

# Developmental and Tissue-Specific Involvement of Peroxisome Proliferator-Activated Receptor- $\alpha$ in the Control of Mouse Uncoupling Protein-3 Gene Expression

Neus Pedraza,\* Meritxell Rosell,\* Joan Villarroya, Roser Iglesias, Frank J. Gonzalez, Gemma Solanes, and Francesc Villarroya

Departament de Bioquímica i Biologia Molecular (N.P., M.R., J.V., R.I., G.S., F.V.), Universitat de Barcelona, E-08028 Barcelona, Spain; and Laboratory of Metabolism (F.J.G.), National Cancer Institute, Bethesda, Maryland 20892

**Uncoupling protein-3 (UCP3) is a member of the mitochondrial carrier family expressed preferentially in skeletal muscle and heart. It appears to be involved in metabolic handling of fatty acids in a way that minimizes excessive production of reactive oxygen species. Fatty acids are powerful regulators of UCP3 gene transcription. We have found that the role of peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ) on the control of UCP3 gene expression depends on the tissue and developmental stage. In adults, UCP3 mRNA expression is unaltered in skeletal muscle from PPAR $\alpha$ -null mice both in basal conditions and under the stimulus of starvation. In contrast, UCP3 mRNA is down-regulated in adult heart both in fed and fasted PPAR $\alpha$ -null mice. This occurs despite the increased levels of free fatty acids caused by fasting in PPAR $\alpha$ -null mice. In neonates, PPAR $\alpha$ -null mice show impaired UCP3**

**mRNA expression in skeletal muscle in response to milk intake, and this is not a result of reduced free fatty acid levels. The murine UCP3 promoter is activated by fatty acids through either PPAR $\alpha$  or PPAR $\delta$  but not by PPAR $\gamma$  or retinoid X receptor alone. PPAR $\delta$ -dependent activation could be a potential compensatory mechanism to ensure appropriate expression of UCP3 gene in adult skeletal muscle in the absence of PPAR $\alpha$ . However, among transcripts from other PPAR $\alpha$  and PPAR $\delta$  target genes, only those acutely induced by milk intake in wild-type neonates were altered in muscle or heart from PPAR $\alpha$ -null neonates. Thus, PPAR $\alpha$ -dependent regulation is required for appropriate gene regulation of UCP3 as part of the subset of fatty-acid-responsive genes in neonatal muscle and heart. (Endocrinology 147: 4695–4704, 2006)**

**U**NCOUPLING PROTEIN-3 (UCP3) is a mitochondrial protein similar to the thermogenic UCP1 protein present in brown adipose tissue. UCP3 is expressed preferentially in skeletal muscle and brown fat and to a minor extent in heart and white adipose tissue (1). Although this protein appears to be capable of lowering mitochondrial membrane potential, its precise biological function is a matter of debate. Various reports suggest that it can play a role in mitochondrial fatty acid metabolism (2) as well as in the control of mitochondrial reactive oxygen species production (3). In any case, UCP3 appears to be involved in the regulation of biological processes associated with mitochondrial energy metabolism, and accordingly, UCP3 gene expression is tightly regulated under physiological conditions associated with major changes in fuel metabolism, such as the early neonatal period (4).

Birth leads to a major change in nutrient use for energy metabolism. In rodents, there is a sudden change after delivery from a glucose-based fetal nutrition to a lipid-based

diet coming from milk intake. Thus, the metabolic adaptation to birth requires the induction of hepatic gluconeogenesis to maintain glycemia and the activation of lipid oxidation in most tissues to use this fuel for energy metabolism. In neonates, extrahepatic tissues such as skeletal muscle or heart switch from the use of glucose to the use of fatty acids and ketone bodies as the major fuel source instead of glucose (5). These metabolic events, which resemble those elicited by fasting in adults, are induced in neonates by the fed state, because they result from the sudden imbalance between glucose and lipid availability in the transition from the fetal to the neonatal period.

UCP3 gene expression is very low in skeletal muscle during the fetal period, and it is dramatically induced after birth under the stimulus of the rise of circulating free fatty acids (FFA) because of the initiation of milk intake (6). In fact, FFA levels determine UCP3 gene expression in skeletal muscle regardless of their origin and the feeding status of mice. This is exemplified in adult rodents, in which it is fasting that up-regulates UCP3 mRNA expression in response to the rise in circulating FFA, in this case derived from lipolysis of stored triacylglycerols in white adipose tissue (7).

Acute treatment of adults or neonates with fibrates, drugs capable of activating peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ) or PPAR $\alpha$  plus PPAR $\delta$  (also known as PPAR $\beta$ ) were shown to mimic the effects of fatty acids on UCP3 gene expression in skeletal muscle (6, 8). Studies on the transcriptional control of the human UCP3 gene established that both PPAR $\alpha$  and PPAR $\delta$  can activate UCP3 gene tran-

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\* N.P. and M.R. contributed equally to this work.

Abbreviations: ChiP, Chromatin immunoprecipitation; CPT, carnitine palmitoyl transferase; FFA, free fatty acids; MCAD, medium-chain acyl-CoA dehydrogenase; PDK4, pyruvate dehydrogenase kinase-4; PGC1 $\alpha$ , peroxisome proliferator-activated receptor-coactivator 1 $\alpha$ ; PPAR $\alpha$ , peroxisome proliferator-activated receptor- $\alpha$ ; RXR, retinoid X receptor; UCP3, uncoupling protein-3.

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scription and mediate fatty acid effects through a PPAR-responsive element in the proximal region of the promoter (9). However, PPAR $\alpha$  is more potent than PPAR $\delta$  in transactivating the human UCP3 gene promoter, and in rodents, Wy14,643, a PPAR $\alpha$ -specific activator, was also capable of maximal induction of UCP3 mRNA expression (10, 11). This led to the proposal that PPAR $\alpha$  could be the preferential mediator of UCP3 gene activation in response to fatty acids. In contrast, studies using myogenic cell lines differentiated in culture, such as C2C12 or L6 cells, indicated that the UCP3 gene was particularly sensitive to PPAR $\delta$  agonists. However, the relevance of these observations in relation to UCP3 gene regulation *in vivo* is unclear considering the abnormal repression in PPAR $\alpha$  expression in these cell models with respect to skeletal muscle (10).

Mice with a targeted disruption of the PPAR $\alpha$  gene (PPAR $\alpha$ -null) provide a unique tool to establish the involvement of this nuclear receptor in the control of UCP3 gene expression *in vivo* in response to fatty acids. Previous reports have shown that basal expression and responsiveness of the UCP3 gene to induction by fasting in adults is unaltered in skeletal muscle of PPAR $\alpha$ -null mice, but it is down-regulated in heart (12–14). In the present study, we report that PPAR $\alpha$  is required for the induction of UCP3 gene in response to milk intake in both skeletal muscle and heart from neonatal mice. This indicates that the compensatory mechanisms to mediate fatty-acid-dependent responsiveness of the UCP3 are activated during skeletal muscle development, whereas they are never able to compensate the absence of PPAR $\alpha$  in heart or in neonatal muscle.

## Materials and Methods

### Materials

Oleic acid, Wy14,643 (pirinixic acid), phytanic acid, GW501516, and bezafibrate were obtained from Sigma Chemical Co. (St. Louis, MO). Rosiglitazone was from Cayman (Ann Arbor, MI). AGN 194204 was a kind gift from Dr. R. Chandraratna (Allergan, Irvine, CA).

### Animals

The care and use of mice were in accordance with the European Community Council Directive 86/609/EEC and approved by the Experimental Animal Ethics Committee of the University of Barcelona. For studies in PPAR $\alpha$ -null mice, heterozygous females carrying the corresponding targeted deletion (15) were mated with heterozygous males, and the day of gestation was determined by the presence of vaginal plugs. For studies in neonates, pups were studied at birth (considered to be the time at which pups had been born but had not yet started suckling) and 8 and 16 h after birth. In adult mice, the effects of fasting were determined by food withdrawal for 30 h. Mice were killed by decapitation, and blood was collected into heparinized tubes and centrifuged to obtain plasma. Hearts and whole muscle from the leg in neonates and gastrocnemius muscle from adult mice were extracted and frozen in liquid nitrogen. Fed and fasted animals, as well as wild-type compared with homozygous gene-disrupted mice, were taken from the same litter in each experiment, and at least three different litters per experiment were analyzed.

### Quantitative real-time RT-PCR

Total RNA was extracted from heart and skeletal muscle using Tripure (Roche, Indianapolis, IN). The quality and concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm, and the RNA integrity was confirmed by electrophoresis in agarose/MOPS gel. The RNA was treated with DNase I to remove genomic DNA

(DNA-free; Ambion, Austin, TX). RT was performed in 20  $\mu$ l, using random hexamer primers (Applied Biosystems, Foster City, CA) and 0.5  $\mu$ g RNA. PCR were conducted in duplicate for increased accuracy. Each 25  $\mu$ l of reaction mixture contained 1  $\mu$ l cDNA, 12.5  $\mu$ l TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM probes, and 900 nM primers from Assays-on-Demand Gene Expression Assay Mix or Assays-by-Design Gene Expression Assay Mix (Applied Biosystems). The TaqMan Gene Expression Assays used were as follows: UCP3, Mm00494074\_m1; PPAR $\alpha$ , Mm 00440939\_m1; carnitine palmitoyl transferase 1b (CPT-1b), Mm00487200; CPT-II, Mm00487202; medium-chain acyl-CoA dehydrogenase (MCAD), Mm00431611; peroxisome proliferator-activated receptor-coactivator 1 $\alpha$  (PGC-1 $\alpha$ ), Mm00447183; UCP2, Mm00495907; pyruvate dehydrogenase kinase-4 (PDK4), Mm00443325\_m1; and 18S rRNA, Hs99999901\_s1. Primers and probe for the detection of PPAR $\delta$  were designed (Custom TaqMan Gene Expression Assays; Applied Biosystems), and the sequences were GCCCCG-GAGCTCAATGG (forward) and TGGTCCAGCAGGGAGGAA (reverse) and the FAM-labeled probe was CTGTGCAGACCTCTCC. The amplification was performed as follows: 2 min at 50 C, 10 min at 95 C, and then 40 cycles each at 95 C for 15 sec and 60 C for 60 sec in the ABI/Prism 7700 Sequence Detection System. Controls with no RNA, primers, or reverse transcriptase were included in each set of experiments. Each sample was run in duplicate, and the mean value of the duplicate was used to calculate the mRNA expression of the gene of interest and the housekeeping reference gene (18S rRNA). The amount of the gene of interest in each sample was normalized to that of the reference control using the comparative ( $2^{-\Delta\text{CT}}$ ) method following the manufacturer's instructions.

### Immunoblot assay of UCP3 protein levels

Mitochondrial preparations were obtained from neonatal muscle as already reported (8). Samples of mitochondrial protein were mixed with equal volumes of 2 $\times$  SDS loading buffer, incubated at 90 C for 5 min, and subjected to SDS-PAGE (12% wt/vol gel). Protein (40  $\mu$ g) was transferred to polyvinylidene difluoride membranes, and immunodetection was performed using a rabbit affinity-pure UCP3 antiserum (Chemicon AB3046; Chemicon, Temecula, CA). It was used at a 1:1000 dilution, and detection was achieved by the use of a horseradish-peroxidase-coupled antirabbit secondary antibody (sc-2004; Santa Cruz Biotechnology, Santa Cruz, CA) and an enhanced chemiluminescence (ECL) detection kit (Amersham, Piscataway, NJ). Blots were stripped and probed with an antibody for subunit IV of cytochrome c oxidase (A-6409; Molecular Probes, Eugene, OR). The sizes of the proteins were estimated using protein molecular mass standards (Bio-Rad, Richmond, CA).

### Plasma metabolites

Plasma FFA levels were quantified using a colorimetric acyl-CoA synthase and acyl-CoA oxidase-based method (NEFA C; Wako Chemicals, Neuss, Germany). Plasma glucose levels were determined with a glucose-oxidase-based test (Accutrend; Roche). Plasma  $\beta$ -hydroxybutyrate levels were quantified using a spectrophotometric  $\beta$ -hydroxybutyrate dehydrogenase-based assay (Sigma).

### Cell culture and transient transfection assays

Myoblastic L6 cells were obtained from American Type Culture Collection (Rockville, MD) and were grown in DMEM containing 10% fetal bovine serum. Transfection experiments were carried out in L6 cells at 50% confluence by using FuGene6 Transfection Reagent (Roche) and were performed according to the manufacturer's instructions. For L6 transfection, each point was assayed in triplicate in a six-well plate. Each transfection contained 1.5  $\mu$ g of the plasmid 2mUCP3-Luc in which the region from -1946 to +60 of the mouse UCP3 gene drives the expression of the cDNA for firefly (*Photinus pyralis*) luciferase, as reported elsewhere (16). The plasmid 2mUCP3-PPREmut-Luc, containing point mutations at the putative peroxisome proliferator response element (CC instead of AG at sites -51 and -50, G instead of C at site -45, and TA instead of GG at sites -43 and -42), was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and checked by direct DNA sequencing. When indicated, transfection assays contained also 0.3

$\mu$ g of the mammalian expression vectors pCMV-MyoD (17), pRSV-RXR $\alpha$  (18), pSG5-PPAR $\alpha$  (19), pSG5-PPAR $\delta$ , and pSG5-PPAR $\gamma$  (20) and 3 ng pRL-CMV (Promega, Madison, WI), an expression vector for the sea pansy (*Renilla reniformis*) luciferase used as an internal transfection control. Cells were incubated for 48 h after transfection, and when indicated, cells were treated for 24 h before harvest with or without 10  $\mu$ M Wy14,643, 100  $\mu$ M bezafibrate, 1  $\mu$ M GW501516, 1  $\mu$ M AGN194204, 30  $\mu$ M phytanic acid, 10  $\mu$ M rosiglitazone, and 500  $\mu$ M oleic acid.

Firefly luciferase and Renilla luciferase activities were measured in a Turner Designs Luminometer (model TD20/20) using the Dual Luciferase Reporter assay system kit (Promega). Homogenates from cells were prepared with 500  $\mu$ l passive lysis buffer (Promega). Cells were lysed in agitation for 15 min, and 20  $\mu$ l of homogenate was used for measurement. Luciferase activity elicited by UCP3 promoter constructs was normalized for variation in transfection efficiency using Renilla luciferase as an internal standard.

### EMSA

The cDNAs for mouse PPAR $\alpha$  and PPAR $\delta$  and human retinoid X receptor- $\alpha$  (RXR $\alpha$ ) were transcribed and translated *in vitro* using the TNT Quick-Coupled Transcription/Translation Systems (Promega). The sequence of the double-stranded oligonucleotide used in EMSA corresponds to positions –58 to –25 of the mouse UCP3 gene (see Fig. 4B for the sequence). The <sup>32</sup>P-labeled oligonucleotide (10,000–20,000 cpm) was incubated for 30 min at 25 C with 5  $\mu$ l of *in vitro*-transcribed/translated proteins. Reactions were carried out in a volume of 20  $\mu$ l containing 10 mM Tris-HCl (pH 8.0), 0.05% Nonidet P-40, 1 mM dithiothreitol, 40 mM KCl, 6% glycerol, and 2  $\mu$ g of poly(dI)·(dC). Samples were analyzed by electrophoresis at 4 C in nondenaturing 5% polyacrylamide gels in 0.5 $\times$  TBE (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA). The specificity of the binding was determined by including 100-fold molar excess of unlabeled oligonucleotide as competitor.

### Chromatin immunoprecipitation (ChIP) assay

L6 cells were transfected with 2mUCP3-Luc (containing 1946 bp of the promoter) or 2mUCP3-PPREmut-Luc in the presence of PPAR $\delta$  expression vector and treated with oleic acid. ChIP was performed basically as described elsewhere (16). The precleared chromatin solutions were incubated overnight at 4 C with 2  $\mu$ g anti-PPAR $\delta$  antibody (Santa Cruz sc-1983) or an equal amount of an unrelated Ig (Santa Cruz sc-9314). DNA obtained after phenol-chloroform extraction was used for PCR analysis. The primers used for the amplification of the promoter region containing the DR-1 element are CCGTCTCTCTCTCCCCCTC (forward) and GTTGTCTCTGCTGTCCCTGG (reverse).

### Statistical analysis

Where appropriate, statistical analysis was performed by Student's *t* test; significance is indicated in the text.

## Results

### Differential regulation of UCP3 mRNA expression in heart and skeletal muscle in adult wild-type and PPAR $\alpha$ -null mice

To analyze the effects of the lack of expression of PPAR $\alpha$  in skeletal muscle on the regulation of UCP3 gene, UCP3 mRNA levels were measured in gastrocnemius skeletal muscle in adult PPAR $\alpha$ -null mice in comparison with their control littermates (Fig. 1A). The results showed that under fed conditions, UCP3 mRNA levels were similar between both genotypes. The increase in UCP3 mRNA in response to 30 h of starvation was also similar in control and PPAR $\alpha$ -null mice (around 5-fold). The increase of UCP3 mRNA levels resulting from starvation was also not impaired in tibialis anterior or soleus muscles from PPAR $\alpha$ -null mice (data not shown).

UCP3 mRNA levels were analyzed in heart under the

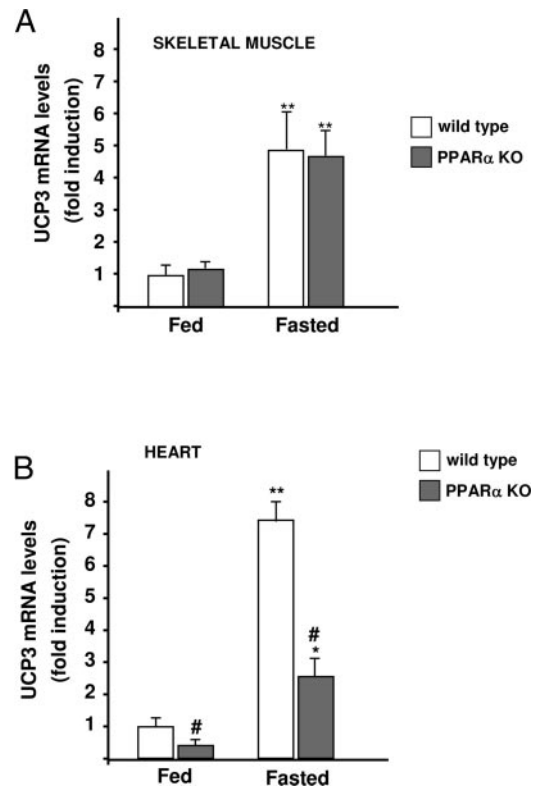


FIG. 1. UCP3 mRNA levels in skeletal muscle and heart from wild-type and PPAR $\alpha$ -null adult mice. Eight-week-old mice were used in basal conditions (fed) or under 30 h of starvation (fasted). Gastrocnemius skeletal muscle or heart was isolated, and UCP3 mRNA expression was analyzed by quantitative RT-PCR as described in *Materials and Methods*. Bars indicate the mean  $\pm$  SEM of six to 10 mice per group coming from at least three different litters. Data are shown as fold induction with respect to values in wild-type fed mice. Significant differences between fed and fasted mice are shown: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; comparison between wild-type and PPAR $\alpha$ -null mice: #,  $P < 0.05$ .

same experimental conditions. UCP3 mRNA abundance was significantly reduced (around 40%) in fed PPAR $\alpha$ -null mice with respect to fed controls (Fig. 1B). When mice were starved for 30 h, the levels of UCP3 mRNA in heart increased more than 7-fold in controls, whereas in PPAR $\alpha$ -null mice, the induction was significantly lower with respect to fed PPAR $\alpha$ -null mice (around 5-fold), and the total amount of UCP3 mRNA was just 30% of the levels reached in the heart of the starved control littermates. These findings are similar to other reports (12, 13) and had been interpreted either as indicating a minor role for PPAR $\alpha$  in the control of skeletal muscle UCP3 gene expression *in vivo* or as evidence of the existence of compensatory mechanisms acting preferentially in muscle to maintain normal regulation of UCP3 gene expression.

To analyze the alterations in circulating metabolites related to energy metabolism, glucose, FFA, and ketone bodies ( $\beta$ -hydroxybutyrate) were determined in plasma (Table 1). Although glucose levels were similar in wild-type and PPAR $\alpha$ -null mice in fed conditions and the levels of glucose decrease in both genotypes because of starvation, in PPAR $\alpha$ -null mice, the levels of glucose were statistically lower than in wild-type mice. FFA levels were similar under fed con-



**TABLE 1.** Serum metabolites in adult wild-type and PPAR $\alpha$ -null mice: effects of fasting

	Fed		Fasted	
	Wild type	PPAR $\alpha$ -null	Wild type	PPAR $\alpha$ -null
Glucose (mM)	9.8 $\pm$ 1.1	8.5 $\pm$ 1	4.2 $\pm$ 0.6 <sup>b</sup>	2.8 $\pm$ 0.3 <sup>b</sup>
FFA ( $\mu$ M)	650 $\pm$ 85	770 $\pm$ 20	825 $\pm$ 27 <sup>a</sup>	1558 $\pm$ 102 <sup>b,c</sup>
$\beta$ -Hydroxybutyrate (mM)	0.2 $\pm$ 0.01	0.2 $\pm$ 0.04	1.2 $\pm$ 0.1 <sup>a</sup>	0.39 $\pm$ 0.14 <sup>c</sup>

Statistical significance of differences between fed and fasted mice is shown as <sup>a</sup>  $P < 0.05$  and <sup>b</sup>  $P < 0.01$  and that between wild-type and PPAR $\alpha$ -null mice at any feeding status as <sup>c</sup>  $P < 0.05$ .

ditions in both genotypes, but in response to starvation, FFA from PPAR $\alpha$ -null were significantly higher than in wild-type mice.  $\beta$ -Hydroxybutyrate levels showed no differences in fed conditions between wild-type and PPAR $\alpha$ -null mice. As expected,  $\beta$ -hydroxybutyrate levels increased in response to starvation, but this response was strongly impaired in PPAR $\alpha$ -null mice, being 60% less than that in their wild-type littermates. The pattern of alterations in the metabolic response to starvation in PPAR $\alpha$ -null mice is in agreement with previous reports on the characterization of the phenotype of these mice (21, 22).

#### UCP3 mRNA expression in skeletal muscle and heart depends on PPAR $\alpha$ during the perinatal period

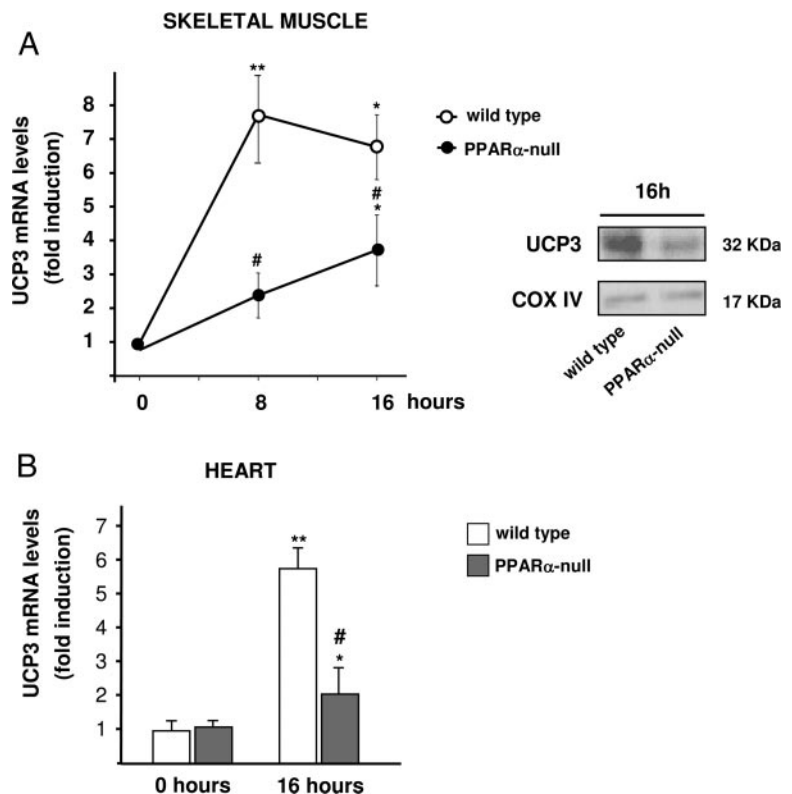
To study the role of PPAR $\alpha$  on UCP3 gene regulation in the perinatal period, hind-limb muscle and heart were obtained from wild-type and PPAR $\alpha$ -null mice just after birth (0 h, no suckling) and at 8 and 16 h after birth, with pups suckling (Fig. 2A, *left*). As expected, the levels of UCP3 mRNA in skeletal muscle of wild-type mice increased rapidly after birth, 8-fold after 8 h, and remained high 16 h later. In PPAR $\alpha$ -null mice, UCP3 mRNA levels increased to a mi-

nor extent; they were just 2-fold and 3-fold higher 8 and 16 h after birth, respectively.

The reduction in UCP3 gene expression in muscle from PPAR $\alpha$ -null neonates occurred also at the protein level (Fig. 2A, *right*). The regulation of UCP3 by PPAR $\alpha$  in heart was similar to adults (Fig. 2B). In wild-type mice, 16 h after birth, UCP3 mRNA levels increased 5-fold with respect to pups at birth. In the PPAR $\alpha$ -null pups, the increase observed on UCP3 mRNA at 16 h was just 2-fold, significantly lower than in wild-type mice. These results indicate a major impact of the lack of PPAR $\alpha$  in skeletal muscle of neonatal mice with respect to adults, whereas the behavior of UCP3 gene expression in heart was similarly impaired as in adult PPAR $\alpha$ -null mice.

The levels of glucose, FFA, and ketone bodies were also measured in plasma of wild-type and PPAR $\alpha$ -null mice 16 h after birth (Table 2). Glucose and FFA levels did not show any statistically significant difference between both groups of mice, whereas the levels of the  $\beta$ -hydroxybutyrate showed a remarkable 75% reduction in PPAR $\alpha$ -null with respect to wild-type pups. Unaltered levels of FFA in PPAR $\alpha$ -null mice indicate that there is no impairment in their suckling behav-

FIG. 2. UCP3 gene expression in skeletal muscle and heart from wild-type and PPAR $\alpha$ -null neonates. Pups were studied immediately after birth (0 h, no feeding) or 8 and 16 h after birth. Quantitative RT-PCR was performed to assess UCP3 mRNA levels in hind limb and heart from neonates. Results are the mean  $\pm$  SEM of four to six mice per group coming from at least three different litters. Data are shown as fold induction with respect to values in wild-type, 0-h-old pups. Significant differences between values at 8 or 16 h after birth with respect to values at 0 h are shown: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; comparison between wild-type and PPAR $\alpha$ -null mice at every time point of development: #,  $P < 0.05$ . A, *Right*, A representative immunoblot of equal amounts (40  $\mu$ g) of skeletal muscle mitochondrial protein from 16-h-old wild-type and PPAR $\alpha$ -null mice probed with UCP3 and subunit IV of cytochrome c oxidase (COX IV) antibodies (see *Materials and Methods*). Data are representative of three independent experiments.



**TABLE 2.** Serum metabolites in neonatal wild-type and PPAR $\alpha$ -null mice

	Wild type	PPAR $\alpha$ -null
Glucose (mM)	3.17 $\pm$ 0.54	2.98 $\pm$ 0.81
FFA ( $\mu$ M)	746 $\pm$ 103	946 $\pm$ 245
$\beta$ -Hydroxybutyrate (mM)	0.70 $\pm$ 0.38	0.18 $\pm$ 0.09 <sup>a</sup>

Serum was obtained from 16-h-old pups. Statistical significance of differences between wild-type and PPAR $\alpha$ -null mice are shown as <sup>a</sup> $P < 0.01$ .

ior, because FFA in neonatal blood are highly dependent on the extent of milk intake. Thus, lowered UCP3 mRNA in PPAR $\alpha$ -null pups could not be caused by the exposure of skeletal muscle and heart to lower levels of circulating FFA and should be caused by a lack of responsiveness of the tissues. Similarly to adults, PPAR $\alpha$ -null pups showed lowered circulating ketone bodies, which is indicative of a reduced hepatic oxidation of fatty acids, in this case coming from milk intake. This is in agreement with the reported impairment in gene expression for ketogenic enzymes in perinatal liver of PPAR $\alpha$ -null mice (23).

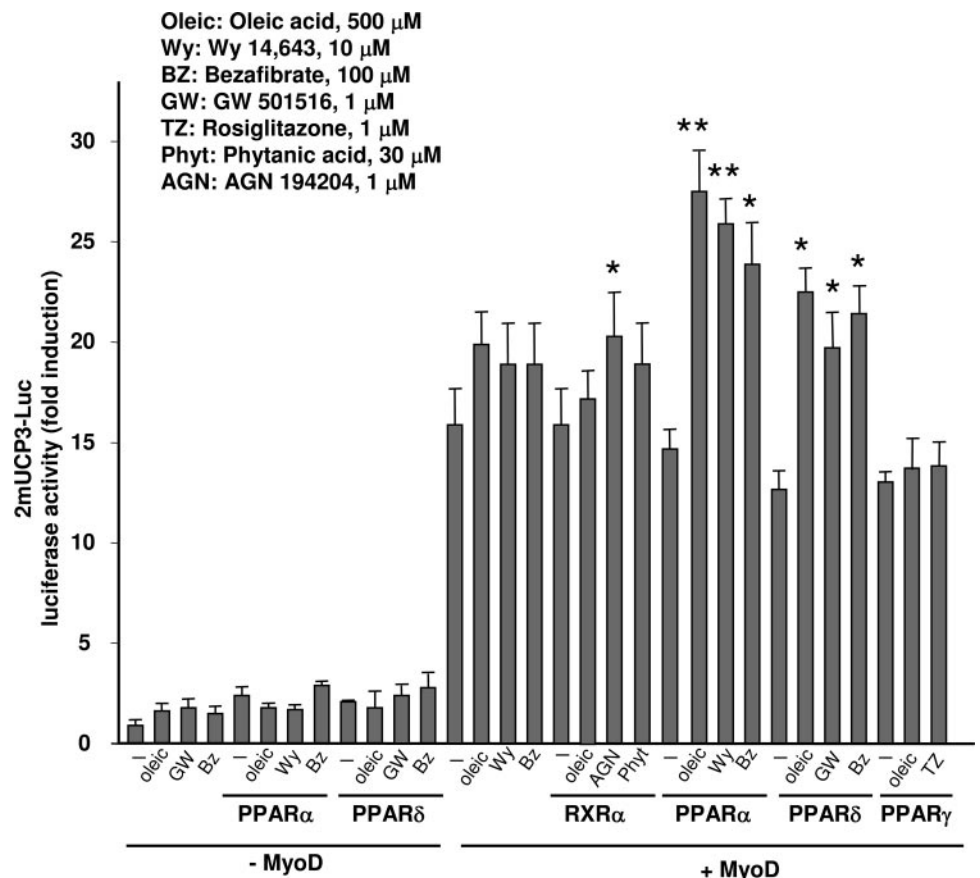
*PPAR $\alpha$  and PPAR $\delta$  can mediate fatty-acid-dependent activation of mouse UCP3 gene transcription*

We undertake the analysis of the responsiveness of the mouse UCP3 gene transcription to fatty acids and the involvement of PPAR $\alpha$  with respect to other nuclear receptors. For this purpose, a construct containing around 2 kb of the mouse UCP3 promoter fused to luciferase reporter gene was

transiently transfected to L6 myogenic cells. In the absence of cotransfected MyoD, the mouse UCP3 gene promoter showed a very low activity and was not sensitive to induction by fatty acids or PPAR $\alpha$  and PPAR $\delta$  agonists (Fig. 3). In the presence of cotransfected MyoD, the mouse UCP3 promoter activity increased around 15-fold and became sensitive to the induction by fatty acids. Thus, in the presence of MyoD, induction of UCP3 mouse promoter by oleic acid was observed when PPAR $\alpha$  or PPAR $\delta$  but not PPAR $\gamma$  was cotransfected. In addition, in the presence of PPAR $\alpha$ , treatment of the cells with PPAR $\alpha$  agonists (Wy 14,643 and bezafibrate) increased the activity of the promoter similarly to what is observed for oleic acid. Also, the addition of PPAR $\delta$  agonists (GW 501516 or bezafibrate), when cotransfected with PPAR $\delta$ , increased the activity of the promoter significantly. Because the induction by fatty acids could be mediated by RXR (24), parallel experiments were performed cotransfecting this nuclear receptor and treating the cells with oleic acid and also with RXR agonists (phytanic acid and AGN 194204). The specific RXR ligand AGN 194204 caused a small but significant induction of the UCP3 promoter. No statistically significant effects due to oleic or phytanic acid were observed, indicating that the ligand-dependent activation of RXR alone, via a potential homodimer or heterodimer with receptors other than PPARs, is not mediating mouse UCP3 regulation in response to fatty acids, at least *in vitro*.

Sequence analysis of the 5' noncoding region of the mouse UCP3 gene indicated the presence at position  $-51/-38$  of a one-base-spaced direct repeat (AGGTTTCAGGTCA) identi-

**FIG. 3.** Mouse UCP3 promoter activity in response to oleic acid in muscle cells. Effects of PPARs and RXR. Transient transfection assays of the 2mUCP3-Luc construct, containing 2 kb of the promoter region of mouse UCP3 gene fused to luciferase reporter gene, were performed in L6 muscle cells. When indicated, 0.3  $\mu$ g MyoD expression vector was cotransfected and also, when indicated, with 0.3  $\mu$ g PPAR $\alpha$ ,  $\delta$ , or  $\gamma$  and RXR $\alpha$  expression vectors. Treatments with agonists were performed for 24 h. Results are the mean  $\pm$  SEM of at least three independent experiments performed in triplicate. All samples cotransfected with MyoD expression vectors show significantly higher luciferase activity than non-cotransfected ones ( $P < 0.001$ ). Significant differences resulting from addition of the agonists respect to their relative controls are shown: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

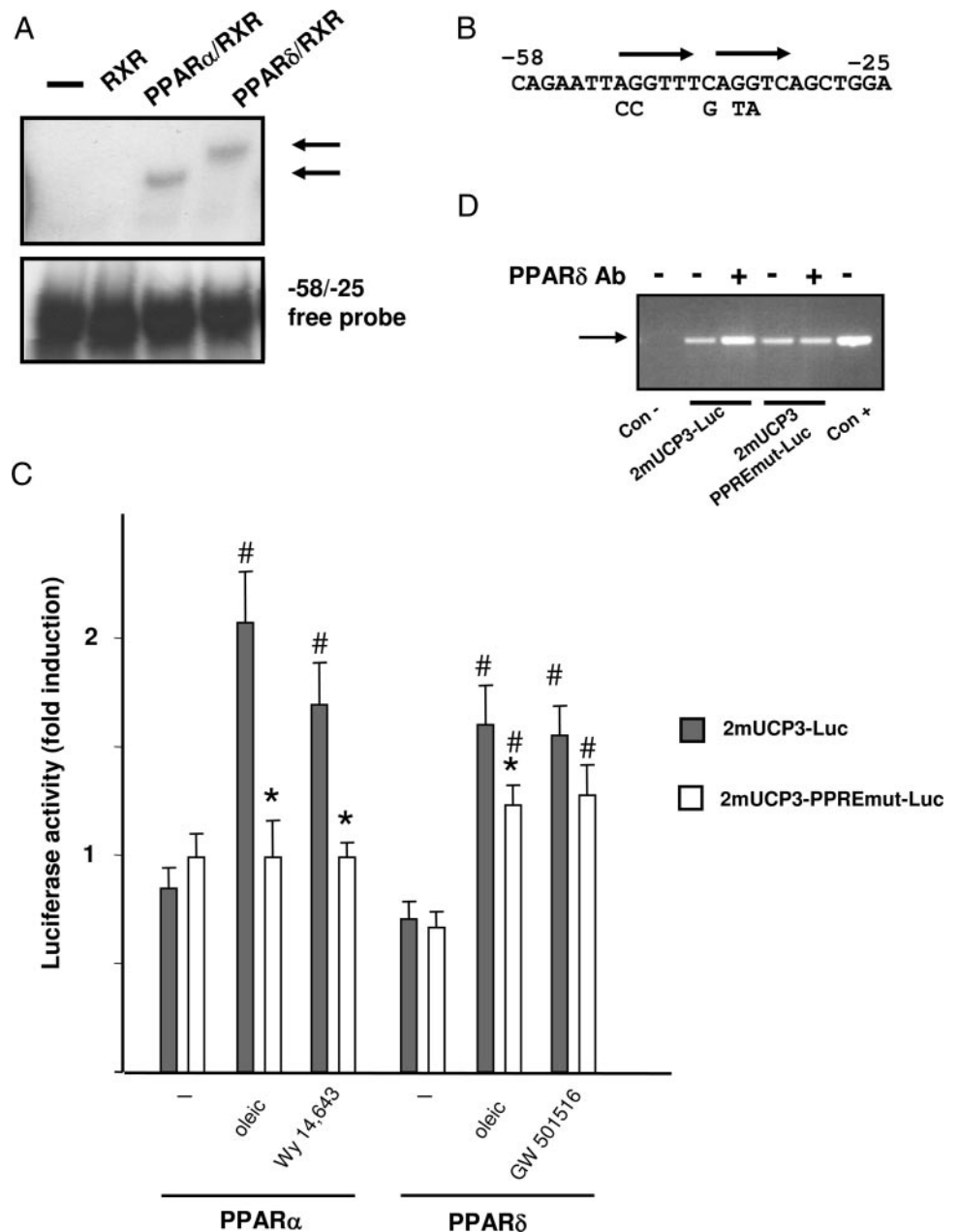


cal to that previously identified in the human UCP3 promoter to mediate fatty acid effects through PPARs (9). EMSA analysis revealed that this site was capable of a high-affinity binding to PPAR $\alpha$ /RXR and PPAR $\delta$ /RXR heterodimers but not to RXR alone (Fig. 4A).

A version of the plasmid construct containing the 2-kb mouse UCP3 gene promoter including point mutations at the -51/-38 region (Fig. 4B) was obtained. The mutations introduced disrupted the capacity of PPAR $\delta$ /RXR and PPAR $\alpha$ /RXR to bind this site as determined by EMSA (not shown). This mutated construct lost completely the capacity of PPAR $\alpha$ -mediated activation by oleic acid, and it was totally unresponsive to the PPAR $\alpha$ -specific activator Wy 14,643 (Fig. 4C). In addition, the mutated construct showed also a reduced capacity of being activated by oleic acid in the pres-

ence of transfected PPAR $\delta$ , although some extent of activation remained. The same behavior was observed for the PPAR $\delta$ -specific activator GW 501516.

Experiments of ChiP were performed to corroborate the binding of PPAR $\delta$  to this region (Fig. 4D). Nuclear extracts from L6 where obtained from cells transfected either with 2mUCP3-Luc or 2mUCP3-PPREmut-Luc. Results showed an enrichment of the PCR product of the region containing the DR-1 region when the PPAR $\delta$  antibody was added to the extracts from the cells transfected with the wild-type construct. In contrast, no enrichment was observed when the antibody was added in cells transfected with the mutated construct. These results indicate that PPAR $\delta$  binds *in vivo* the DR-1 located at -51/-38 of the mouse UCP3 gene promoter.



**FIG. 4.** A proximal site binding PPARs is involved in mediating the responsiveness of the mouse UCP3 promoter to oleic acid and PPAR agonists in muscle cells. **A**, EMSA of the binding of *in vitro* translated PPAR $\alpha$  and - $\delta$  and RXR $\alpha$ . Arrows indicate the specific retarded bands due to PPAR $\alpha$ /RXR $\alpha$  and PPAR $\delta$ /RXR $\alpha$  heterodimers. **B**, Sequence of the mouse UCP3 promoter at -58/-25, letters at the bottom indicate the point mutation changes introduced to suppress PPAR binding. **C**, Transient transfection assays of the 2mPPREmut-UCP3-Luc construct in comparison with the wild-type 2mUCP3-Luc. Plasmids were transiently transfected into L6 cells as in Fig. 3. Transfections included 0.3  $\mu$ g MyoD expression vector and, when indicated, 0.3  $\mu$ g PPAR $\alpha$  or PPAR $\delta$  expression vectors. Treatments with agonists were performed for 24 h. Results are the mean  $\pm$  SEM of at least three independent experiments performed in triplicate and are expressed as fold activity respect to basal activity in the absence of PPARs or agonists. #, Statistically significant ( $P < 0.05$ ) differences resulting from oleic acid or PPAR agonists in every construct; \*, those between constructs at any experimental condition. **D**, ChiP analysis of PPAR $\delta$  binding to the mouse UCP3 gene promoter. ChiP was performed on L6 cells transfected with the 2mUCP3-Luc or 2mUCP3-PPREmut-Luc constructs. The arrow indicates the PCR product from mouse UCP3 promoter. Con-, No DNA; Con+, positive plasmid amplification. The results are representative of three independent experiments.

*Gene expression of PPAR $\delta$  and PPAR $\alpha$  in neonatal and adult skeletal muscle and heart*

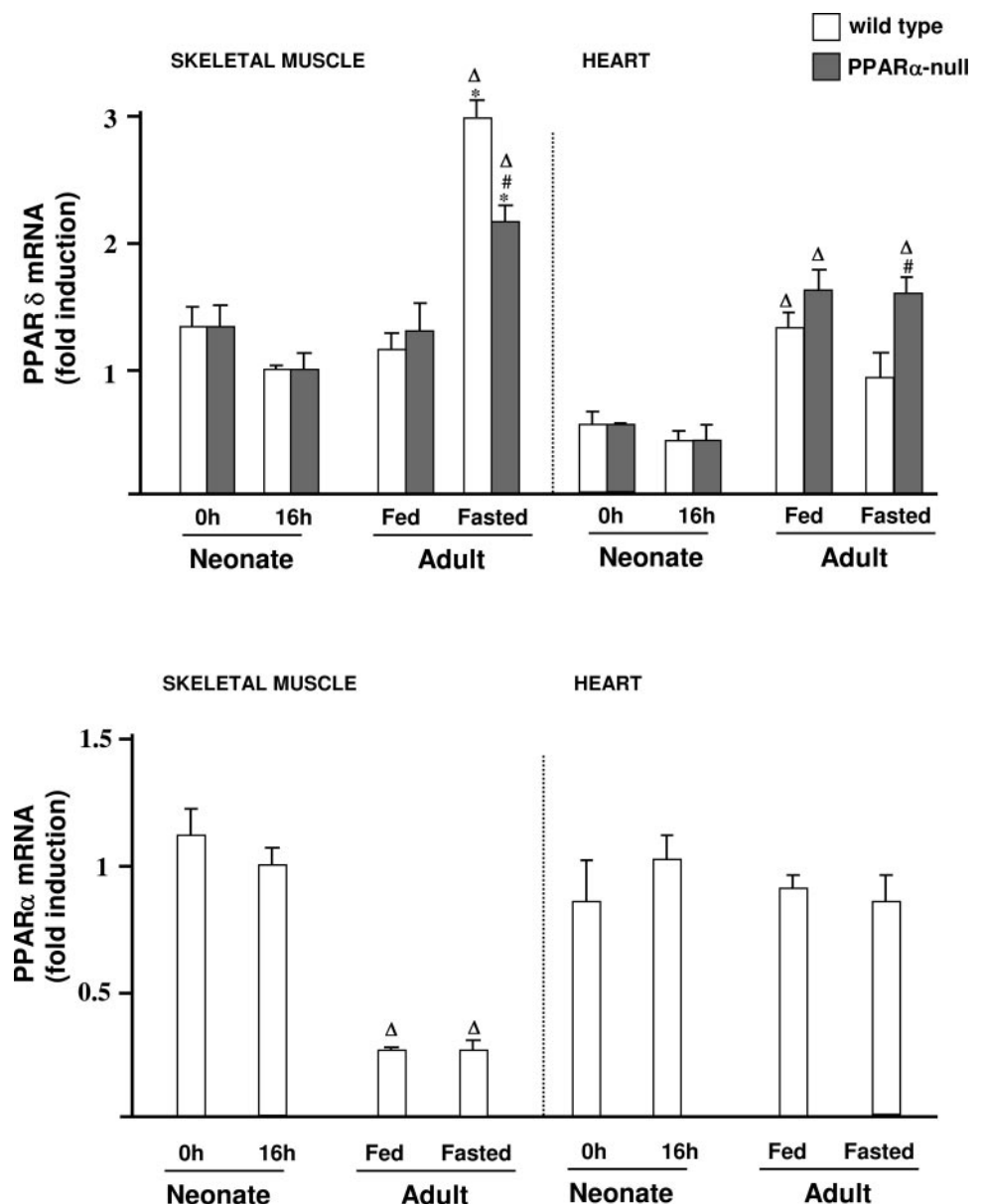
The results above suggest that the sensitivity of the UCP3 gene transcription to PPAR $\delta$  can constitute a major mechanism for compensation of the lack of PPAR $\alpha$ . However, this mechanism appears not to be operational in heart but to be enough for compensating UCP3 mRNA expression at least in adult skeletal muscle. PPAR $\delta$  gene expression studies were undertaken in wild-type and PPAR $\alpha$ -null mice in the situations studied before (Fig. 5). In skeletal muscle, PPAR $\delta$  mRNA levels were slightly decreased in neonates 16 h after birth. No differences were observed between wild-type and PPAR $\alpha$ -null either at birth or 16 h later. PPAR $\delta$  mRNA abundance was similar in adult and neonatal skeletal muscle. PPAR $\delta$  mRNA expression was induced as a consequence of starvation in skeletal muscle from wild-type mice and, to a minor extent, in PPAR $\alpha$ -null mice. In heart, there were also

no differences in PPAR $\delta$  mRNA levels between wild-type and PPAR $\alpha$ -null neonates. Adults showed a higher expression of PPAR $\delta$  mRNA with respect to neonates. Starvation caused a slight decrease in PPAR $\delta$  mRNA levels in wild-type mice. Thus, results indicate that there were no major changes in PPAR $\delta$  mRNA expression resulting from the lack of PPAR $\alpha$  in skeletal muscle and heart.

Gene expression analysis of PPAR $\alpha$  revealed that neonatal skeletal muscle expressed around 3-fold more PPAR $\alpha$  mRNA than adult skeletal muscle. This difference did not occur in heart. Starvation of adult mice did not cause changes in PPAR $\alpha$  mRNA levels either in skeletal muscle or in heart.

The study was extended to determine PGC-1 $\alpha$  mRNA expression during development. PGC-1 $\alpha$  mRNA relative abundance in muscle from neonatal (16-h-old) pups was  $1.7 \pm 0.3$ -fold with respect to that in adult skeletal muscle, and in neonatal heart, it was  $2.7 \pm 0.4$ -fold with respect to that

FIG. 5. PPAR $\delta$  mRNA and PPAR $\alpha$  mRNA levels in skeletal muscle and heart from wild-type and PPAR $\alpha$ -null neonates and adults. Gastrocnemius skeletal muscle (adults) or hind-limb muscle (neonates) and heart were dissected, and the mRNA levels for PPAR $\delta$  and PPAR $\alpha$  were analyzed by quantitative RT-PCR as described in *Materials and Methods*. Bars indicate the mean  $\pm$  SEM of four to 10 mice per group coming from at least three different litters. Results are expressed as fold induction with respect to values in neonatal (16-h-old) mice. For every genotype, significant differences between neonates (16 h old) and adults either fed or fasted are shown:  $\Delta$ ,  $P < 0.05$ ; \*,  $P < 0.05$  for significant differences due to fasting in adults; #,  $P < 0.05$  for those between wild-type and PPAR $\alpha$ -null mice.





in adult heart. Similar findings have been reported in cardiac mouse development (25). Levels of PGC-1 $\alpha$  transcripts were higher in heart than in skeletal muscle, in both neonates and adults. This is in agreement with previous reports in adult mice (26). PGC-1 $\alpha$  transcript levels were not significantly modified in muscle or heart from PPAR $\alpha$ -null mice, either in neonates or in adults (not shown).

#### Differential effects of PPAR $\alpha$ invalidation on the expression of PPAR $\alpha$ and PPAR $\delta$ target genes

To determine whether the reduction in UCP3 mRNA expression in neonatal muscle and heart from PPAR $\alpha$ -null mice corresponds to a general impairment in the expression of PPAR $\alpha$  target genes, additional gene expression analysis was performed on neonates. Genes involved in fatty acid catabolism, especially those whose transcripts were up-regulated in transgenic mouse models of overexpression of PPAR $\alpha$  in skeletal muscle, were analyzed (27). In wild-type mice, transcript levels for UCP2, CPT-II, and PDK4 were dramatically up-regulated in heart from 16-h-old, suckling pups, with respect to the those before initiation of suckling (0 h), whereas no postnatal induction was observed for CPT-I $\beta$  or MCAD mRNA. In skeletal muscle, only UCP2 mRNA levels showed postnatal induction. In skeletal muscle and heart from PPAR $\alpha$ -null neonates, the postnatal induction of UCP2 mRNA was impaired (Fig. 6). CPT-II mRNA and PDK4 mRNA levels were down-regulated only in postnatal heart and unaffected in muscle. No effects were observed in CPT-I $\beta$  or MCAD mRNA levels in skeletal muscle or heart from PPAR $\alpha$ -null mice, in accordance with the low sensitivity of these genes to PPAR $\alpha$ -dependent regulation *in vivo* (27). UCP2 mRNA, the only transcript lowered in postnatal muscle, was not significantly altered in skeletal muscle from adult PPAR $\alpha$ -null mice either in fed ( $1.2 \pm 0.4$ -fold change) or in fasted conditions ( $1.5 \pm 0.7$ -fold change). However, in heart, UCP2 mRNA levels were significantly reduced in fasted ( $0.23 \pm 0.1$ -fold change with respect to wild-type,  $P < 0.05$ ) but not in fed ( $0.8 \pm 0.2$ -fold change with respect to wild-type) PPAR $\alpha$ -null mice, similarly to UCP3 mRNA.

#### Discussion

In the present study, we describe a differential impact of the lack of PPAR $\alpha$  on UCP3 gene expression depending on the tissue and the stage of development. Thus, UCP3 gene expression in heart is systematically impaired both in neonates and adults, either fed or fasted, when mice lack PPAR $\alpha$ . In contrast, skeletal muscle from PPAR $\alpha$ -null adult mice shows unaltered UCP3 gene expression in basal conditions and in response to starvation. However, because the increase in FFA concentration is considered the signal inducer for UCP3 mRNA expression in response to fasting in muscle, it is noteworthy that the higher increase in FFA levels observed in fasted PPAR $\alpha$ -null mice results just in an equal induction of UCP3 mRNA with respect to wild-type mice. Previous data in rodents under different physiological and experimental situations indicate a very close association between the levels of circulating FFA and skeletal muscle UCP3 mRNA levels (4). The differential relationship between FFA and UCP3 mRNA levels in PPAR $\alpha$ -null mice may indicate

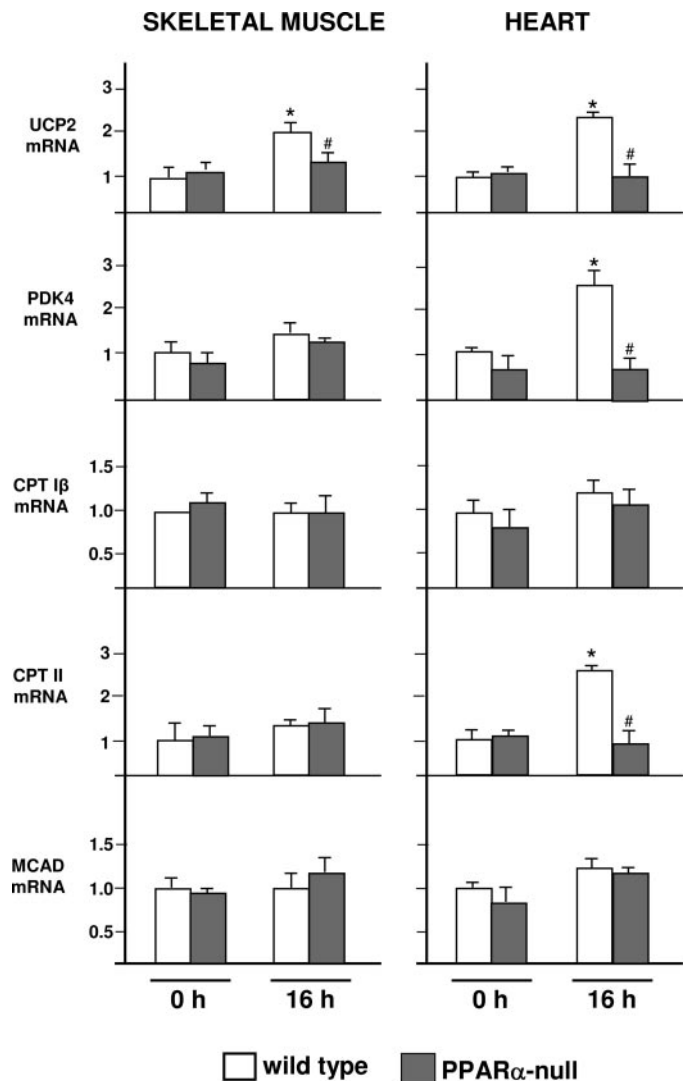


FIG. 6. Gene expression analysis of genes involved in fatty acid metabolism in skeletal muscle and heart from wild-type and PPAR $\alpha$ -null neonates. mRNA was isolated from hind limb and heart from neonates just after birth (0 h) or after 16 h being with their mothers (16 h), and quantitative RT-PCR was performed as described in *Materials and Methods* to analyze the levels of expression of UCP2, PDK4, CPT I $\beta$ , CPT II, and MCAD. Results are shown as fold induction with respect to values in wild-type mice at 0 h, and bars indicate the mean  $\pm$  SEM of four to six mice per group. For every genotype, significant differences between neonates at 0 and 16 h are shown: \*,  $P < 0.05$ ; #, significant differences between wild-type and PPAR $\alpha$ -null mice at  $P < 0.05$ .

that despite UCP3 mRNA levels being preserved, there is some extent of impairment or resistance in the responsiveness of UCP3 gene expression to fatty acids because of the lack of PPAR $\alpha$ .

In contrast with adults, the UCP3 mRNA induction in neonates as a consequence of initiation of suckling is severely impaired not only in heart but also in skeletal muscle from PPAR $\alpha$ -null mice. Lowered UCP3 mRNA in PPAR $\alpha$ -null pups is not caused by lower levels of circulating FFA, which were unaltered. Thus, it appears that PPAR $\alpha$  is particularly required for UCP3 regulation in response to fatty acids in the neonatal period.



The present findings on the transcriptional regulation of the mouse UCP3 gene promoter indicate a similar behavior with respect to the human promoter (9); it is dependent on MyoD, and responsiveness to fatty acids can be mediated by PPAR $\alpha$  or PPAR $\delta$ . This is in contrast with the responsiveness of the UCP3 gene to thyroid hormones, which is remarkably different between humans and mice (16). In fact, despite the large divergence in overall promoter sequence and functional structure between humans and mice (28), the one-base-spaced direct repeat previously identified in the human UCP3 promoter to mediate fatty acid effects through PPARs is completely conserved in the mouse proximal promoter region at position  $-51/-38$ . We found that, like in humans, it binds PPAR $\delta$ /RXR and PPAR $\alpha$ /RXR heterodimers as well as being involved in mediating the responsiveness to fatty acids via PPARs. This identical sequence is also present in the proximal region of the UCP3 genes from all the other mammalian species in which the 5' noncoding region sequence is available in databases (accession number AF168989 for *Rattus norvegicus*, AY523564 for *Phodopus sungorus*, Nw876273 for *Canis familiaris*, and Nw92833 for *Bos taurus*). However, it should be noted that in contrast with PPAR $\alpha$ , some responsiveness to PPAR $\delta$  remains in mutated constructs at this site, thus indicating the possibility of additional mechanisms of PPAR $\delta$ -dependent activation of the mouse UCP3 gene.

Overall, the present findings indicate that the requirement of PPAR $\alpha$  for the regulation of UCP3 gene expression is tissue and development specific, and PPAR $\alpha$  plays an especially critical role in the perinatal period. Compensatory mechanisms other than PPAR $\alpha$  activation for fatty-acid-dependent regulation of UCP3 gene transcription are present in adults to ensure normal UCP3 mRNA expression when muscle is exposed to high levels of circulating FFA, but they are not active in adult heart or in the heart and skeletal muscle during the neonatal period. Although other mechanisms of fatty-acid-dependent regulation of transcription cannot be excluded, PPAR $\delta$  is a likely candidate to mediate this alternative pathway of regulation of UCP3 gene expression, considering its activity on UCP3 gene promoter regulation in response to fatty acids. However, PPAR $\delta$ -mediated compensatory mechanisms of control of UCP3 gene expression would not require up-regulation of PPAR $\delta$  mRNA levels, which are unaltered in different tissues and developmental stages of mice lacking PPAR $\alpha$ . However, the high expression of PPAR $\alpha$  mRNA in neonatal skeletal muscle with respect to adults may be consistent with a more critical requirement of PPAR $\alpha$  to mediate gene expression responsiveness to fatty acids in muscle during the perinatal period.

The fact that a gene such as UCP2, sharing dual PPAR $\alpha$ - and PPAR $\delta$ -dependent regulation like UCP3 (27, 29–31), also shows altered gene expression in heart and in neonatal muscle but not in adult muscle supports the notion of a lack of compensatory effects of PPAR $\delta$ -dependent pathways in muscle from neonates. However, the unaltered expression of the transcripts for PDK4 and CPT-II, which are also targets of both PPAR $\alpha$  and PPAR $\delta$  regulation in adult muscle (27, 29, 31), suggests other potential mechanisms to explain the tissue- and development-dependent impact of PPAR $\alpha$  deficiency in the UCP3 and UCP2 genes. Only those genes involved in lipid catabolism whose expression is significantly

induced in muscle or heart when milk intake begins were affected in neonates by the lack of PPAR $\alpha$ . It cannot be excluded that the differential origin and composition of fatty acids stimulating gene expression of UCP3 and UCP2 genes when they come from milk (neonates) or from lipolysis in white fat (starved adults) act differently on the PPAR $\alpha$ - and PPAR $\delta$ -dependent mechanisms of regulation of gene expression in muscle and heart. Another aspect to be considered is the potential differential requirement of coactivators in muscle and heart from neonates with respect to adults to mediate PPAR-dependent regulation of UCP3 gene and other genes involved in lipid catabolism. Our present data do not indicate lower levels of PGC-1 $\alpha$ , at least at the transcript level, in neonates with respect to adults, but the opposite. P300, another PPAR $\alpha$  coactivator involved in UCP3 promoter regulation in humans, has been reported to be more abundant in neonates than in adults (32). PGC-1 $\alpha$  and p300 expression are higher in heart than in skeletal muscle at any time of development. Thus, these data do not suggest that limiting amounts of these coactivators could explain the specific impairment of gene expression in skeletal muscle from PPAR $\alpha$ -null neonates or in heart with respect to muscle. However, an extensive analysis of developmental regulation in muscle and heart of the multiple coactivators of PPARs reported to date should be undertaken to settle this issue.

On the other hand, although the precise physiological function is still a matter of debate, UCP3 appears to be involved in metabolic handling of fatty acids in muscle and heart in a way that minimizes lipotoxicity and excessive production of reactive oxygen species (33, 34). In fact, its pattern of regulation reported here in relation to neonatal development and PPAR $\alpha$ -dependent pathways is shared in muscle only by the other gene involved in similar functions, UCP2. Genes involved in lipid catabolism but without relation to mitochondrial bioenergetics or reactive oxygen species regulation, such as PDK4 or CPT-II, are altered in parallel with UCP genes only in heart. In this sense, modulation of UCP3 gene expression through drugs acting on PPARs could influence muscle oxidative metabolism and would be expected to act in parallel upon UCP2 gene expression. UCP3 gene promoter studies described above as well as studies *in vivo* using transgenic mice overexpressing PPAR $\alpha$  or PPAR $\delta$  in skeletal muscle indicate that both types of PPAR receptors can induce UCP3 gene expression in adults (27, 29). Present findings indicate that in other physiological situations such as early development, UCP3 is much more dependent of PPAR $\alpha$  activation. Moreover, despite that UCP3 mRNA levels are lower in heart than in skeletal muscle, recent data are indicative of a relevant role of UCP3 in human heart in relation to the partitioning of metabolic fuels (35), and present results point to PPAR $\alpha$  as the receptor unequivocally mediating UCP3 gene regulation in response to fatty acids or fibrates in rodent heart.

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Address all correspondence and requests for reprints to: Dr. G. Solanes, Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, Avda Diagonal 645, E-08028 Barcelona, Spain. E mail: gsolanes@ub.edu.

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