Down-regulation of acyl-CoA oxidase gene expression and increased NF-κB activity in etomoxir-induced cardiac hypertrophy

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Abstract  Activation of nuclear factor-κB (NF-κB) is required for hypertrophic growth of cardiomyocytes. Etomoxir is an irreversible inhibitor of carnitine palmitoyltransferase I (CPT-I) that activates peroxisome proliferator-activated receptor α (PPARα) 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 β-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-κB was increased in heart after 10 days of etomoxir treatment. Overall, the findings here presented show that etomoxir treatment may induce cardiac hypertrophy via myocardial oxidative stress and NF-κB activation.—Cabrero, À., M. Merlos, J. C. Laguna, and M. Vázquez Carrera. Down-regulation of acyl-CoA oxidase gene expression and increased NF-κB activity in etomoxir-induced cardiac hypertrophy. J. Lipid Res. 2003. 44: 388–398.

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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 (α, δ/β, and γ) have been identified to date. PPARα 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). PPARδ is ubiquitously expressed, and PPARγ 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-κB, nuclear factor κB; PGC-1, PPARγ coactivator 1; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; UCP-3, uncoupling protein 3.
bind potential competitors such as homodimers of other nuclear receptors including RXRα, chicken ovalbumin upstream promoter transcription factor II (COP1-TP-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 PPARα (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 COP1-TP 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 β-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 PPARα (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 β-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 PPARα, and of several of its target genes involved in mitochondrial β-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 κB (NF-κB). Overall, the results presented here suggest that etomoxir increases oxidative stress in cardiomyocytes, leading to NF-κB 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 β-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 ± 1°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 etomoxir (dissolved in 0.5% carboxymethyl 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°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°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 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 MgCl₂, 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 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 3 μl of the RT reaction, 1.2 mM MgCl₂, 200 μM dNTPs, 1.25 μg dNTP (3,000 Ci/mmol, Amersham), 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 oil. To avoid unspecific annealing, cDNA and Taq polymerase were separated from primers and dNTPs by using a layer of paraffin oil.

The sequences of the sense and antisense primers used for amplification were: PPARα, 5′-GGCTTCGAGGGGTCCTGTAC-3′ and 5′-AAATGACCTGCGGACAGTGA-3′; CPT-I, 5′-TTCTGTCGACCCCAAGGTC-3′ and 5′-ATGCCAGAATGCATTCTGG-3′; PGC-1, 5′-TCCAGAGCTGCTACAGACCT-3′ and 5′-TCCTGACGTCTGTTTGCCTGG-3′; RXRα, 5′-AGCTATATTGCGCAGAATTG-3′ and 5′-CATTGGCAGTGTTGTCCACCGG-3′; CTE, 5′-CAGCCCAACCGGAGTAAAAGC-3′ and 5′-CTTGGAGCCACATCG-3′; RXRα, 5′-CTTGGACGCTGTTGTCCTCA-3′ and 5′-TTGAGCTTGTTGTTCGTTCTTGCA-3′; RXRα, 5′-GGCTTCGAGGGGTCCTGTAC-3′ and 5′-AAATGACCTGCGGACAGTGA-3′; CPT-I, 5′-TCCTGACGTCTGTTTGCCTGG-3′; RXRα, 5′-AGCTATATTGCGCAGAATTG-3′ and 5′-CATTGGCAGTGTTGTCCACCGG-3′; CTE, 5′-CAGCCCAACCGGAGTAAAAGC-3′ and 5′-CTTGGAGCCACATCG-3′; RXRα, 5′-CTTGGACGCTGTTGTCCTCA-3′ and 5′-TTGAGCTTGTTGTTCGTTCTTGCA-3′; RXRα, 5′-GGCTTCGAGGGGTCCTGTAC-3′ and 5′-AAATGACCTGCGGACAGTGA-3′; CPT-I, 5′-TCCTGACGTCTGTTTGCCTGG-3′; RXRα, 5′-AGCTATATTGCGCAGAATTG-3′ and 5′-CATTGGCAGTGTTGTCCACCGG-3′; CTE, 5′-CAGCCCAACCGGAGTAAAAGC-3′; and adenosyl phosphoribosyltransferase (APRT), 5′-AGCTTCCGAGGCATCCATCG-3′ and 5′-GAGCCATCTTGCCCGCTGGTTC-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°C, PCR was performed for 20 (MCAD), 22 (GPX), 25 (UCP-2, PGC-1), 25 (UCP-3, PPARα, BAX), 26 (RXRα and RXRγ), 27 (CTE), and 28 (M-CPT-I, ACO) cycles. Each cycle consisted of denaturation at 92°C for 1 min, primer annealing at 60°C (except 58°C for ACO), 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 films to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (PPARα: 645 bp; M-CPT-I: 222 bp; MCAD: 216 bp; UCP-3: 179 bp; UCP-2: 471 bp; ACO: 195 bp; CTE: 224 bp; RXRα: 202 bp; RXRγ: 220 bp; PGC-1: 228 bp; GPX:

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in ice-cold low salt buffer (25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA). Supernatants were discarded and pellets were suspended in the homogenization step and centrifuged (10,000 × g, 15 min). Homogenates were incubated for 10 min on ice and centrifuged (25,000 × g, 15 min). Supernatants were discarded and pellets were suspended in ice-cold low salt buffer (25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 20 mM KCl, 20 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 MgCl₂, 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 MgCl₂, 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 mixed. Supernatants were collected in microfuge tubes and stored in aliquots at −80°C.

**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 MgCl₂, 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). For supershift assays, antibodies were added after incubation with labeled probe for a further 30 min at room temperature. Western-blot analysis

**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 modulate 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*    |
| Heart weight/body weight (mg/g) | 0.46 ± 0.04 | 0.45 ± 0.04 | 0.50 ± 0.06 | 0.62 ± 0.09*    |
| 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.

* P < 0.05.
Fig. 1. Effects of etomoxir (100 mg/kg/day) for either 1 (A) or 10 days (B) on the expression of ACO, MCAD, CTE, M-CPT-I, UCP-3, UCP-2, and PPARα mRNA levels in heart. A representative autoradiogram and the quantification of the APRT-normalized mRNA levels are shown. Data are expressed as mean ± SD of four or five mice. * P < 0.05 compared with control experiments. Exposition times for ACO mRNA levels were adjusted in order to better observe differences caused by drug treatment on days 1 and 10.
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 β-oxidation of fatty acids and its transcription is controlled by PPARα (4). The second, MCAD, is also a PPARα-target gene that catalyzes a rate limiting step in the mitochondrial β-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 PPARα 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 β-oxidation system MCAD and muscle-type carnitine palmitoyl-transferase (M-CPT-I), being the latter a PPARα-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 PPARα (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 PPARα-independent manner, since this is the only PPARα-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 PPARγ or COUP-TF II, may regulate CTE in heart (16). Given the crucial role of PPARα 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. PPARα mRNA levels were not modified after 10 days of etomoxir treatment, indicating that changes in PPARα expression were not responsible for the effects of etomoxir.

Effects of etomoxir treatment for 10 days on RXR and PPARγ coactivator 1 mRNA levels in heart

The induction of PPARα-target genes, such as ACO and MCAD, by PPARα activators is reduced in livers of RXRα-deficient mice (21). Therefore, in order to study whether reduced availability of the PPARα heterodimeric partner RXRα was responsible for the reduced transcriptional activity of the ACO gene in 10-days etomoxir-treated mice, we determined the transcript levels of RXRα and RXRγ. Eto-moxir 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 PPARγ coactivator 1 (PGC-1), which directly interacts with PPARα and has been postulated as a regulator of mitochondrial β-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 RXRα, RXRγ, 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 ± SD of four or five mice. * \( P < 0.05 \) compared with control experiments.
Etomoxir treatment for 10 days, but not for one day, enhanced the DNA binding activity of cardiac nuclear proteins to a PPRE probe

EMSAs were performed to examine the interaction of PPAR with its cis-regulatory element using a 32P-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 mutant PPRE 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 PPARα-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 PPARα, PPARδ, or PPARγ to the PPRE probe was observed in supershift assays using specific antibodies (data not shown). In addition to PPARα, 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 32P-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

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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 32P-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 32P-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 32P-labeled Oct-1 nucleotide.
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 β-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 H₂O₂ is critical to protect the heart against oxidative stress, and GPX appears to act as a key enzyme degrading H₂O₂ in the cytosol of cardiac myocytes (23). Moreover, transcript levels of GPX are induced in skeletal muscle cells treated with pro-oxidants (menadione and H₂O₂) (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-κB 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-κB was altered by etomoxir. The NF-κB 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-κB oligonucleotide to incubation mixtures. In agreement with the reported antagonism of NF-κB by PPAR activation (4), binding activity of NF-κB was reduced in cardiac nuclear extracts from etomoxir-treated mice for 1 day (Fig. 8B). In contrast, NF-κB 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-κB 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-κB.
DISCUSSION

Pharmacological inhibition of CPT-I was proposed as a therapeutic option for the treatment of type 2 diabetes mellitus, since increased fatty acid β-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 β-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-κB 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 PPARα 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 agreement 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 (~2-fold) after 5 days of treatment. Therefore, in short treatments (1–5 days), when cardiac hypertrophy is not present, etomoxir activates PPARα in heart, which results in an increase in the expression of PPARα target genes. The mechanism by which inhibitors of CPT-I, such as etomoxir, activate PPARα 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 PPARα target genes involved in mitochondrial β-oxidation is abolished, without changes in PPARα 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 PPARα-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
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 β-oxidation system correlates with enhanced cardiac oxidative stress, suggesting that it may be involved. Peroxisomal β-oxidation is implicated in the degradation of proinflammatory molecules such as leukotriene B4 and 8(β)-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 H2O2 in liver (39). In this study we show that when ACO mRNA levels are down-regulated, GPX mRNA levels are increased, suggesting increased H2O2 levels. In fact, it has been reported that GPX mRNA levels increase in skeletal muscle cells treated with H2O2 (24). Elimination of H2O2 is critical to protect heart tissue against oxidative stress, because superoxide is converted to H2O2 by superoxide dismutase, and in the presence of cardiac myocytes, H2O2 forms hydroxyl radical, one of the most toxic oxygen free radicals. In the myocardium, GPX plays a predominant role in the scavenging of H2O2, given that catalase, the other major H2O2-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, H2O2 induces dimerization or oligomerization of ASK1 and activates its kinase activity (42), supporting a role for H2O2 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, PPARα 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 (30, 51). Further, the presence of several Sp binding sites in the promoter region of M-CPT-I, MCAD (7), and PPARγ 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-κB 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.

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