Fatty Acid Homeostasis and Induction of Lipid Regulatory Genes in Skeletal Muscles of Peroxisome Proliferator-activated Receptor (PPAR) α Knock-out Mice

EVIDENCE FOR COMPENSATORY REGULATION BY PPARδ*

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Deborah M. Muoio§, Paul S. MacLean, David B. Lang†, Shi Li‡, Joseph A. Houmard¶, James M. Way¶, Deborah A. Winegar§, J. Christopher Corton**, G. Lynis Dohm¶, and William E. Kraus‡

From the §Departments of Medicine and Cell Biology, Duke University Medical Center, Durham, North Carolina 27710, the ¶Department of Biochemistry and the Human Performance Laboratory, East Carolina University, Greenville, North Carolina 27858, the §§Departments of Metabolic Diseases and Nuclear Receptor Biology, GlaxoSmithKline, Research Triangle Park, North Carolina 27709, and the ††Chemical Industry Institute of Toxicology Centers for Health Research, Research Triangle Park, North Carolina 27709

Ablation of peroxisome proliferator activated receptor (PPAR) α, a lipid-activated transcription factor that regulates expression of β-oxidative genes, results in profound metabolic abnormalities in liver and heart. In the present study we used PPARα knockout (KO) mice to determine whether this transcription factor is essential for regulating fuel metabolism in skeletal muscle. When animals were challenged with exhaustive exercise or starvation, KO mice exhibited lower serum levels of glucose, lactate, and ketones and higher nonesterified fatty acids than wild type (WT) littermates. During exercise, KO mice exhausted earlier than WT and exhibited greater rates of glycogen depletion in liver but not skeletal muscle. Fatty acid oxidative capacity was similar between muscles of WT and KO when animals were fed and only 28% lower in KO muscles when animals were starved. Exercise-induced regulation and starvation-induced regulation of pyruvate-dehydrogenase kinase 4 and uncoupling protein 3, two classical and robustly responsive PPARα target genes, were similar between WT and KO in skeletal muscle but markedly different between genotypes in heart. Real-time quantitative PCR analyses showed that unlike in liver and heart, in mouse skeletal muscle PPARδ is severalfold more abundant than either PPARα or PPARγ. In both human and rodent myocytes, the highly selective PPARδ agonist GW742 increased fatty acid oxidation about 2-fold and induced expression of several lipid regulatory genes, including pyruvate-dehydrogenase kinase 4 and uncoupling protein 3, responses that were similar to those elicited by the PPARα agonist GW647. These results show redundancy in the functions of PPARα and PPARδ as transcriptional regulators of fatty acid homeostasis and suggest that in skeletal muscle high levels of the δ subtype can compensate for deficiency of PPARα.

Peroxisome proliferator activated receptors (PPARs) α, δ, and γ comprise a family of nuclear hormone receptors that regulate systemic fatty acid metabolism via ligand-dependent transcriptional activation of target genes (1). Strong evidence indicates that their endogenous ligands consist of fatty acids and/or lipid metabolites and that they function to mediate adaptive metabolic responses to changes in systemic fuel availability (1, 2). PPARα, which is expressed most abundantly in tissues that are characterized by high rates of fatty acid oxidation (FAO), is considered the primary subtype that mediates lipid-induced activation of FAO genes (3). This premise is based largely on studies of PPARα knockout (KO) mice, which, compared with wild type (WT) littermates, exhibit low rates of β-oxidation and abnormal accumulation of neutral lipids in both cardiac and hepatic tissues (4, 5). The metabolic phenotype of KO mice is associated with decreased expression of FAO genes and failure of liver and heart to induce β-oxidative pathways in response to physiological or pharmacological perturbations in lipid metabolism (4–6). Taken together, these studies indicate that, at least in rodents, PPARα plays an essential role in maintaining lipid homeostasis in liver and heart by modulating both constitutive and inducible expression of genes that regulate fatty acid catabolism.

Skeletal muscle is also a major site of fatty acid catabolism in mammals. Similar to other oxidative organs, mRNA expression of muscle genes that promote selective utilization of lipid substrates is augmented during physiological states that are associated with increased systemic delivery of free fatty acids, such as exercise and starvation (7–10). Furthermore, many of these same muscle genes are also up-regulated by in vivo administration of PPARα-selective drugs (11, 12), prompting widespread speculation that fatty acid-induced activation of PPARα plays a critical role in mediating the adaptive response of muscle to starvation and exercise. PPARα protein expression is increased by exercise training (13, 14) and induced during myocyte differentiation, coincident with increased β-oxidative capacity (15). Additionally, we recently showed that PPARα...
regulates fatty acid utilization and mRNA expression of several FAO genes in primary human skeletal muscle cells (HSkMC) (15).

We hypothesized that deficiency of PPARs might have profound consequences on skeletal muscle fuel metabolism and gene regulation. Pyruvate-dehydrogenase kinase 4 (PDHK4) and uncoupling protein 3 (UCP3) represent two muscle target genes that are robustly induced by PPARα agonists (11, 12), starvation (8, 10), and exercise (7, 9) and that are proposed to play key roles in mediating adaptive adjustments in muscle substrate selection. To investigate the requirement for PPARα in regulating muscle fatty acid metabolism and transcriptional activation of PDHK4 and UCP3, we evaluated metabolic and gene regulatory responses to 24 h of starvation and endurance exercise, as well as in vitro FAO, in muscles from PPARα KO mice and WT littermates. Contrary to our hypothesis, here we report that skeletal muscles from KO mice exhibited only minor changes in fatty acid homeostasis and, moreover, that neither constitutive nor inducible mRNA expression of known PPARα target genes was negatively affected by its absence. We also found that skeletal muscle expressed high levels of PPARδ and that in both rodent and human skeletal muscle cells, activation of the δ-subtype increased FAO as well as mRNA levels of several classical PPARα target genes. These results, which are the first to show significant overlap in the functions of PPARα α and δ, indicate that both subtypes play important roles in mediating lipid-induced regulation of β-oxidative pathways. We propose that high levels of PPARδ might compensate for the lack of PPARα in the skeletal muscles of KO mice.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin, carnitine, and sodium oleate were from Sigma. Fetal bovine serum and Hanks’ balanced salt solution were from Invitrogen. Heat-inactivated horse serum was from HyClone (Logan, UT). Dulbecco’s low glucose modified Eagle’s medium was from Invitrogen. Human skeletal muscle cell SingleQuots were obtained from Dr. Peter Whittaker Inc. (Walkersville, MD). Biocoat/H23041 Invitrogen. Human skeletal muscle cell SingleQuots.

**Northern Analyses**—RNA was isolated from quick frozen muscle samples using the TRIzol (Life Technologies) reagent according to the manufacturer’s instructions as described previously (23). Total RNA (10 μg/sample) was fractionated on a 1.2% agarose, 2 μm formaldehyde gel and then electrotransferred to a Hybond N1 membrane (Amersham Biosciences). Following UV cross-linking, the filters were prehydrized for 30 min at 80 °C in ExpressHyb (CLONTECH Laboratories, Inc., Palo Alto, CA) and then hybridized 2 h at 68 °C in ExpressHyb buffer containing 50% formamide, 0.1× SSC, 0.1% SDS, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% casein, 0.1 μM digoxigenin-11-dUTP (Roche), and 0.5% bovine serum albumin. After 60 min at 37 °C, the reactions were terminated by adding 50 μl of 70% percollic acid. The CO2 and ASM produced during the incubation were measured as described previously (21).

**Experimental Animals**—Male C57BL/6 mice were used to generate the TAM mice (C57BL/6) on the C57BL/6 background. Mice were housed under controlled temperature and lighting (20 °C, 12 h-light/12 h-dark cycle) with free access to food and water. For starvation experiments, the food was removed at 10:00 a.m. the morning prior to collection of blood and tissue samples, which were harvested 24 h later along with samples from fed/obese and exercised mice.

**Exercise Protocol**—The mice were accustomed to the treadmill (Stanhope SAT 2000) with 5-min bouts of mild exercise (8 m/min) on three consecutive days prior to the experiment. The exercise test consisted of an incremental treadmill regimen during which mice ran at 10 m/min for the first 60 min. The speed was then increased 0.75 m/min at 15-min intervals, up to 13 m/min at 2 h. Mice in the 2-h exercise group were removed and immediately sacrificed for tissue and blood collection. The remaining mice continued running, and the treadmill speed was increased 1.0 m/min at 15-min intervals until the animals could no longer sustain the exercise. The exhausted mice were removed and immediately sacrificed for tissue and blood collection.

**In Vitro Fatty Acid Oxidation Studies**—Intact soleus and extensor digitorum longus muscles from the fed mice were removed between 9:00 and 11:00 a.m. under anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine), cleaned free of adipose and connective tissue, and added to 1.0 ml of a modified Krebs-Ringer bicarbonate buffer containing 1.0 mM [1-14C]oleate (1.0 μCi/ml), 1.0 mM carnitine, and 1.0 mM bovine serum albumin as described previously (19). After 90 min the incubation medium was transferred to new dishes, and the oleate oxidation rates (nmol/mg muscle weight/h) were determined by measuring production of 14C-labeled acid-soluble metabolites (ASM), a measure of tricarboxylic acid cycle intermediates and acetyl esters (incomplete oxidation) (20), and [1-14C]oleate (complete oxidation) trapped in 200 μl of 1 N sodium hydroxide using a modified 48-well microtiter plate (Costar, Cambridge, MA) (21).

Mixed gastrocnemius muscles were removed from mice that were fed or starved for 24 h. Approximately 90 mg of tissue was minced thoroughly with scissors in 300 μl of a modified sucrose-EDTA medium containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, as described previously (22). The reactions were initiated by adding 40 μl of whole homogenates to 160 μl of the incubation buffer, pH 7.4, yielding final concentrations of 0.2 mM oleate ([1-14C]oleate at 0.5 μCi/ml), 100 mM sucrose, 10 mM Tris-HCl, 5 mM potassium phosphate, 80 mM sodium malate, 1 mM magnesium chloride, 2 mM L-carnitine, 0.1 mM malate, 2 mM ATP, 0.05 mM coenzyme A, 1 mM dithiothreitol, 0.2 mM EDTA, and 0.5% bovine serum albumin. After 60 min at 37 °C, the reactions were terminated by adding 50 μl of 70% percollic acid. The CO2 and ASM produced during the incubation were measured as described previously (21).

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and then stored at −80°C. All of the assays were performed in triplicate.

**Real Time Quantitative PCR**—Total RNA was prepared using the TRIzol reagent, treated with DNase I (Ambion) and quantified using the RiboGreen™ RNA quantitation kit (Molecular Probes, Eugene, OR). RTQ-PCR was performed using an ABI PRISM 7700 sequence detection system instrument and software (PerkinElmer Life Sciences). Primer/probe sets were designed using the manufacturer’s software and the sequences available in the GenBank™ data base. The sequences for the rat genes were: PPARα, F: GTTATTAGGAGACCAAGGCAC, R: GGGTGCCGGATGATGATC, P: ACATAAGTCAATGGAAAATTTCCAGGCCCA; PPARγ, F: ACGATGTGGTGGTTGATGATG, R: CACTGCATCATCTGGCATG, P: TTGGGAAACACCGTTCACGC; UCP3, F: TGGCCTCCCAGTGGAT, R: GGGCTTCTGGATCTGGCT, P: TAAAGACCGGTAAGTAAACGC; CT, F: TCGGAAAACACCGTTCACGC, R: GGGCTCTGGATCTGGCT, P: TCGGAAAACACCGTTCACGC.

The sequences for the mouse genes were: PPARα, F: ACGATGTGGTGGTTGATGATG, R: GGGCTTCTGGATCTGGCT, P: TCGGAAAACACCGTTCACGC; PPARγ, F: CCATTCCCACCAAC, R: AAAGGCACTTCCTTATCCATCA, P: TCGGAAAACACCGTTCACGC.

The sequences were used for analysis of human genes were published previously (15). RNA samples were normalized for comparison by determining 18 S rRNA levels by RTQ-PCR. PPAR subtype expression in mouse tissues was analyzed by generating six-point serial standard curves using total RNA from heart, liver, and skeletal muscle. Estimates of relative mRNA abundance (arbitrary units) were determined by the ratio of calculated units of RNA from each tissue. The calculations were made using the portion of the curve for which the plot of the log input amount versus the Ct (cycle threshold) differences resulted in a slope of approximately 0, indicating that the amplicon efficiencies were approximately equal. Relative quantitation of PPAR target genes in cultured myocytes was calculated by using the 2^−ΔCT formula, in which ΔCt equals the difference between Cts values for vehicle (Me2SO) and drug-treated cells. This formula was validated for each primer/probe set using six-point serial standard curves as described previously (24).

**Statistics**—Statistical analyses were performed using JMP Statistical Software (SAS, Cary, NC). Two- and three-way ANOVAs were performed using a standard least squares model to test both the main and interaction effects of genotype × starvation or exercise × time (where appropriate) on serum parameters and mRNA expression. In HSKMC and L6 myocytes differences in FAO rates and mRNA expression (Cy values) were analyzed by Student’s t test for paired data.

**RESULTS**

**Metabolic Responses to Exercise and Starvation Are Altered in PPARα KO Mice**—Consistent with earlier reports (4), we found that the body weights of older KO mice (23.4 ± 0.73 g) were greater than their WT littermates (21.7 ± 0.98 g). The effects of PPARα ablation on muscle fuel metabolism were first evaluated during *in vivo* studies that compared the metabolic responses to 24 h of starvation with that of endurance exercise. We found that when KO mice were challenged by 24 h of starvation, they exhibited increased serum nonesterified fatty acids (NEFA) and hypoketonemia (Fig. 1, A and B) but showed no changes in serum glycerol (not shown) compared with WT. These results are consistent with previous studies (4) and support the premise that ablation of PPARα severely impairs the capacity of the liver to clear and oxidize fatty acids. Moreover, because starvation fully depletes liver glycogen levels (Fig. 2), gluconeogenesis becomes the primary pathway for sustaining blood glucose. KO mice exhibited hypoglycemia, consistent with reduced liver gluconeogenic capacity, and lower serum lactate levels, suggesting increased dependence on this substrate as a gluconeogenic precursor (Fig. 1, C and D). These metabolic abnormalities observed in KO mice after 24 h of starvation are remarkably similar to those exhibited in response to exercise, and as predicted, time to exhaustion (180 ± 35 versus 150 ± 15 min; p < 0.05) and total distance run (1.80 versus 1.37 km; p < 0.01) were greater in WT compared with KO, respectively, indicating that KO mice are less tolerant of endurance exercise. Notably, depletion of liver rather than muscle glycogen corresponded with exhaustion in both groups and interaction effects of genotype × starvation or exercise × time.
Two-way ANOVA.‡ and fed/rested/H11021 p and the significant differences (H11021). Gen content was measured in liver (H11021) and muscle (H11021) from WT and KO quariceps muscle. In fed/rested mice, mRNA levels of PDHK4 dictated, PPAR genotypes. These findings suggested that, contrary to our pre-

Fig. 2. Glycogen depletion in skeletal muscle and liver. Glyco-
gen content was measured in liver (A) and muscle (B) from WT and KO mice that were fed/rested, exercised for 2 h, exercised to exhaustion, or starved for 24 h. The values are the means ± S.E. from 6–8 animals, and the significant differences (p < 0.05) between WT versus KO mice (a) and fed/rested versus starved/exercised mice (*) were analyzed by two-way ANOVA. (Fig. 2). Moreover, estimated rates of liver glycogen depletion were ~50% greater in null compared with WT mice, whereas in contrast, muscle glycogen depletion rates were similar between genotypes. These findings suggested that, contrary to our prediction, PPARα ablation might not increase the reliance of muscle on glucose substrate.

In Vitro Fatty Acid Oxidation Rates in Muscles of PPARα KO Compared with WT Mice—To evaluate changes in muscle fatty acid oxidative capacity directly, we performed in vitro experiments using skeletal muscles comprised of red (soleus), white (extensor digitorum longus), or mixed (gastrocnemius) fiber types. In the fed state, rates of muscle FAO were similar between WT and null mice (Table I), regardless of the fiber composition, implying that PPARα is not required to maintain constitutive activity of muscle FAO enzymes. When animals were starved for 24 h, FAO rates were 28% lower in muscle homogenates from KO compared with WT mice, but neutral lipid accumulation, analyzed by Oil Red O staining, was not different between genotypes (not shown). These results suggested that the metabolic consequences of PPARα deficiency in skeletal muscle are much less severe than those previously shown in liver and heart (4, 5, 9).

PPARα-independent Regulation of Target Genes in Skeletal Muscle—Next, we evaluated both constitutive and inducible mRNA expression of classical PPARα target genes in mixed quadriceps muscle. In fed/rested mice, mRNA levels of PDHK4 and UCP3 were similar in muscles from KO compared with WT mice (Fig. 3). In WT, exercise increased muscle mRNA levels of UCP3 and PDHK4 86 and 60%, respectively, whereas starvation increased expression of both genes five to eight times. Surprisingly, the exercise- and/or starvation-induced up-regulation of these genes was either similar or greater (UCP3) in muscles from KO mice, indicating that PPARα is not required for these physiological responses. In comparison, PPARα gene ablation attenuated constitutive and/or inducible expression of the same transcripts in heart (Fig. 4).

PPAR δ Subtype Expression in Fatty Acid Oxidative Tissues—Our results in Figs. 3 and 4, taken together with those from previous studies that investigated liver and heart, revealed tissue-specific differences in PPARα-dependent regulation of FAO and target gene expression. Using RTQ-PCR, we evaluated whether the requirement for PPARα might be associated with distinct expression patterns of the three PPAR subtypes in various oxidative tissues (Fig. 5). RTQ-PCR detected the nonfunctional α transcript in tissues from KO mice because our mPPARα primer/probes produce an amplicon from exon 7 that occurs before the targeted disruption in exon 8 (18). We found that, compared with skeletal muscle, PPARα mRNA levels were 7- and 19-fold enriched in heart and liver, respectively, whereas PPARδ mRNA abundance was similar among oxidative tissues. However, in skeletal muscle, expression level of the δ-subtype was severalfold greater than either PPARα or PPARγ. PPARδ expression decreased 68% in liver of KO, whereas PPARγ, which was the least abundant subtype in all three tissues from WT mice, increased 2–3-fold in the heart and liver, but not the skeletal muscle, of KO mice.

The PPARδ-selective Agonist GW742 Increases Fatty Acid Oxidation in Cultured Skeletal Muscle Cells—The present results, as well as those from other animal studies, indicate that at the mRNA level, PPARδ is the most abundant subtype in rodent skeletal muscle. In human skeletal muscle we have observed high levels of PPARα and δ and low levels of PPARγ.2 Taken together, these findings suggest that PPARδ might play a major role in regulating oxidative metabolism in both rodent and human skeletal muscle. To address this possibility we evaluated the effects of a novel PPARδ agonist, GW742, on FAO and gene expression in mature myotubes.

Table I

| Fatty acid oxidation in isolated muscles | WT | KO |
|----------------------------------------|----|----|
| Soleus                                 | 151 ± 4.0 | 160 ± 6.2 |
| EDL                                    | 145 ± 10.4 | 127 ± 8.6 |

| Fatty acid oxidation in homogenates of gastrocnemius | WT | KO |
|------------------------------------------------------|----|----|
| Fed                                                  | 6.74 ± 0.69 | 7.21 ± 0.55 |
| Starved                                              | 8.99 ± 0.30a | 6.42 ± 0.35a |

2 D. M. Muio, P. S. MacLean, D. B. Lang, S. Li, J. A. Houmard, J. M. Way, D. A. Winegar, J. C. Corton, G. L. Dohm, and W. E. Kraus, unpublished data.
derived from rat L6 myocytes or primary human myoblasts. These muscle cell systems were selected because they express PPARs $\alpha$ and $\delta$ at levels that are comparable with rodent and human skeletal muscle in vivo (not shown). In both rodent and human myotubes, 48 h of treatment with 0–100 nM GW742 increased $[^{14}C]$oleate oxidation dose-dependently up to $\sim$2.5-fold ($p < 0.001$) (Fig. 6A). The maximal effect of the PPAR$\alpha$ agonist was nearly identical to that of the $\alpha$ agonist GW647, whereas the PPAR$\gamma$ agonist GW929 did not effect FAO (Fig.

**Fig. 3.** Absence of PPAR$\alpha$ does not affect expression of lipid regulatory genes in quadriceps muscle. Total RNA from quadriceps (Quad) muscle of PPAR$\alpha$ KO mice and age-matched WT littersmates was prepared as described under "Experimental Procedures." mRNA levels of PDHK4 (A) and UCP3 (B) in muscles of mice that were fed/rested (F), exercised for 2 h (E), and starved for 24 h (S) were quantified by Northern blot analysis. The values are the means ± S.E. from 7–9 animals, and the differences between WT and KO mice ($\dagger$) and fed/rested versus exercised/starved mice (*) were analyzed by two-way ANOVA.

**Fig. 4.** Absence of PPAR$\alpha$ attenuates expression of lipid regulatory genes in heart. Total RNA from heart of PPAR$\alpha$ KO mice and age-matched WT littermates was prepared as described under "Experimental Procedures." mRNA levels of PDHK4 (A) and UCP3 (B) in heart of mice that were fed/rested (F), exercised for 2 h (E), and starved for 24 h (S) were quantified by Northern blot analysis. The values are the means ± S.E. from 6–8 animals, and the differences between WT and KO mice ($\dagger$) and fed/rested versus exercised/starved mice (*) were analyzed by two-way ANOVA.
Most remarkably, activation of either PPARα or δ resulted in increased mRNA expression of several genes that promote FAO (15). To investigate possible redundancy in skeletal muscle PPARα and δ target genes, here we used RTQ-PCR to perform similar evaluations in human and rodent myotubes that were treated with agonists selective for either PPARδ (GW742) or PPARα (GW647). The results in Table II show that PPARδ-mediated increases in myotube mRNA expression of FAO genes were similar to that of PPARα. Most remarkably, activation of either α or δ increased PDHK4 mRNA expression ~30-fold. Both treatments also increased mRNA expression of malonyl-CoA decarboxylase (MCD) and the muscle isoform of carnitine palmitoyltransferase 1 (CPT1), both of which code for proteins that increase muscle fatty acid uptake and/or β-oxidation. Induction of these genes in drug-treated myotubes relative to vehicle controls was highly consistent among cells obtained from different subjects and similar in myotubes treated with GW647 compared with GW742. In contrast, changes in UCP3 mRNA expression were highly variable and significant only in cells that were treated with the PPARδ agonist.

Similar to HSkMC, in L6 myotubes both the α (GW647) and δ (GW742) agonists increased mRNA abundance of PDHK4 ~24-fold (Table III). Both agonists also increased mCPT1 and UCP3, but notably, in L6 myocytes induction of these genes was severalfold more robust than in the HSkMC. Together, these results suggest that gene- and species-specific elements modulate the physiological induction of PPAR targets. In results not shown, the PPARγ agonist, GW929, did not affect the mRNA levels of any of the genes analyzed.

**DISCUSSION**

PPARα is expressed most abundantly in highly oxidative tissues where it plays a key role in activating pathways of β-oxidation (2). Conversely, PPARγ, which is expressed primarily in adipose tissue, promotes adipocyte differentiation and induces genes involved in lipogenesis (26). The function of PPARδ, which is expressed ubiquitously, is less certain, although recent data implicate this subtype as a regulator of cholesterol metabolism (17) and adiposity (27). Skeletal muscle expresses all three PPAR subtypes, but their distinct roles in regulating muscle energy metabolism are unknown. In the present investigation we used PPARα KO mice to determine whether the α-subtype is essential for maintaining skeletal muscle lipid homeostasis. The mice were challenged by exercise or 24 h of starvation, two physiological stresses that increase systemic delivery of fatty acid and induce adaptive changes in muscle fuel metabolism. In contrast to starvation, a long term stress in which lipid catabolism increases most markedly in the liver, exhaustive exercise represents a short term stress in which high rates of skeletal muscle FAO are critical for sustaining prolonged work. Although we found that KO mice were less tolerant of exercise, they still performed better than expected given our hypothesis that ablotion of PPARα would produce severe defects in muscle fatty acid oxidative capacity.

The findings that glycogen depletion rates were greater in liver but not in muscle of KO mice and that in vitro muscle FAO rates were similar between genotypes suggested that exercise intolerance was due to metabolic perturbations in liver and heart rather than skeletal muscle. Interestingly, exhaustive exercise depleted muscle glycogen only 50%, whereas depletion of liver glycogen reached nearly 100%, corresponding with the onset of hypoglycemia in both genotypes (not shown). These data implicate liver fuel metabolism as a limiting factor during exhaustive, low intensity exercise.

Previous studies using KO mice have provided convincing evidence that, in rodents, PPARα plays an obligatory role in regulating lipid homeostasis in liver and heart. In these oxidative tissues, absence of functional PPARα decreases mitochondrial FAO ~70% (28–30), a finding that is explained by reduced constitutive and/or inducible expression levels of several fatty acid catabolic enzymes including MCD (30) and PDHK4 (31). Furthermore, when KO mice are challenged by physiological or pharmacological perturbations in fatty acid metabolism, cardiac and hepatic tissues accumulate abnormally high amounts of neutral lipids (4, 32). In contrast, the present study found that skeletal muscles of KO mice exhibited minor abnormalities in FAO only when animals were starved and showed no evidence of neutral lipid accumulation. KO muscles also maintained constitutive and inducible mRNA levels of the PPARα target genes, PDHK4 and UCP3. Although exercise starvation induced stress hormones probably contribute to the physiological regulation of these genes, PPARα is widely considered a primary mediator because administration of PPARα-selective drugs, as well as treatments that elevate serum NEFA in fed animals, mimic the effects of starvation on UCP3 and PDHK4 gene expression (10, 12, 33). Surprisingly, starvation-induced up-regulation of muscle UCP3 was actually more robust in KO mice, which corresponded with higher serum NEFA. This result is consistent with previous studies describing a tight correlation between systemic NEFA and changes in skeletal muscle UCP3 mRNA levels (33); however, it is inconsistent with speculation that PPARα is an essential mediator of these changes. In contrast, when we examined the same target genes in the heart, deficiency of PPARα decreased the constitutive levels of UCP3 and diminished exercise- and starvation-induced increases in both UCP3 and PDHK4. Thus, importantly, these results are the first to show that skeletal and cardiac muscles, both of which are highly oxidative tissues, exhibit distinct requirements for PPARα.

Tissue-specific differences in the metabolic consequences of PPARα deficiency might be related to the relative abundance of...
Selective agonists of PPARs α and δ have similar effects on expression of lipid regulatory genes in primary HSkMC

Total RNA was isolated and quantified by RTQ-PCR as described under “Experimental Procedures,” and differences in Cq values between treated and control cells were analyzed by Student’s t test for paired data. Gene expression levels in day 8 myotubes that were treated for 48 h with 10 nM GW742 or 1.0 μM GW647, relative to vehicle (Me2SO) controls, are presented as the means ± S.E. of cells from nine different subjects analyzed in quadruplicates.

| Fold change vs. Me2SO control | GW647 (α) p | GW742 (δ) p |
|-------------------------------|-------------|-------------|
| CPT1                          | 2.2 ± 0.3   | <0.001      | 2.8 ± 0.5   | <0.005      |
| MCD                           | 2.1 ± 0.2   | <0.001      | 2.3 ± 0.1   | <0.001      |
| PDHK4                         | 29.2 ± 4.6  | <0.001      | 37.4 ± 7.0  | <0.001      |
| UCP3                          | 1.9 ± 0.5   | 0.10        | 4.2 ± 2.0   | <0.05       |

FIG. 6. Effects of PPAR agonists on fatty acid oxidation in cultured myocytes. After 6 days in DFM, mature rat L6 or primary human myotubes were treated for 48 h with vehicle (Me2SO) or 0.10–100 nM of the PPARδ agonist GW742 (A) and maximal doses of GW647 (1.0 μM), GW742 (10 nM), and GW929 (100 nM), agonists selective for PPARs α, δ, or γ, respectively (B). Day 8 myotubes were then incubated for 3 h at 37°C in DFM with 100 μM 14C-oleate and 0.25% bovine serum albumin. Oleate oxidation (nmol/g/h) was determined by measuring production of 14C-labeled CO2 and ASM as described under “Experimental Procedures.” All of the assays were performed in triplicate, and the values are the means ± S.E. from 3 subjects (human myocytes) or two experiments (L6 myocytes) (A) and 3–10 subjects (B) as indicated. The differences between vehicle and drug-treated cells were analyzed by Student’s t test for paired data and one-way ANOVA. *p < 0.05; **p < 0.001 versus Me2SO-treated controls.

the three subtypes in various oxidative tissues. We found predominant expression of PPARα in liver, whereas PPARs α and δ were similarly abundant in heart. In contrast, PPARδ was the major subtype expressed in mouse quadriceps muscle. These results, obtained by RTQ-PCR, are similar to studies that used RNase protection assays to evaluate expression levels of the PPAR subtypes in mouse (34) and rat (35) tissues. Low levels of PPARδ in oxidative tissues from WT are consistent with the notion that this subtype does not play a major role in regulating FAO. On the contrary, increased PPARγ in liver and heart of KO mice is consistent with its function as an activator of lipogenesis and glycerolipid synthesis and, similar to other reports (36), implicates elevated γ levels as an early response in the progression of hepatic and cardiac lipotoxicity.

The results from several in vivo studies have implied a role for the PPARs in mediating fatty acid-induced regulation of muscle metabolism, but only recently has direct evidence emerged showing that PPAR-selective drugs induce expression of lipid regulatory genes in cultured skeletal myocytes (15, 35, 37, 38). Still, there is little information on whether the three skeletal muscle PPAR subtypes target similar or distinct metabolic pathways. The results of our study using PPARδ KO mice suggested that PPARδ might play a role in regulating muscle lipid homeostasis. This hypothesis was supported by experiments in cultured myocytes showing that the highly selective PPARδ agonist, GW742, increased FAO and induced expression of several lipid regulatory genes. In both human and rat myotubes the metabolic and gene regulatory effects of the δ agonist were similar to those observed when cells were treated with the PPARα agonist. Our results are consistent with recent studies showing that activation of PPARδ induces mRNA expression of UCP2 and UCP3 in human and L6 myotubes, respectively. Skeletal muscle UCPs, which are mitochondrial membrane proteins that uncouple oxidative phosphorylation, are proposed to facilitate FAO and/or protect muscle against damaging by-products of oxidative metabolism (39, 40). Additionally, we found that PPARδ activation also increased mRNA expression of PDHK4, CPT1, and MCD. PDHK4 functions by phosphorylating and inactivating pyruvate dehydrogenase, the multienzyme complex that catalyzes the irreversible oxidation of pyruvate to acetyl-CoA (41). Increased expression and activity of PDHK4, which occurs in response to exercise (7), starvation (8), and a low carbohydrate diet (42), is thought to promote fatty acid oxidation by decreasing glucose-derived acetyl-CoA. CPT1 catalyzes the initial and rate-limiting step in the transport of fatty acid into mitochondria, whereas MCD disposes of the potent CPT1 inhibitor, malonyl-CoA. Thus, in the aggregate, activation of these genes increases catabolism of fatty acids by promoting their entry into the mitochondria and by relieving competition between carbohydrate and lipid-derived acetyl-CoA. Importantly, this study is the first to demonstrate that PPARδ is capable of inducing multiple pathways that cooperatively promote FAO, a function that is generally assigned to PPARα.

Because PPARδ binds to many of the same fatty acids and lipid metabolites as the other PPAR subtypes, it has been presumed to play a role in fatty acid homeostasis, although...
Skeletal Muscle Metabolism in PPARα Knockout Mice

Total RNA was isolated and quantified by RT-Q-PCR as described under “Experimental Procedures,” and differences in Ct values between treated and control cells were analyzed by Student’s t test for paired data. Gene expression levels in day 8 myotubes that were treated for 48 h with 10 nM GW742 or 1.0 μM GW647, relative to vehicle (Me2SO) controls, are presented as the means ± S.E. of cells from two separate experiments analyzed in quadruplicates.

| Gene | p GW647 (α) | p GW647 (β) |
|------|-------------|-------------|
| CPT1 | 0.4 ± 0.5   | 1.3 ± 1.2   |
| MCD  | 3.3 ± 0.24  | 2.0 ± 0.37  |
| PDHK4| 23.3 ± 3.3  | 25.6 ± 2.5  |
| UCP3 | 3.0 ± 0.25  | 3.8 ± 2.7   |

**Table III**

Selective agonists of PPARs α and δ have similar effects on expression of lipid regulatory genes in L6 myocytes

data confirming this hypothesis have remained elusive because of the absence of potent and selective agonists. The results of the present study, which used a novel agonist that is 1000-fold selective for the δ subtype, demonstrate that activation of PPARδ promotes fatty acid catabolism in skeletal muscle, thus supporting a role for this subtype in regulating whole body lipid metabolism. Consistent with our findings, a close analogue of GW742 with equivalent PPARδ activity was recently shown to decrease serum triglycerides and insulin and increase HDL cholesterol in obese rhesus monkeys (17). Changes in serum lipid profiles were at least partly explained by induction of genes that promote reverse cholesterol transport, but our data suggest that increased skeletal muscle lipid uptake and catabolism might have contributed to the therapeutic effects of the treatment.

All three PPAR subtypes bind to DNA as obligate heterodimers with the retinoic acid receptor RXR, and the preferred DNA-binding site, referred to as the DR-1 motif, is the same for each of the PPAR/RXR heterodimer pairs (1). Because PPARα and δ bind to the same response element within a given target promoter, conceivably, full activation of these two distinct transcription factors, as occurs when the receptor binds to its chemicogenic engineered agonist in vitro, might produce nearly identical responses. However, considerable data, including phenotypic differences between PPARα- and PPARδ-null mice (43), indicate that these transcription factors play distinct physiological roles in vivo. Our studies in KO mice showed that PPARδ is indispensable for UCP3 regulation in heart, despite high expression levels of PPARδ, indicating that properties other than mRNA abundance contribute to the tissue-specific roles of these transcription factors. In addition to tissue distribution, specificity of function might be conferred by mechanisms that impart subtype-specific regulation of receptor activity, such as differences in their binding affinities for endogenous ligands (44, 45). Transcriptional activity of PPARs α and γ is also regulated by phosphorylation of their transcriptional activation domains (44, 46–48) and by tissue-specific interactions with co-activators/repressors (47). Less is known about PPARδ, but the activity of this subtype is likely to exhibit similar mechanisms of regulation.

In summary, the results from our experiments using cultured myocytes show overlap in the functions of PPARs α and δ as transcriptional regulators of FAO. Taken together with previous studies, our data from PPARδ KO mice indicate that the essential and/or permissive roles of each subtype in controlling expression FAO genes in vivo depend on complex physiological interactions that exhibit tissue- and target gene specificity. A full accounting of the distinct and cooperative roles of PPARs α and δ in regulating lipid homeostasis, and perhaps other metabolic pathways, will require combined loss of function experiments in both cell culture and tissue-specific knockout mouse models. Information from such studies should have important implications for treating energy metabolic diseases such as obesity, hyperlipidemia, and type 2 diabetes.

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Fatty Acid Homeostasis and Induction of Lipid Regulatory Genes in Skeletal Muscles of Peroxisome Proliferator-activated Receptor (PPAR) α Knock-out Mice:

EVIDENCE FOR COMPENSATORY REGULATION BY PPAR δ

Deborah M. Muoio, Paul S. MacLean, David B. Lang, Shi Li, Joseph A. Houmard, James M. Way, Deborah A. Winegar, J. Christopher Corton, G. Lynis Dohm and William E. Kraus

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