

# Down-regulation of acyl-CoA oxidase gene expression and increased NF- $\kappa$ B activity in etomoxir-induced cardiac hypertrophy

Àgatha Cabrero, Manuel Merlos, Juan C. Laguna, and Manuel Vázquez Carrera<sup>1</sup>

Unitat de Farmacologia, Departament de Farmacologia i Química Terapèutica, Facultat de Farmàcia, Universitat de Barcelona, Spain

**Abstract** Activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) is required for hypertrophic growth of cardiomyocytes. Etomoxir is an irreversible inhibitor of carnitine palmitoyltransferase I (CPT-I) that activates peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) and induces cardiac hypertrophy through an unknown mechanism. We studied the mRNA expression of genes involved in fatty acid oxidation in the heart of mice treated for 1 or 10 days with etomoxir (100 mg/kg/day). Etomoxir administration for 1 day significantly increased (4.4-fold induction) the mRNA expression of acyl-CoA oxidase (ACO), which catalyzes the rate-limiting step in peroxisomal  $\beta$ -oxidation. In contrast, etomoxir treatment for 10 days dramatically decreased ACO mRNA levels by 96%. The reduction in ACO expression in the hearts of 10-day etomoxir-treated mice was accompanied by an increase in the mRNA expression of the antioxidant enzyme glutathione peroxidase and the cardiac marker of oxidative stress bax. Moreover, the activity of the redox-regulated transcription factor NF- $\kappa$ B was increased in heart after 10 days of etomoxir treatment. **Overall, the findings here presented show that etomoxir treatment may induce cardiac hypertrophy via increased cellular oxidative stress and NF- $\kappa$ B activation.**—Cabrero, À., M. Merlos, J. C. Laguna, and M. Vázquez Carrera. **Down-regulation of acyl-CoA oxidase gene expression and increased NF- $\kappa$ B activity in etomoxir-induced cardiac hypertrophy.** *J. Lipid Res.* 2003. 44: 388–398.

**Supplementary key words** nuclear factor  $\kappa$ B • oxidative stress • PPAR • heart

Energy demand of the heart depends on the oxidation of a variety of substrates, mainly fatty acids and glucose. This process is regulated during development and under various physiological and pathophysiological conditions depending on the substrate availability (1, 2). Thus, during the fetal period, cardiac metabolism relies on glucose, whereas after birth, myocardial energy increasingly switches from glucose to fatty acids oxidation, the latter being the

major source of energy in the adult mammalian heart. In addition, during the development of cardiac hypertrophy in rodents and humans, a dramatic reduction in fatty acid oxidation is detected, since a shift in the source of energy is observed from fatty acids to glucose (1). The adjustments of cardiac metabolism to the substrate availability seem to involve changes in the transcriptional control of genes implicated in the transport and metabolism of fatty acids and glucose, which in turn are under the control of a class of transcription factors called peroxisome proliferator-activated receptors (PPARs). Three different PPAR subtypes ( $\alpha$ ,  $\delta/\beta$ , and  $\gamma$ ) have been identified to date. PPAR $\alpha$  is expressed primarily in tissues with a high level of fatty acid catabolism, such as liver, brown fat, kidney, heart, and skeletal muscle (3, 4). PPAR $\beta$  is ubiquitously expressed, and PPAR $\gamma$  has a restricted pattern of expression, mainly in white and brown adipose tissues, whereas other tissues such as skeletal muscle and heart contain limited amounts (3). PPARs are activated by ligands, such as naturally occurring fatty acids, which are activators of all three PPAR subtypes (5, 6). In addition to fatty acids, several synthetic compounds, such as fibrates and thiazolidinediones, bind and activate specific PPAR subtypes. In order to be transcriptionally active, PPARs need to heterodimerize with the retinoid X receptor (RXR). PPAR-RXR heterodimers bind to DNA-specific sequences called peroxisome proliferator response elements (PPREs), consisting of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (AGGTCA) separated by one nucleotide (DR-1). This direct repeat is known to

Abbreviations: ACO, acyl-CoA oxidase; COUP-TF II, chicken ovalbumin upstream promoter transcription factor II; CTE, cytosolic acyl-CoA thioesterase; GPX, glutathione peroxidase; MCAD, medium-chain acyl-CoA dehydrogenase; M-CPT-I, muscle-type carnitine palmitoyltransferase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PGC-1, PPAR $\gamma$  coactivator 1; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; UCP-3, uncoupling protein 3.

<sup>1</sup>To whom correspondence should be addressed.  
e-mail: mvaz@farmacia.far.ub.es

Manuscript received 26 July 2002 and in revised form 15 October 2002.

Published, JLR Papers in Press, November 4, 2002.

DOI 10.1194/jlr.M200294JLR200

bind potential competitors such as homodimers of other nuclear receptors including RXR $\alpha$ , chicken ovalbumin upstream promoter transcription factor II (COUP-TF-II), also called apolipoprotein A-I regulatory protein, ARP-1), and hepatic nuclear factor-4 (HNF-4) (4).

It has been previously reported that development of pressure overload-induced ventricular hypertrophy in mice, which involves a shift in the substrate utilization from fatty acids to glucose, is associated with deactivation of PPAR $\alpha$  (7, 8). Further, it has been shown that the energy substrate switch observed in cardiac hypertrophy involves reactivation of fetal transcriptional control via members of the Sp and COUP-TF families of transcription factors (7). These changes may account for the down-regulation of enzymes involved in fatty acid oxidation. However, little is known about the effects of several drugs leading to cardiac hypertrophy. In fact, several studies have shown that inhibition of mitochondrial  $\beta$ -oxidation by carnitine palmitoyltransferase I (CPT-I) inhibitors, which were developed for the treatment of type 2 diabetes mellitus, is sufficient to cause cardiac hypertrophy (9). In order to delineate the molecular mechanisms involved in etomoxir-induced cardiac hypertrophy, we treated mice with this irreversible inhibitor of CPT-I. Treatment for 1 day with this drug, which in addition activates PPAR $\alpha$  (10), resulted in a significant increase in the mRNA expression of acyl-CoA oxidase (ACO), the gene that catalyzes the rate-limiting step in peroxisomal  $\beta$ -oxidation. In contrast, a dramatic reduction in ACO mRNA levels was observed after etomoxir administration for 10 days. On the other hand, the mRNA levels of PPAR $\alpha$ , and of several of its target genes involved in mitochondrial  $\beta$ -oxidation, were not significantly modified by etomoxir after 10 days of treatment. The fall in ACO expression after 10 days of etomoxir treatment was accompanied by increased activity of the redox-regulated transcription factor, nuclear factor  $\kappa$ B (NF- $\kappa$ B). Overall, the results presented here suggest that etomoxir increases oxidative stress in cardiomyocytes, leading to NF- $\kappa$ B activation, which is required for the hypertrophic growth of cardiomyocytes (11). The negative correlation in heart between enhanced oxidative stress and the reduction ACO expression suggests that peroxisomal  $\beta$ -oxidation may be involved in cardiac hypertrophy after etomoxir treatment.

## MATERIALS AND METHODS

### Animals and treatment

Twenty male Swiss mice from Harlan (Barcelona, Spain) were used. They were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature ( $21 \pm 1^\circ\text{C}$ ) and fed a standard diet. The mice were randomly distributed into two groups. Each group was administered, respectively, either 0.5% carboximethyl cellulose (control group) or 100 mg/kg/day of etomoxir (dissolved in 0.5% carboximethyl cellulose) per os once a day for either 1 or 10 days (1 ml/100 g of body weight). Food and water were given ad libitum. Twenty-four hours after the last administration, mice were killed under pentobarbitone anesthesia to collect blood samples and to isolate hearts. Blood

samples, obtained by cardiac puncture, were collected in EDTA tubes, and plasma was obtained by centrifugation at 2,200 *g* for 10 min at  $4^\circ\text{C}$ . Plasma glucose (Roche, Barcelona, Spain), triglycerides (Sigma), and nonesterified fatty acids (Wako, Germany) levels were determined with colorimetric test. Hearts were rapidly removed, washed in ice-cold 0.9% NaCl, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . Animal handling and disposal were performed in accordance with law 5/1995, 21st July, of the Generalitat de Catalunya.

### RNA preparation and analysis

Total RNA was isolated by using the Ultraspec reagent (Biotech, Houston). Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR). cDNA was synthesized from RNA samples by mixing 0.5  $\mu\text{g}$  of total RNA, 125 ng of random hexamers as primers in the presence of 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies), 20 units of RNAsin (Life Technologies), and 0.5 mM of each dNTP (Sigma) in a total volume of 20  $\mu\text{l}$ . Samples were incubated at  $37^\circ\text{C}$  for 60 min. A 5  $\mu\text{l}$  aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers.

Each 25- $\mu\text{l}$  PCR reaction contained 5  $\mu\text{l}$  of the RT reaction, 1.2 mM MgCl<sub>2</sub>, 200  $\mu\text{M}$  dNTPs, 1.25  $\mu\text{Ci}$  [<sup>32</sup>P]dATP (3,000 Ci/mmol, Amersham), 1 unit of Taq polymerase (Ecogen, Barcelona, Spain), 0.5  $\mu\text{g}$  of each primer, and 20 mM Tris-HCl, pH 8.5. To avoid unspecific annealing, cDNA and Taq polymerase were separated from primers and dNTPs by using a layer of paraffin (reaction components contact only when paraffin fuses, at  $60^\circ\text{C}$ ). The sequences of the sense and antisense primers used for amplification were: PPAR $\alpha$ , 5'-GGCTCGGAGGGCTCTGTCATC-3' and 5'-ACATGCACTGGCAGCAGTGGA-3'; M-CPT-I, 5'-TTC-ACTGTGACCCGACGCGG-3' and 5'-AATGGACGCCCCATGGAGA; MCAD, 5'-TCGAAAGCGGCTCACAAGCAG-3' and 5'-CACCGCAGCTTTCGGGAATGT-3'; UCP-3, 5'-GGAGCCATGG-CAGTGACCTGT-3' and 5'-TGTGATGTTGGGCCAAGTCCC-3'; UCP-2, 5'-AACAGTTCTACACCAAGGGC-3' and 5'-AGCATGG-TAAGGGCACAGTG-3'; ACO, 5'-ACTATATTTGGCCAATTTTGTG-3' and 5'-TGTGGCAGTGGTTTCCAAGCC-3'; CTE, 5'-CAGCCACCCCGAGGTAAGG-3' and 5'-CCTTGAGGCCATCCTTGGTCA-3'; RXR $\alpha$ , 5'-GCTCTCCAACGGGTCGAGGCT-3' and 5'-TGGGTGTGGTGGGTACCGACA-3'; RXR $\gamma$ , 5'-CCATG AAGACATGCCGGTGA-5' and 5'-CCCCTGGCAGATGACCTGGT-3'; PGC-1 5'-CCCCTGGATGAAGACGGATTG-3' and 5'-GTGGGTGTGGTTTGTGTCATG-3'; GPX, 5'-CGGCACAGTC-CACCGTGTATG-3' and 5'-ACTGATTGCACGGGAAACCGA-3'; BAX, 5'-GGCCACCAGCTCTGAACAGA-3' and 5'-AGCTGCC-ACCCGGAAGAAGAC-3'; and adenosyl phosphoribosyl transferase (APRT), 5'-AGCTTCCCGGACTTCCCCATC-3' and 5'-GACCACTTTCTGCCCCGGTTC-3'. PCR was performed in an MJ Research Thermocycler equipped with a peltier system and temperature probe. After an initial denaturation for 1 min at  $94^\circ\text{C}$ , PCR was performed for 20 (MCAD), 22 (GPX), 23 (UCP-2, PGC-1), 25 (UCP-3, PPAR $\alpha$ , BAX), 26 (RXR $\alpha$  and RXR $\gamma$ ), 27 (CTE), and 28 (M-CPT-I, ACO) cycles. Each cycle consisted of denaturation at  $92^\circ\text{C}$  for 1 min, primer annealing at  $60^\circ\text{C}$  (except  $58^\circ\text{C}$  for ACO), and primer extension at  $72^\circ\text{C}$  for 1 min and 50 s. A final 5-min extension step at  $72^\circ\text{C}$  was performed. Five microliters of each PCR sample was electrophoresed on a 1 mm thick 5% polyacrylamide gel. The gels were dried and subjected to autoradiography using Kodak X-ray films to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (PPAR $\alpha$ : 645 bp; M-CPT-I: 222 bp; MCAD: 216 bp; UCP-3: 179 bp; UCP-2: 471 bp; ACO: 195 bp; CTE: 224 bp; RXR $\alpha$ : 202 bp; RXR $\gamma$ : 220 bp; PGC-1: 228 bp; GPX:

210 bp; BAX: 256 bp; and APRT: 329 bp). Preliminary experiments were carried out with varying amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, relative quantification of mRNA was assessed by the RT-PCR method used in this study (12). Radioactive bands were quantified by videodensitometric scanning (Vilbert Lourmat Imaging). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

### Isolation of nuclear extracts

Crude nuclear extracts were isolated using the Dignam method (13) with the modifications described by Helenius et al. (14). Frozen hearts were weighed, transferred to Corning tubes, and ice-cold hypotonic buffer (1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 mM HEPES, pH 7.9) was added to each sample. The volume was proportional to the weight of the tissue so as to give 15% homogenates. The tissues were left to thaw in an ice bath and homogenized (2 × 5 s) using a Polytron (Kinematica, Germany). Homogenates were incubated for 10 min on ice and centrifuged (25,000 g, 15 min, 4°C). Pellets were washed once with the same volume of hypotonic buffer used in the homogenization step and centrifuged (10,000 g, 4°C, 15 min). Supernatants were discarded and pellets were suspended in ice-cold low salt buffer (25% glycerol, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 20 mM KCl, 20 mM Hepes, pH 7.9) using half of the volume of the hypotonic buffer. Nuclear proteins were released by adding high-salt buffer (25% glycerol, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 1.2 M KCl, 20 mM Hepes, pH 7.9) drop-by-drop using half of the volume of the low-salt buffer. Samples were incubated on ice for 30 min. During incubation, the tubes were smoothly mixed frequently. Samples were centrifuged (25,000 g, 30 min, 4°C), and supernatants were collected in microfuge tubes and stored in aliquots at -80°C. The protein concentration of the nuclear extracts was then measured.

### Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed using double-stranded oligonucleotides (Santa Cruz Biotechnology) for the consensus binding sites of PPRE (5'-CAAACTAG-GTCAAAGGTCA-3'), mutant PPRE (5'-CAAACTAGCACAAA-GCACA-3'), Sp1 (5'-ATTCGATCGGGCGGGCGAGC-3'), and Oct-1 (5'-TGTCGAATGCAAATCACTAGAA-3'). The NF-κB consensus oligonucleotide (5'AGTTGAGGGACTTTCCAGGC-3') was from Promega. Oligonucleotides were labeled in the following reaction: 1 μl of oligonucleotide (20 ng/μl), 2 μl of 5× kinase buffer, 5 units of T4 polynucleotide kinase, and 3 μl of [<sup>32</sup>P]ATP (3,000 Ci/mmol at 10 mCi/ml) incubated at 37°C for 1 h. The reaction was stopped by adding 90 μl of TE buffer (10 mM Tris-HCl pH 7.4 and 1 mM EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Pharmacia) according to the manufacturer's

instructions. Eight micrograms of crude nuclear proteins were incubated for 15 min on ice in binding buffer [10 mM Tris-HCl pH 8.0, 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA pH 8.0, 5% glycerol, 5 mg/ml BSA, 100 μg/ml tRNA, and 50 μg/ml poly(dI-dC)], in a final volume of 15 μl. Labeled probe (~50,000 cpm) was added and the reaction was incubated for 20 min at room temperature. Where indicated, specific competitor oligonucleotide was added before the addition of labeled probe and incubated for 10 min on ice. For supershift assays, antibodies were added after incubation with labeled probe for a further 30 min at room temperature. Protein-DNA complexes were resolved by electrophoresis at 4°C on a 5% acrylamide gel and subjected to autoradiography. Antibodies against COUP-TF II and p65 were from Santa Cruz Biotechnology.

### Western-blot analysis

Crude nuclear extracts (40 μg) from hearts were subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene fluoride transfer membranes (Millipore), and immunological detection was performed using a goat polyclonal antibody raised against COUP-TF II (dilution 1:1,000). Detection was achieved using the enhanced chemiluminescence (ECL) detection system (Amersham). Blots were also incubated with a rabbit antibody against β-tubulin (dilution 1:5,000) (Boehringer Mannheim), used as a control of equal abundance of nuclear extracts in the samples. Size of detected proteins was estimated using protein molecular mass standards (Life Technologies).

### Statistical analyses

Results are expressed as means ± SD of four or five mice. Significant differences were established by Student's *t*-test using the computer program GraphPad Instat.

## RESULTS

### Effects of etomoxir in cardiac hypertrophy

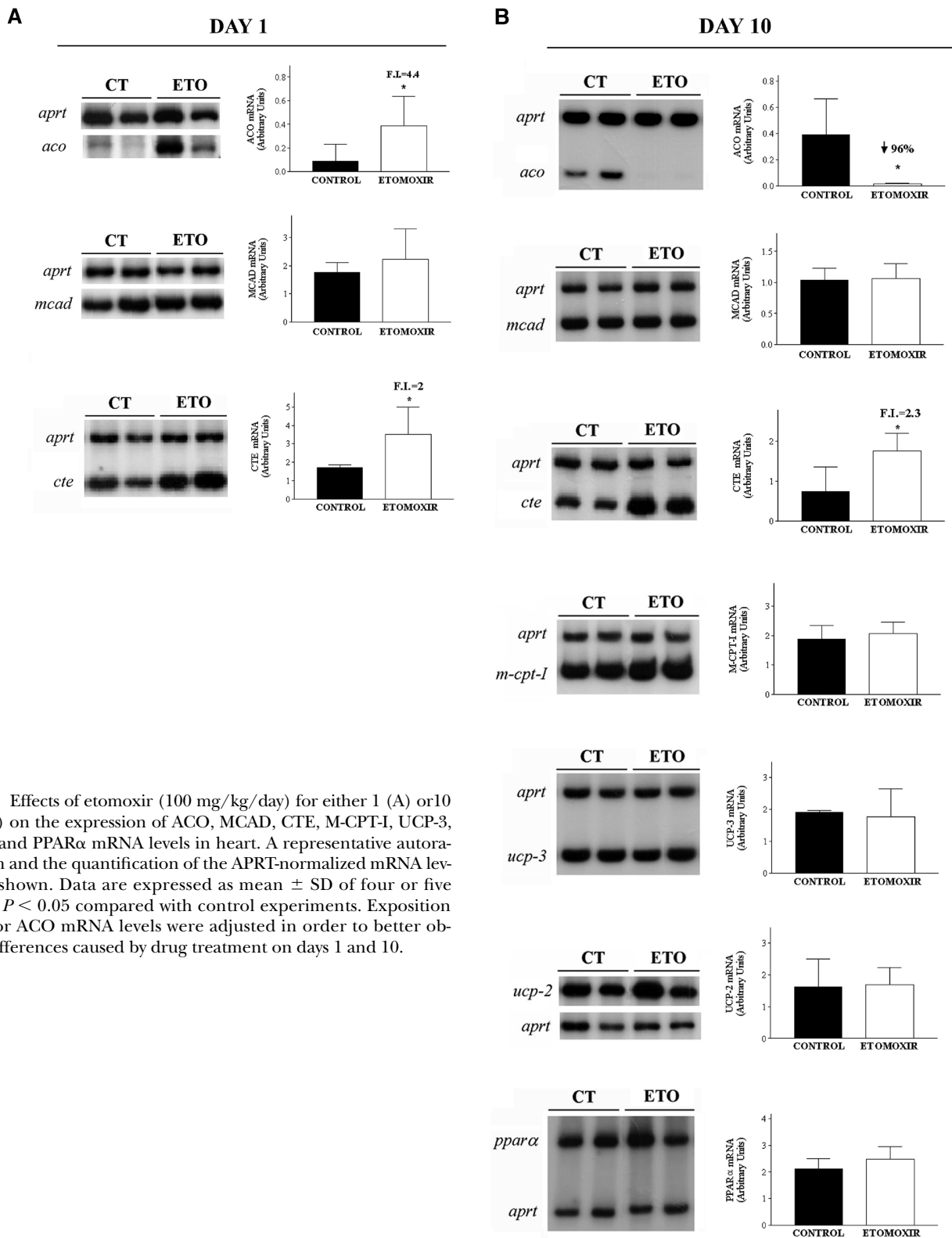
First, we investigated the effect of the CPT-I inhibitor etomoxir on the ratio of heart weight to body weight of mice to assess cardiac hypertrophy. Etomoxir administration (100 mg/kg/day) to mice for 1 day did not cause cardiac hypertrophy. In contrast, 10 days of etomoxir treatment resulted in a significant increase in both the net heart weight (20%) and the ratio of heart weight to body weight (24%) compared with the control group, indicating the presence of cardiac hypertrophy (Table 1). On the other hand, we assessed the effects of etomoxir on plasma glucose levels. In the nonfasted normoglycemic mice used in this study, drug treatment did not modify glucose levels compared with the control group.

TABLE 1. Etomoxir effects on cardiac hypertrophy and plasma glucose levels

	Control	1 Day Etomoxir	Control	10 Days Etomoxir
Body weight (g)	32.6 ± 2.8	32.7 ± 3.1	39.9 ± 5.5	39.1 ± 1.6
Heart weight (g)	0.15 ± 0.007	0.15 ± 0.015	0.20 ± 0.02	0.24 ± 0.03 <sup>a</sup>
Heart weight/body weight (mg/g)	0.46 ± 0.04	0.45 ± 0.04	0.50 ± 0.06	0.62 ± 0.09 <sup>a</sup>
Plasma glucose (mg/dl)	—	—	99 ± 8	93 ± 11

Mice were treated for one or 10 days with either 0.5% carboxymethyl cellulose (control group) or 100 mg/kg/day of etomoxir (dissolved in 0.5% carboxymethyl cellulose). Values are means ± SD of five animals per group.

<sup>a</sup> *P* < 0.05.



### ACO mRNA levels in heart of etomoxir-treated mice for one or 10 days

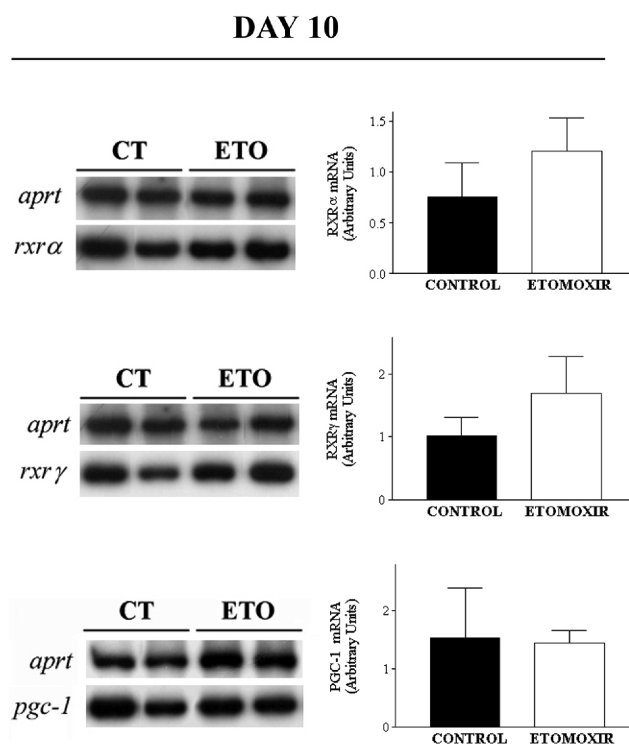
We first examined the effects of etomoxir treatment for either 1 or 10 days on the mRNA levels of ACO and medium chain acyl-CoA (MCAD) genes. The former catalyzes the rate-limiting step of peroxisomal  $\beta$ -oxidation of fatty acids and its transcription is controlled by PPAR $\alpha$  (4). The second, MCAD, is also a PPAR $\alpha$ -target gene that catalyzes a rate limiting step in the mitochondrial  $\beta$ -oxidation of medium-chain fatty acyl-thioesters (4). In the heart of 1-day etomoxir-treated mice, the mRNA levels of ACO were significantly increased (4.4-fold induction,  $P < 0.05$ ) compared with control mice (Fig. 1A). Transcript levels of MCAD were not modified by this short treatment with etomoxir. These results are in agreement with previous studies showing that etomoxir (50 mg/kg body weight) administration for 1 day to mice caused a 4.5-fold induction in ACO mRNA levels in heart, whereas MCAD expression was not modified (15). Therefore, these results confirm the validity of our etomoxir treatment. Since inhibition by etomoxir of the transport of long-chain fatty acyl-CoA compounds into the mitochondria increases its accumulation in the cytoplasm, we also studied the effects of this drug on cytosolic acyl-CoA thioesterase (CTE). CTE catalyzes the hydrolysis of acyl-CoAs to free fatty acids and CoA, and as a consequence, it is an important mediator in cellular processes regulated by intracellular levels of nonesterified fatty acids and acyl-CoAs. In the heart of 1-day etomoxir-treated mice, the mRNA levels of CTE were 2-fold higher ( $P < 0.05$ ) than in control mice. The changes in CTE mRNA after a single etomoxir administration are consistent with the reported regulation of this gene by PPAR $\alpha$  in heart (16).

When we examined the expression of ACO in the heart of 10-days etomoxir-treated mice, a dramatic reduction was observed in ACO mRNA (96% reduction,  $P < 0.05$ ) (Fig. 1B) compared with control mice. In contrast, etomoxir treatment did not affect the mRNA expression of two genes of the mitochondrial  $\beta$ -oxidation system MCAD and muscle-type carnitine palmitoyl-transferase (M-CPT-I), being the latter a PPAR-target gene (17, 18) that catalyzes the entry of long-chain fatty acids into the mitochondrial matrix (18). Similarly, the expression of uncoupling protein 3 (UCP-3) and UCP-2, mitochondrial carriers localized in the inner mitochondrial membrane that have been implicated in fatty acid utilization and are regulated by PPAR $\alpha$  (19), was not affected by the treatment. CTE mRNA levels were 2.3-fold higher in etomoxir-treated mice compared with the control group. This finding, in agreement with a previous report (16), suggests that CTE is also induced in a PPAR $\alpha$ -independent manner, since this is the only PPAR $\alpha$ -target gene that is up-regulated after 10 days of etomoxir treatment. In fact, it has been postulated that other lipid-activated nuclear receptors such as PPAR $\delta$  or COUP-TF II, may regulate CTE in heart (16). Given the crucial role of PPAR $\alpha$  in the control of cardiac lipid metabolism (20) and the previous results showing down-regulation of this transcription factor during the development of pressure over-

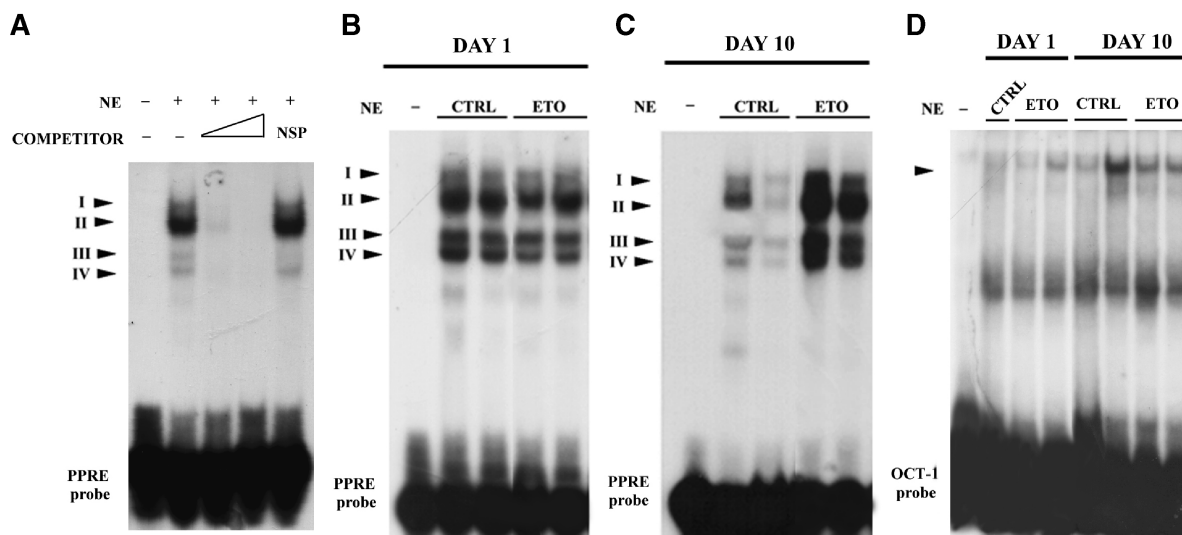
load-induced ventricular hypertrophy in mice (7, 8), we finally studied whether the effects of etomoxir treatment in heart were mediated by reduced expression of this transcription factor. PPAR $\alpha$  mRNA levels were not modified after 10 days of etomoxir treatment, indicating that changes in PPAR $\alpha$  expression were not responsible for the effects of etomoxir.

### Effects of etomoxir treatment for 10 days on RXR and PPAR $\gamma$ coactivator 1 mRNA levels in heart

The induction of PPAR $\alpha$ -target genes, such as ACO and MCAD, by PPAR $\alpha$  activators is reduced in livers of RXR $\alpha$ -deficient mice (21). Therefore, in order to study whether reduced availability of the PPAR $\alpha$  heterodimeric partner RXR was responsible for the reduced transcriptional activity of the ACO gene in 10-days etomoxir-treated mice, we determined the transcript levels of RXR $\alpha$  and RXR $\gamma$ . Etomoxir treatment for 10 days did not modify the mRNA expression of these transcription factors in heart compared with control mice (Fig. 2). In addition, we studied the mRNA expression of PPAR $\gamma$  coactivator 1 (PGC-1), which directly interacts with PPAR $\alpha$  and has been postulated as a regulator of mitochondrial  $\beta$ -oxidation (22). PGC-1 mRNA was not altered in the heart of 10-days etomoxir-treated mice. All these findings make unlikely a role for RXR and PGC-1 in the changes observed after etomoxir treatment for 10 days.



**Fig. 2.** Effects of etomoxir (100 mg/kg/day) for 10 days on the expression of RXR $\alpha$ , RXR $\gamma$ , and PGC-1 mRNA levels in heart. A representative autoradiogram and the quantification of the APRT-normalized mRNA levels are shown. Data are expressed as mean  $\pm$  SD of four or five mice. \*  $P < 0.05$  compared with control experiments.



**Fig. 3.** Binding of cardiac nuclear proteins to a peroxisome proliferator response element (PPRE) probe is induced by etomoxir treatment for 10 days but not for 1 day. **A:** Autoradiograph of EMSA performed with a  $^{32}\text{P}$ -labeled PPRE nucleotide and crude nuclear protein extract (NE) shows four specific complexes (I to IV) based on competition with a molar excess of unlabeled probe but not by an equivalent amount of a mutant PPRE oligonucleotide (NSP). Autoradiograph of EMSA performed with a  $^{32}\text{P}$ -labeled PPRE nucleotide and NE from hearts of control and etomoxir-treated mice for 1 (B) or 10 days (C). Exposition times were adjusted in order to better observe differences caused by drug treatment on days 1 and 10. **D:** Autoradiograph of EMSA performed with a  $^{32}\text{P}$ -labeled Oct-1 nucleotide.

#### Etomoxir treatment for 10 days, but not for one day, enhanced the DNA binding activity of cardiac nuclear proteins to a PPRE probe

EMSA were performed to examine the interaction of PPAR with its *cis*-regulatory element using a  $^{32}\text{P}$ -labeled PPRE probe and cardiac nuclear extracts from control and etomoxir-treated mice. The PPRE probe formed four complexes with cardiac nuclear proteins (complexes I to IV, Fig. 3A). The four complexes represented specific PPRE-protein interactions, since they were competed with a molar excess of unlabeled probe but not by an equivalent amount of a nonspecific competitor oligonucleotide (NSP). These results suggest that several endogenous cardiac nuclear proteins bind PPRE. In nuclear extracts from hearts of 1-day etomoxir-treated mice, no significant changes were observed in DNA binding activity to the PPRE probe compared with control animals (Fig. 3B). In contrast, despite the lack of induction in the transcriptional rate of PPAR $\alpha$ -target genes after etomoxir treatment for 10 days, this drug increased the binding of cardiac proteins for the PPRE *cis*-element (Fig. 3C), resulting in an increase of specific complexes. No changes were observed in the DNA binding of cardiac proteins from control and etomoxir-treated mice to an Oct-1 probe, indicating that the increase observed for the PPRE probe was specific (Fig. 3D). No increased binding of PPAR $\alpha$ , PPAR $\delta$ , or PPAR $\gamma$  to the PPRE probe was observed in supershift assays using specific antibodies (data not shown). In addition to PPAR $\alpha$ , previous studies have shown a role for Sp1 in the changes observed in the pressure overload-induced cardiac hypertrophy (7). Thus, we also evaluated whether etomoxir induced changes in the binding of cardiac nuclear proteins to a  $^{32}\text{P}$ -labeled Sp1 probe. Competition studies performed with a molar excess of unlabeled

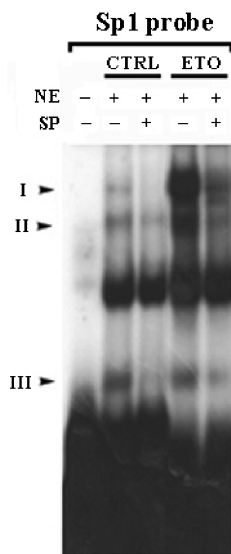
probe revealed that three bands (I, II, and III) represented specific DNA-protein interactions (Fig. 4). Etomoxir treatment resulted in increased binding of cardiac nuclear proteins to the Sp1 probe, as shown by the increase formation of bands representing complexes I and II. These data seem to involve Sp1 transcription factor in the changes caused by etomoxir.

#### Effects of etomoxir on COUP-TF II protein levels in the heart

The fall in the transcriptional rate of the ACO gene, together with the increased interaction of the PPRE probe with cardiac proteins in etomoxir-treated mice, suggests that drug treatment induces the expression of a transcriptional repressor in the heart. Given that the DR-1-type present in the PPRE is capable of interacting *in vitro* with multiple nuclear receptors, including homodimers of the transcriptional repressor COUP-TF (4), we determined whether expression of COUP-TF II parallels the increased interaction of the PPRE probe in etomoxir-treated mice. Protein levels of COUP-TF II were assessed by the Western blot technique. Nuclear levels of COUP-TF II were not modified after 1 day of etomoxir treatment compared with control animals (Fig. 5A). However, protein levels of COUP-TF II were higher in nuclear protein extract of hearts of 10-days etomoxir-treated mice compared with control mice samples (Fig. 5B).

#### Lack of increased binding of the transcriptional repressor COUP-TF II to a PPRE probe in cardiac nuclear extracts of 10-day etomoxir-treated mice

When supershift assays were performed with COUP-TF II antibody and cardiac nuclear extracts from control and treated mice for 10 days, the supershifted bands observed

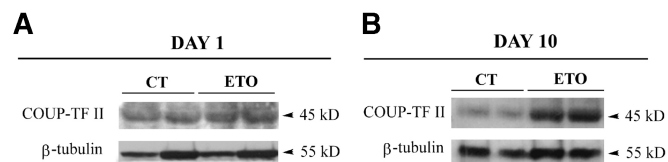


**Fig. 4.** Binding of cardiac nuclear proteins to an Sp1 probe is induced by etomoxir treatment for 10 days. Autoradiograph of EMSA performed with a  $^{32}\text{P}$ -labeled Sp1 nucleotide and crude nuclear protein extract (NE) from hearts of control mice and etomoxir-treated mice for 10 days. When indicated, a 20-fold molar excess of either specific unlabeled (SP) Sp1 oligonucleotide was included.

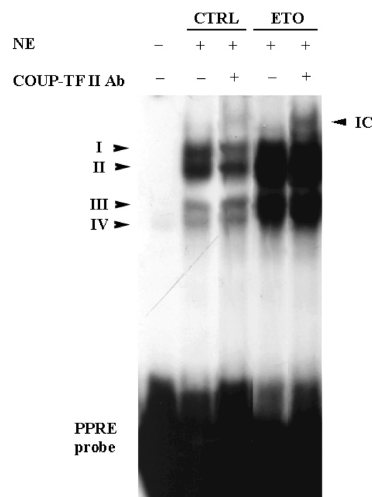
showed similar levels of COUP-TF bound to the PPRE probe in cardiac nuclear extracts from etomoxir-treated mice compared with control mice (**Fig. 6**). This finding makes unlikely a role for this transcriptional repressor in the changes observed after etomoxir treatment for 10 days.

#### Etomoxir treatment for 10 days enhanced the mRNA levels of markers of cardiac oxidative stress

Since peroxisomal  $\beta$ -oxidation is involved in the degradation of proinflammatory molecules and increased inflammatory response generates reactive oxygen species (ROS), we determined the mRNA levels of two markers of oxidative stress, glutathione peroxidase (GPX) and bax. Elimination of  $\text{H}_2\text{O}_2$  is critical to protect the heart against oxidative stress, and GPX appears to act as a key enzyme degrading  $\text{H}_2\text{O}_2$  in the cytosol of cardiac myocytes (23). Moreover, transcript levels of GPX are induced in skeletal



**Fig. 5.** Cardiac nuclear levels of COUP-TF II are increased in hearts of etomoxir treated mice for 10 days but not for 1 day. A representative autoradiograph of a Western blot performed with 40  $\mu\text{g}$  of cardiac crude nuclear protein extract from etomoxir-treated mice for 1 (A) or 10 days (B). Protein mass markers are displayed on the right in kilodaltons (kDa).  $\beta$ -tubulin levels are shown as a control of equal abundance of nuclear extracts in the samples.

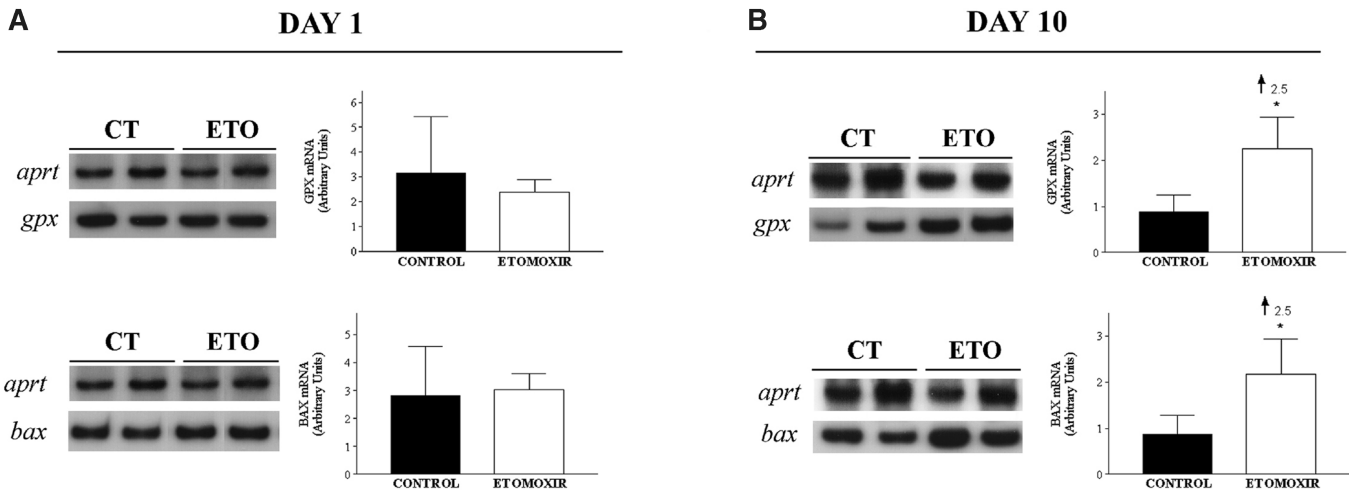


**Fig. 6.** Identification of COUP-TF II in cardiac nuclear proteins complexes by antibody recognition experiments. The autoradiograph depicted represents EMSA antibody recognition studies performed with a PPRE probe and anti-COUP-TF II antibody (Ab). Cardiac nuclear proteins from control and 10 days etomoxir-treated mice were used. The positions of the supershifted immune complexes are indicated.

muscle cells treated with pro-oxidants (menadione and  $\text{H}_2\text{O}_2$ ) (24). In the hearts of 1-day etomoxir-treated mice, GPX mRNA levels were not modified, whereas a 3.2-fold induction was observed in the hearts of 10-days etomoxir-treated mice (**Fig. 7A**). The mRNA levels of bax, a proapoptotic member of the bcl-2 family, are increased in response to apoptotic levels of oxidative stress in cardiac myocytes (25–27). Interestingly, bax transcripts increased in the hearts of 10-days but not in the hearts of 1-day etomoxir treated mice (**Fig. 7B**).

#### NF- $\kappa\text{B}$ activity is increased in hearts from 10-day etomoxir-treated mice

We finally studied whether the activity of the redox-regulated and oxidant stress-activated transcription factor NF- $\kappa\text{B}$  was altered by etomoxir. The NF- $\kappa\text{B}$  probe formed three complexes with cardiac nuclear proteins (complexes I to III, **Fig. 8A**). Specificity of the three DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF- $\kappa\text{B}$  oligonucleotide to incubation mixtures. In agreement with the reported antagonism of NF- $\kappa\text{B}$  by PPAR activation (4), binding activity of NF- $\kappa\text{B}$  was reduced in cardiac nuclear extracts from etomoxir-treated mice for 1 day (**Fig. 8B**). In contrast, NF- $\kappa\text{B}$  binding activity, mainly of specific complex I, increased in cardiac nuclear extracts from 10-days etomoxir-treated mice compared with control animals (**Fig. 8C**). Characterization of NF- $\kappa\text{B}$  was performed by incubating nuclear extracts with antibody directed against the p65 subunit. Addition of anti-p65 antibody completely supershifted complex I and reduced the formation of complex II, showing that these incubation mixtures contained NF- $\kappa\text{B}$ .



**Fig. 7.** Effects of etomoxir (100 mg/kg/day) for 10 days on the expression of GPX and bax mRNA levels in heart. A representative autoradiogram and the quantification of the APRT-normalized mRNA levels are shown. Data are expressed as mean  $\pm$  SD of four or five mice. \*  $P < 0.05$  compared with control experiments.

## DISCUSSION

Pharmacological inhibition of CPT-I was proposed as a therapeutic option for the treatment of type 2 diabetes mellitus, since increased fatty acid  $\beta$ -oxidation, as a result of elevated plasma free fatty acids, may contribute to insulin resistance (28). Etomoxir is an irreversible inhibitor of CPT-I, and, therefore of fatty acid  $\beta$ -oxidation, which has a potent hypoglycemic activity in fasted mice (29) and patients (30). However, the clinical development of etomoxir was discontinued due to presence of cardiac hypertrophy (31, 32). In this study we sought to determine the molecular mechanisms responsible for the metabolic changes observed in etomoxir-induced cardiac hypertrophy. Our results show that during etomoxir-induced cardiac hypertrophy, there is a dramatic reduction in ACO mRNA that is accompanied by an increase in NF- $\kappa$ B activity.

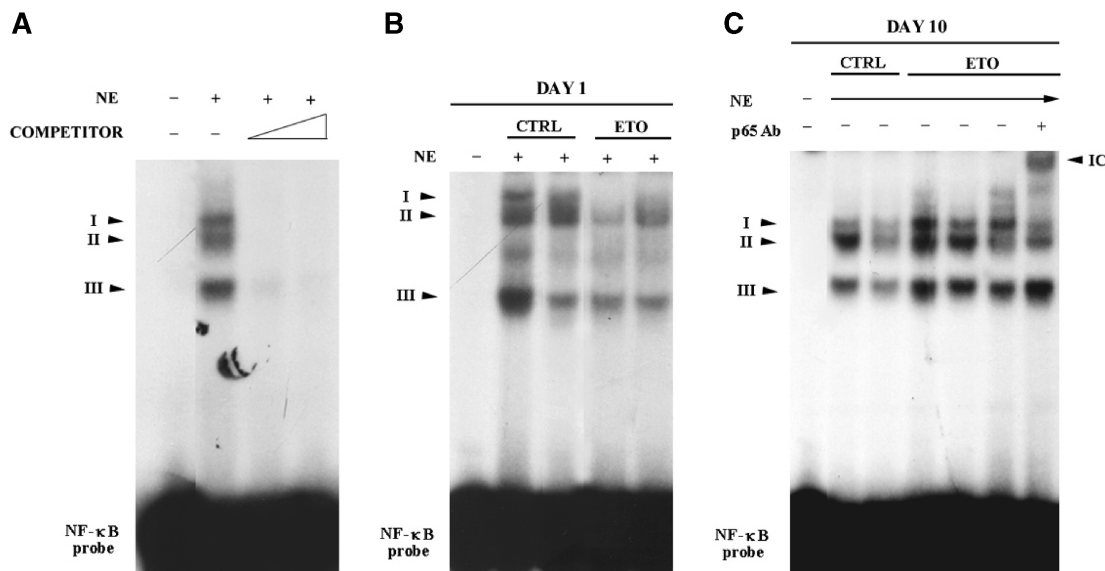
In the present work, etomoxir administration to mice for 10 days resulted in cardiac hypertrophy, as previously reported (33). However, the cardiac hypertrophy, achieved after 10 days of etomoxir treatment (24% increase in the ratio of heart weight to body weight) should be considered in its initial stage, since it has been reported that longer treatments at the same dose do not always result in cardiac hypertrophy (33). In addition, other models of cardiac hypertrophy, such as the pressure overload-induced cardiac hypertrophy, may induce a rapid and higher degree of hypertrophy, achieving a 39% increase in the ratio of left ventricle to body weight (34). In the etomoxir-induced cardiac hypertrophy model used in this work, ACO mRNA levels were nearly abolished, whereas the expression of either PPAR $\alpha$ s or several of their target genes, such as M-CPT-I, MCAD, or UCP-3, were not affected. In contrast, in hearts of 1-day etomoxir-treated mice, a high increase in ACO mRNA levels was observed compared with control animals. The effects of 1-day etomoxir treatment on ACO expression in heart are in agree-

ment with those reported by Djouadi et al. (15). They showed the effects of etomoxir (50 mg/kg/day) administration for either 1 or 5 days on ACO and MCAD mRNA levels in heart. ACO mRNA levels increased 4.5-fold after 1 day of treatment and 2-fold after 5 days, showing a tendency to decrease, whereas MCAD mRNA levels only increased ( $\sim$ 2-fold) after 5 days of treatment. Therefore, in short treatments (1–5 days), when cardiac hypertrophy is not present, etomoxir activates PPAR $\alpha$  in heart, which results in an increase in the expression of PPAR $\alpha$  target genes. The mechanism by which inhibitors of CPT-I, such as etomoxir, activate PPAR $\alpha$  includes direct binding to this receptor (10), and, indirectly, as metabolic inhibitors, may lead to the accumulation of endogenous fatty acid ligands. In contrast, in longer treatments leading to cardiac hypertrophy, the expression of ACO mRNA levels falls, whereas the induction of PPAR $\alpha$  target genes involved in mitochondrial  $\beta$ -oxidation is abolished, without changes in PPAR $\alpha$  mRNA levels.

The data presented here do not establish the mechanisms involved in ACO down-regulation in heart after etomoxir treatment. It is likely that the enhanced sensitivity to insulin caused by etomoxir may contribute to the reduction in ACO expression without affecting other PPAR $\alpha$ -target genes. This is supported by the fact that peroxisomal fatty acid oxidation is inhibited at a much lower insulin concentration than is mitochondrial oxidation (35).

Interestingly, in this study we show that etomoxir treatment results in increased cardiac oxidative stress. This finding is in agreement with previous results reported by our group in C2C12 skeletal muscle cells (36). Several factors may be involved in increased oxidative stress after etomoxir treatment. Thus, inhibition of CPT-I by etomoxir prevents the entrance of palmitoyl-CoA into mitochondria, leading to its accumulation in the cytoplasm. Because palmitoyl-CoA is a precursor of sphingolipid synthesis, etomoxir treatment may result in enhanced ceramide





**Fig. 8.** Nuclear NF-κB activity is reduced in hearts from 1-day etomoxir-treated mice but it is increased in 10-day etomoxir-treated mice. A: Autoradiograph of EMSA performed with a  $^{32}\text{P}$ -labeled NF-κB nucleotide and crude nuclear protein extract (NE) shows three specific complexes (I to III), based on competition with a molar excess of unlabeled probe from etomoxir-treated mice for 10 days. Autoradiograph of EMSA performed with a  $^{32}\text{P}$ -labeled NF-κB nucleotide and NE from hearts of control and etomoxir-treated mice for 1 (B) or 10 (C) days. When indicated, cardiac nuclear extracts were incubated with an antibody recognizing the NF-κB subunit p65. Supershifted immune complex (IC) is denoted.

synthesis and apoptosis (37). However, we have demonstrated that ceramides do not cause apoptosis in C2C12 skeletal muscle cells after etomoxir treatment (36). In addition, increased CTE mRNA levels in hearts of 10-days etomoxir-treated mice may reduce the accumulation of fatty acyl-CoA in cardiomyocytes. Here we show that down-regulation of ACO, the rate-limiting step of the peroxisomal  $\beta$ -oxidation system correlates with enhanced cardiac oxidative stress, suggesting that it may be involved. Peroxisomal  $\beta$ -oxidation is implicated in the degradation of proinflammatory molecules such as leukotriene B4 and 8(*S*)-hydroxyeicosatetraenoic acid. Leukotriene B4 is a potent chemoattractant that induces ROS generation (38). Therefore, it is likely that reduced degradation of these agents in 10-days etomoxir-treated mice may lead to an increased inflammatory response that generates ROS. In agreement with this, the ACO knockout mouse presented elevated levels of  $\text{H}_2\text{O}_2$  in liver (39). In this study we show that when ACO mRNA levels are down-regulated, GPX mRNA levels are increased, suggesting increased  $\text{H}_2\text{O}_2$  levels. In fact, it has been reported that GPX mRNA levels increase in skeletal muscle cells treated with  $\text{H}_2\text{O}_2$  (24). Elimination of  $\text{H}_2\text{O}_2$  is critical to protect heart tissue against oxidative stress, because superoxide is converted to  $\text{H}_2\text{O}_2$  by superoxide dismutase, and in the presence of cardiac myocytes,  $\text{H}_2\text{O}_2$  forms hydroxyl radical, one of the most toxic oxygen free radicals. In the myocardium, GPX plays a predominant role in the scavenging of  $\text{H}_2\text{O}_2$ , given that catalase, the other major  $\text{H}_2\text{O}_2$ -scavenging enzyme, shows a very low activity (40). The induction in the transcript levels of bax also correlates with ACO down-regulation and suggests that apoptotic levels of oxidative stress are reached in etomoxir-treated hearts. It is important to


note that NF-κB and apoptosis signal-regulating kinase 1 (ASK1) have been recently involved in the cardiomyocyte hypertrophy induced by G-protein-coupled receptor agonist (41). Interestingly,  $\text{H}_2\text{O}_2$  induces dimerization or oligomerization of ASK1 and activates its kinase activity (42), supporting a role for  $\text{H}_2\text{O}_2$  as a mediator of cardiac hypertrophy.

It is well known that cellular activation of PPARs has been demonstrated to antagonize the activity of NF-κB (4), probably through competition by limiting amounts of essential transcription coactivators such as CBP/p300 or SRC-1. Therefore, PPAR $\alpha$  activation by etomoxir can explain the reduction in NF-κB binding activity in cardiac nuclear extracts after 1 day of treatment. However, NF-κB binding activity in cardiac nuclear extracts was increased after 10 days of treatment. The data here presented indicate that after 10 days of etomoxir treatment, an increase in oxidative cellular stress appears in cardiomyocytes, resulting in NF-κB activation. Therefore, treatment for 10 days with etomoxir results in NF-κB activation in heart, similar to the effects attained by peroxisome proliferators in liver (43–45).

Further, in the present study we show that etomoxir treatment results in a strong increase in the protein levels of the PPAR-transcriptional repressor, COUP-TF II. According to previous studies (46, 47), the mechanism by which COUP-TF antagonizes PPAR signaling involves competitive occupation of the DR-1 present in the PPRE. However, we were unable to show increased binding of COUP-TF to the PPRE *cis*-element, making unlikely a role for this transcription factor in the effects caused by etomoxir. This idea is supported by the different effects observed after etomoxir treatment in ACO and MCAD

genes. The expression of the former was nearly abolished by etomoxir, whereas the second was not modified by the treatment, although it has been reported that MCAD down-regulation in hypertrophied hearts through COUP-TF (7). The mechanism involved in the increased nuclear levels of COUP-TF II is unknown, but it has been speculated that a post-translational modification such as phosphorylation could be implicated (48). It is noteworthy that inhibition of CPT-I by etomoxir leads to accumulation of fatty acyl-CoA derivatives, which have been implicated in the regulation of the activity of several kinases (49), affording a possible explanation for the increase in COUP-TF II levels.

Previous studies performed in the pressure overload-induced hypertrophy mouse heart have proposed a role for the Sp family of transcription factors in cardiac hypertrophy (7). In the present study, the binding of cardiac nuclear proteins to an Sp1 probe was increased in etomoxir-treated mice compared with control mice, suggesting that Sp1 or proteins competing for Sp1 binding sites (such as early growth response-1 gene, *egr-1*) are also involved in cardiac hypertrophy induced by etomoxir. The role of the Sp family transcription factors in cardiac hypertrophy is not well characterized. They usually act as transcriptional activators, but in addition, they may repress gene expression in a promoter context-dependent manner (50, 51). Further, the presence of several Sp binding sites in the promoter region of M-CPT-I, MCAD (7), and PPAR $\alpha$  genes (52) suggests a role for Sp proteins in the coordinate regulation of genes involved in cardiac fatty acid oxidation.

In summary, we have shown increased cellular oxidative stress and NF- $\kappa$ B activity in hearts of etomoxir-treated mice. It remains to study whether the increase in cellular oxidative stress in cardiomyocytes occurs as a result of ACO down-regulation. 

We thank the Language Advisory Service of the University of Barcelona for helpful assistance. This study was partly supported by grants from the FPCNL, Ministerio de Ciencia y Tecnología of Spain (SAF00-0201), and FIS (00/1124). We also thank the Generalitat de Catalunya for grants 1998SGR-33 and 2000SGR-45. À.C. was supported by a grant from the Ministerio de Educación of Spain.

## REFERENCES

1. Van Bilsen, M., G. J. van der Vusse, and R. S. Reneman. 1998. Transcriptional regulation of metabolic processes: implications for cardiac metabolism. *Pflügers Arch.* **437**: 2–14.
2. Stanley, W. C., G. D. Lopaschuk, and J. G. McCormack. 1997. Regulation of energy substrate metabolism in the diabetic heart. *Cardiovasc. Res.* **34**: 25–33.
3. Braissant, O., F. Foufelle, C. Scotto, M. Dauça, and W. Wahli. 1996. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology.* **137**: 354–366.
4. Desvergne, B., and W. Wahli. 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr. Rev.* **20**: 649–688.
5. Kliewer, S. A., S. A. Sundseth, S. A. Jones, P. J. Brown, G. B. Wisely, C. S. Koble, P. Devchand, W. Wahli, T. M. Willson, J. M. Lenhard, and J. M. Lehmann. 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc. Natl. Acad. Sci. USA.* **94**: 4318–4328.
6. Krey, G., O. Braissant, F. L'Horsset, E. Kalkhoven, M. Perroud, M. G. Parker, and W. Wahli. 1997. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. *Mol. Endocrinol.* **11**: 779–791.
7. Sack, M. N., D. L. Disch, H. A. Rockman, and D. P. Kelly. 1997. A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophy growth program. *Proc. Natl. Acad. Sci. USA.* **94**: 6438–6443.
8. Barger, P. M., J. M. Brandt, T. C. Leone, C. J. Weinheimer, and D. P. Kelly. 2000. Deactivation of peroxisome proliferator-activated receptor- $\alpha$  during cardiac hypertrophic growth. *J. Clin. Invest.* **105**: 1723–1730.
9. Vetter, R., and H. Rupp. 1994. CPT-1 inhibition by etomoxir has a chamber-related action on cardiac sarcoplasmic reticulum and isomyosins. *Am. J. Physiol.* **267**: H2021–H2099.
10. Forman, B. A., J. Chen, and R. M. Evans. 1997. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPARgamma. *Proc. Natl. Acad. Sci. USA.* **94**: 4312–4317.
11. Purcell, H. N., G. Tang, C. Yu, F. Mercurio, J. A. DiDonato, and A. Lin. 2001. Activation of NF-kappa B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. *Proc. Natl. Acad. Sci. USA.* **98**: 6668–6673.
12. Freeman, W. M., S. J. Walker, and E. V. Vrana. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques.* **26**: 112–125.
13. Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**: 1475–1489.
14. Helenius, M., M. Hänninen, S. Lehtinen, and A. Salminen. 1996. Aging-induced up-regulation of nuclear binding activities of oxidative stress responsive NF-kB transcription factor in mouse cardiac muscle. *J. Mol. Cell. Cardiol.* **28**: 487–498.
15. Djouadi, F., C. J. Weinheimer, J. E. Saffitz, C. Pitchford, J. Bastin, F. J. Gonzalez, and D. P. Kelly. 1998. A gender-related defect in lipid metabolism and glucose homeostasis in peroxisome proliferator-activated receptor alpha-deficient mice. *J. Clin. Invest.* **102**: 1083–1091.
16. Hunt, M. C., P. J. G. Lindquist, J. M. Peters, F. J. Gonzalez, U. Diczfalusy, and S. E. Alexson. 2000. Involvement of the peroxisome proliferator-activated receptor alpha in regulating long-chain acyl-CoA thioesterases. *J. Lipid Res.* **41**: 814–823.
17. McGarry, J. D., and N. F. Brown. 1997. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur. J. Biochem.* **244**: 1–14.
18. Mascaró, C., E. Acosta, J. A. Ortiz, P. F. Marrero, F. G. Hegardt, and D. Haro. 1998. Control of human muscle-type carnitine palmitoyltransferase I gene transcription by peroxisome proliferator-activated receptor. *J. Biol. Chem.* **273**: 8560–8563.
19. Ricquier, D., and F. Bouillad. 2000. The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *Biochem. J.* **345**: 161–179.
20. Djouadi, F., J. M. Brandt, C. J. Weinheimer, T. C. Leone, F. J. Gonzalez, and D. P. Kelly. 1999. The role of the peroxisome proliferator-activated receptor alpha (PPAR alpha) in the control of cardiac lipid metabolism. *Prostaglandins Leukot. Essent. Fatty Acids.* **60**: 339–343.
21. Wan H-J, Y. Y. Cai, W. Lungo, P. Fu, J. Locker, S. French, H. M. Su-cov. 2000. Peroxisome proliferator-activated receptor alpha-mediated pathways are altered in hepatocyte-specific retinoid X receptor alpha-deficient mice. *J. Biol. Chem.* **275**: 28285–28290.
22. Vega, R. B., J. M. Huss, and D. P. Kelly. 2000. The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol. Cell. Biol.* **20**: 1868–1876.
23. Igarashi, J., M. Nishida, S. Hoshida, N. Yamashita, H. Kosaka, M. Hori, T. Kuzuya, and M. Tada. 1998. Inducible nitric oxide synthase augments injury elicited by oxidative stress in rat cardiac myocytes. *Am. J. Physiol.* **274**: C245–C252.
24. Franco, A. A., R. S. Odom, and T. A. Renado. 1999. Regulation of antioxidant enzyme gene expression in response to oxidative

stress and during differentiation of mouse skeletal muscle cells. *Free Radic. Biol. Med.* **27**: 1122–1132.

25. Siwik, D. A., J. D. Tzortzis, D. R. Pimental, D. L. Chang, P. J. Pagano, K. Singh, D. B. Sawyer, and W. S. Clucci. 1999. Inhibition of copper-zinc superoxide dismutase induces cell growth, hypertrophic phenotype, and apoptosis in neonatal rat cardiac myocytes in vitro. *Circ. Res.* **85**: 147–153.
26. Nakamura, K., K. Fushimi, H. Kouchi, K. Mihara, M. Miyazaki, T. Ohe, and M. Namba. 1998. Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor- $\alpha$  and angiotensin II. *Circulation.* **98**: 794–799.
27. Pimentel, D. R., J. K. Amin, L. Xiao, T. Miller, J. Viereck, J. Oliver-Krasinski, R. Baliga, J. Wang, D. A. Siwik, K. Singh, P. Pagano, W. S. Colucci, and D. B. Sawyer. 2001. Reactive oxygen species mediate amplitude-dependent hypertrophic and apoptotic responses to mechanical stretch in cardiac myocytes. *Circ. Res.* **89**: 453–460.
28. Foley, J. E. 1992. Rationale and application of fatty acid oxidation inhibitors in treatment of diabetes mellitus. *Diabetes Care.* **15**: 773–784.
29. Wolf, H. P. O. 1992. Possible new therapeutic approach in diabetes mellitus by inhibition of carnitine palmitoyltransferase I (CPTI). *Horm. Metab. Res.* **26** (Suppl.): 62–67.
30. Ratheiser, K., B. Schneeweib, W. Waldhausl, P. Fasching, A. Korn, P. Nowotny, M. Rohac, and H. P. Wolf. 1991. Inhibition by etomoxir of carnitine palmitoyltransferase I reduces hepatic glucose production and plasma lipids in non-insulin-dependent diabetes mellitus. *Metabolism.* **40**: 1185–1190.
31. Bressler, R., R. Gay, J. G. Copeland, J. J. Bahl, J. Bedotto, and S. Goldman. 1989. Chronic inhibition of fatty acid oxidation: new model of diastolic dysfunction. *Life Sci.* **44**: 1897–1906.
32. Anderson, R. C., M. Balestra, P. A. Bell, R. O. Deems, W. S. Fillers, J. E. Foley, J. D. Fraser, W. R. Mann, M. Rudin, and E. B. Vilhauer. 1995. Antidiabetic agents: a new class of reversible carnitine palmitoyltransferase I inhibitors. *J. Med. Chem.* **38**: 3448–3450.
33. Yotsumoto, T., T. Naitoh, M. Kitahara, and N. Tsuruzoe. 2000. Effects of carnitine palmitoyltransferase I inhibitors on hepatic hypertrophy. *Eur. J. Pharmacol.* **398**: 297–302.
34. Rogers, J. H., P. Tamirisa, A. Kovacs, C. J. Weinheimer, M. Courtois, K. J. Blumer, D. P. Kelly, A. J. Muslin. 1999. RGS4 causes increased mortality and reduced cardiac hypertrophy in response to pressure overload. *J. Clin. Invest.* **104**: 567–576.
35. Hamel, F. G., R. G. Bennett, J. L. Upward, and W. C. Duckworth. 2001. Insulin inhibits peroxisomal fatty acid oxidation in isolated rat hepatocytes. *Endocrinology.* **142**: 2702–2706.
36. Cabrero, A., M. Alegret, R. M. Sánchez, T. Adzet, J. C. Laguna, and M. Vázquez Carrera. 2002. Increased reactive oxygen species production down-regulates peroxisome proliferator-activated  $\alpha$  pathway in C2C12 skeletal muscle cells. *J. Biol. Chem.* **277**: 10100–10107.
37. Paumen, M. B., Y. Ishida, M. Muramatsu, M. Yamamoto, and T. Honjo. 1997. Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. *J. Biol. Chem.* **272**: 3324–3329.
38. Woo, C-H., H-J. You, S-H. Cho, Y-W. Eom, J-S. Chun, Y-J. Yoo, and J-H. Kim. 2002. Leukotriene B4 stimulates Rac-ERK cascade to generate reactive oxygen species that mediates chemotaxis. *J. Biol. Chem.* **277**: 8572–8578.
39. Fan, C. Y., J. Pan, N. Usuda, A. V. Yeldandi, M. S. Rao, and J. K. Reddy. 1998. Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor alpha natural ligand metabolism. *J. Biol. Chem.* **273**: 15639–15645.
40. Doroshov, J. H., G. Y. Locker, and C. E. Myers. 1980. Enzymatic defenses of the mouse heart against reactive oxygen metabolites. *J. Clin. Invest.* **65**: 128–135.
41. Hirotsu, S., K. Otsu, K. Nishida, Y. Higuchi, T. Morita, H. Nakayama, O. Yamaguchi, T. Mano, Y. Matsumura, H. Ueno, M. Tada, and M. Hori. 2002. Involvement of nuclear factor- $\kappa$ B and apoptosis signal-regulating kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. *Circulation.* **105**: 509–515.
42. Gotoh, Y., and J. Cooper. 1998. Reactive oxygen species and dimerization-induced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor- $\alpha$  signal transduction. *J. Biol. Chem.* **273**: 17477–17482.
43. Li, Y., L. K. Leung, H. P. Glauert, and B. T. Spear. 1996. Treatment of rats with the peroxisome proliferator ciprofibrate results in increased liver NF- $\kappa$ B activity. *Carcinogenesis.* **17**: 2305–2309.
44. Nilakantan, V., B. T. Spear, and H. P. Glauert. 1998. Liver-specific catalase expression in transgenic mice inhibits NF- $\kappa$ B activation and DNA synthesis induced by the peroxisome proliferator ciprofibrate. *Carcinogenesis.* **19**: 631–637.
45. Tharappel, J. C., M. L. Cunningham, B. T. Spear, and H. P. Glauert. 2001. Differential activation of hepatic NF- $\kappa$ B in rats and hamsters by the peroxisome proliferators Wy-14,643, gemfibrozil, and dibutyl phthalate. *Toxicol. Sci.* **62**: 20–27.
46. Miyata, K. S., B. Zhang, S. L. Marcus, J. P. Capone, and R. A. Rachubinski. 1993. Chicken ovalbumin upstream promoter transcription factor (COUP-TF) binds to a peroxisome proliferator-responsive element and antagonizes peroxisome proliferator-mediated signaling. *J. Biol. Chem.* **268**: 19169–19172.
47. Baes, M., H. Castelein, L. Desmet, and P. E. Declercq. 1995. Antagonism of COUP-TF and PPAR  $\alpha$ /RXR  $\alpha$  on the activation of the malic enzyme gene promoter: modulation by 9-cis RA. *Biochem. Biophys. Res. Commun.* **215**: 338–345.
48. Marcus, S. L., J. P. Capone, and R. A. Rachubinski. 1996. Identification of COUP-TFII as a peroxisome proliferator response element binding factor using genetic selection in yeast: COUP-TFII activates transcription in yeast but antagonizes PPAR signaling in mammalian cells. *Mol. Cell. Endocrinol.* **120**: 31–39.
49. Faergeman, N. J., and J. Knudsen. 1997. Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signaling. *Biochem. J.* **323**: 1–12.
50. Li, R., Z. Hodny, K. Luciakova, P. Barath, and D. Nelson. 1996. Sp1 activates and inhibits transcription from separate elements in the proximal promoter of the human adenine nucleotide translocase 2 (ANT2) gene. *J. Biol. Chem.* **271**: 18925–18930.
51. Tang, Q-Q., M-S. Jiang, and D. Lane. 1999. Repressive effect of Sp1 on the C/EBP $\alpha$  gene promoter: role in adipocyte differentiation. *Mol. Cell. Biol.* **19**: 4855–4865.
52. Gearing, K. L., A. Crickmore, and A. Gustafsson. 1994. Structure of the mouse peroxisome proliferator activated receptor alpha gene. *Biochem. Biophys. Res. Commun.* **199**: 255–263.