial ATP synthase subunit ATP5J2 (49% decrease,  $p = 0.04$ ), and the adenine nucleotide translocator 1 (47% decrease,  $p = 0.04$ ) (Fig. 4B). Notably, we observed no change in mitochondrial DNA copy number during 72 h of palmitate treatment.



**FIGURE 3. Long chain-saturated fatty acids decrease expression of PGC-1 $\alpha$  and - $\beta$  in C2C12 myotubes.** mRNA expression of PGC-1 $\alpha$  (left panel) and  $\beta$  (right) was assessed in C2C12 myotubes incubated with 500  $\mu$ M caprylic acid (C8:0), lauric acid (C12:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), and oleic acid (C18:1) for 16 h (A) and 500  $\mu$ M palmitate or eicosapentanoic acid (EPA) (B). C, time course of palmitate (500  $\mu$ M) effects on PGC-1 $\alpha$  (left) and PGC-1 $\beta$  (right). D, effect of palmitate on PGC-1 $\alpha$  promoter activity. C2C12 myotubes were cotransfected with the pSV- $\beta$ -galactosidase, empty pGL3basic reporter gene vector (pGL3), or pGL3basic reporter gene vector subcloned with the 2-kilobase PGC-1 $\alpha$  promoter (-2533 to +78) (pGL3-PGC-1) and differentiated for 3 days. Myotubes were treated for 16 h with 500  $\mu$ M palmitate or BSA, and luciferase and  $\beta$ -galactosidase activity was measured in cell lysates. Reporter expression is normalized to  $\beta$ -galactosidase. Activity in cells transfected with pGL3-PGC-1 is normalized to that of cells transfected with pGL3. Data are mean  $\pm$  S.E. \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ .

To assess the effects on mitochondrial function, we measured oxygen consumption rate after palmitate exposure (250  $\mu$ M). Although we observed a modest but significant increase at 24 h, oxygen consumption rate was significantly decreased by 48 h of palmitate exposure (40% decrease,  $p = 0.008$ , Fig. 4C).

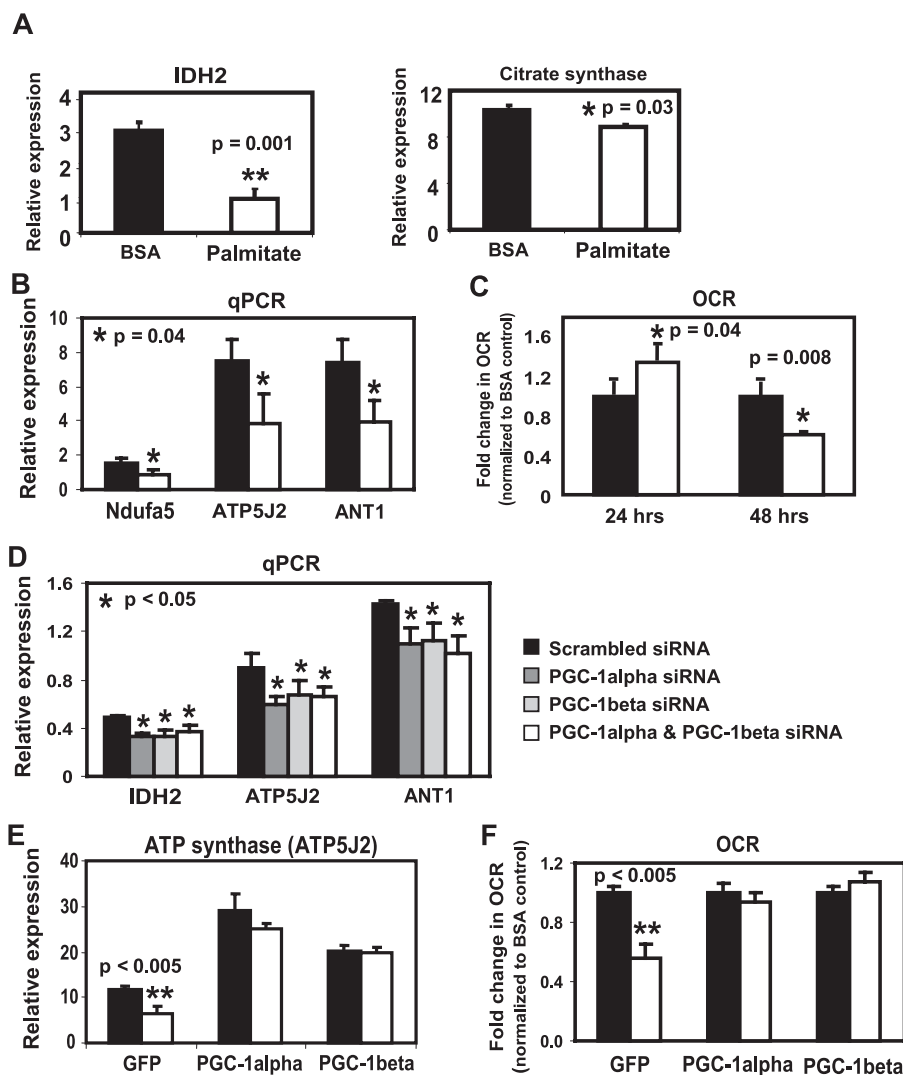
**Palmitate Effects on Mitochondrial Expression and Function Are PGC-1-dependent**—We next assessed the PGC-1 dependence of the effects of palmitate on oxidative gene expression and metabolism using both siRNA and adenoviral overexpression strategies. As expected, siRNA targeted against PGC-1 $\alpha$  and/or - $\beta$  (reducing expression by 92 and 84%, respectively) significantly decreased expression of multiple tricarboxylic acid cycle and oxidative phosphorylation genes, (Fig. 4D,  $p < 0.05$  for all). Conversely, adenoviral overexpression of either PGC-1 $\alpha$  or PGC-1 $\beta$  (8.5-fold protein overexpression) significantly increased basal levels of a representative oxidative phosphorylation gene, ATP synthase subunit ATP5J2. More importantly, PGC-1 completely ablated the effect of palmitate on ATP5J2 expression (Fig. 4E), indicating that palmitate-induced inhibitory effects on mitochondrial gene

expression are mediated by PGC-1. In addition, fatty acids that did not affect expression of PGC-1 (e.g. oleate and methyl palmitate) likewise did not affect expression of ATP5J2 (not shown). Finally, overexpression of either PGC-1 $\alpha$  or - $\beta$  also completely reversed the effect of palmitate to decrease oxygen consumption rate at 48 h (Fig. 4F).

**Rosiglitazone, Metformin, and AICAR Increase PGC-1 Expression**—C2C12s were treated with rosiglitazone, metformin, or AICAR, a potent activator of 5'-AMP-activated protein kinase, which is a key enzyme activated by exercise (30). Both metformin and AICAR increased basal PGC-1 $\alpha$  expression by 3.6-fold ( $p < 0.001$ ) and 4.6-fold ( $p < 0.001$ ), respectively (Fig. 5A). To determine whether these compounds could reverse the inhibitory effect of palmitate on PGC-1 expression, cells were treated for 30 min before incubation with palmitate or BSA. Rosiglitazone, metformin, and AICAR each significantly increased PGC-1 $\alpha$  expression in the presence of palmitate by 2-, 4.7-, and 6.8-fold, respectively ( $p < 0.001$  for all), and both rosiglitazone and AICAR reversed the inhibitory effect of palmitate (Fig. 5A). Similarly, AICAR increased basal expression of PGC-1 $\beta$  by 1.7-fold ( $p = 0.008$ ) and reversed the

inhibitory effect of palmitate (Fig. 5B). Rosiglitazone also increased PGC-1 $\beta$  expression in cells treated with palmitate by 42% ( $p = 0.04$ ) (Fig. 5B). These changes in PGC-1 expression occurred in parallel with improved insulin sensitivity, as measured by insulin-stimulated Akt Ser-473 phosphorylation (Fig. 5C, AICAR). Interestingly, despite the effects of leptin to increase AMP kinase (31) and basal levels of PGC-1 $\alpha$ , leptin (1 ng/ml) was unable to reverse palmitate effects. Although these data suggest a potential role of 5'-AMP-activated protein kinase pathways to reverse palmitate-mediated effects (32, 33, 42), this may be indirect, as we observed no effect of palmitate on phosphorylation of 5'-AMP-activated protein kinase or ACC either in the basal state or in AICAR-treated cells.

To determine whether the positive effects of rosiglitazone and metformin on PGC-1 expression also extended to the *in vivo* state, we treated 8-week-old *Lep<sup>ob</sup>/Lep<sup>ob</sup>* (ob/ob) or *Lep<sup>ob</sup>/+* (ob/+) control mice with rosiglitazone or metformin for 3 months. Rosiglitazone increased PGC-1 $\alpha$  mRNA expression by 77% ( $p = 0.048$ ) and PGC-1 $\beta$  by 59% ( $p = 0.01$ ) in ob/ob



**FIGURE 4. Palmitate-induced decreases in mitochondrial gene expression and function are PGC-1 dependent.** Expression of tricarboxylic acid cycle genes isocitrate dehydrogenase 2 (*IDH2*) and citrate synthase (A) electron transport chain genes NADH-ubiquinone oxidoreductase 1 $\alpha$  subcomplex 5 (*Ndufa5*) (complex I), mitochondrial ATP synthase subunit (*ATP5J2*) (complex V), and adenine nucleotide translocator 1 (*ANT1*) was assessed by real-time PCR in C2C12 myotubes incubated with 500  $\mu$ M palmitate (white bars) or BSA (black bars) for 16 h (B). qPCR, quantitative PCR. C, O<sub>2</sub> consumption rate (OCR) after 24 and 48 h of 500  $\mu$ M palmitate treatment. D, mitochondrial gene expression in C2C12s transfected on day 3 of differentiation with 100 nM scrambled control siRNA, PGC-1 $\alpha$ , and/or PGC-1 $\beta$  siRNA and harvested 48 h later. Effect of adenoviral expression of green fluorescent protein (GFP) or PGC-1 $\alpha$  or - $\beta$  on palmitate effects on ATP synthase expression (16 h) (E) and oxygen consumption rate (48 h) (F) in C2C12s. Data are the mean  $\pm$  S.E. \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ .

mice (supplemental Fig. 3) in parallel with improvements in insulin sensitivity. Rosiglitazone also increased PGC-1 $\alpha$  expression in high fat-fed mice by 87% ( $p = 0.02$ , not shown). Similar trends were observed for metformin in ob/ob mice, increasing PGC-1 $\alpha$  by 53% ( $p = 0.10$ ) and PGC-1 $\beta$  by 45% ( $p = 0.06$ ), respectively. Interestingly, these compounds had no effect on PGC-1 expression in ob/+ controls.

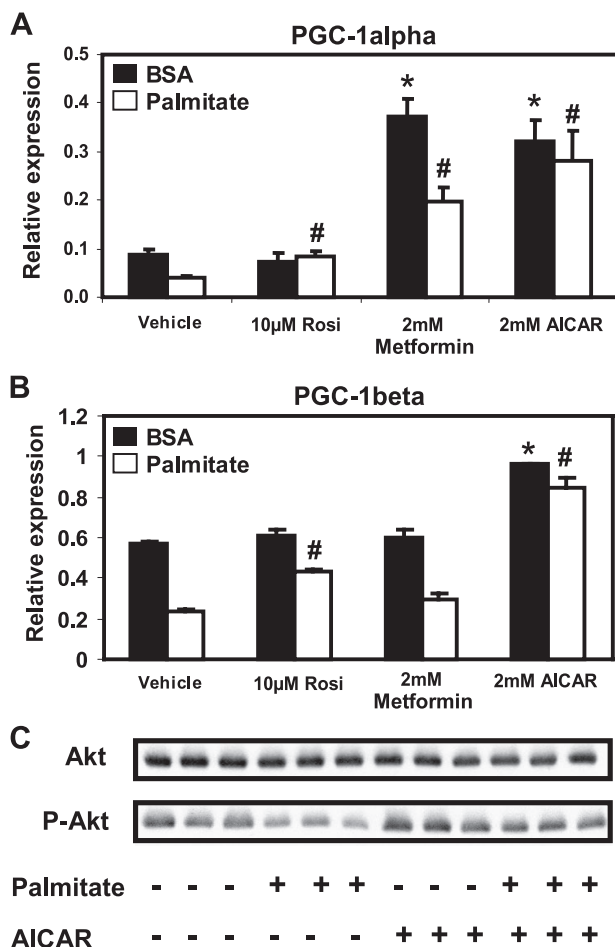
**Mechanisms Mediating the Effects of Palmitate**—One common mechanism mediating repression of transcription involves the modification of histones. We observed a dramatic reversal of palmitate effects with the histone deacetylase class I/II inhibitor trichostatin A, increasing basal PGC-1 $\alpha$  expression by 2.5-fold ( $p < 0.005$ ) and in palmitate-treated cells by 6.3-fold ( $p = 0.02$ , Fig. 6). These findings implicate a role for

histone modification in mediating the effects of palmitate on PGC-1 expression (34).

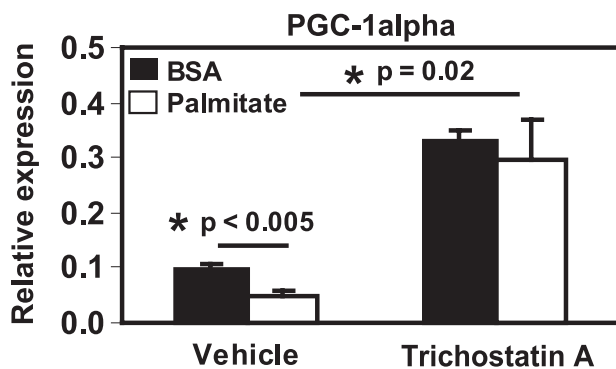
To further elucidate the upstream mechanisms mediating the effects of palmitate, we pretreated C2C12 myotubes with pathway-specific compounds before the addition of palmitate and determined the effects on palmitate-induced decreases in PGC-1 $\alpha$  mRNA expression or promoter activity. Compounds selected were chosen based on their (a) influence on expression and/or activity of pathways linked to PGC-1 expression, (b) activation by fatty acids, and/or (c) links to insulin resistance.

We observed no effect of (a) inhibition of classical nutrient-sensing pathways, the hexosamine biosynthetic pathway (azaserine) (23), or the mTOR/S6 kinase pathway (rapamycin) (25) and no change in phosphorylation of mTOR or S6 kinase, (b) inhibition of synthesis of ceramides (3-*O*-methyl-sphingomyelin, fumonisin B1), products of fatty acid metabolism associated with insulin resistance (35), (c) agonists of PPAR  $\beta/\delta$  (GW501516) (36) or PPAR $\alpha$  (WY-14,643) and no alterations in expression of these nuclear receptors, (d) activation of cAMP signaling pathway (forskolin) (37) and no change in expression of transducer of regulated cAMP-response element-binding protein (CREB)-binding proteins (38) or CREB phosphorylation (10), (e) inhibition of NF- $\kappa$ B-dependent pathways (sodium salicylate), known to be activated by fatty acids and in humans with diabetes (39, 43), and (f) antioxidant treatment (*N*-acetylcysteine) (40). Although *N*-acetylcysteine reversed palmitate induction of oxidative stress genes (peroxiredoxin 1 and superoxide dismutase 2.2-fold ( $p = 0.005$ ) and 2.3-fold ( $p = 0.02$ ) respectively), it did not alter expression of PGC-1.

Calcineurin and calcium-dependent protein kinases are known to positively regulate expression of PGC-1 $\alpha$  by activation of the transcription factors cAMP-response element-binding protein and MEF2 (34, 38, 41). Although we observed no effect of the calcineurin inhibitor cyclosporin A, expression of MEF2C and MEF2A was significantly decreased by palmitate. In addition, MEF2 DNA binding was decreased (electrophoretic mobility shift assay, not shown).



**FIGURE 5. Modulation of PGC-1 expression by rosiglitazone, metformin, and AICAR.** C2C12 myotubes incubated with 10  $\mu$ M rosiglitazone (Rosi), 2 mM metformin, or 2 mM AICAR 30 min before treatment with 500  $\mu$ M palmitate (16 h). Expression of PGC-1 $\alpha$  (A) and PGC-1 $\beta$  (B) was assessed by real-time PCR. C, anti-phospho-Akt (P-Akt) and total Akt Western blot of cell lysates treated with vehicle or AICAR 2 mm before BSA or palmitate exposure. Data are the means  $\pm$  S.E. The asterisk indicates  $p < 0.05$  for the effect of rosi, metformin, or AICAR as compared with control during BSA exposure; # indicates  $p < 0.05$  for effects in the presence of palmitate.



**FIGURE 6. Inhibition of histone deacetylation reverses palmitate effects on PGC-1 $\alpha$  expression.** PCR analysis of PGC-1 $\alpha$  expression in C2C12 myotubes treated with trichostatin A (class I/II histone deacetylase inhibitor) or vehicle control 30 min before the addition of BSA or palmitate for 16 h. Data are the means  $\pm$  S.E.

**Role of p38 MAPK-dependent Pathways in Mediating Palmitate Effects**—The MAP kinase signaling family has also been linked to regulation of PGC-1 (42–44). We, therefore, investigated whether p38, extracellular signal-related kinase, or c-Jun-

NH<sub>2</sub>-terminal kinase pathways were involved in mediating palmitate effects. Palmitate exposure did increase p38 MAPK phosphorylation from 30 min to 16 h; this stimulation was completely inhibited by preincubation with the p38 $\alpha/\beta$  MAPK inhibitor SB203580 (representative data are shown at 1 h, Fig. 7A). Importantly, SB203580 completely prevented palmitate-induced decreases in PGC-1 expression (Fig. 7B). Similar effects were observed with 2 additional distinct p38 $\alpha/\beta$  MAPK inhibitors, SB202910 and PD169316 (Fig. 7B). By contrast, there was no effect of MEK (MAPK-extracellular signal-related kinase) or c-Jun-NH<sub>2</sub>-terminal kinase inhibition (PD98059, SP600125) (Fig. 7C), respectively. The three p38 MAPK inhibitors also prevented palmitate-induced decreases in PGC-1 $\beta$  and ATP synthase subunit ATP5J2 mRNA expression (Fig. 7B).

Because pharmacological inhibitors are often nonspecific, we transfected C2C12s with siRNA directed against p38 $\alpha$  MAPK and 24 h later treated cells overnight with 500  $\mu$ M palmitate or BSA. Transfection efficiency was monitored using siGLO, a non-coding fluorescent siRNA (Fig. 7D). Expression of p38 $\alpha$  MAPK was reduced by 55% ( $p = 0.02$ ) at the RNA level and 58% at the protein level ( $p = 0.003$ ), and phosphorylation of p38 MAPK was reduced by 72% ( $p = 0.001$ ) (Fig. 7E). Importantly, this reduction in p38 $\alpha$  MAPK prevented both palmitate-induced phosphorylation of p38 MAPK (Fig. 7F) and palmitate-induced reductions in PGC-1 $\alpha$ , PGC-1 $\beta$ , and ATP synthase mRNA expression (Fig. 7G), confirming a mechanistic role for p38 MAPK-dependent pathways in mediating the effects of palmitate.

## DISCUSSION

Multiple lines of evidence support the hypothesis that mitochondrial dysfunction is a key feature of insulin resistance and type 2 diabetes (7, 8) and may contribute to reduced lipid oxidation, accumulation of intramyocellular lipid (2), and other features of insulin resistance (45). We hypothesize that genetic and environmental risk factors may decrease expression of PGC-1 $\alpha$  and PGC-1 $\beta$  and mitochondrial gene expression and function (46).

In our studies expression of PGC-1 $\alpha$  and - $\beta$  was reduced in skeletal muscle from mice with either genetic or diet-induced obesity. These data are in parallel with the recent observation that muscle expression of PGC-1 and mitochondrial genes is decreased in healthy humans after only 3 days of high fat diet (18). Such responses are likely to be influenced by genetic background, as high fat feeding did not alter PGC-1 expression in obesity-resistant A/J mice.

Differential expression in the setting of obesity may be a consequence of insulin resistance or associated hyperinsulinemia. We did not observe any effect of experimental insulin resistance on PGC-1 expression in myotubes, a finding in accord with the normal PGC-1 expression in muscle insulin receptor knock-out mice (MIRKO) despite marked muscle insulin resistance (47). Although specific mechanisms underlying insulin resistance *in vivo* may influence transcriptional effects on PGC-1, these data suggest that muscle insulin resistance may not be a primary factor mediating decreased PGC-1.

Nutrient excess may also contribute to obesity-linked decreases in PGC-1. Excess nutrients, including glucose, fatty

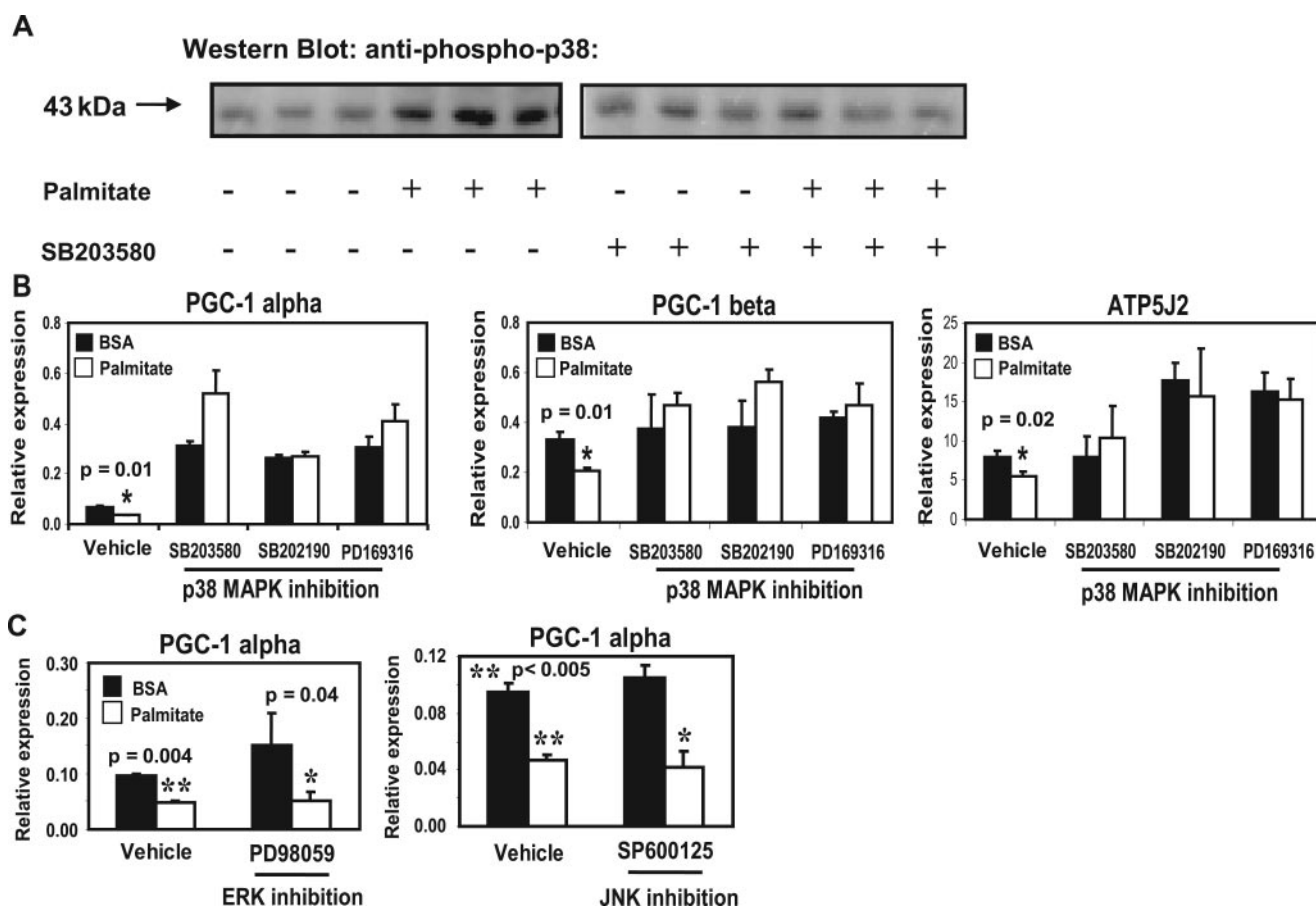
## Role of Obesity and FFA in PGC-1 Expression

acids, and amino acids, can modulate signal transduction and gene transcription via nutrient-sensing pathways including the mTOR (48) and hexosamine biosynthetic pathways (23), contributing to insulin resistance (24). However, high concentrations of glucose, glucosamine, or amino acids did not modulate PGC-1 expression in differentiated myotubes. This was not entirely unexpected, since PGC-1 expression is not altered in insulin-deficient streptozotocin diabetic mice despite severe hyperglycemia (47).

Free fatty acids (FFA) have emerged as an important link between obesity, insulin resistance, and DM (26). Elevated plasma FFA levels in obese and type 2 diabetic subjects correlate with intramyocellular lipid and insulin resistance (2, 28), and infusion of fatty acids in both rodents and humans induces insulin resistance in muscle (27). Interestingly, infusion of a fatty acid mixture into healthy humans for 24 h (plasma FFA, 1.73 mM) also decreases expression of muscle PGC-1 $\alpha$  and oxidative phosphorylation genes (17). Conversely, reduction of plasma FFA with acipimox, a long-acting antilipolytic drug, reduces insulin resistance (49), and lowering of FFA by nicotinic acid increases expression of PGC-1 $\alpha$  in humans (50). We

now demonstrate that the long chain saturated fatty acids palmitate and stearate potently decrease both PGC-1 $\alpha$  and PGC-1 $\beta$  mRNA expression. Short chain, unsaturated, omega-3 and non-metabolizable FA have no effect. These data suggest that FA-induced decreases in PGC-1 expression are dependent on their structure or specific oxidation products. Our data are in agreement with other studies demonstrating FA effects on PGC-1 expression in muscle cells (44, 51). Differences observed in effects of specific FA may be cell type- or species-specific.

Palmitate-induced-reduced PGC-1 expression was also accompanied by reduced expression of many mitochondrial tricarboxylic acid cycle and oxidative phosphorylation genes. At a functional level, oxygen consumption was initially increased after 24 h of palmitate treatment, likely due to increased substrate availability and oxidation; however, with sustained palmitate exposure, oxygen consumption rate decreases, possibly reflecting the down-regulation of oxidative phosphorylation genes. Interestingly, siRNA-mediated reduction in PGC-1 $\alpha$  or  $\beta$  expression resulted in decreased expression of tricarboxylic acid cycle and oxidative phosphorylation genes. Furthermore, adenoviral overexpression of PGC-1 $\alpha$  or



**FIGURE 7. Inhibition of p38 MAPK reverses the effects of palmitate on PGC-1 and downstream mitochondrial gene expression in C2C12 myotubes.** *A*, Western blot of p38 MAPK phosphorylation in C2C12 myotubes treated for 1 h with BSA or 500  $\mu$ M palmitate in the presence or absence of 10  $\mu$ M SB203580. *B*, mRNA expression of PGC-1 $\alpha$  and  $\beta$ , and ATP5J2 in C2C12 myotubes treated with 500  $\mu$ M palmitate for 16 h in the presence or absence of p38 MAPK inhibitors SB203580, SB202190, or PD169316 (10  $\mu$ M). *C*, effect of extracellular signal-related kinase (ERK; 20  $\mu$ M PD98059) or c-Jun-NH<sub>2</sub>-terminal kinase (JNK) inhibition (20  $\mu$ M SP600125) on PGC-1 $\alpha$  mRNA expression. *D*, light micrograph (*left panel*) and fluorescence micrograph (*right*) from C2C12 myotubes transfected on day 3 of differentiation with 100 nM fluorescent siGLO (non-coding siRNA); 200 $\times$  magnification. *E*, efficacy of p38 $\alpha$  siRNA, as demonstrated by PCR and anti-p38 $\alpha$  and anti-phospho-p38 Western blots (*C*, control cells; *TC*, transfection control (reagent only); *Scr*, scrambled non-coding control siRNA; *p38*, p38 siRNA). *qPCR*, quantitative PCR. *F*, efficacy of p38 $\alpha$  siRNA to inhibit palmitate-induced p38 $\alpha$  phosphorylation. *G*, effect of p38 $\alpha$  siRNA on palmitate-induced reductions in PGC-1 $\alpha$  and  $\beta$  and ATP synthase mRNA expression. Data are the means  $\pm$  S.E. \* $p$  < 0.05; \*\* $p$  < 0.005.

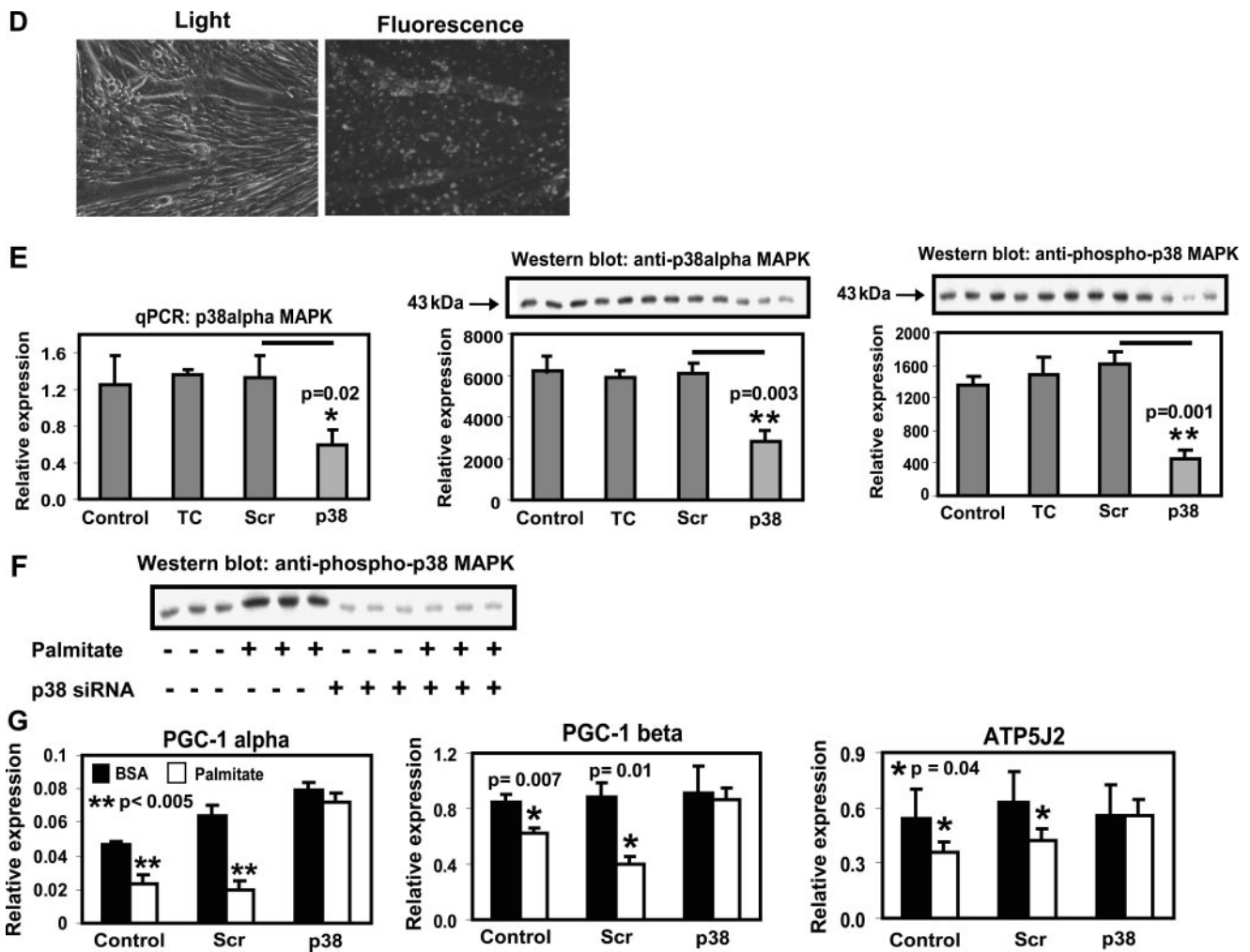


FIGURE 7—continued

$-\beta$  reversed palmitate effects to decrease both mitochondrial gene expression and oxygen consumption rate. Taken together, these data indicate that palmitate-induced mitochondrial changes are mediated by PGC-1-dependent pathways. These data support the concept that excess long chain saturated fatty acids within muscle may be responsible at least in part for the reductions in PGC1 $\alpha$  and  $-\beta$  and mitochondrial dysfunction associated with DM.

**Role of Type 2 Diabetes Therapies**—Vigorous exercise, metformin, and rosiglitazone are potent activators of AMP kinase (32, 33) and PGC-1 expression (15, 52, 53), and all reduce diabetes risk (54, 55). Consistent with these findings, metformin and the PPAR $\gamma$  agonist rosiglitazone increased PGC-1 expression in *Lep<sup>ob</sup>/Lep<sup>ob</sup>* mice in parallel with improvements in insulin sensitivity. Similar effects were observed with rosiglitazone in mice with diet-induced obesity.

Rosiglitazone also reversed the effects of palmitate in myotubes. These effects may also be related to its activity as a PPAR $\gamma$  agonist, since the PGC-1 $\alpha$  promoter does contain a PPAR response element. Interestingly, we found no effect of PPAR $\beta/\delta$  or  $\alpha$  agonists to reverse the effects of palmitate despite recent data indicating activation of PPAR $\beta/\delta$  by exercise (56) and a direct role for PPAR $\beta/\delta$  in regulating PGC-1 $\alpha$  and mitochondrial gene expression (36). Further experiments will be required

to determine whether the effects of rosiglitazone to reverse palmitate transcriptional effects are direct or indirect.

The 5'-AMP-activated protein kinase activator AICAR increased basal PGC-1 $\alpha$  and PGC-1 $\beta$  expression and reversed palmitate effects in C2C12 myotubes. Surprisingly, palmitate had no effect on phosphorylation of 5'-AMP-activated protein kinase or ACC; this may be related to the more chronic experimental time course and/or a greater transcriptional effect, as compared with phosphorylation of 5'-AMP-activated protein kinase *per se*.

**What Are the Mechanisms by Which Palmitate Inhibits PGC-1 Expression?**—PGC-1 $\alpha$  promoter activity was dramatically reduced by palmitate exposure, indicating inhibition of transcription by palmitate. Our data suggest that one mechanism mediating palmitate-induced inhibition of native PGC-1 expression is histone deacetylation, as indicated by the reversal of effects by histone deacetylase I/II inhibition. Although these data are also consistent with the role of histone acetylation to regulate PGC-1 $\alpha$  expression and transcriptional activity (34, 57), further studies are required to determine the specific effect of palmitate on histone modification and transcription.

**Role of Nutrient-sensing Pathways**—Although nutrient-sensing pathways can regulate lipid effects (58), our data do not

## Role of Obesity and FFA in PGC-1 Expression

support a mechanistic role for the hexosamine or mTOR nutrient-sensing pathways in palmitate effects on PGC-1.

Adipocyte secretion of leptin and other adipokines may also be considered a systemic nutrient-sensing pathway regulating whole-body metabolism. We demonstrate a modest effect of leptin to increase PGC-1 expression in myotubes, suggesting that obesity-related leptin deficiency (as in *ob/ob* mice) or resistance (as with high fat feeding) may contribute to alterations in PGC-1 expression *in vivo*. However, at a cellular level, pharmacological doses of leptin were unable to reverse the inhibitory effects of palmitate. Moreover, both metformin and rosiglitazone were effective in leptin-deficient mice. Thus, we cannot completely exclude a contribution of leptin resistance in mediating palmitate effects on PGC-1 expression; however, leptin resistance is unlikely to play a major role.

Adiponectin may also contribute to whole-body insulin sensitivity and mitochondrial biogenesis (59). Because adiponectin levels increased in C57BL6 mice during high fat feeding, when PGC-1 levels were lower, it is unlikely that adiponectin deficiency plays a role in this setting *in vivo*. However, we cannot exclude a possible contribution of adiponectin resistance in this setting.

**Role of Products of Fatty Acid Metabolism**—Interestingly methyl palmitate had no effect on PGC-1 expression, suggesting that a specific fatty acyl-CoA moiety or metabolite is necessary for FFA effects on PGC-1 expression. Reversal of palmitate effects by CPT-1 inhibition also indicates that a mitochondrial oxidative metabolite or byproduct may mediate these effects (60). We observed no effect of inhibition of ceramide synthesis (35). Another potential candidate would be fatty acid oxidation-linked increases in reactive oxygen species (40). High fat feeding induces oxidative stress in muscle (61), and PGC-1 ameliorates the effects of reactive oxygen species (62). Palmitate did increase expression of genes induced by oxidative stress. However, whereas antioxidant therapy reversed these effects, it did not prevent the effects of palmitate on PGC-1. Thus, an unidentified product of oxidative fatty acid metabolism may be playing a mechanistic role.

**p38 MAPK-dependent Pathways**—PGC-1 $\alpha$  activity may be controlled post-translationally by p38 MAP kinase phosphorylation (42, 43), leading to protein stabilization and increased transcriptional activity. In mouse muscle, activation of upstream MKK3/6 and p38 MAPK after either acute or endurance exercise is associated with increased PGC-1 mRNA and protein expression (63).

Although these data implicate a positive role for p38 activation in the regulation of PGC-1 expression and/or function in muscle, we now demonstrate that sustained activation of p38 MAPK by saturated fatty acids occurs in parallel with reductions in PGC-1 (64, 65). Moreover, using (a) three independent inhibitors of p38 MAPK activity, and (b) siRNA-mediated reduction in p38 $\alpha$  MAPK expression, we show that p38 $\alpha$  MAPK can mediate palmitate-induced decreases in both PGC-1 $\alpha$  and PGC-1 $\beta$  expression. Although we have been unable to evaluate PGC-1 expression at a protein level due to the low endogenous levels of PGC-1 (42), the palmitate-induced reduction in mitochondrial gene expression and function and its reversal by p38 MAPK inhibition indicates that

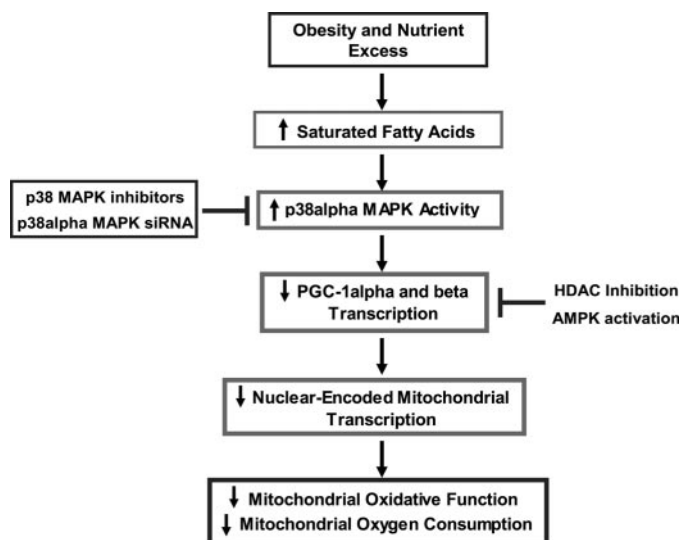


FIGURE 8. Potential mechanisms mediating effects of obesity and over-nutrition to decrease PGC-1 transcription and mitochondrial gene expression and function. HDAC, histone deacetylase; AMPK, 5'-AMP-activated protein kinase.

palmitate also impairs PGC-1 transcriptional activity. Interestingly, our data contrast with a recent study in which extracellular signal-related kinase-dependent pathways contributed to palmitate-mediated decreases in PGC-1 $\alpha$ ; however, we saw no impact of MEK1/2 (MAPK-extracellular signal-related kinase 1/2) or c-Jun-NH<sub>2</sub>-terminal kinase inhibition (44).

p160<sup>MYB</sup> has been implicated in negative regulation of PGC-1 $\alpha$  transcriptional activity, a process that can be reversed by p38 MAPK phosphorylation (66). Although p160<sup>MBP</sup> expression was increased by palmitate (45%,  $p = 0.01$ , not shown), we saw no effect of p160<sup>MBP</sup> siRNA on PGC-1 expression or transcriptional activity on mitochondrial targets or p38 MAPK expression (data not shown). Thus, p160<sup>MBP</sup> does not appear to mediate palmitate effects in this setting.

Taken together, our data support that sustained activation of p38 MAPK may serve as a negative transcriptional regulator of both PGC-1 $\alpha$  and PGC-1 $\beta$  and downstream mitochondrial gene expression. Such effects of sustained p38 MAPK activation may also be both tissue- and stimulus-specific, with the net effect dependent upon additional interacting pathways. In this context, it is interesting that basal p38 MAPK phosphorylation is increased in adipocytes from type 2 diabetic subjects, in association with decreased IRS-1 and GLUT4 content (67). In addition, high fat feeding in rats increases p38 MAPK phosphorylation in heart (68), and p38 activation is associated with hepatic insulin resistance (69). Overexpression of p38 $\alpha$  MAPK also exerts deleterious proinflammatory effects in the heart (70). Thus, it will be important to determine whether high fat feeding similarly leads to elevated p38 MAPK activity in muscle.

In summary, we demonstrate that sustained cellular exposure to saturated FA reduces expression of PGC-1 $\alpha$  and - $\beta$  and mitochondrial oxidative gene expression and reduces cellular oxygen consumption rates. Such responses may initially be adaptive to limit oxidative stress or accumulation of products of incomplete oxidation. However, with chronic FA excess, reductions in PGC-1 expression, particularly when coupled with

inactivity and inadequate fatty acid turnover, may ultimately induce and perpetuate metabolic inflexibility and insulin resistance.

We recognize that the precise effects of obesity- and palmitate-induced decreases in PGC-1 expression on insulin action and metabolism in skeletal muscle remains unknown. Although whole-body PGC-1 $\alpha$  ablation in mice decreases mitochondrial gene expression and number in skeletal muscle (71), mice are unexpectedly protected from obesity, perhaps due to marked increases in locomotor activity associated with central nervous system lesions. PGC-1 $\beta$  ablation (72–74) decreases mitochondrial gene expression but does not influence whole-body insulin sensitivity (74). Thus, tissue-specific and developmental models of PGC-1 $\alpha$  and/or  $\beta$  deficiency in mice will be required to fully elucidate the impact of muscle PGC-1 on skeletal muscle insulin sensitivity.

In conclusion, saturated fatty acids mediate decreased expression of PGC-1 $\alpha$ , PGC-1 $\beta$ , and oxidative phosphorylation genes as well as impaired mitochondrial function (Fig. 8). These effects appear to be largely mediated at a transcriptional level via byproducts of fatty acid oxidation and p38 MAPK pathway activation. Reversal with known insulin sensitizers, AMP kinase activation, and inhibition of histone deacetylase and p38 MAP kinase suggests potential pathways which could be targeted to improve skeletal muscle metabolic defects associated with insulin resistance and diabetes.

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