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PPARδ agonism activates fatty acid oxidation via PGC-1α but does not increase mitochondrial gene expression and function

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The peroxisome proliferator-activated receptor delta (PPARδ) is a regulator of lipid metabolism and has been shown to induce fatty acid oxidation (FAO). PPARδ transgenic and knockout mice indicate an involvement of PPARδ in regulating mitochondrial biogenesis and oxidative capacity; however, the precise mechanisms by which PPARδ regulates these pathways in skeletal muscle remain unclear. In this study, we determined the effect of selective PPARδ agonism with the synthetic ligand, GW501516, on FAO and mitochondrial gene expression *in vitro* and *in vivo*. Our results show that activation of PPARδ by GW501516 led to a robust increase in mRNA levels of key lipid metabolism genes. Mitochondrial gene expression and function were not induced under the same conditions. Additionally, the activation of PDK4 transcription by PPARδ was coactivated by PGC-1α. PGC-1α, but not PGC-1β, was essential for full-activation of CPT1b and PDK4 gene expression via PPARδ agonism. Furthermore, the induction of FAO by PPARδ agonism was completely abolished in the absence of both PGC-1α and PGC-1β. Conversely, PGC-1α-driven FAO was independent of PPARδ. Neither GW501516 treatment nor knockdown of PPARδ affect PGC-1α-induced mitochondrial gene expression in primary myotubes. These results demonstrate that pharmacological activation of PPARδ induces FAO via PGC-1α. However, PPARδ agonism does not induce mitochondrial gene expression and function. PGC-1α-induced FAO and mitochondrial biogenesis appear to be independent of PPARδ.

The peroxisome proliferator-activated receptors (PPARs) are ligand-modulated transcription factors that form heterodimers with retinoid X receptors (RXRs) (1). Certain PPARs regulate transcription in response to fatty acids and are potential therapeutic targets for metabolic disorders (1). Three PPAR family members have been identified. PPARα is primarily expressed in the liver, but also in heart, kidney and brown fat, where it regulates fatty acid catabolism. PPARγ is highly expressed in adipose tissue and is a key regulator of adipogenesis and insulin sensitivity. The expression of PPARδ is more ubiquitous, with relatively high levels in metabolically active tissues such as muscle, liver, and adipose tissue (2). Selective activation of PPARδ by agonists has been shown to improve glucose

metabolism and insulin sensitivity in mouse models of obesity and insulin resistance. These effects are mainly attributed to the agonists' capacity to activate fatty acid transport and oxidation (1). In addition, recent reports suggest that activation of PPARδ increases mitochondrial gene expression and function. The strongest evidence stems from the muscle-specific VP16-PPARδ transgenic mice in which a constitutively active fusion protein is overexpressed specifically in the muscle using the skeletal α-actin promoter (3). These mice exhibit enhanced mitochondrial gene expression in association with a fiber type switch from fast glycolytic type II fibers to slow oxidative type I fibers. Type I fibers utilize fatty acids as their main fuel and are more fatigue resistant. Concordantly, PPARδ transgenic mice display enhanced exercise endurance when compared to the wild-type mice. However, these studies were done with a PPARδ fusion protein, so it is not yet clear to what extent those results represent the physiological function of the native PPARδ protein.

A mouse model overexpressing the native PPARδ in muscle was also reported (4). These mice exhibit a much milder phenotype with respect to mitochondrial oxidative capacity when compared to the VP16-PPARδ mice. Nevertheless, overexpression of the native PPARδ increased the percentage of oxidative fibers that are succinate dehydrogenase-positive in tibialis anterior (TA) muscle correlating with an increase in citrate synthase (CS) and β-hydroxy-CoA dehydrogenase activities.

PPARγ coactivator-1 alpha (PGC-1α) and beta (PGC-1β) are important physiological transcriptional regulators of oxidative metabolism and mitochondrial biogenesis. PGC-1α is induced in a tissue-specific manner under conditions such as cold, fasting and exercise. It promotes mitochondrial biogenesis, oxidative capacity and FAO by increasing the expression and activation of various transcription factors including mitochondrial transcription factor A (mtTFA), nuclear respiration factor (NRF) 1 and 2, PPARs and estrogen-related receptor alpha (ERRα) (5,6). The PGC-1α knockout (KO) mice display reduced mitochondrial function and oxidative capacity (7), whereas overexpression of PGC-1α increases both (8). Interestingly, PGC-1α expression was not increased in muscle from either the VP16-PPARδ transgenic mice or the native PPARδ transgenic mice, leading to the assumption that PPARδ might act downstream or independent of PGC-1α (3,4).

Recently, Schuler M et al. reported that the muscle-specific PPARδ KO mice display less oxidative fibers associated with reduced oxidative capacity and mitochondrial gene expression (9). In contrast to the findings in the PPARδ transgenic mouse models, the expression levels of PGC-1α and mtTFA were reduced in the PPARδ KO muscles. Thus, the authors suggested that PPARδ acts upstream of PGC-1α and induces PGC-1α expression in muscle.

Genetic mouse models are very useful but have their limitations with respect to the prediction of pharmacological effects. Although activation of PPARδ by agonists, such as GW501516, has been shown to improve glucose tolerance and insulin resistance, the impact on mitochondrial biogenesis and function is not well characterized (3,16). In addition, the underlying molecular mechanisms of PPARδ activation on lipid metabolism and mitochondrial oxidative capacity are not fully understood based on the phenotyping results obtained from the PPARδ transgenic

and KO mouse models (3,9). Given the mitochondrial phenotypes of the PPARδ mouse models, it is tempting to speculate that pharmacological activation of PPARδ exerts beneficial effects on obesity and insulin resistance in a way that mimics what is happening during physical exercise. We therefore investigated first whether pharmacological activation of the endogenous PPARδ induces mitochondrial biogenesis and enhances mitochondrial function in muscle. Second, we used genetic tools to investigate the mechanisms of PPARδ in regulating energy metabolism, specifically the interdependence of PPARδ and PGC-1 transcriptional coactivators in the regulation of FAO, mitochondrial gene expression and function.

Experimental Procedures

Primary Mouse Myoblast Isolation and Differentiation- Primary muscle cells were isolated from 2-to 3-week-old FVB mice, the PGC-1α wild-type and knockout mice (7) as described (10). Myoblasts were grown and differentiated as described previously (11). Differentiated myotubes were treated with DMSO or 100 nM GW501516 for 24 hr prior to being harvested or subjected to any analyses.

For differentiation of C2C12 myoblasts, cells were grown to confluency at which point the medium was changed to differentiation medium (DMEM, 2 % horse serum). Cells were transduced with indicated adenovirus and harvested at 72 h post-infection. Cells were treated with DMSO or 100 nM GW501516 for the last 24 hrs.

Adenoviral Transduction- Adenoviruses expressing two mouse PPARδ shRNAs with the sequences, GGAGCATCCTCACCGGCAA and GCAGCTGGTCACTGAGCAT, were generated by Welgen (Worcester, MA). In the PPARδ knockdown experiments, these two viruses were used at 1:1 ratio. The adenoviruses expressing PGC-1β shRNA, PGC-1α, and GFP were described previously (12,13). Primary muscle cells were transduced at day 2 or 3 post-differentiation with various adenoviruses as indicated to either overexpress or knock down the gene(s) of interest. The concentrations of adenoviruses used for a single viral transduction were PPARδ-shRNA: $1x10^9$ particles/ml; PGC-1β-shRNA: $0.45x10^9$ particles/ml. The control adenovirus was used in the same concentration as the virus of interest. For double viral transduction, the concentrations used were: PGC-1α: $0.3x10^9$ particles/ml; GFP: $0.3x10^9$ particles/ml; PPARδ-shRNA: $0.8x10^9$ particles/ml; PGC-1β-shRNA: $0.225x10^9$ particles/ml. For knockdown of PPARδ and overexpression of PGC-1α, the cells were first transduced with PPARδ-shRNA adenovirus and subsequently with PGC-1α adenovirus on the following day. The medium was changed daily.

RNA Extraction and Real-Time PCR Analysis of Gene Expression- Total RNA was extracted from tissues and cells using TRIzol (Invitrogen) and subjected to TaqMan analysis as described previously (11). The relative mRNA expression levels were calculated by comparing the target genes to 18S rRNA and were expressed as means ± SEM of the fold change relative to the control, which was set at 1. The TaqMan primer/probe sets for all of the genes examined were obtained from Applied Biosystems (Foster City, CA).

Nuclear Fractionation and Western Blotting- Nuclear fractionation was done as previously described (6). Fifty micrograms of nuclear extract or whole cell lysate was

used for Western blot analysis. Proteins of interest were detected with specific antibodies: anti-Cytochrome C clone 7H8.2C12 (abcam) at 1:200 dilution, anti-UCP3 (Affinity Bioreagent), anti-PDK4 clone RB3041 (Agent), anti- Complex IV subunit (MitoScience), anti-Tubulin (abcam), anti-PGC-1α (Chemicon) at 1:1000 dilution. The secondary antibody, HRP-conjugated goat anti-rabbit IgG, was obtained from Pierce (Rockford, IL) and used at 1:10,000 dilution. The immunoblot was developed by using enhanced chemiluminescence with Super Signal West Dura Chemiluminescence kit from Pierce.

Measurement of Citrate Synthase Activity- Citrate synthase (CS) activity was determined in cell lysates as previously described (14). Each sample was assayed in triplicate. CS activity was normalized for protein concentration and expressed as means ± SEM.

Fatty Acid Oxidation Measurements- Fatty acid oxidation assay was performed as described (15).

PDK4 reporter gene assay- Hela cells were cultured in DMEM supplemented with 10% FBS in 5% CO_2. Cells (7×10^4) were plated in a 96-well plate and transfected the next day with the PDK4 reporter (24) and the Renilla control plasmid along with various expression vectors as indicated. One day after transfection, cells were treated with 100 nM GW501516 or DMSO for 24 hrs. Luciferase activities were measured at 48 hr post-transfection as described previously (11).

Animal studies- All mice used in this study were purchased from the Jackson Labs (Bar Harbor, ME) and group housed (n =4 per cage) in a temperature and humidity-controlled facility with a 12-h light/dark cycle and free access to water and regular mouse chow. Obese (*ob/ob*) nine week-old male mice were randomly divided into three treatment groups: vehicle control (0.5% carboxymethylcellulose/2% Tween-80), GW501516 (3 mg/kg, 10 mg/kg, and 30 mg/kg) and treated once daily via oral gavage for a total of 3 weeks. After 19 days of treatment and after a 4 hour fast, blood samplings (50-100 μl) were obtained via retro-orbital bleed. Plasma concentrations of glucose, triglycerides and nonesterified free fatty acids (NEFA) were determined using a clinical auto-analyzer (Olympus AU4000, Melville, NY). Plasma insulin was determined using a kit from Meso Scale Discovery (MSD, Gaithersburg, MD). An oral glucose tolerance test (OGTT) was performed on day 21 after the mice were fasted for 4 hour. On day 22 and approximately 4 hour after receiving the last dose, mice were sacrificed and muscle tissues were collected and snap-frozen in liquid nitrogen for gene expression analysis.

The lean C57BL/6 male mice were randomly divided into two treatment groups: vehicle control and GW501516 (30 mg/kg) and treated once daily via oral gavage for a total of 4 weeks. After 4 weeks and approximal 6 hour after receiving the last dose, mice were sacrificed and muscle tissues were collected and snap-frozen in liquid nitrogen for gene expression and protein analysis.

RESULTS

PPARδ agonism induces FAO but not mitochondrial gene expression in primary mouse myotubes. We used the PPARδ agonist GW501516 to activate the endogenous PPARδ protein in primary mouse myotubes. To ensure that effects are PPARδ-dependent, we first established experimental conditions in which GW501516-induced effects could only be

observed in the presence of PPARδ in the muscle cells. To accomplish this, adenoviral-mediated knockdown of PPARδ was performed in primary muscle cells using two shRNAs designed specifically against PPARδ. The mRNA level of PPARδ was reduced 80% in the cells transduced with the adenoviral PPARδ-shRNAs (Fig. 1A). GW501516 treatment resulted in a robust activation of several PPARδ target genes involved in the FAO pathway and uncoupling such as carnitine palmitoyltransferase 1b (CPT1b), which catalyzes the esterification of acyl-CoA to form acyl-carnitine, the rate limiting step of mitochondrial FAO, pyruvate dehydrogenase kinase 4 (PDK4), which plays an important role in switching the fuel source from glucose to fatty acids by inactivating pyruvate dehydrogenase, uncoupling protein 3 (UCP3), which induces FAO *in vivo* and *in vitro*. The induction of the mRNA expression of these genes was completely abolished when PPARδ expression was knocked down (Fig. 1B). Consistent with the mRNA expression, the protein levels of PDK4 and UCP3 were also increased with GW501516 treatment and this induction was blocked following knockdown of PPARδ (Fig. 1C). Correlating with the induction of gene expression in the FAO pathway, GW501516 treatment increased palmitate oxidation rates, which were completely prevented with the knockdown of PPARδ (Fig. 1D). These results demonstrate that the GW501516-induced effects on gene expression and FAO rate are PPARδ dependent. Thus we used these experimental conditions for all the subsequent experiments described in this study.

We next examined whether activation of PPARδ could promote mitochondrial gene expression as it has been reported in the muscle-specific VP16-PPARδ transgenic mice, but not in the transgenic mice overexpressing the native PPARδ protein (3). As shown in Fig. 1E, the mRNA levels of the key mitochondrial transcription regulators, PGC-1α, PGC-1β and ERRα were not increased by GW501516 treatment. Furthermore, there was no increase observed in the mRNA or protein levels of several mitochondrial proteins, components of the electron transport chain (ETC) and the citric acid cycle (TCA cycle) including cytochrome c (Cyt C), cytochrome c oxidase subunit I, IV, Va (Cox I, Cox IV, Cox5a), isocitrate dehydrogenase 3, alpha subunit (IDH3α) (Fig. 1C, E). GW501516 treatment also did not increase CS activity, indicative of mitochondrial function (Fig. 1F). Interestingly, knockdown of PPARδ slightly, but significantly reduced the mRNA levels of ERRα and Cox5a (Fig. 1E). However, knockdown of PPARδ did not alter the protein levels of CytC, CoxI and CoxIV as well as CS activity. (Fig. 1C, F). These results indicate that PPARδ agonism increases the gene expression and rate of FAO, however, it does not increase mitochondrial gene expression and function in primary muscle cells.

GW501516 treatment improves glucose homeostasis and induces lipid metabolism but not mitochondrial gene expression in vivo. It was previously reported that GW501516 increased mitochondrial number and expression of some mitochondrial genes *in vivo* (3,16). However, we did not observe increased mitochondrial gene expression and CS activity in the GW501516-treated muscle cells *in vitro* (Fig 1C, E, F). It is possible that additional factors required for GW501516 to exert its effect on mitochondrial biogenesis are missing in the *in vitro* experimental system (i.e., primary muscle cells). To rule out this possibility, we

investigated the effect of GW501516 on mitochondrial gene expression in the genetically obese and insulin resistant *ob/ob* mice. The mice were gavaged daily with 10 or 30 mg/kg (mpk) of GW501516 or vehicle. At day 19, blood samples were taken from the mice after 4 hr fasting. Plasma glucose, insulin, triglycerides and non-esterified fatty acids (NEFA) were measured and shown in Fig. 2A. GW501516 treatment significantly and dose-dependently reduced fasting plasma glucose in the mice. Insulin, triglycerides and NEFA were reduced at 30 mpk. To further investigate the effect of GW501516 on glucose homeostasis in these mice, an oral glucose tolerance test (OGTT) was performed at day 21 after 4 hr fasting. Glucose tolerance was significantly improved by GW501516 treatment in a dose-dependent manner with 45% and 59% reduction of glucose AUC (Area under the curve) from the basal at 10 mpk and 30 mpk, respectively (Fig. 2B). These results demonstrate that GW501516 at both doses efficaciously improved glucose homeostasis *in vivo*. To determine whether GW501516 treatment has an effect on muscle PPARδ target genes and mitochondrial gene expression in these mice, the mRNA levels of CPT-1b, PDK4 and UCP3 were measured in both tibialis anterior (TA) and quadriceps muscles. Consistent with results from the primary mouse myotubes (Fig. 1B), mRNA levels of these genes were significantly increased in both muscles in a dose-dependent manner with the GW501516 treatment (Fig. 2C). In contrast, the mRNA levels of PGC-1α, PGC-1β, CytC, Cox5a and IDH3α were not increased in either of the muscles (Fig. 2D). VP16-PPARδ mice exhibited a fiber type switch from glycolytic to more oxidative fibers concurrent with the upregulation of mitochondrial genes. To determine whether there was any fiber type conversion induced by the GW501516 treatment, the mRNA expression levels of several myosin heavy chain (MHC) isoforms, MHCI, IIa, IId/x, and IIb, were measured in the muscles from the mice treated with either vehicle, 10 or 30 mpk of GW501516 for 3 weeks. MHCI is preferentially expressed in the slow twitch oxidative type I fiber while MHCIIa, IId/x and IIb are preferentially expressed in the fast twitch type II fibers, with former being more oxidative and latter being more glycolytic. As shown in Fig. 2E, the expression of these MHC isoforms was not altered by the GW501516 treatment at either 10 or 30 mkg indicating that there was no fiber type switch in these mice. Chronic administration of GW501516 at 10 mpk was reported to cause hepatomegaly in mice previously (16). In our study, we did not observe hepatomegaly in the *ob/ob* mice when they were dosed with 10 and 30 mpk of GW501516 for three weeks. Nevertheless, we carried out another study where the *ob/ob* mice were dosed with 3 mpk for three weeks. The fasted blood glucose was significantly lower in the GW501516 treated mice compared with the control (Suppl Fig.). The expression levels of PDK4 and UCP3 in muscle were significantly increased by the treatment but there was no change in the expression of PGC-1α, ERRα, and the mitochondrial proteins, Cyt C, IDH3a and Cox5a (Suppl Fig.) which is similar to the results in Figure 2. We also investigated the effect of GW501516 on lipid metabolism and mitochondrial oxidative capacity in the lean mice. Both the mRNA and protein levels of PKD4 and UCP3 were significantly increased by the GW501516 treatment in the gastrocnemius muscle in the lean mice (Fig. 3A, B). However, the mRNA expression of

PGC-1α, PGC-1β, ERRα, CytC, IDH3α, and Cox5a was not increased by the GW501516 treatment (Fig. 3C). The protein levels of CoxI and CoxIV were also not increased by GW501516 (Fig. 3 B). CS activity was not altered in gastrocnemius cell lysates from the GW501516-treated mice (Fig. 3D). The mRNA expression of MHCI, IIa, IId/x and IIb was not altered by the GW501516 treatment indicating there was no fiber type conversion (Fig. 3E). These results are similar to the findings in the ob/ob mice (Fig. 2). Taken together, pharmacological activation of PPARδ with GW501516 improved glucose homeostasis and increased the expression of PKD4, CPT-1b and UCP3. However, it did not promote mitochondrial gene expression in skeletal muscle *in vivo*.

Loss of PGC-1α reduces PPARδ-driven FAO. PGC-1α functions as a transcriptional regulator in a number of metabolic pathways by interacting and/or modulating the activity of various transcription factors and nuclear hormone receptors (5). To determine whether PGC-1α plays a role in PPARδ-mediated FAO, primary myoblasts were isolated from PGC-1α wild-type (WT) (17) and knockout (KO) mice and differentiated into myotubes (7). As expected, in the KO cells PGC-1α mRNA was not detected and the expression levels of ERRα, CytC, Cox5a and IDH3α were reduced 30 to 60% (Fig. 4A) concurrent with a reduction of CS activity (Fig. 4B) indicating an impairment of mitochondrial function in the PGC-1α null cells. To compare the effect of GW501516 on the expression of the PPARδ target genes, both PGC-1α WT and KO cells were treated with either DMSO or GW501516. The mRNA and protein levels of PPARδ target genes as well as FAO rates were determined. PDK4 and UCP3 mRNA levels were comparable in the PGC-1α WT and KO cells while CPT-1b mRNA level was reduced in the KO cells without the GW501516 treatment (Fig. 4C). The mRNA levels of these three genes were induced in both WT and PGC-1α null cells, however, the expression levels of CPT-1b and PDK4 were significant lower in the PGC-1α null cells compared with the WT cells upon stimulation with GW501516 (Fig. 4C). The mRNA expression level of UCP3 was comparable with the GW501516 treatment in both WT and KO cells (Fig. 4C) suggesting that the induction of UCP3 by GW501516 was independent of PGC-1α. Consistent with the mRNA expression levels, PDK4 protein was increased by GW501516 treatment in the WT cells, however, this increase was diminished in the PGC-1α null cells (Fig. 4D), UCP3 protein was increased by GW501516 treatment to a similar extent in the WT cells compared with the PGC-1α null cells (Fig. 4D). Palmitate oxidation with the GW501516 treatment was 30% lower in the PGC-1α KO muscle cells compared with the WT controls, indicating that loss of PGC-1α impairs PPARδ-induced FAO (Fig. 4E). Similar results were obtained in the primary mouse myotubes following knockdown of PGC-1α (data not shown). These results indicate that the effects of pharmacological activation of PPARδ on PDK4 and CPT-1b expression as well as fat oxidation depend on PGC-1α.

PGC-1a potentiates PPARδ-induced CPT1b and PDK4 gene expression. The induction of CPT-1b and PDK4 by PPARδ agonism appears to be dependent on PGC-1α. To determine whether PGC-1α coactivates PPARδ-mediated PDK4 transcription directly, a PDK4 promoter-driven luciferase reporter (24) was transfected into HeLa cells along with

various expression vectors as indicated in Fig. 5A. Cells were treated with either vehicle or 100 nM of GW501516 for 24 hrs. PPARδ activated PDK4 transcription only in the presence of GW501516 treatment indicating PPARδ-induced PDK4 transcription is ligand-dependent (Fig. 5A, lane 4 vs. 3; lane 3 vs. 1). PGC-1α robustly increased PDK4 transcription regardless of GW501516 treatment (lanes 5, 6 vs. lanes 1, 2). Interestingly, PPARδ and PGC-1α increased PDK4 transcription synergistically only with the GW501516 treatment (Fig. 5A, lane 8 vs. 7; lane 7 vs. 5). The results demonstrate that PPARδ induced-PDK4 transcription is coactivated by PGC-1α in a ligand-dependent manner. To further confirm this ligand-dependent coactivation of the PPARδ target gene transcription by PGC-1α, the endogenous expression levels of PDK4 and CPT-1b were measured in C2C12 myotubes transduced with an adenovirus expressing either GFP or PGC-1α. As shown in Fig. 5B, PGC-1α mRNA was markedly elevated in the myotubes transduced with the PGC-1α adenovirus compared with the cells transduced with GFP adenovirus. The mRNA levels of CPT-1b, PDK4 and UCP3 were significantly increased by the GW501516 treatment alone in C2C12 myotubes (Fig. 5C). This data is consistent with the results obtained in the primary mouse myotubes (Fig 1B). Overexpression of PGC-1α alone resulted in a significant increase of CPT-1b and PDK4 expression demonstrating that PGC-1α was able to induce PDK4 and CPT-1b gene expression independent of PPARδ agonism (Fig. 5A, C). Overexpression of PGC-1α combined with GW501516 treatment led to a robust synergistic induction of PDK4 and CPT-1b mRNA expression (Fig. 5C) in agreement with the synergistic activation of PDK4 (Fig. 5A) and CPT-1b promoters by PPARδ and PGC-1α (19). UCP3 expression was only induced by the GW501516 treatment but not by PGC-1α overexpression (Fig. 5C). This data, together with the results in Fig. 4 indicate that UCP3 induction by PPARδ is independent of PGC-1α. PGC-1α overexpression led to a robust induction of ERRα, CytC and Cox5a expression in the cells. This induction was not further enhanced by the treatment with GW501516 (Fig. 5D). The results demonstrate that PGC-1α potentiates PPARδ-induced PDK4 and CPT-1b expression in a PPARδ ligand-dependent manner while PGC-1α-induced mitochondrial biogenesis in these cells is independent of PPARδ agonism.

PGC-1β knockdown does not affect PPARδ-driven FAO. Loss of PGC-1α reduced, but not completely abolished GW501516-induced FAO (Fig. 4E) raising the possibility that other coactivators, such as PGC-1β, might also contribute to PPARδ-mediated FAO. To test this hypothesis, primary muscle cells were transduced with a PGC-1β-shRNA or a control adenovirus. PGC-1β mRNA level was reduced 80% and PGC-1β protein was completely abolished in the PGC-1β-shRNA transduced cells (Fig. 6A, D). Knockdown of PGC-1β resulted in a significant reduction of CytC and Cox5a mRNA levels (Fig. 6A). CS activity was also decreased indicating that mitochondrial function was reduced (Fig. 6B) by the loss of PGC-1β in the cells. Unlike PGC-1α, depletion of PGC-1β expression did not impair the induction of CPT-1b, PDK4 and UCP3 mRNA expression by GW501516 treatment (Fig. 6C). PDK4 and UCP3 proteins were also increased by GW501516 treatment to a similar extent in the PGC-1β knockdown cells compared with the control cells (Fig 6D). The induction of palmitate

oxidation rates by GW501516 were also comparable in both PGC-1β knockdown and the control cells (Fig. 6E). These results indicate that knockdown of PGC-1β alone does not affect PPARδ-mediated gene expression and FAO rate or that PGC-1α compensates for the loss of PGC-1β.

Reduction of both PGC-1α and PGC-1β completely abolishes PPARδ-mediated FAO in muscle cells. To investigate whether PGC-1α and PGC-1β have overlapping functions in regulating PPARδ-mediated FAO, we knocked down PGC-1β expression in the PGC-1α KO myotubes to generate cells lacking both proteins. PGC-1α KO cells display already a 40-60% reduction of mitochondrial gene expression when compared to the WT cells (Fig. 4A). A reduction of PGC-1β in the PGC-1α KO cells resulted in an additional 50 to 60% decrease in the mRNA levels of CytC, IDH3α and Cox5a (Fig. 7A). Protein levels of CoxI, and CoxIV were markedly reduced with the loss of PGC-1β in the PGC-1α KO cells (Fig. 7D), indicating that mitochondrial gene expression is strongly dependent on PGC-1β in the absence of PGC-1α. The CS activity was further reduced with the knockdown of PGC-1β (Fig. 7B). Expression levels of CPT1b, PDK4 and UCP3 were significantly reduced at the basal condition when PGC-1β was knocked down (Fig. 7C), but the protein level of PDK4 was not affected. Loss of both PGC-1β in addition to PGC-1α did not further impair the induction of these genes upon PPARδ activation by GW501516 (Fig. 7C). In addition to gene expression, the fatty acid rates were measured in the muscle cells lacking either both PGC-1α/β or only PGC-1α. Surprisingly, GW-induced palmitate oxidation was completely abolished in the cells lacking both PGC-1α and PGC-1β (Fig. 7E) despite a compareable increase in GW501516-induced gene expression (Fig. 7C). Similar results were obtained in primary muscle cells where both PGC-1α and PGC-1β were knocked down by shRNAs (data not shown). These results indicate that both PGC-1α and PGC-1β are essential for the induction of fat oxidation induced by PPARδ agonism.

Knockdown of PPARδ does not affect PGC-1α-mediated FAO and mitochondrial gene expression. PGC-1α has been shown to drive oxidative metabolism and to induce FAO through coactivation of ERRα and PPARα (18). However, PGC-1α was still able to induce FAO, to a less extent, in the cells lacking these two proteins, suggesting that other factor(s) might mediate PGC-1α-induced FAO (18). PPARδ is a conceivable candidate since it interacts with PGC-1α and its transcriptional activity is enhanced by PGC-1α (19, 20, Fig 5A). To determine whether PPARδ contributes to the regulation of FAO mediated by PGC-1α, primary myotubes were co-transduced with an adenovirus expressing PGC-1α and a PPARδ-shRNA to knockdown PPARδ expression. PGC-1α protein levels were elevated in the cells transduced with the PGC-1α adenovirus (Fig. 8A), while PPARδ mRNA levels were markedly reduced in the cells transduced with the PPARδ-shRNA adenovirus (Fig. 8B). Overexpression of PGC-1α increased the mRNA levels of the PPARδ target genes CPT-1b and PDK4 to a similar extent at both the mRNA and protein levels in the PPARδ knockdown cells compared with the control cells (Fig. 8C, D). The FAO rate was increased by PGC-1α overexpression and this induction was not attenuated when PPARδ was knocked down (Fig 8E). PGC-1α overexpression led to a robust increase in

the expression of ERRα, CytC, Cox5a and IDH3α mRNA suggesting there is an increase of mitochondrial biogenesis in the cells (Fig. 8F). This induction was modestly, but significantly reduced when PPARδ was knocked down. However, the protein levels of CytC, CoxI and CoxIV as well as CS activity were not affected by PPARδ knockdown (Fig. 8D, G). Thus knockdown of PPARδ did not significantly affect the ability of PGC-1α to increase fat oxidation and mitochondrial biogenesis. The data in Fig 5D showed that PPARδ agonism did not enhance PGC-1α-induced mitochondrial gene expression. Taken together, these results suggest that PGC-1α-driven mitochondrial biogenesis and FAO are not dependent on PPARδ.

DISCUSSION

In this study, the effects of pharmacological activation of PPARδ on lipid metabolism and mitochondrial gene expression were examined *in vitro* and *in vivo*. As expected, activation of PPARδ with GW501516 resulted in a robust activation of genes involved in lipid metabolism (CPT1b, PDK4, and UCP3) *in vivo* and *in vitro* as well as increased FAO rate in primary muscle cells. However, PPARδ agonism failed to increase mitochondrial gene expression and function *in vitro* and *in vivo* (Fig. 1, 2, 3). Therefore, the effects of pharmacological activation of the endogenous PPARδ do not fully recapitulate the effects observed in the VP16-PPARδ transgenic mouse or the muscle-specific PPARδ KO mouse model, in which mitochondrial biogenesis was strongly induced or decreased, respectively (3). In addition, GW501516 treatment did not cause a fiber type switch as it has been seen in the VP16-PPARδ transgenic and muscle-specific PPARδ mouse model and could be developmentally regulated. Despite the lack of increased mitochondrial function, activation of PPARδ improved glucose homeostasis in the obese and insulin resistant *ob/ob* mice (Fig. 2), confirming its potency in ameliorating metabolic disorders (1). These data suggest that the effects of PPARδ activity on glucose homeostasis are most likely mediated by increased lipid oxidation rates or other not yet known PPARδ effects which are not dependent on an increase in muscle mitochondrial gene expression.

We also investigated the molecular mechanisms by which activation of PPARδ regulates lipid metabolism. PGC-1α has been shown to enhance the transcriptional activity of PPARs in luciferase assays (19, 20). However, it was not known, to what extent this coactivation is essential for the action of PPARδ on endogenous target gene expression. Our data revealed that PGC-1α, but not PGC-1β, was required for the full activation of CPT1b and PDK4 by PPARδ (Fig. 4 and 5). Since PPARδ mRNA levels were not altered in the PGC-1α KO primary myotubes, nor in the muscle of either the PGC-1α KO mice or the MCK-PGC-1α transgenic mice (unpublished data), it is unlikely that PGC-1α regulates PPARδ expression directly. Thus, the impact of PGC-1α on PPARδ-mediated effects is most likely through coactivation of this receptor. This is supported by the synergistic action of PGC-1α and GW501516 on these genes (Fig. 5C). In addition, our data show that PGC-1α directly coactivates PDK4 promoter via PPARδ in a ligand-dependent manner (Fig. 5A). Others had reported a similar effect of PGC-1α on the CPT-1b promoter (19). Interestingly, UCP3 appears

to be a PPARδ-specific target gene whose expression was induced by PPARδ agonism in a PGC-1α-independent manner (Fig. 4, 5).

We further investigated the roles of PGC-1 coactivators in mediating FAO induced by GW501516. Deletion of PGC-1α, but not PGC-1β, significantly reduced the induction of FAO rate induced by GW501516 (Fig. 4, 6). Surprisingly, GW-induced fat oxidation was completed abolished in the absence of both PGC-1α and PGC-1β proteins despite an induction of CPT-1b, PDK4 and UCP3 expression comparable to the PGC-1α KO cells (Fig. 7). We believe that a strong reduction of mitochondrial function in the absence of both PGC-1 coactivators might contribute to this. Under normal conditions where PGC-1 proteins are expressed, activation of PPARδ triggers an increase in fatty acid β-oxidation through upregulation of the key FAO target genes. PGC-1α enhances the action of PPARδ on CPT1b and PDK4 in a PPARδ-ligand dependent manner. In addition, PGC-1 proteins are essential to maintain mitochondrial gene expression, including genes involved in lipid metabolism, oxidative phosphorylation (OXOPHOS) and TCA cycle to ensure the proper function of mitochondria. The final product of β-oxidation is metabolized in the TCA cycle, which yields reducing agents that are subsequently oxidized in the electron transport chain (ETC) to generate ATP in the functional mitochondria (Fig. 9A). In the absence of PGC-1 proteins, PPARδ activation can still increase the expression of its target genes to a certain extent. However, mitochondrial oxidative capacity, such as TCA cycle and ETC activities, is discordantly reduced which in turn hindering an increase in fatty acid β-oxidation (Fig. 9B). Similarly, Koves et al. (21) suggested that high lipid supply, under conditions of low PGC-1α, could provoke a disconnection between mitochondrial β-oxidation and the TCA cycle which might contribute to the pathogenesis of insulin resistance. Our data support the hypothesis that PGC-1α furnishes the mitochondria to cope with high lipid load by coordinately regulating fatty acid β-oxidation, TCA cycle and electron transport chain activity (21,22).

Although PGC-1α is able to coactivate PPARδ on the CPT1b and PDK4 gene, PPARδ does not seem to be essential for PGC-1α-mediated gene expression, including CPT-1b, PDK4 and mitochondrial genes. Knockdown of PPARδ did not affect the transcriptional program induced by PGC-1α nor did reduce PGC-1α-mediated mitochondrial function in primary myotubes. Our results indicate that PGC-1α does not require the presence of PPARδ to activate mitochondrial biogenesis which is most likely mediated through ERRα (18).

In summary, our study shows that activation of the endogenous PPARδ using a synthetic ligand increased lipid oxidation, and improved glucose metabolism and insulin sensitivity. However, PPARδ agonism failed to induce mitochondrial gene expression and function *in vitro* and *in vivo which* was confirmed by another study (25) published during the revision of this manuscript. In addition, PGC-1α coactivates PDK4 transcription via PPARδ in a ligand-dependent manner. PGC-1α is required for full activation of FAO by PPARδ in muscle cells. Furthermore, both PGC-1α and PGC-1β are essential for PPARδ-driven fat oxidation. We hypothesize that PGC-1α and PGC-1β provide a functional mitochondrial environment to allow an increased FAO rate through activation of PPARδ (Fig. 9). Conversely, PGC-1α-mediated

mitochondrial biogenesis and fatty oxidation appears to be independent of PPARδ.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. GW501516-induced effects on FAO and mitochondrial gene expression in primary myotubes. Primary myotubes were infected with PPARδ-shRNAs or a control-shRNA adenovirus. At 48 hr post-transduction, the cells were treated with 100 nM GW501516 (23) or DMSO for 24 hr. Total RNA was extracted from cells and the mRNA levels of the indicated genes were determined by quantitative PCR (Q-PCR) (*A, B and E*). *D*. Cells were labeled with ^{14}C-palmitate and FAO rates were determined. The FAO rate in the control cells treated with DMSO was set at 1 and the FAO rates under other conditions were expressed as fold change relative to that in the control cells treated with DMSO. *C*. Cells were lysed in RIPA buffer and protein lysate from each sample was used for Western blotting. *F*. Cells were lysed and CS activities were determined. Data are mean ± SEM; n=3, *p≤0.05 for DMSO vs. GW, #p≤0.05 for control vs. PPARδ-shRNA.

Fig. 2. GW501516-induced effects on glucose homeostasis and muscle mitochondrial gene expression in *ob/ob* mice. *ob/ob* mice were treated once daily with 10 mpk or 30 mpk

GW501516 for 3 weeks. *A.* Plasma glucose, insulin and NEFA were measured at day 19 post-treatment after 4 hr fast (n=8). *B.* OGTT and glucose AUC from basal vs. vehicle , n=8, *p≤0.05. *C & D. E.* Tibilias anterior and quadriceps were isolated, RNA was extracted and Q-PCR was performed to determine the mRNA levels of the indicated genes. Data are mean ± SEM, n=7-8, *p≤0.05 for vehicle vs. GW501516.

Fig. 3. GW501516-induced effects on muscle mitochondrial gene expression in lean C57BL/6 mice. Mice were gavaged once daily with 30 mpk GW501516 for 4 weeks. Gastrocnemius was isolated, RNA was extracted and Q-PCR was performed to determine the mRNA levels of the indicated genes (*A, C, E*). *B.* Protein was extracted from gastrocnemius muscle isolated from mice. Protein lysate from each sample was used for Western Blotting. *D.* Citrate synthase activity was determined in the protein lysate from gastrocnemius muscles. Data are mean ± SEM, n=7-8, *p≤0.05 for vehicle vs. GW.

Fig. 4. GW501516-mediated gene expression and FAO in PGC-1α KO myotubes. Primary WT and PGC-1α KO myotubes were treated with 100 nM GW501516 for 24 hr. *A. C.* RNA was extracted and the mRNA levels for the indicated genes were measured by Q-PCR. *B.* Cells were lysed and CS activity was measured. *D.* Cells were lysed in RIPA buffer and protein lysate was subjected to Western blot analysis. *E.* Cells were labeled with ^{14}C-palmitate and FAO rates were measured. Data are mean ± SEM, n=3, *p≤0.05 for DMSO vs. GW, #p≤0.05 for WT vs. PGC-1α KO.

Fig. 5. Effect of PGC-1α on GW501516-mediated FAO and mitochondrial gene expression. *A.* Hela cells were transfected with PDK4-luciferase reporter and the indicated expression vectors for 24 hr followed by a 24 hr treatment with either DMSO or GW501516 (100 nM). The luciferase activity, indicative of PDK4 transcription, was measured. Data are mean ± SEM, n=8, *p≤0.05 for DMSO vs. GW. #p≤0.05 for the comparison as indicated. *B-D.* C2C12 myotubes were transduced with either the PGC-1α or GFP adenovirus for 48 h and then treated with DMSO or 100 nM GW501516 for 24 hr. RNA was extracted and the mRNA levels of the indicated genes were measured by Q-PCR. Data are mean ± SEM, n=3, *p≤0.05 for DMSO vs. GW, #p≤0.05 for GFP vs. PGC-1α.

Fig. 6. Effect of PGC-1β knockdown on GW501516-mediated gene expression and FAO. Primary myotubes were infected with the PGC-1β-shRNA or a control adenovirus for 72 hr and then treated with either 100 nM GW501516 or DMSO for the last 24 hr. *A & C,* RNA was extracted and the mRNA levels of the indicated genes were measured by Q-PCR. *B.* Cells were lysed and CS activities were determined. *D.* Cells were lysed in RIPA buffer. Protein lysate from each sample was subjected to Western blot analysis. *E.* Cells were labeled with ^{14}C-palmitate and FAO rates were measured. The FAO rate in the control/DMSO was set at 1 and the FAO rates under other conditions were expressed as fold change relative to the control/DMSO. Data are mean ± SEM, n=3, *p≤0.05 for DMSO vs. GW, #p≤0.05 for control vs. PGC-1β-shRNA

Fig. 7. The effect of knockdown PGC-1β on GW501516-mediated gene expression and FAO in the PGC-1α KO myotubes. The PGC-1α null myotubes were transduced with PGC-1β-shRNA or a control adenovirus for 72 h and then treated with DMSO or 100 nM GW501516 (23) for the last 24 hr. *A & C,* RNA was extracted and the mRNA levels of the indicated genes were measured by Q-PCR. *B.* Cells were lysed and CS activities were determined. *D.* Cells were lysed in RIPA buffer an 10-40 ng protein extract was used for Western blotting. *E.* Cells were labeled with ^{14}C-palmitate and FAO rates were measured. The FAO rate in the control/DMSO was set at 1 and the FAO rates under other conditions were expressed as fold change relative to the control/DMSO. Data are mean ± SEM, n=3, *p≤0.05 for DMSO vs. GW, #p≤0.05 for control vs. PGC-1β-shRNA

Fig. 8. Effect of PPARδ knockdown on PGC-1α-mediated FAO and mitochondrial gene expression. Primary myotubes were transduced with PPARδ-shRNA or a control adenovirus. The next day the cells were transduced with either the PGC-1α or GFP adenovirus for 48 hr. *A.* Cells were lysed and subjected to Western blot analysis to measure PGC-1α protein levels. *B. C. E. F.* RNA was extracted and the mRNA levels of the indicated genes were measured by Q-PCR. *D.* Cells were labeled with ^{14}C-palmitate and FAO rates were measured. *E.* Cells were lysed in RIPA buffer an 10-40 ng protein extract was used for Western blotting *G.* Cells were lysed and CS activities were determined. Data are mean ± SEM, n=3, *p≤0.05 for GFP vs. PGC-1α, #p≤0.05 for control vs. PPARδ-shRNA.

Fig. 9. A proposed model by which PGC-1 coactivators and PPARδ regulate FAO. In the presence of PGC-1 coactivators, the mitochondria are furnished with a functional β-oxidation, TCA cycle and electron transport chain (ETC). PGC-1α and PPARδ activate CPT1b and PDK4 genes synergistically in a ligand-dependent manner, which results in an increase in fatty acid β-oxidation. The fatty acids are completely oxidized to CO_2 to generate ATP in the functional mitochondria. *B.* In the absence of PGC-1 coactivators, PPARδ-driven PDK4 and CPT1b gene expression is reduced due to lack of coactivation by PGC-1α. Other genes important for β-oxidation and components of the TCA cycle and ETC are markedly decreased in the absence of PGC-1s, which in turn may hinder fat oxidation mediated by PPARδ agonism.

Figure 1

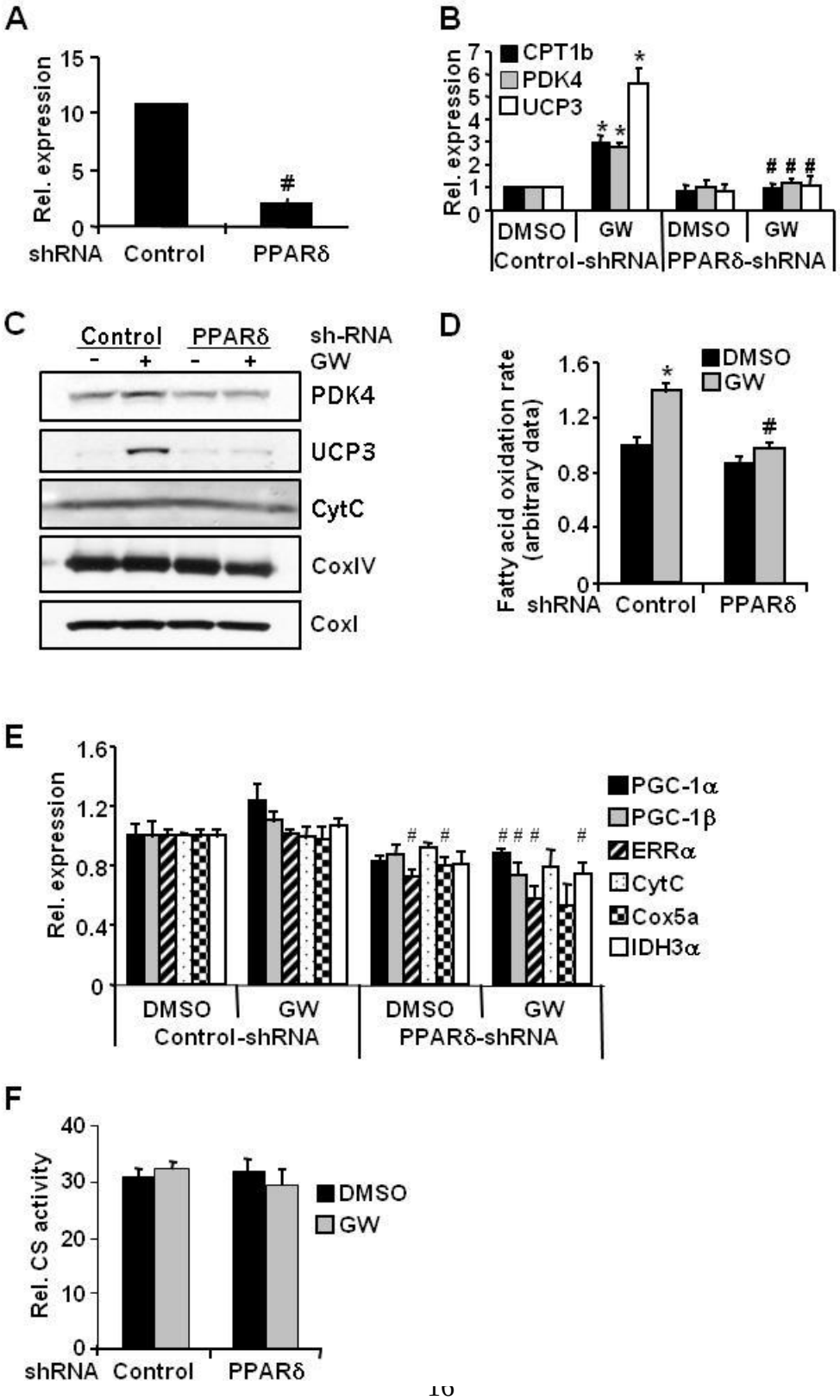
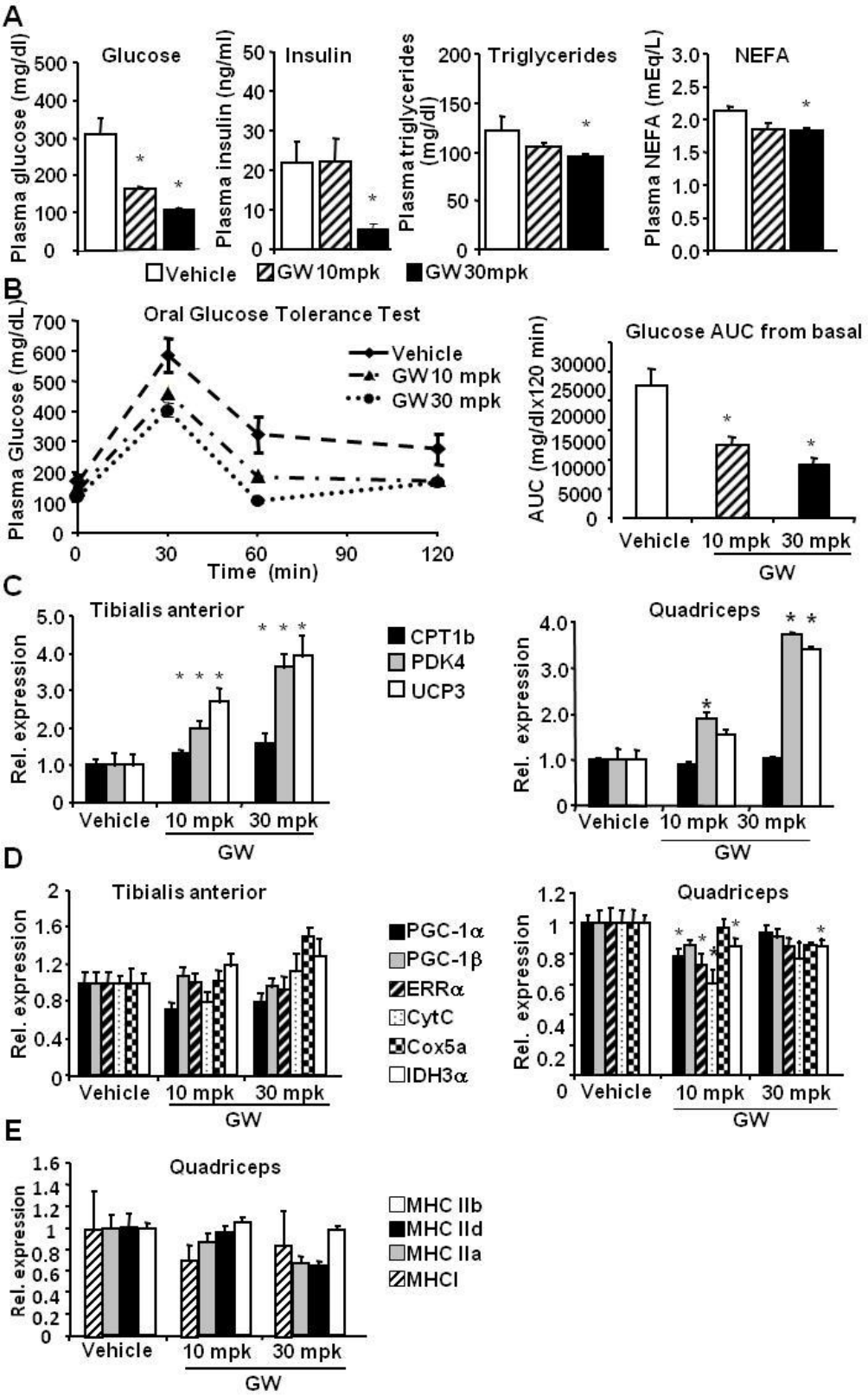


Figure 2



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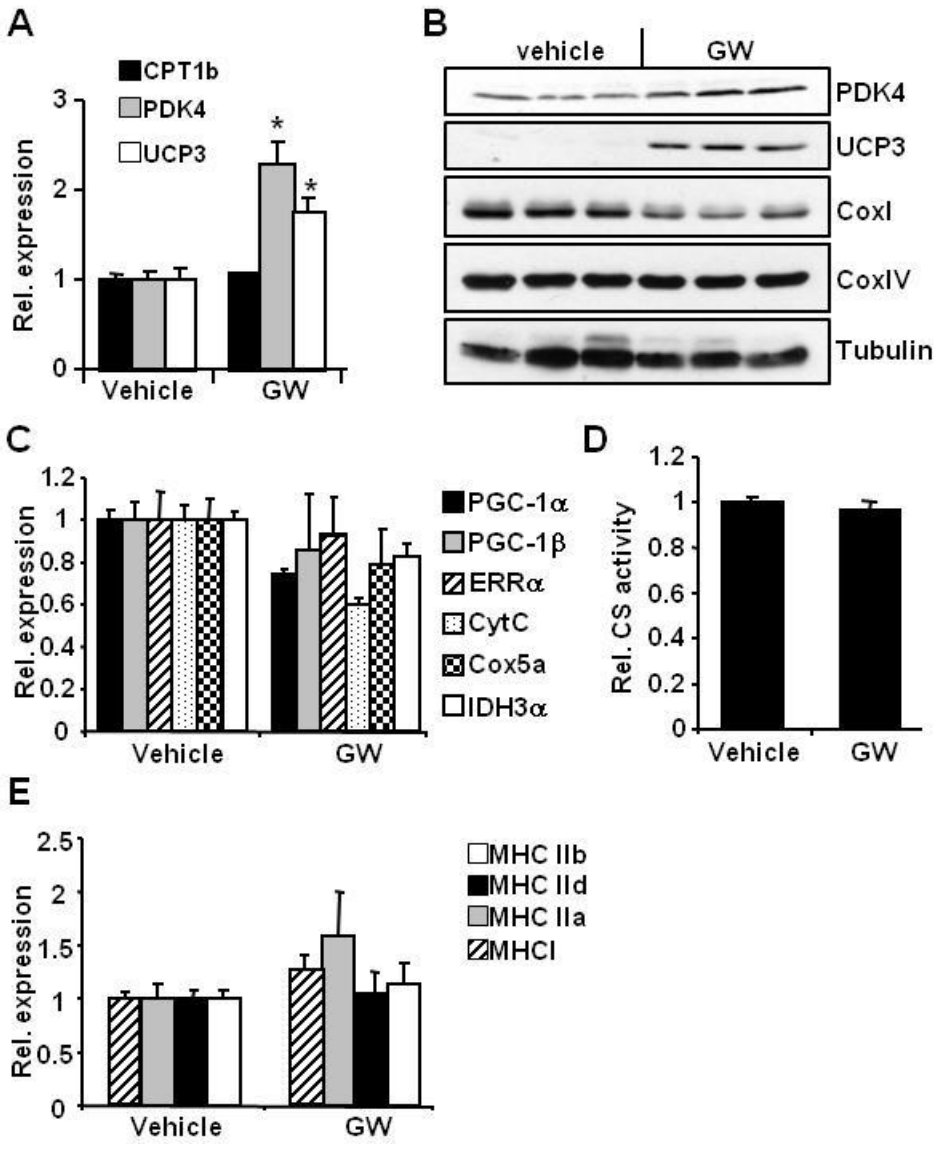


Figure 4

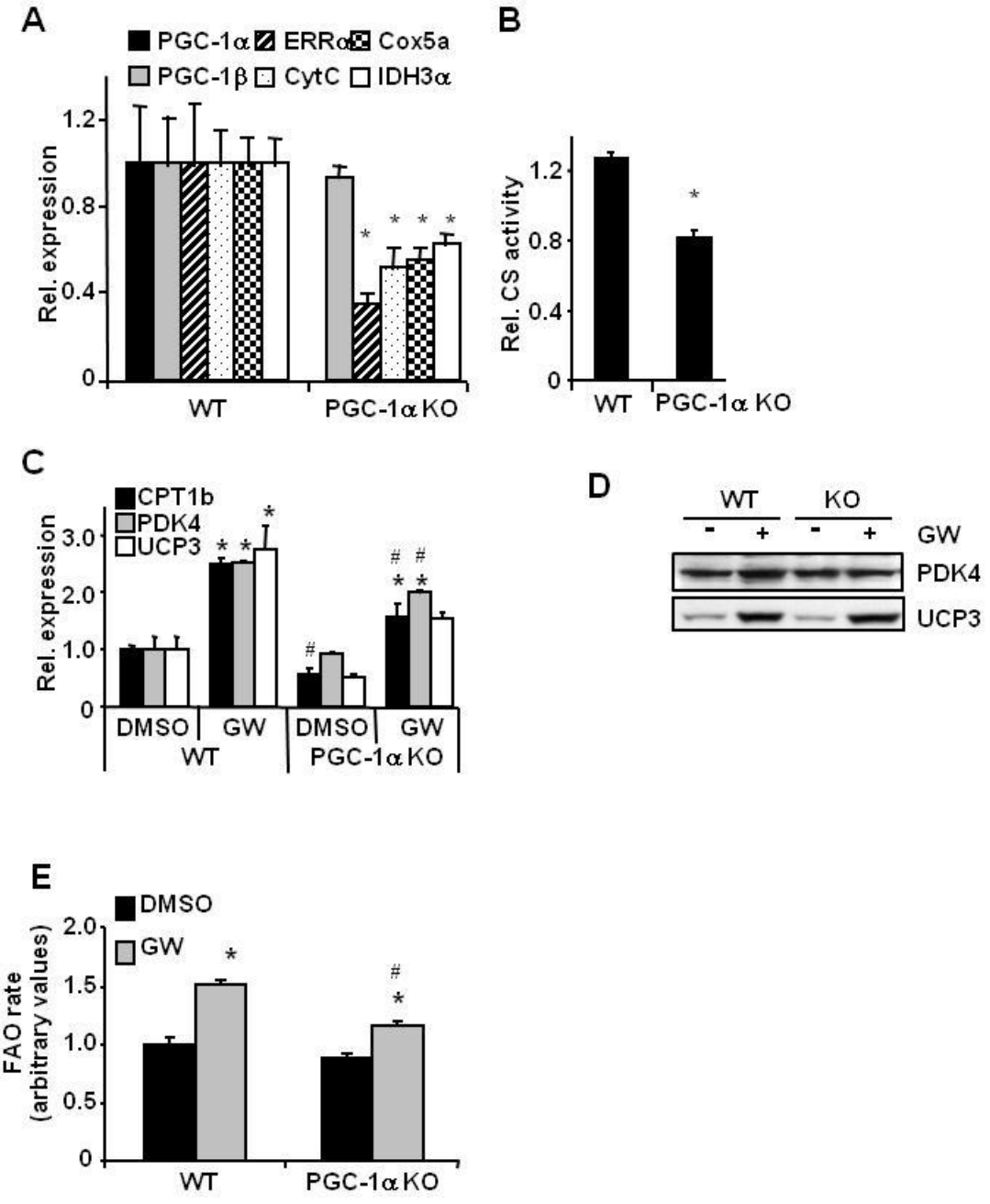


Figure 5

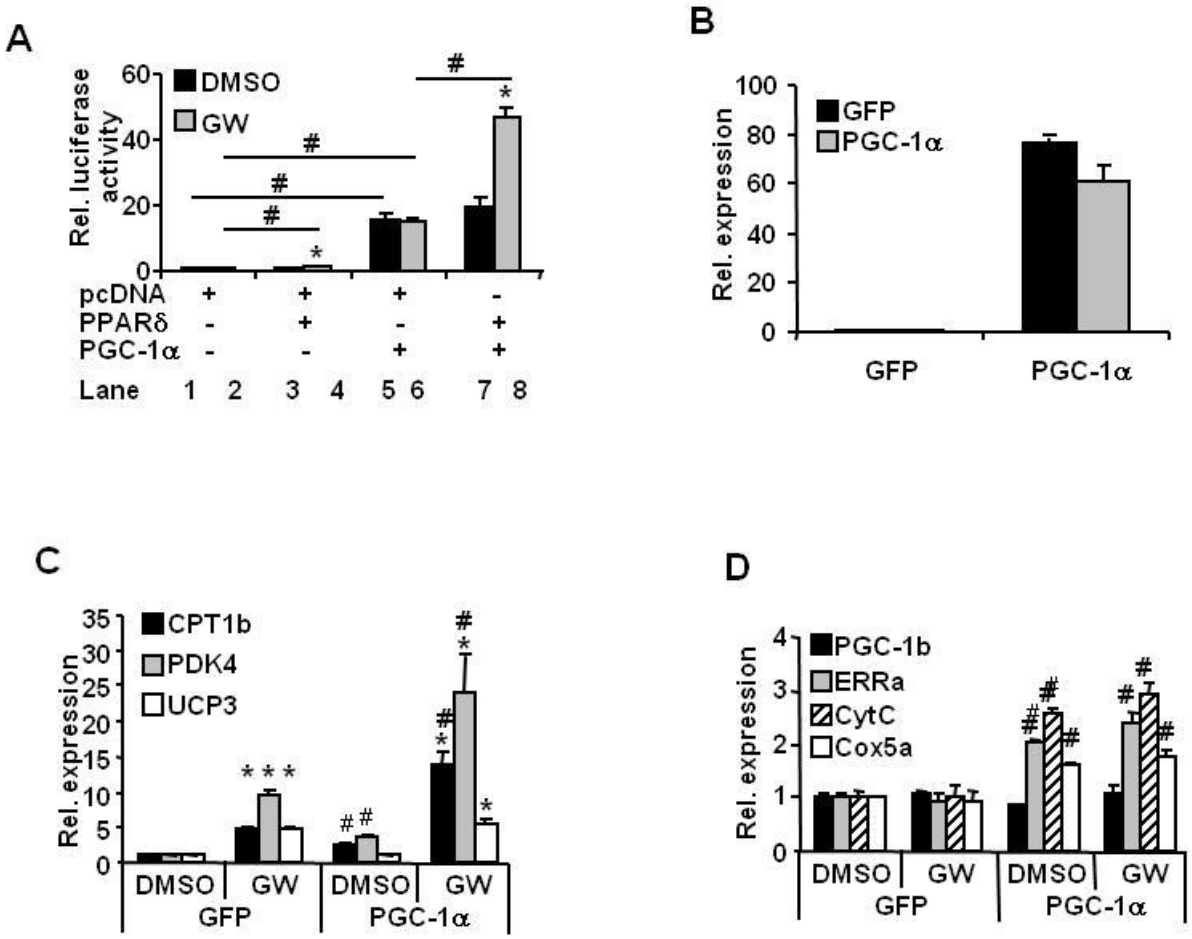


Figure 6

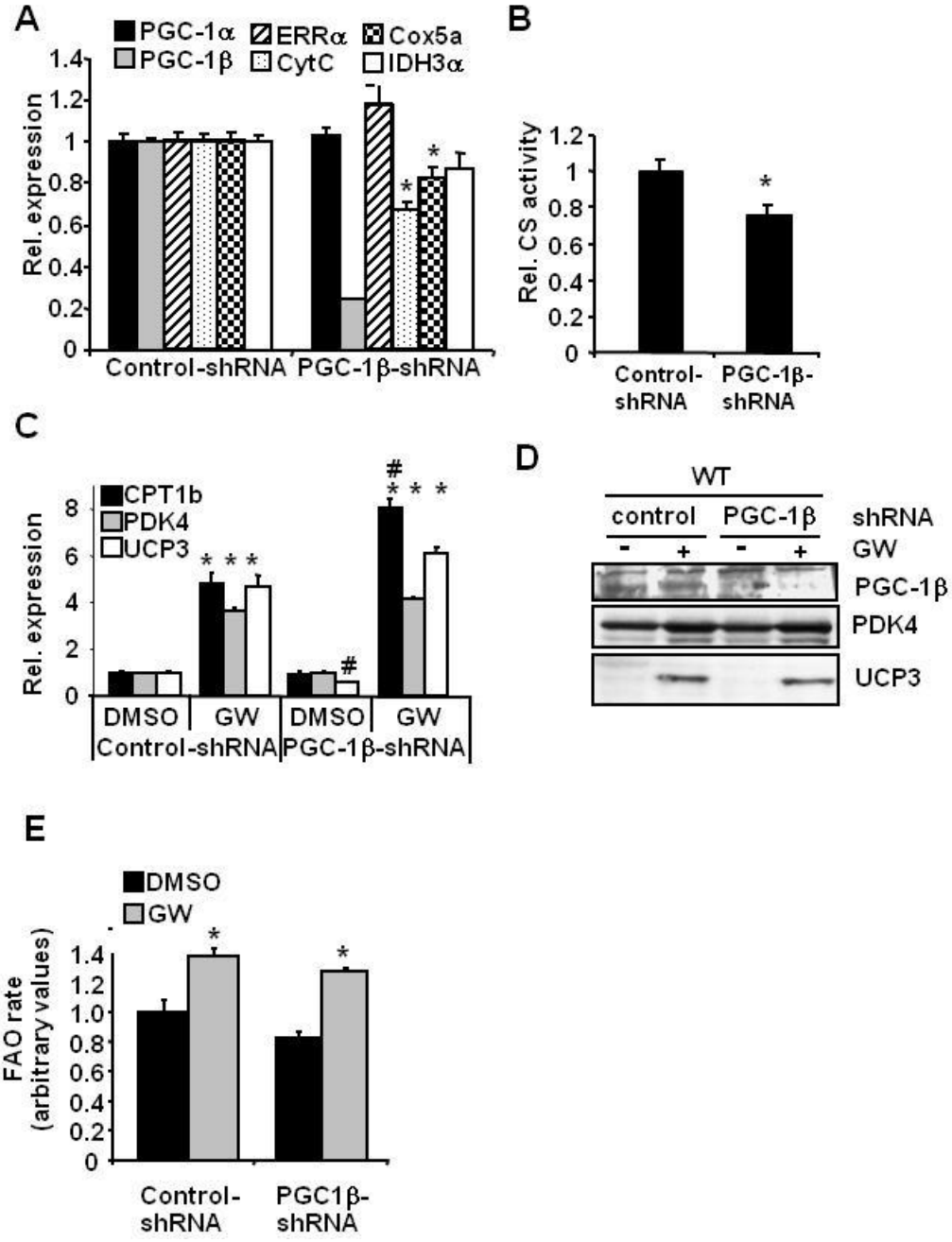


Figure 7

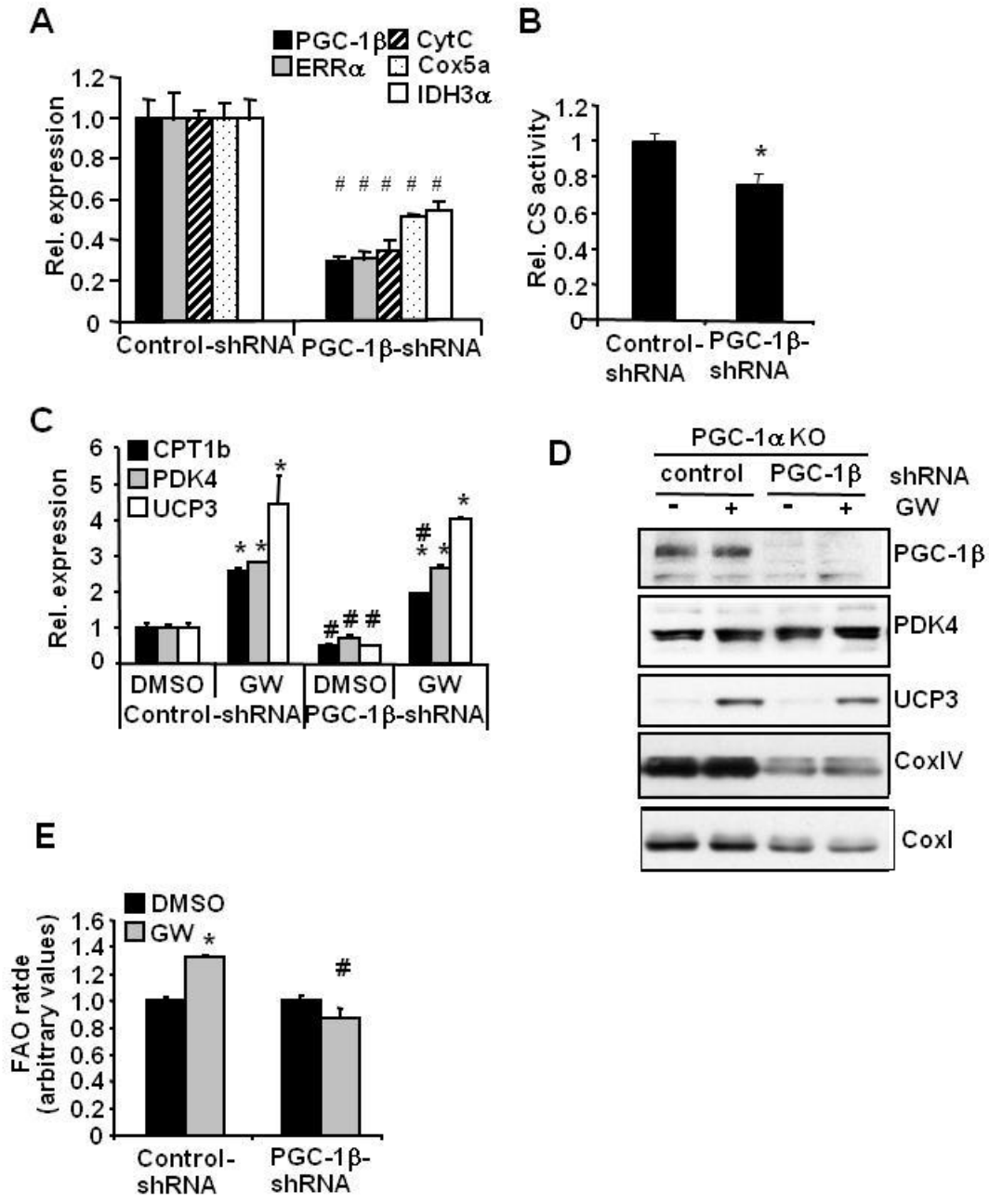


Figure 8

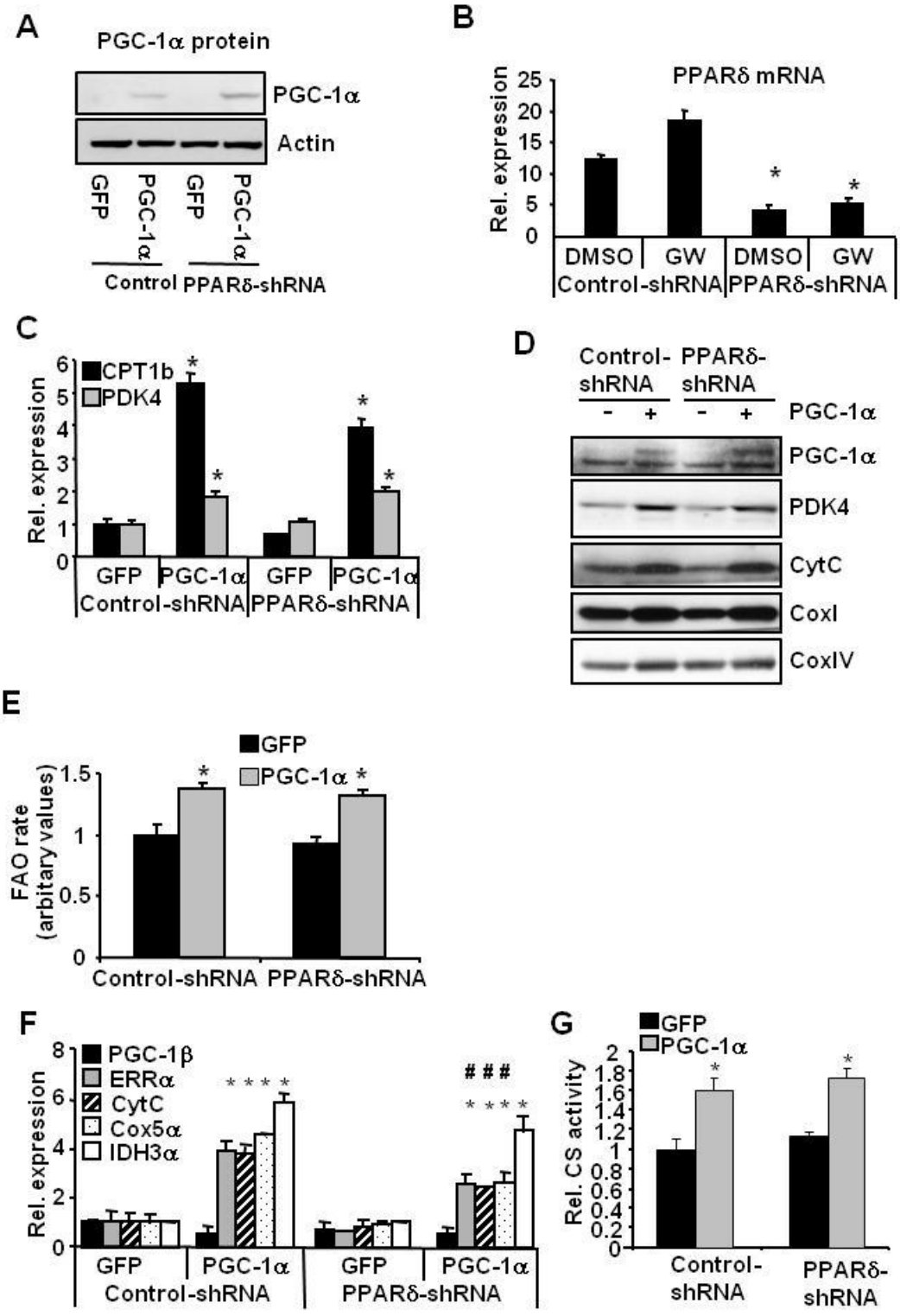
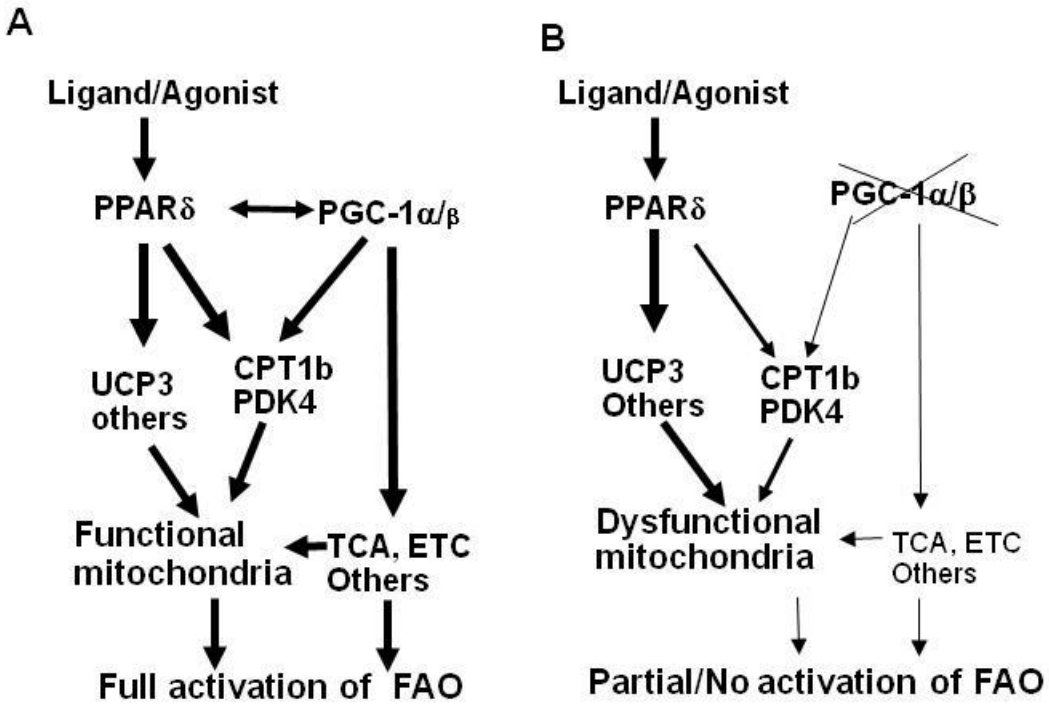


Figure 9



PPARdelta agonism activates fatty acid oxidation via PGC-1alpha but does not increase mitochondrial gene expression and function

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