

Down-Regulation of Acyl-CoA Oxidase Gene Expression in Heart of Troglitazone-Treated Mice through a Mechanism Involving Chicken Ovalbumin Upstream Promoter Transcription Factor II

ÀGATHA CABRERO, MIREIA JOVÉ, ANNA PLANAVILA, MANUEL MERLOS, JUAN C. LAGUNA, and MANUEL VÁZQUEZ-CARRERA

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

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ABSTRACT

Cardiac expression of genes involved in fatty acid metabolism may suffer alterations depending on the substrate availability. We studied how troglitazone, an antidiabetic drug that selectively activates peroxisome proliferator-activated receptor γ (PPAR γ), affected the expression of several of these genes. A single-day troglitazone administration (100 mg/kg/day) did not significantly alter plasma free fatty acids or triglyceride levels. In contrast, a 10-day period of troglitazone treatment significantly reduced plasma free fatty acids and triglyceride levels by 74% ($P < 0.001$) and 56% ($P < 0.01$), respectively. Cardiac mRNA expression of acyl-CoA oxidase (ACO) increased (8.3-fold induction) after 1-day troglitazone treatment, whereas after 10 days of treatment ACO mRNA levels were dramatically reduced (98% reduction, $P < 0.02$), as well as those of uncoupling protein 3 (41% reduction, $P = 0.05$). The mRNA expression of PPAR α and several PPAR target genes, such as medium chain

acyl-CoA dehydrogenase or fatty acid translocase were not altered after 10 days of troglitazone treatment, whereas muscle-type carnitine palmitoyltransferase I increased 1.7-fold ($P < 0.05$). The reduction in ACO expression in the hearts of 10-day troglitazone-treated mice was accompanied by an increase in the protein levels of the transcriptional repressor chicken ovalbumin upstream promoter transcription factor II (COUP-TF II). Electrophoretic mobility shift assays performed with COUP-TF II antibody to examine its interaction with a labeled peroxisome proliferator response element probe showed enhanced binding of COUP-TFII in cardiac nuclear extracts from troglitazone-treated mice for 10 days but not in the control nuclear extracts. Overall, the findings presented here show that 10 days of troglitazone treatment decreased expression of the ACO gene through a mechanism involving the transcriptional repressor COUP-TF II.

Constant pump function of the heart requires a high-energy demand, which is mainly satisfied by fatty acids and glucose. The oxidation of fatty acids and glucose covers, respectively, 65 and 30% of the energy demand of the adult heart (Van Bilsen et al., 1998). The heart, in contrast to other tissues such as the brain, adapts its metabolism to substrate availability. For example, in cardiac hypertrophy and heart failure, a dramatic reduction in fatty acid oxidation is de-

tected, because a shift in the source of energy is observed from fatty acids to glucose (Van Bilsen et al., 1998). In contrast, diabetes induces a rapid change in substrate preference of the heart characterized by a decrease in glucose uptake and utilization, and an increase in fatty acid oxidation (Stanley et al., 1997). In these processes, 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. Transport and metabolism of these substrates are controlled by a class of transcription factors called peroxisome proliferator-activated receptors (PPARs). Three different PPAR subtypes (α , δ/β , and γ) have been identified to date. PPAR α is expressed primarily in tissues that have a

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ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; RXR, retinoid-X-receptor; PPPE, peroxisome proliferator response element; COUP-TF II, chicken ovalbumin upstream promoter transcription factor II; ACO, acyl-CoA oxidase; RT, reverse transcription; PCR, polymerase chain reaction; ANF, atrial natriuretic factor; M-CPT-I, muscle-type carnitine palmitoyl-transferase; MCAD, medium chain acyl-CoA dehydrogenase; UCP-3, uncoupling protein 3; FAT/CD36, fatty acid translocase; CTE, cytosolic acyl-CoA thioesterase; PGC-1, peroxisome proliferator-activated receptor- γ coactivator 1; bp, base pair; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; NF- κ B, nuclear factor- κ B.

high level of fatty acid catabolism such as liver, brown fat, kidney, heart, and skeletal muscle (Braissant et al., 1996; Desvergne and Wahli, 1999). PPAR δ/β is ubiquitously expressed, and PPAR γ 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 (Braissant et al., 1996). PPARs are activated by ligands, such as naturally occurring fatty acids, which are activators of all three PPAR subtypes (Kliwer et al., 1997; Krey et al., 1997). In addition to fatty acids, several synthetic compounds bind and activate specific PPAR subtypes. Thiazolidinediones (troglitazone, ciglitazone, and rosiglitazone), which bind and selectively activate PPAR γ (Forman et al., 1995; Lehmann et al., 1995), are a novel class of insulin-sensitizing agents with potent antidiabetic activity in vivo. To be transcriptionally active, PPARs need to heterodimerize with the retinoid-X-receptor (RXR). PPAR-RXR heterodimers binds to DNA-specific sequences called peroxisome proliferator-response elements (PPRE), 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 bind potential competitors such as homodimers of other nuclear receptors, including RXR α , chicken ovalbumin upstream promoter transcription factor II (COUP-TF-II, also called apolipoprotein A1 regulatory protein) and hepatic nuclear factor-4 (Desvergne and Wahli, 1999).

Recently, it has been reported that cardiac hypertrophic growth, which involves a shift in the substrate utilization from fatty acids to glucose, is associated with deactivation of PPAR α (Barger et al., 2000). These results suggest that reduced activity of this transcription factor may account for the down-regulation of enzymes involved in fatty acid oxidation. Similarly, in a previous study (Cabrero et al., 2000) we reported that in C2C12 myotubes, cultured in the presence of glucose but in the absence of fatty acids, troglitazone strongly repressed the mRNA expression of genes involved in fatty acid oxidation. The reduction in the expression of these genes correlated with decreased PPAR α expression after thiazolidinedione treatment, suggesting that the impaired expression of this transcription factor was involved in the effects of these drugs. It is noteworthy that in the presence of oleic acid in the culture medium, the effects of troglitazone on PPAR α mRNA levels were reversed. Here, we examine whether troglitazone induces similar changes in the heart of mice. Drug administration for 1 day strongly increased acyl-CoA oxidase (ACO) mRNA levels in heart, whereas after 10 days of treatment, when a fall in plasma values of free fatty acids and triglycerides was detected, a dramatic reduction in its transcript levels was observed. Interestingly, troglitazone treatment strongly increased DNA-protein binding activity of cardiac nuclear proteins to a PPRE probe in gel mobility shift assays after 10 days but not after 1 day of troglitazone treatment. In addition, drug treatment for 10 days strongly increased COUP-TF II protein levels in heart, suggesting that increased amounts of this known transcriptional repressor were responsible for the reduced expression of the ACO gene. In fact, antibody supershift studies showed enhanced binding of COUP-TF II to the PPRE probe in the specific complexes from 10-day troglitazone-treated mice. Overall, the results presented here suggest that COUP-TF II is involved in the reduction of ACO expression in hearts of troglitazone-treated mice.

Materials and Methods

Materials. Troglitazone was kindly provided by Glaxo Wellcome (Greenford, UK).

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% carboxymethyl cellulose (control group) or 100 mg/kg/day of troglitazone (dissolved in 0.5% carboxymethyl cellulose). Each compound was administered per os once a day for 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 2,200g for 10 min at 4°C . Hearts were rapidly removed, washed in ice-cold 0.9% NaCl, frozen in liquid nitrogen, and stored at -80°C . Animal handling and disposal were performed in accordance with law 5/1995, 21 July, of the Generalitat de Catalunya.

Plasma Determinations. Plasma cholesterol (Roche Diagnostics, Barcelona, Spain), triglycerides (Sigma, Madrid, Spain), nonesterified fatty acids (Wako Pure Chemicals, Neuss, Germany), and glucose (Roche Diagnostics) concentration were determined by colorimetric tests.

RNA Preparation and Analysis. Total RNA was isolated by using the Ultraspec reagent (Biotecx, Houston, TX). Relative levels of specific mRNAs were assessed by the reverse transcription (RT)-polymerase chain reaction. Complementary DNA was synthesized from RNA samples by mixing 0.5 μg 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_2 , 10 mM dithiothreitol, 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA), 20 U of RNasin (Invitrogen), and 0.5 mM each dNTP (Sigma-Aldrich, St. Louis, MO) in a total volume of 20 μl . Samples were incubated at 37°C for 60 min. A 5- μl aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers.

Each 25- μl PCR reaction contained 5 μl of the RT reaction, 1.2 mM MgCl_2 , 200 μM dNTPs, 1.25 μCi of [^{32}P]dATP (3,000 Ci/mmol; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK), 1 unit of *Taq* polymerase (Ecogen, Barcelona, Spain), 0.5 μg 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°C). The sequences of the sense and antisense primers used for amplification are shown in Table 1. PCR was performed in a thermocycler (MJ Research, Watertown, MA) equipped with a peltier system and temperature probe. After an initial denaturation for 1 min at 94°C , PCR was performed for 18 (ANF), 20 (MCAD and FAT/CD36), 23 (PGC-1), 25 (UCP-3 and PPAR α), 26 (RXR α and RXR γ), 27 (CTE), and 28 (M-CPT-I and ACO) cycles. Each cycle consisted of denaturation at 92°C for 1 min, primer annealing at 60°C , and primer extension at 72°C for 1 min and 50 s. A final 5-min extension step at 72°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 film to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (ANF, 271 bp; PPAR α , 645 bp; M-CPT-I, 222 bp; MCAD, 216 bp; UCP-3, 179 bp; ACO, 195 bp; FAT/CD36, 256 bp; CTE, 224 bp; RXR α , 202 bp; RXR γ , 220 bp; PGC-1, 228 bp; and APRT, 329 bp). Preliminary experiments were carried out with various amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Thus, cDNA amplification was performed in comparative and semiquantitative conditions (Freeman et al., 1999). Radioactive bands were quantified by video-densitometric scanning (Vil-

Electrophoretic Mobility Shift Assays (EMSAs). EMSAs were performed using double-stranded oligonucleotides (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for the consensus binding sites of PPRE (5'-CAAACCTAGGTCAAAGGTCA-3'), mutant PPRE (5'-CAAACCTAGCACAAGGCACA-3'), and Oct-1 (5'-TGTCGAATG-

ANF	
Sense	5'–GCAGGCCCTGAGTGAGCAGAC–3'
Antisense	5'–CCGGAAGCTGTTGCAGCCTAG–3'
PPAR α	
Sense	5'–GGCTCGGAGGGCTCTGTATC–3'
Antisense	5'–ACATGCACTGGCAGCAGTGA–3'
M-CPT-1	
Sense	5'–TTCCTGTGACCCAGACGGG–3'
Antisense	5'–AATGGACCAAGCCCATGGAGA–3'
MCAD	
Sense	5'–TCGAAAGCGGCTCACAAGCAG–3'
Antisense	5'–CACCGCAGCTTTCGGAATGT–3'
UCP-3	
Sense	5'–GGAGCCATGGCAGTGACCTGT–3'
Antisense	5'–TGTGATGTTGGGCCAAGTCCC–3'
ACO	
Sense	5'–ACTATATTTGGCCAAATTTTGTG–3'
Antisense	5'–TGTGGCAGTGGTTTCCAAGCC–3'
FAT/CD36	
Sense	5'–AATGGCACAGATGCAGCCTCC–3'
Antisense	5'–TGCCAATGTCCAGCACACCAT–3'
CTE	
Sense	5'–CAGCCACCCCGAGGTAAAAGG–3'
Antisense	5'–CCTTGAGGCCATCCTTGGTCA–3'
RXR α	
Sense	5'–GCTCTCCAACGGGTCGAGGCT–3'
Antisense	5'–TGGGTGTGTGGGTACCGACA–3'
RXR γ	
Sense	5'–CCATGAAGACATGCCGGTGA–5'
Antisense	5'–CCCAGTGCAGAAATGACCTGGT–3'
PGC-1	
Sense	5'–CCCCTGGATGAAGACGGATTG–3'
Antisense	5'–GTGGGTGTGGTTTGCTGCATG–3'
APRT	
Sense	5'–AGCTTCCCGGACTTCCCCATC–3'
Antisense	5'–GACCACTTTCTGCCCGGTTTC–3'

Statistical Analyses. Results are usually expressed as means \pm S.D. of four or five mice. Significant differences were established by Student's *t* test using the computer program Instat (GraphPad Software Inc., San Diego, CA).

Troglitazone Differently Modifies ACO mRNA Levels in Heart after 1 or 10 Days of Treatment. We first exam-

ined the effects of troglitazone for 1 day on the mRNA levels of ACO and MCAD genes. ACO catalyzes the rate-limiting step of peroxisomal β -oxidation of fatty acids and its transcription is controlled by PPAR α (Desvergne and Wahli, 1999). MCAD is also a PPAR α -target gene that catalyzes a rate-limiting step in the mitochondrial β -oxidation of medium-chain fatty acyl-thioesters (Desvergne and Wahli, 1999). In the heart of 1-day troglitazone-treated mice the mRNA levels of ACO seemed increased (8.3-fold induction, $P < 0.05$) in relation to control mice (Fig. 2A). In contrast, MCAD transcript levels were not modified by a single administration of troglitazone. We also studied the effects of this drug on CTE, which catalyzes the hydrolysis of acyl-CoAs to free fatty acids and CoA, and so 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 troglitazone-treated mice the mRNA levels of CTE were not modified. These findings indicate that, although PPAR γ is expressed at low levels in heart compared with adipose tissue (Braissant et al., 1996; Vidal-Puig et al., 1997), in vivo administration of troglitazone increases ACO mRNA levels, confirming previous results showing that troglitazone induced transcriptional activity of neonatal cardiac myocytes transfected with a (ACO-PPRE)₃-TK-Luc reporter plasmid (Takano et al., 2000). In contrast, MCAD and CTE showed a different behavior after troglitazone treatment, suggesting that the activity of PPAR γ may depend on the nature of the PPRE, as

suggested previously (Marcus et al., 1993). When we examined the expression of ACO in the heart of 10-day troglitazone-treated mice, a dramatic reduction was observed (98% reduction, $P < 0.02$) (Fig. 2B) compared with control mice. In contrast, troglitazone treatment did not affect the mRNA expression of either MCAD or CTE. Similarly, the expression of the fatty acid translocase (FAT/CD36), a membrane-associated protein that facilitates the uptake of long-chain fatty acids into cells and is up-regulated after PPAR γ activation (Desvergne and Wahli, 1999), was not modified by 10-day troglitazone treatment (Fig. 2B). Muscle-type carnitine palmitoyl-transferase (M-CPT-I), a PPAR-target gene (McGarry and Brown, 1997; Mascaró et al., 1998) that catalyzes the entry of long-chain fatty acids into the mitochondrial matrix (McGarry and Brown, 1997), was significantly increased (1.7-fold induction, $P < 0.05$) after troglitazone treatment for 10 days in relation to control animals. Interestingly, the expression of uncoupling protein 3 (UCP-3), a mitochondrial carrier localized in the inner mitochondrial membrane, which has been implicated in fatty acid utilization and it is a PPAR α target-gene (Ricquier and Bouillad, 2000), was reduced (41%, $P = 0.05$) after troglitazone treatment (Fig. 2B). Given the important role of PPAR α in the control of cardiac lipid metabolism (Djouadi et al., 1999) and the previous results showing down-regulation of this transcription factor after troglitazone treatment in C2C12 myotubes (Cabrero et al., 2000), we finally studied whether the effects of troglita-

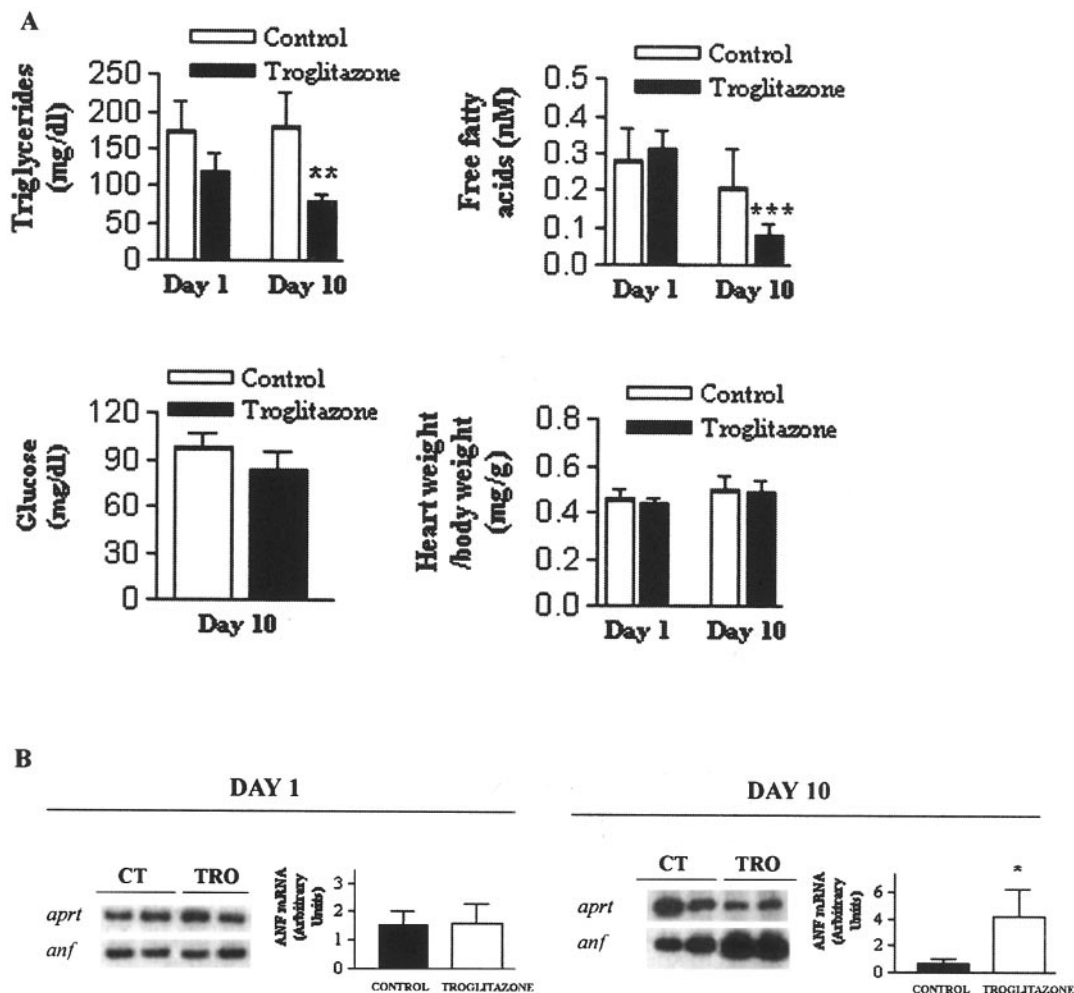


Fig. 1. A, troglitazone effects on plasma triglycerides, non-esterified fatty acids, glucose, and on cardiac weight. Mice were treated for 1 or 10 days with either 0.5% carboxymethyl cellulose (control group) or 100 mg/kg/day of troglitazone (dissolved in 0.5% carboxymethyl cellulose). Values are means \pm S.D. of five animals per group. B, effects of troglitazone (100 mg/kg/day) for either 1 or 10 days on the expression of ANF mRNA levels in heart. A representative autoradiogram and the quantification of the APRT-normalized mRNA levels is shown. Data are expressed as mean \pm S.D. of four or five mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

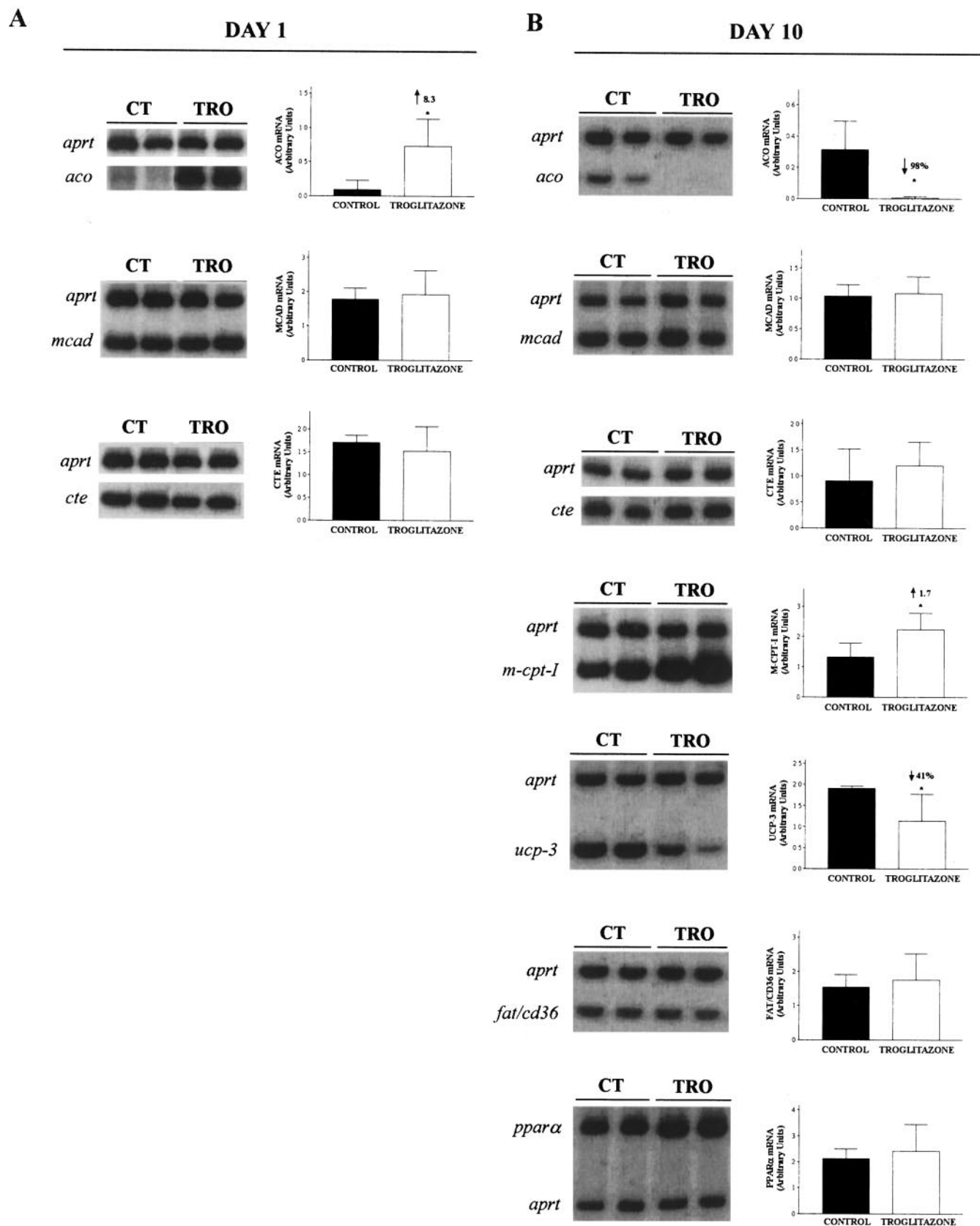


Fig. 2. Effects of troglitazone (100 mg/kg/day) for either 1 (A) or 10 days (B) on the expression of ACO, MCAD, CTE, M-CPT-I, UCP-3, FAT/CD36, and PPAR α mRNA levels in heart. A representative autoradiogram and the quantification of the APRT-normalized mRNA levels are shown. Data are expressed as mean \pm S.D. of four or five mice. *, $P < 0.05$ compared with control experiments.

zone treatment on ACO expression were mediated by reduced expression of this transcription factor. The mRNA amounts of this PPAR subtype were not modified after 10 days of troglitazone treatment (Fig. 2B), suggesting that changes in PPAR α expression were not responsible for the effects of troglitazone. Similarly, no changes were observed in PPAR γ transcript levels in heart after troglitazone treatment for either 1 or 10 days (data not shown).

Effects of Troglitazone Treatment for 10 Days on RXR and PGC-1 mRNA Levels in Heart. To study whether reduced availability of the PPAR α heterodimeric partner RXR was responsible for the reduced transcriptional activity of the ACO gene in 10 days troglitazone-treated mice, we determined the transcript levels of RXR α and RXR γ . Troglitazone treatment for 10 days did not modify the mRNA expression of these transcription factors in heart compared with control mice (Fig. 3). In addition, we studied the mRNA expression of PGC-1, which directly interacts with PPAR α , and it has been postulated as a regulator of mitochondrial β -oxidation (Vega et al., 2000). PGC-1 mRNA was not altered in the heart of 10-day troglitazone-treated mice. All these findings suggest that RXR and PGC-1 are unlikely to be involved in the changes observed after troglitazone treatment for 10 days.

DNA Binding Activity of Cardiac Nuclear Proteins to a PPRE Probe Was Induced in 10-Day but Not in 1-Day Troglitazone-Treated Mice. EMSAs were performed to examine the interaction of PPARs with its *cis*-regulatory element using a 32 P-labeled PPRE probe and cardiac nuclear extracts from control and troglitazone-treated mice for 1 or 10 days. The PPRE probe formed four complexes with cardiac

nuclear proteins (Fig. 4A, complexes I to IV). Competition studies performed with a molar excess of unlabeled probe revealed that the four complexes represented specific PPRE-protein interactions. These results suggest that several endogenous cardiac nuclear proteins bind to PPRE. In nuclear extracts from hearts of 1-day troglitazone-treated mice no significant changes were observed in DNA binding activity to the PPRE probe compared with control animals (Fig. 4B). In contrast, despite the lack of induction in the transcriptional rate of PPAR-target genes after troglitazone treatment for 10 days, this drug increased the binding of cardiac proteins to the PPRE *cis*-element (Fig. 4C), resulting in an increase of specific complexes, mainly of complex II. No changes were observed in the DNA binding of cardiac proteins from control and troglitazone-treated mice to an Oct-1 probe, indicating that the increase observed for the PPRE probe was specific (Fig. 4D).

Supershift studies performed using antibodies against PPAR α , PPAR δ/β , and PPAR γ (Fig. 4, E–G) demonstrated that the incubation mixtures contained the three different PPAR subtypes.

Troglitazone Treatment Increases COUP-TF II Protein Levels in Heart. The fall in the transcriptional rate of the ACO gene together with the increased interaction of the PPRE probe with cardiac proteins in troglitazone-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 (Desvergne and Wahli, 1999), we determined whether expression of COUP-TF II parallels the increased interaction of the PPRE probe in troglitazone-treated mice. Protein levels of COUP-TF II were assessed by the Western blot technique. Nuclear levels of COUP-TF II were only slightly increased after 1 day of troglitazone treatment compared with control animals (Fig. 5A). However, after 10 days of troglitazone treatment a strong induction in COUP-TF II protein levels in heart was observed compared with control mice samples (Fig. 5B). These findings show that through the time course of troglitazone treatment, there is a good correlation between the fall in plasma lipids and the induction in COUP-TF II protein levels.

Increased Binding of the Transcriptional Repressor COUP-TF II to a PPRE Probe in Cardiac Nuclear Extracts of 10-Day Troglitazone-Treated Mice. In EMSAs performed with a PPRE oligonucleotide and cardiac nuclear extracts from control and treated mice for 10 days, a reduction in the intensity of complex II was observed in the presence of COUP-TF II antibody, indicating enhanced levels of COUP-TF bound to the PPRE probe in cardiac nuclear extracts from troglitazone-treated mice (Fig. 6). This finding supports a role for this transcriptional repressor in the changes observed after troglitazone treatment for 10 days.

Discussion

The mammalian heart may adjust its metabolism depending on the substrate availability through changes in the transcriptional control of genes involved in the transport and metabolism of fatty acids and glucose (Van Bilsen et al., 1998). We have recently reported that thiazolidinediones down-regulate the expression of genes involved in fatty acid

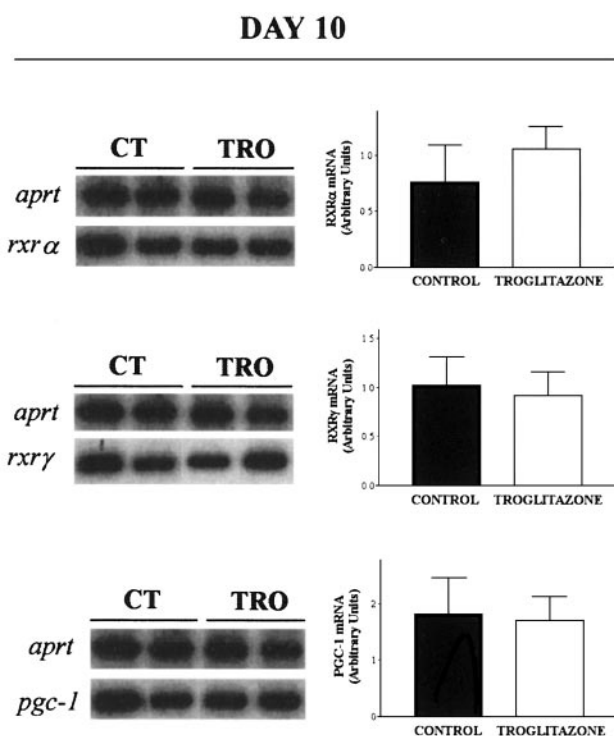


Fig. 3. Effects of troglitazone (100 mg/kg/day) for 10 days on the expression of RXR α , RXR γ , and PGC-1 mRNA levels in heart. A representative autoradiogram and the quantification of the APRT-normalized mRNA levels are shown. Data are expressed as mean \pm S.D. of four or five mice. *, $P < 0.05$ compared with control experiments.

oxidation in C2C12 myotubes cultured in the presence of glucose but in the absence of exogenous fatty acids (Cabrero et al., 2000). These changes correlated well with impaired expression of PPAR α and were reverted by addition of fatty acids to the culture medium, suggesting that different fuel substrate utilization modifies the regulation of the fatty acid oxidation system. In the normoglycemic mice used in this work troglitazone treatment for 10 days, but not for 1 day, significantly reduced plasma free fatty acids and triglycerides, whereas glucose levels were not altered. Thus, 10 days of troglitazone treatment induced a change in plasma substrate availability, resulting in a high ratio of glucose to fatty acids. Under these conditions, troglitazone caused a dramatic reduction in ACO mRNA levels, without changes in the expression of PPAR α . The reduction in ACO expression after 10 days of troglitazone treatment was accompanied by increased

protein levels of COUP-TF II, suggesting that this PPAR-transcriptional repressor was involved.

According to previous studies (Tsai and Tsai, 1997), the mechanism by which COUP-TF antagonizes PPAR signaling involves competitive occupation of the DR-1 present in the PPRE. We observe increased binding of COUP-TF to the PPRE *cis*-element, suggesting a role for this transcription factor in the effects caused by troglitazone. However, different effects were observed after troglitazone treatment in ACO and MCAD genes. The expression of the former was nearly abolished by troglitazone, whereas the second was not modified by the treatment. The data here presented do not allow us to know the reasons for the lack of effect of troglitazone on MCAD compared with ACO expression. However, differences in the nature of their PPRE could be involved. In fact, it has been reported that the PPREs containing two

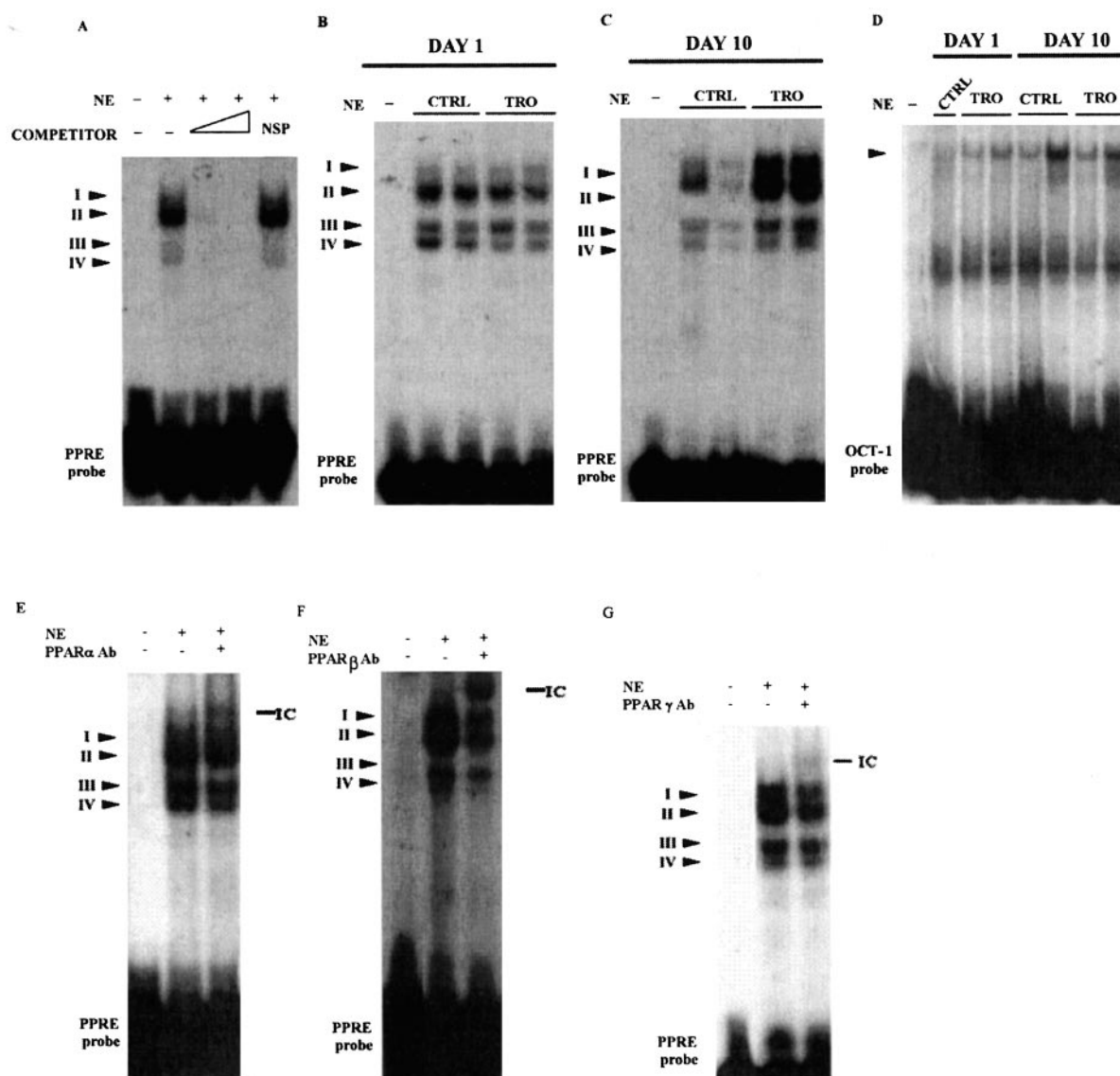


Fig. 4. Binding of cardiac nuclear proteins to a PPRE probe is induced by troglitazone treatment for 10 days but not for 1 day. A, autoradiograph of EMSA performed with a 32 P-labeled PPRE nucleotide and crude nuclear protein extract (NE) shows four specific complexes (I to IV), based on competition with a molar excess of unlabeled probe but not by an equivalent amount of a mutant PPRE oligonucleotide (NSP). Autoradiograph of EMSAs performed with a 32 P-labeled PPRE nucleotide and NE from hearts of control and troglitazone-treated mice for 1 (B) or 10 days (C). D, autoradiograph of EMSA performed with a 32 P-labeled Oct-1 nucleotide. E to G, identification of PPAR subtypes in cardiac nuclear proteins from hearts. Antibodies to PPAR α , PPAR β/δ , PPAR γ , and Oct-1 were added when indicated. Supershifted immune complexes (IC) are denoted.

divergent half sites in the DR-1, such as that present in PPRE-MCAD, are less able to bind the transcriptional repressor COUP-TF II compared with the ACO-PPRE (Palmer et al., 1995). Likewise, the expression of MCAD is strongly reduced in the heart of PPAR α knockout mice, whereas ACO expression was not affected (Watanabe et al., 2000), suggesting that MCAD expression strongly depends on PPAR α expression. Therefore, the PPRE present in the MCAD gene may be more specific for PPAR α -RXR α than the PPRE present in the ACO gene. In cardiac hypertrophy induced by pulmonary artery banding the reduction in MCAD is accompanied by an increase in the expression of COUP-TF II and a strong reduction in the expression of PPAR α (Sack et al., 1997). In our study, we have observed an induction in the expression of COUP-TF II, whereas the expression of PPAR α at the mRNA level was unchanged. Therefore, given the strong dependence of MCAD on PPAR α levels, the presence of normal levels of PPAR α in hearts of troglitazone-treated mice may be responsible for the unchanged MCAD expression.

Alternative mechanisms not directly involving PPAR may also participate in the regulation of ACO gene expression

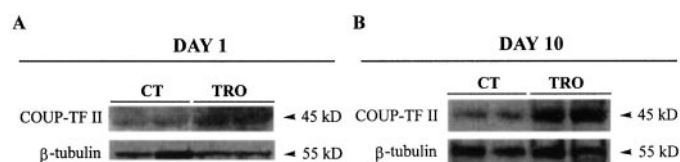


Fig. 5. Cardiac nuclear levels of COUP-TF II are increased in hearts of troglitazone treated mice for 10 days. A representative autoradiograph of a Western blot performed with 40 μ g of cardiac crude nuclear protein extract from troglitazone-treated mice for 1 (A) or 10 days (B). Protein mass markers are displayed on the right in kilodaltons. β -Tubulin levels are shown as a control of equal abundance of nuclear extracts in the samples.

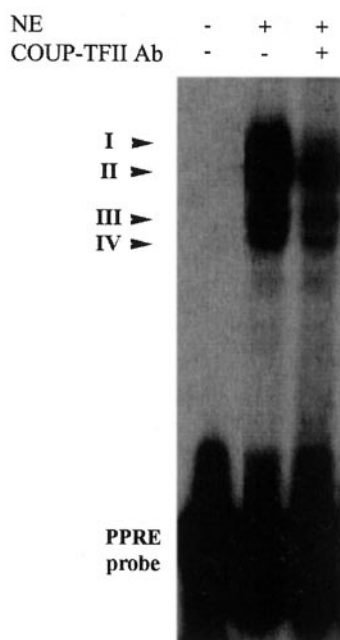


Fig. 6. Identification of COUP-TF II in cardiac nuclear proteins complexes by antibody recognition experiments. The autoradiograph depicted represent EMSA antibody recognition studies performed with a PPRE probe and anti-COUP-TF II antibody (Ab). Cardiac nuclear proteins from control and 10 days troglitazone-treated mice were used.

after troglitazone treatment. Thus, it is likely that the enhanced sensitivity to insulin caused by troglitazone may contribute to the reduction in ACO expression without affecting other PPAR-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 (Hamel et al., 2001).

In contrast to ACO and MCAD, M-CPT-I expression was increased by 10-day troglitazone treatment. Although the up-regulation of this gene may be mediated through PPAR γ activation by troglitazone, the lack of effect of troglitazone on the well known PPAR γ target gene FAT/CD36 make this possibility unlikely. On the other hand, it is well known that increased glucose entry to skeletal myocytes and white adipocytes, increases the concentration of malonyl-CoA, a known inhibitor of CPT-I (McGarry et al., 1989). Because troglitazone increases glucose utilization it is likely that up-regulation of M-CPT-I was a compensatory mechanism for its inhibition caused by malonyl-CoA, similar to the increased expression of M-CPT-I in heart after treatment with the CPT-I inhibitor etomoxir (Brandt et al., 1998). In addition, it has been recently reported that troglitazone treatment results in a down-regulation in heart of the mRNA of malonyl-CoA decarboxylase, the gene that catalyzes the degradation of malonyl-CoA (Young et al., 2001). Therefore, it is likely that the decreased malonyl-CoA decarboxylase expression may also contribute to the up-regulation of M-CPT-I, as a mechanism to compensate inhibition of this enzyme by malonyl-CoA. Finally, the reduction in plasma free fatty acids levels after troglitazone treatment may account for the fall in UCP-3 transcripts, as reported previously (Ricquier and Bouillad, 2000).

In the present study, troglitazone treatment for 10 days caused cardiac hypertrophy, in agreement with previous studies showing that all thiazolidinediones have been associated with cardiac hypertrophy in animal studies, at doses far exceeding those recommended for therapeutic use (Ghazzi et al., 1997). In addition, it has been reported that although troglitazone does not initiate hypertrophy, it can sensitize cardiomyocytes to growth effects of serum (Bell and McDermott, 2000), indicating that the actions of this drug may promote changes favoring increased growth of these cells under some circumstances. In contrast, recent studies have shown that thiazolidinediones inhibit cardiac hypertrophy (Yamamoto et al., 2001, Asakawa et al., 2002). These differences could be attributed to the different concentrations of thiazolidinediones used. Thus, it is well known that at low concentrations PPAR γ activators antagonize the activity of NF- κ B (Desvergne and Wahli, 1999), whereas in our study we found increased NF- κ B activation (A. Cabrero, M. Vázquez-Carrera, unpublished data) in hearts after 10 days of troglitazone treatment. Because NF- κ B activation is required for the hypertrophic growth of cardiomyocytes (Purcell et al., 2001), the different effects of thiazolidinediones on cardiac hypertrophy may depend on the concentration used.

It is still a matter of controversy whether changes in intracellular substrate and metabolite levels in cardiomyocytes are the consequence or the reason for the shift of cardiac metabolism from fatty acids to glucose observed in cardiac hypertrophy (Van Bilsen et al., 1998). In fact, an increase in the activities of several glycolytic enzymes has been reported before cardiac hypertrophy (Taegtmeyer and Overturn, 1998),

suggesting that increased glucose metabolism may induce the metabolic changes observed in cardiac hypertrophy. Because troglitazone improves insulin responsiveness in skeletal muscle by facilitating glucose transport activity, which thereby leads to increased rates of muscle oxidation (Petersen et al., 2000), it is likely that these changes initiate a shift toward a fetal metabolic pattern in the heart, which mainly relies on glucose utilization. This explanation seems not to be in accordance with the absence of a reduction in plasma glucose levels after 10 days of troglitazone treatment. However, it has been recently postulated that normoglycemic animals are protected from troglitazone-induced hypoglycemia by a mechanism that maintains plasma glucose levels through elevation of both gluconeogenesis and glycogenolysis (Dea et al., 2000). Therefore, increased glucose utilization by muscle after troglitazone treatment occurs without changes in plasma glucose levels in normal animals.

In summary, we report that troglitazone treatment results in the adjustment of the expression of genes implicated in peroxisomal fatty acid β -oxidation through a mechanism involving the transcriptional repressor COU-TF II.

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Address correspondence to: Dr. Manuel Vázquez-Carrera, Unitat de Farmacologia, Facultat de Farmàcia, Diagonal 643, E-08028 Barcelona, Spain. E-mail: mvaz@farmacia.far.ub.es