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# Fenofibrate activates the biochemical pathways and the de novo expression of genes related to lipid handling and uncoupling protein-3 functions in liver of normal rats

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## Abstract

Fibrates (anti-hyperlipidemic agents) enhance the mRNA expression of uncoupling protein 2 (UCP2) in the liver and that of uncoupling protein 3 (UCP3) in skeletal muscle in standard-diet-fed rats and induce a de novo expression of UCP3 (mRNA and protein) in the liver of high-fat-fed rats. Here, we report that in the liver of normal rats, fenofibrate induces a de novo expression of UCP3 and a 6-fold increase in UCP2 mRNA, whereas UCP2 protein was not detectable. Indeed, we evidenced an ORF in UCP2 exon 2 potentially able to inhibit the expression of the protein. Fenofibrate increases the expression and activity of hepatic enzymes and cofactors involved in lipid handling and UCP3 activity and, as is the case for UCP3, induces other muscle-specific genes (e.g., Carnitine palmitoyl transferase 1b and Ubiquinone biosynthesis protein COQ7 homolog). In addition, we demonstrated that in mitochondria from fenofibrate-treated rats a palmitoyl-carnitine-induced GDP-sensitive uncoupling takes place, involving UCP3 rather than other uncouplers (i.e., UCP2 and Adenine Nucleotide Translocase). Thus, the liver of fenofibrate-treated standard-diet-fed rat is a useful model for investigations of the biochemical functions of UCP3 and allowed us to demonstrate that fenofibrate programs a gene-expression pattern able to modulate lipid handling and UCP3 activation.

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**Keywords:** Fenofibrate; Uncoupling protein; Adenine nucleotide translocase; Mitochondrial thioesterase I; Mitochondrial uncoupling; Liver

## 1. Introduction

Fenofibrate, a synthetic fibric acid derivative, is a pro-drug that is rapidly hydrolyzed in vivo to form fenofibric acid, which

**Abbreviations:** UCPs, Uncoupling proteins 1, 2, 3; HDMCP, Hepatocellular Carcinoma Downregulated Mitochondrial Carrier Protein; MTE-I, mitochondrial thioesterase I; PPAR, Peroxisome Proliferator-activated Receptor; FA, fatty acid; ROS, reactive oxygen species; ORF, open reading frame; CAT, CarboxyAtracTilate; CoQ, Coenzyme Q; CPT, Carnitine Palmitoyl Transferase; ACS, Acyl CoA Synthetase; LPL, Lipoprotein Lipase; COQ7, Ubiquinone biosynthesis protein COQ7 homolog; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; AdNT, adenine nucleotide translocase

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is a highly effective agent for the treatment of dyslipidemias [1]. Fenofibrate acts as a nuclear peroxisome proliferator-activated receptor  $\alpha^1$  (PPAR- $\alpha$ ) ligand, thereby promoting increased hepatic fatty acid oxidation as well as an increased catabolism and reduced synthesis/secretion of triglycerides [2–6]. Fenofibrate-activated PPAR- $\alpha$  has also been suggested to be involved in the regulation of energy balance on the grounds that it prevents or reduces body-weight gain in rodent genetic models of obesity [7,8] as well as in diet-induced obese rodents [9–12]. Stimulatory effects of PPAR- $\alpha$  agonists on peroxisomal and mitochondrial fatty acid  $\beta$ -oxidation have been described in both liver and muscle [6], and in a recent study, we demonstrated that fenofibrate enhanced hepatic mitochondrial and peroxisomal FA oxidation, leading to a slight increase in resting metabolic rate [9]. Previous studies have also shown that fenofibrate stimulates

the expressions of the mRNAs encoding UCP2 and UCP3 [13–16]. These proteins, which are mitochondrial anion carriers located in the inner membrane, are potentially able to lower the proton electrochemical gradient, permitting H<sup>+</sup> to bypass the ATP synthase complex and so generate heat and waste some energy. However, this mechanism is well established only for UCP1 (present in brown adipose tissue), the physiological role of which is to mediate a regulated, thermogenic proton leak in response either to cold or to diet [17].

For UCP2 and UCP3, however, the above function seems unlikely, and several hypotheses [18–26] have been put forward including: (1) modulating the production of reactive oxygen species (ROS) and limiting or preventing their deleterious effects, (2) being involved in lipid metabolism, (3) preventing lipotoxicity, (4) being involved in glucose homeostasis, and (5) modulating energy expenditure. With regard to UCP3 and its putative involvement in lipid metabolism, a role as a regulator of the use of lipids as a fuel substrate has been postulated in situations in which lipid metabolism predominates [23,24]. In this context, we have shown that, in the liver of fat-fed rats, the *de novo* expression of UCP3 that is induced by fenofibrate treatment is associated with enhancements of both fatty acid metabolism and the expression of mitochondrial thioesterase 1 (MTE-I), an enzyme that catalyzes the hydrolysis of acyl-CoAs to free fatty acid anions and CoASH [16,25]. However, in the presence of the *de novo* expressed UCP3, the observed lack of an enhanced basal uncoupling activity (i.e., in the presence of BSA to chelate endogenous FA) led us to suggest that reducing mitochondrial efficiency is not the primary function of UCP3 in the liver of fat-fed, fenofibrate-treated rats [16]. This conclusion was based on the fact that the interpretation of many of the experiments assumes that UCP3 catalyses a basal proton leak across the inner mitochondrial membrane without the need for other cofactors. This assumption, however, now seems to be incorrect. Indeed, it is becoming increasingly evident (i) that an inducible proton conductance, rather than a basal one, can be catalyzed by UCP3, and that *in vitro* it may be regulated by cofactors such as CoQ, ROS, and lipids (and/or their derivatives) [27,28], and (ii) that in view of the above, UCP3 could display some thermogenic capacities [29].

In the present paper, we addressed this issue by examining, in the liver of normal rats, the effects of fenofibrate on the biochemical pathways and gene-expression pattern of enzymes and cofactors involved both in lipid handling and in the activity of UCP3. To this aim, to attribute the biochemical modifications induced by fenofibrate to UCP3, we had to exclude the possible involvement of UCP2. UCP2 mRNA, in fact, is induced by fenofibrate [14], and in some contexts, UCP2 has been shown to be able to uncouple mitochondria in a GDP-sensitive manner. Since the presence of a UCP2 transcript does not necessarily correspond to UCP2 protein [30], we measured the mitochondrial levels of the protein, and having found it to be absent, we performed direct sequencing of UCP2 cDNA to assess the putative presence of a cis-downregulation mechanism like that already

reported in human and mouse tissues [30]. Moreover, we searched whether the recently cloned hepatocellular carcinoma downregulated mitochondrial carrier protein (HDMCP, a liver-specific anion-carrier endowed with uncoupling properties when ectopically expressed [31]) and adenine nucleotide translocase (AdNT, another mitochondrial carrier with the potential to uncouple mitochondrial respiration [32]) might be involved in the effects exerted by fenofibrate.

In addition, since non-constitutively expressed protein (i.e., UCP3), can be induced by fenofibrate, we asked, whether to support the marked metabolic adaptation in the liver, other proteins of a “non-hepatic” origin might be induced by fenofibrate, too.

## 2. Materials and methods

### 2.1. Materials

A polyclonal antibody raised against the C-terminal region of human UCP3 protein (AB3046) was purchased from Chemicon International (Temecula, CA), while one raised against the C-terminal region of human UCP2 protein (UCP2 C-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Sensitivity and specificity of the above UCP2 and UCP3 antibodies have been examined in several tissues from UCPs knockout mice [33–35]. A mitochondrial thioesterase I (MTE-I) antibody was kindly provided by Dr. Stefan Alexson (Karolinska Institute, Huddinge, Sweden). Fenofibrate and other chemicals were all from Sigma Aldrich S.r.l., (St. Louis, MO) except carboxyatractylate (CAT), which was from Calbiochem (Vwr International Srl, Italy). The primers used for RT-PCR analysis were purchased from Sigma Genosys (Cambridge, UK).

### 2.2. Animals and treatments

Two groups of male Wistar rats (250–300 g) (Charles River, Lecco, Italy) (each consisting of 3 animals) were used throughout: namely, normal rats (referred to as N) and fenofibrate-treated rats (referred to as Fe). For 10 days, Fe rats were treated orally once a day at a dose of 320 mg/Kg body weight with fenofibrate suspended in 1% carboxymethylcellulose [36,37], whereas N rats were dosed with 1% carboxymethylcellulose alone. At the end of the treatment, the rats were anesthetized, then killed by decapitation. The liver was excised, weighed, and either immediately processed for the preparation of mitochondria or immediately frozen in liquid nitrogen and stored at –80 °C for later processing. All experiments were performed in accordance with general guidelines regarding animal experiments and were approved by our institutional committee for animal care.

### 2.3. RNA isolation and RT-PCR assays

Total RNA was isolated using a protocol based on one originally described by Chomczynski and Sacchi [38]. RT-PCR was performed slightly differently from a previous study from us [9], as different primers for UCP2 and other PCR conditions have been used accordingly to [39]. The primers used are listed in Table 1.

Separation of the PCR-products was performed on a 2% agarose gel containing ethidium bromide. The products were readily visualised, and they were quantified by means of a Bio Rad Molecular Imager FX using the supplied software (QuantityOne; Biorad). Expression signals for the genes were normalized with respect to the non-regulated  $\beta$ -actin signal.

### 2.4. Sequence analysis of upstream UCP2 repressor/ORF

Rat liver total RNA was reverse-transcribed as described in the previous section. The UCP2 cDNA fragment comprising the repressor ORF was amplified using the following mouse oligonucleotide primers: sense, 5'-

Table 1  
Oligonucleotide primers used for RT-PCR experiments

Gene	Sense oligonucleotide	Antisense oligonucleotide
UCP3	5'-ATGGATGCCTACAGAACCAT-3'	5'-CTGGGCCACCATCCTCAGCA-3'
UCP2	5'-CCTTCTGCACTCCTGTGTTC-3'	5'-GTGGCCTTGAACCAACCAT-3'
HDMCP	5'-GCCACTCCTTTGCCACCTAC-3'	5'-CACAGCCTCATAAGCCACGA-3'
AdNT1	5'-TGCTCAAGTTCACAGGTTTCAC-3'	5'-CTTCTGTTTGTCTGTGGAATC-3'
AdNT2	5'-GGACAGATTCTCTGGGCTTG-3'	5'-TGGAAATGGCTTTAAGAGAAAAAC-3'
LPL	5'-TCATCAACTGGCTGGAGGAAG-3'	5'-TATGCCTTGTGGGGTTTCT-3'
ACS	5'-AACACGTCAGTGAAGCGATG-3'	5'-AACACATTTGCCCTTTTCAC-3'
CPT1a'	5'-ATGTGGACCTGCATTCTTC-3'	5'-CCTTCAGGAAAGGTGAGTCG-3'
CPT1b	5'-CTCAGCCTCTACGGCAAATC-3'	5'-CTTCTTGATCAGGCCTTTGC-3'
CPT2	5'-GTGGCAAGGAGTTCCTGAAG-3'	5'-TGGTTCATCTGCTGGTATGC-3'
MTE-I	5'-CCTCGTCTTTCGCTGTCCTG-3'	5'-GTGTCCGTCCAGCACCTCCA-3'
HMGR	5'-GAAGCTATGGTTGACGTAAAC-3'	5'-GCTTGCTGAGGTAGAAGGTG-3'
COQ7	5'-TGGAGGAGGACGCTGAGAAG-3'	5'-GTGACACAACCCCAACACC-3'
$\beta$ -actin	5'-TTGTAACCACTGGGACGAT-3'	5'-TAATGTCACGCACGATTTC-3'

CCTTCTGCACTCCTGTGTTC-3'; antisense, 5'-GTGGCCTTGAACCAACCAT-3'. The same oligonucleotides were utilized for direct sequencing using a commercially available sequencing kit (USB Sequenase PCR product sequencing kit; Amersham Life Science Corp, Arlington Heights, IL).

## 2.5. Preparation of mitochondria and assays

Liver mitochondria were isolated by differential centrifugation as previously reported [9] and used immediately either for an assay of respiration rate and membrane potentials or for enzymatic assays, or else immediately stored at  $-80^{\circ}\text{C}$  for later processing. For HPLC analysis, samples of the homogenate were mixed with 0.1 M aqueous SDS, with ethanolic BHT (10 mg/ml) being added to prevent lipid autooxidation without reducing the ubiquinones (as reported by Lang and Packer [40]). Western immunoblot analyses were carried out according to Laemmli [41] as described in [16]. The protein concentration was determined by the method of Hartree [42].

CPT-system activity and acyl-CoA-thioesterase activity were measured spectrophotometrically according to Alexson and Nedergaard [43]. For both enzymes, palmitoyl-CoA was used at saturating concentrations (50  $\mu\text{M}$ ). As CPT-system activity is defined as the carnitine-dependent CoA production due to the overt (outer membrane, CPT1) and latent (inner membrane, CPT2) enzyme activities, it was determined in the presence of both palmitoyl-CoA and saturating carnitine (5 mM).

Mitochondrial aconitase activity was measured according to Hanselden and Fridovich [44]. Briefly, mitochondrial proteins were incubated in 1% Triton x-100 and resuspended in 50 mM Tris-HCl (pH 7.4), 30 mM isocitrate, 0.2 mM NADP, and 2 Units of isocitrate dehydrogenase. Enzyme activity (at 340 nm) corresponds to the active aconitase. Aconitase inhibited by ROS in vivo was reactivated so that total activity could be measured with 0.5 M dithiothreitol, 20 mM  $\text{Na}_2\text{S}$ , and 20 mM ferrous ammonium sulfate. The activity measured after such reactivation corresponds to the total aconitase activity. Mitochondrial CoQ was extracted and quantified by HPLC, as described by Takada et al. [45,46]. The concentrations of CoQ<sub>9</sub> and CoQ<sub>10</sub> obtained represent the sum of the respective quinone and quinol forms. The accurate concentration of each standard solution was determined spectrophotometrically using molar extinction coefficients published previously [47].

## 2.6. Measurement of respiration rate and membrane potential ( $\Delta\Psi$ )

Mitochondrial fatty acid oxidation rate was determined polarographically by means of a Clark-type electrode. Mitochondria were incubated at  $30^{\circ}\text{C}$  in a respiration medium consisting of 80 mM KCl, 50 mM HEPES (pH 7), 1 mM EGTA, 5 mM  $\text{K}_2\text{HPO}_4$ , and 1% BSA, supplemented with 2.5 mM malate and 120  $\mu\text{g}/\text{ml}$  ADP. After a 2–3 min incubation, the reaction was started by the addition of palmitoyl-L-carnitine (40  $\mu\text{M}$ ). Respiration rate and membrane potential were measured simultaneously, the latter being detected using a

triphenylmethylphosphonium (TPMP<sup>+</sup>)-sensitive electrode. To this end, 0.5 mg mitochondrial proteins were incubated in 1 ml standard incubation medium supplemented with 5  $\mu\text{M}$  rotenone, 1  $\mu\text{g}/\text{ml}$  oligomycin, 80 ng/ml nigericin, and 15  $\mu\text{g}/\text{ml}$  carboxyatractylate (CAT), the last being added to exclude the contribution of adenine nucleotide translocase (AdNT). BSA was used to chelate the endogenous FA pool. The electrode was calibrated by means of sequential additions up to 4  $\mu\text{M}$  TPMP<sup>+</sup>. Then, mitochondria were energized using 6 mM succinate, and respiration was titrated with increasing amounts of palmitoyl-carnitine (up to 40  $\mu\text{M}$ ), as previously reported [48]. Palmitoyl-carnitine has been used as substrate for the intramitochondrial production of fatty acid anions (i.e., palmitoyl CoA via acyl-CoA thioesterase activity), while succinate has been used as a saturating substrate in the presence of rotenone to preserve the possible influences on respiration and/or membrane potential due to the formation of the reduced coenzymes produced by complete palmitoyl CoA oxidation. The above determination was performed both in the presence and in the absence of exogenous GDP (500  $\mu\text{M}$ ). At the end of each run, respiration rate and membrane potential were measured after addition of 0.2  $\mu\text{M}$  carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP), which causes complete dissipation of the membrane potential and allows us to evaluate: (i) the amount of TPMP<sup>+</sup> released back into the medium, (ii) any small electrode drift, and (iii) how much respiration can be still stimulated. A TPMP<sup>+</sup>-binding correction of 0.4/( $\mu\text{l}$  per mg protein) was applied for mitochondria from each group of animals.

## 2.7. Statistical analysis

The statistical significance of differences between the two groups of rats was determined using a Student's *t*-test, *P* values less than 0.05 being considered statistically significant.

## 3. Results

### 3.1. Expressions of UCP2, UCP3, HDMC, and AdNT

In the liver of normal rats, fenofibrate treatment induced a de novo expression of UCP3 mRNA (Fig. 1A) and increased the UCP2 mRNA level about 6-fold (Fig. 1A). The latter result seems to be in contrast with our previous paper [16] in which no variation in UCP2 mRNA was observed after fenofibrate administration. However, the contrast may be more apparent than real. Two possible reasons for the apparent discrepancy may be noted as here we used: (1) a different diet regimen (standard diet fed rats instead of high fat diet fed rats) that is known to be able to influence gene expression and (2) different

primers and PCR conditions for analysis of UCP2 mRNA levels, (see Materials and methods). No influence was observed on the mRNA expressions for either HDMCP or AdNT [(both the ubiquitous and muscle-specific isoforms, AdNT2 and AdNT1, respectively) (data not shown). Western blot analysis revealed that the liver mitochondria from Fe rats contained UCP3 protein (Fig. 1B), but UCP2 protein was not detected (Fig. 1B). Direct sequencing of UCP2 cDNA revealed that exon 2 shares a very high homology with the corresponding region of the mouse and human UCP2 genes (Fig. 1C). Pecqueur et al. [30] reported that exon 2 of the mouse UCP2 gene contains a short open reading frame (ORF1) encoding a putative peptide of 36 amino acids that is able to mediate cis-downregulation of the

translation of the gene. Sequence alignment of rat, human, and mouse UCP2 genes provided evidence that exon 2 of rat UCP2 contains an ORF with its sub-region 3 being identical to the human and mouse ones (Fig. 1C), suggesting that an homologous mechanism could be operative in the rat.

### 3.2. Expression of genes and biochemical pathways related to lipid handling

A broad program of genes known to be involved in cellular fatty acid import and utilization was activated by fenofibrate in the liver of Fe rats. The expressions of genes encoding LPL and ACS were upregulated by 36.7- and 9.7-fold, respectively (Fig. 2A and B). In parallel, the expression levels of CPT1a and CPT2 were upregulated by 2.2- and 8.8-fold, respectively (Fig. 3A). It was noteworthy that, as was the case for UCP3, fenofibrate induced a de novo expression of another skeletal muscle-specific gene: CPT1b (Fig. 3A).

As predicted by this expression profile, the mitochondrial CPT-system activity was significantly greater [+115% (Fig. 3B)] in Fe rats than in N rats. The mitochondrial fatty acid  $\beta$ -oxidation rate was significantly higher (+61%) in liver mitochondria isolated from Fe rats than in those from N rats ( $P < 0.05$ ), the actual values [expressed in nAtoms O/min mg protein] being  $90 \pm 12$  and  $146 \pm 43$  for N and Fe, respectively.

Fenofibrate treatment also affected MTE-I expression, increasing both its mRNA [to about 4.5 times that in the untreated controls (Fig. 4A)] and its mitochondrial protein level [to about 5.5 times that in the untreated controls (Fig. 4B)]. As predicted by the expression profile, the mitochondrial acyl-CoA-thioesterase activity was significantly greater [+82% (Fig. 4C)] in Fe rats than in N rats.

### 3.3. Mitochondrial uncoupling

The addition of palmitoyl-carnitine to succinate-energized liver mitochondria from Fe rats simultaneously increased respiration rate and decreased membrane potential (diagnostic of mitochondrial uncoupling) (Fig. 5A, B, D, and E). These effects were not detectable in mitochondria from N rats (Fig. 5A, B, D, and E). The respiration rates of N and Fe

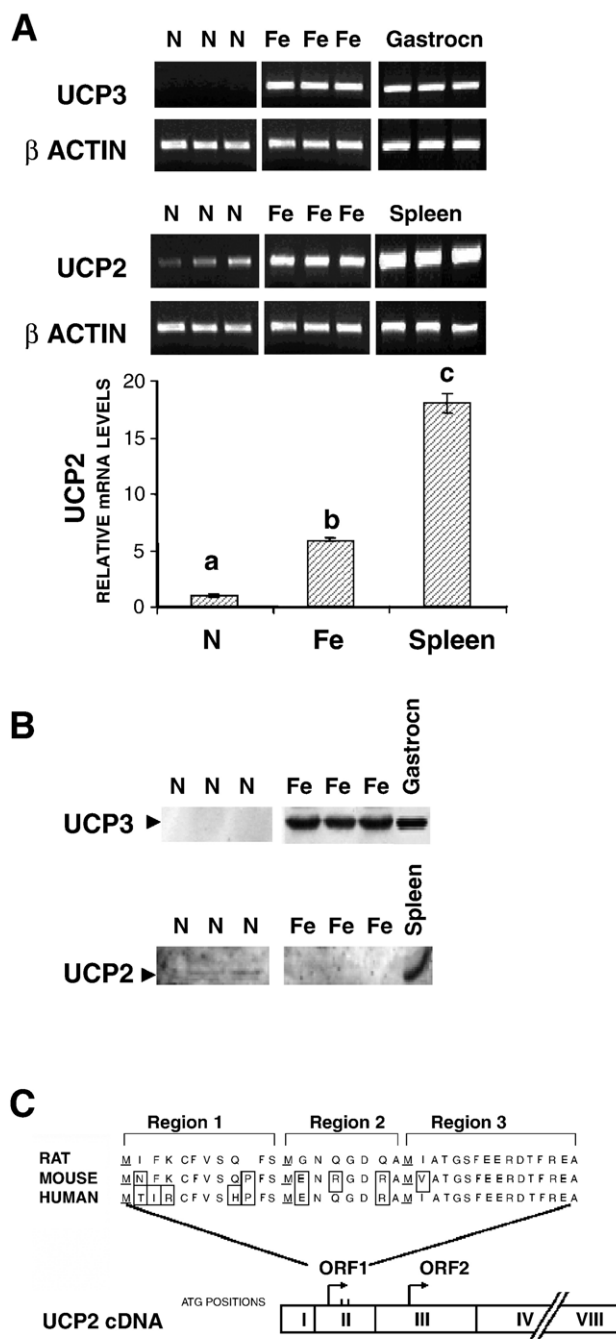


Fig. 1. Uncoupling Protein 3 (UCP3) and Uncoupling Protein 2 (UCP2) mRNA levels and mitochondrial protein density in liver of control (N) and fenofibrate-treated (Fe) rats. (A) RT-PCR-based measurements of UCP3 and UCP2 mRNAs in liver. Gastrocnemius and spleen were used as positive controls for UCP3 and UCP2 expressions, respectively. Lower panel: quantification of the data for UCP2 expression shown in the upper panel. Data are expressed relative to the value obtained for N liver, which was set as 1, and are presented separately for each treatment (as indicated below the bars). Error bars represent S.D. of the mean ( $n=3$ ). Bars labeled with dissimilar letters are significantly different ( $P < 0.05$ ). (B) Western immunoblot analysis of UCP3 and UCP2 protein levels in liver mitochondria. Gastrocnemius and spleen mitochondrial proteins were loaded as UCP3 and UCP2 positive controls, respectively. (C) Amino acid sequence alignment for the repressor peptide encoded by Open Reading Frame 1 (ORF1) of the UCP2 gene of rat, mouse, and human. The three methionines (M) in the repressor are underlined and the three corresponding regions 1, 2, and 3 are marked above. Roman numerals indicate the exons in UCP2 cDNA. Also indicated are the four ATG positions.

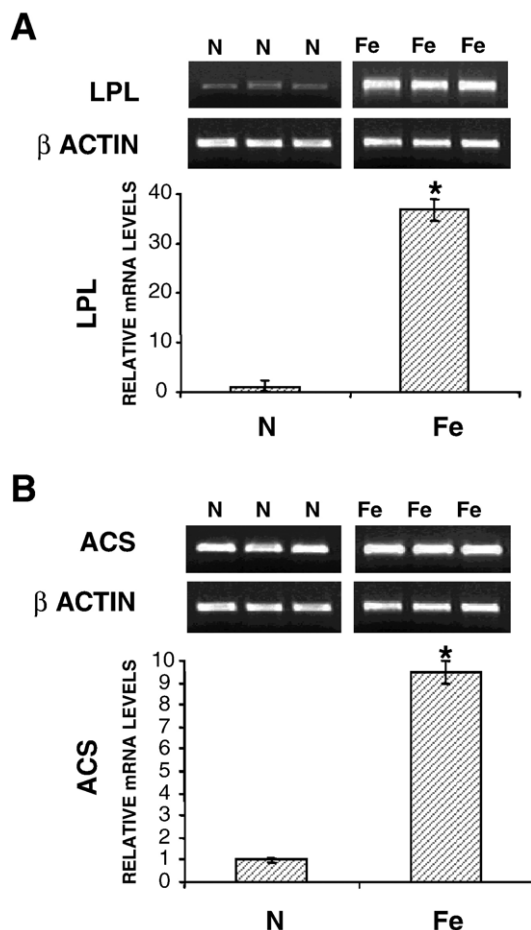


Fig. 2. Lipoprotein Lipase (LPL) and Acyl CoA Synthetase (ACS) mRNA levels in liver of N and Fe rats. RT-PCR-based measurements of LPL (A) and ACS (B) mRNA levels. Lower panels: quantification of the data shown in the upper panels. Data are expressed relative to the value obtained for N liver, which was set as 1, and are presented separately for each treatment (as indicated below the bars). Error bars represent S.D. of the mean ( $n=3$ ). \* $P<0.05$  vs. N.

mitochondria, following the addition of FCCP at the end of each run, increased by about 3 and 4 times, respectively (Fig. 5A and B). At the same time, after FCCP addition the membrane-potential decrease (represented as the TPMP+ release from mitochondria back into the medium) was lower for mitochondria from Fe rats than for those from N rats (Fig. 5A and B, blue vertical line). These data support an uncoupling effect of palmitoyl-carnitine in mitochondria from Fe rats. The changes in both respiration rate and membrane potential induced by palmitoyl-carnitine (whatever its concentration) in Fe mitochondria were completely abolished by GDP (Fig. 5C, D, and E). In contrast, GDP had no effect on mitochondria from N rats (data not shown). Moreover, in the presence of GDP, addition of FCCP led to an increase in respiration rate and a decrease in membrane potential in Fe mitochondria that were greater than the changes observed in the absence of GDP (Fig. 5C and B, blue vertical lines). Taken as a whole, these data demonstrate the involvement of UCP3 in the uncoupling effect of palmitoyl-carnitine in mitochondria from the liver of the Fe rat.

### 3.4. In vivo mitochondrial ROS production

Aconitase activity, measured as an indicator of in vivo ROS production, was significantly higher (+46%) in liver mitochondria isolated from Fe rats than in those from N rats ( $P<0.05$ ), the actual values, expressed in  $\Delta A/\text{min mg protein}$ ,

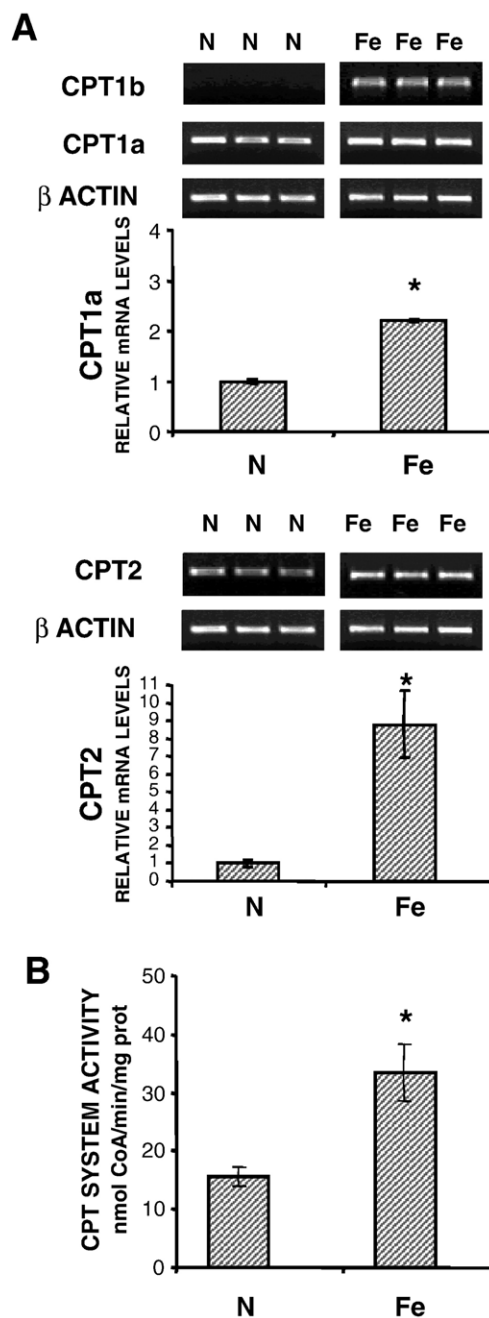


Fig. 3. Carnitine Palmitoyl Transferase (CPT) mRNA levels and total CPT activity in liver of N and Fe rats. (A) RT-PCR-based measurements of the CPT1b, CPT1a, and CPT2 mRNA levels. Lower panels: quantification of the data shown in the upper panels. Data are expressed relative to the value obtained for N liver, which was set as 1, and are presented separately for each treatment (as indicated below the bars). Error bars represent S.D. of the mean ( $n=3$ ). \* $P<0.05$  vs. N. (B) CPT-system activity in mitochondria isolated from liver of N and Fe rats. Results are each the mean  $\pm$  S.E.M. from 4 preparations. \* $P<0.05$  vs. N.

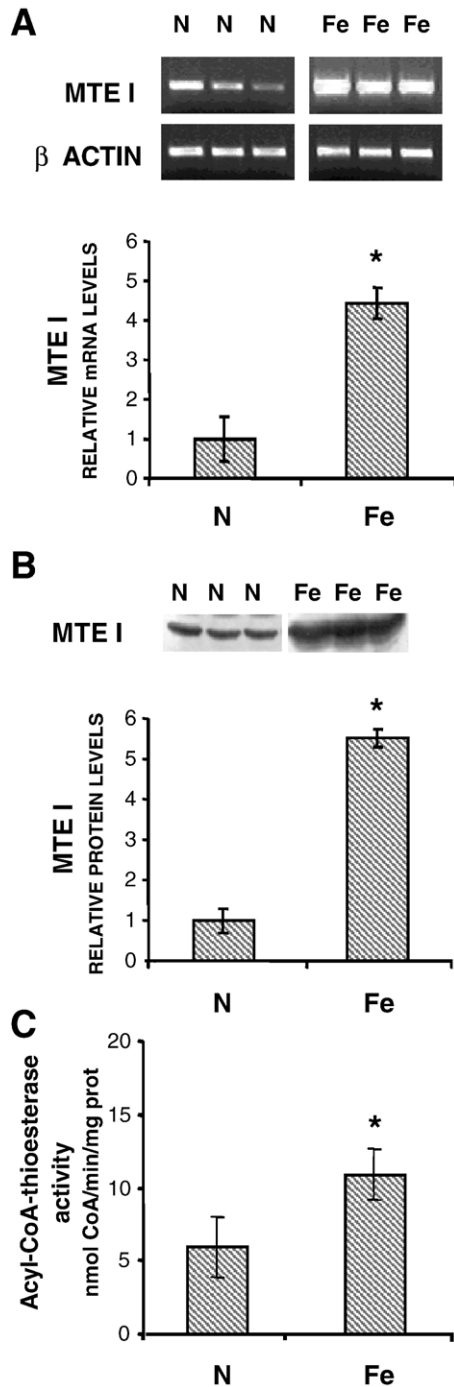


Fig. 4. Mitochondrial Thioesterase 1 (MTE-I) mRNA levels, mitochondrial protein density, and mitochondrial enzyme activity in liver of N and Fe rats. (A) RT-PCR-based measurement of MTE-I mRNA. Lower panel shows quantification of the signals shown in the upper panel (as described in Figs. 1 and 3). (B) Western immunoblot analysis of mitochondrial MTE-I protein levels. Lower panel shows quantification of the signals shown in the upper panel (as described in Figs. 1 and 3). Error bars represent S.D. of the mean ( $n=3$ ). \* $P<0.05$  vs. N. (C) Acyl-CoA-thioesterase activity of liver mitochondria isolated from N and Fe rats. Results are each the mean $\pm$ S.E.M. from 4 preparations. \* $P<0.05$  vs. N.

being  $0.200\pm0.018$  and  $0.290\pm0.035$  for N and Fe, respectively (results are each the mean $\pm$ S.E.M. from 4 preparations,  $P<0.05$  vs. N).

In addition, total aconitase activity, determined after in vitro reactivation, was not different between the liver mitochondria isolated from the two groups (data not shown). This suggests a low degree of in vivo aconitase inhibition by ROS in mitochondria from Fe-treated rats.

### 3.5. Mitochondrial CoQ levels

The amount of mitochondrial total CoQ (CoQ<sub>9</sub>+CoQ<sub>10</sub>) was increased about 3.6-fold by fenofibrate treatment (Fig. 6A). The liver HMGR and COQ7 expression levels were also affected, HMGR expression being significantly reduced by about 17% versus its level in N rats, while COQ7 expression was strongly upregulated (Fig. 6B).

## 4. Discussion

Fenofibrate treatment is known to exert a hypolipidemic effect by enhancing lipid catabolism in the liver, mainly through a PPAR $\alpha$  transcriptional induction of the genes for mitochondrial and peroxisomal oxidative enzymes. Moreover, its concomitant effect on the de novo expression of UCP3 supports a role for such a mitochondrial carrier in the biochemical pathways linked to lipid mobilization and utilization [25,26]. Previous studies indicate that the pharmacological actions of fenofibrate are mediated, at least in part, through a regulation of the transcription of a number of genes involved in lipid and lipoprotein metabolism [1]. As we expected, the present results show that fenofibrate modulates lipid metabolism by reducing the amount of fatty acids available to the liver for triglyceride synthesis, with lipoprotein lipolysis being affected via a strong induction of liver LPL expression. In addition, fenofibrate enhanced the expression of CPT1a and CPT2, and induced, as was also true for UCP3, a de novo expression of the skeletal muscle-specific CPT1b gene, concomitantly stimulating the mitochondrial fatty acid  $\beta$ -oxidation rate. It is well established that CPT1a and CPT1b have markedly different kinetic properties and intrinsic characteristics, with CPT1a having the lowest  $K_m$  for carnitine and the highest IC<sub>50</sub> for malonyl-CoA [49]. The expression pattern of the two isoforms is plastic, and responds both to physiological and developmental signals [50]. The observed de novo expression of CPT1b in the liver following fenofibrate treatment might be interpreted as an additional mechanism that serves to potentiate the mitochondrial uptake of fatty acids in a situation in which the pressure put upon the substrate by fatty acids exceeds their oxidation rate. Indeed, the activity of the CPT system was found to be significantly enhanced (+115%), pointing to a shift in liver cellular metabolism toward a channeling of fatty acids in the direction of an enhanced  $\beta$ -oxidation rate (+61%) at the expense of triacylglycerol synthesis. Moreover, the increased mRNA expression of ACS – an enzyme that prevents efflux of tissue-incorporated fatty acids by converting them into acyl-CoA-derivatives, hence rendering the FA-uptake process unidirectional – further supports the effect of fenofibrate on the biochemical pathways involved in lipid utilization by the

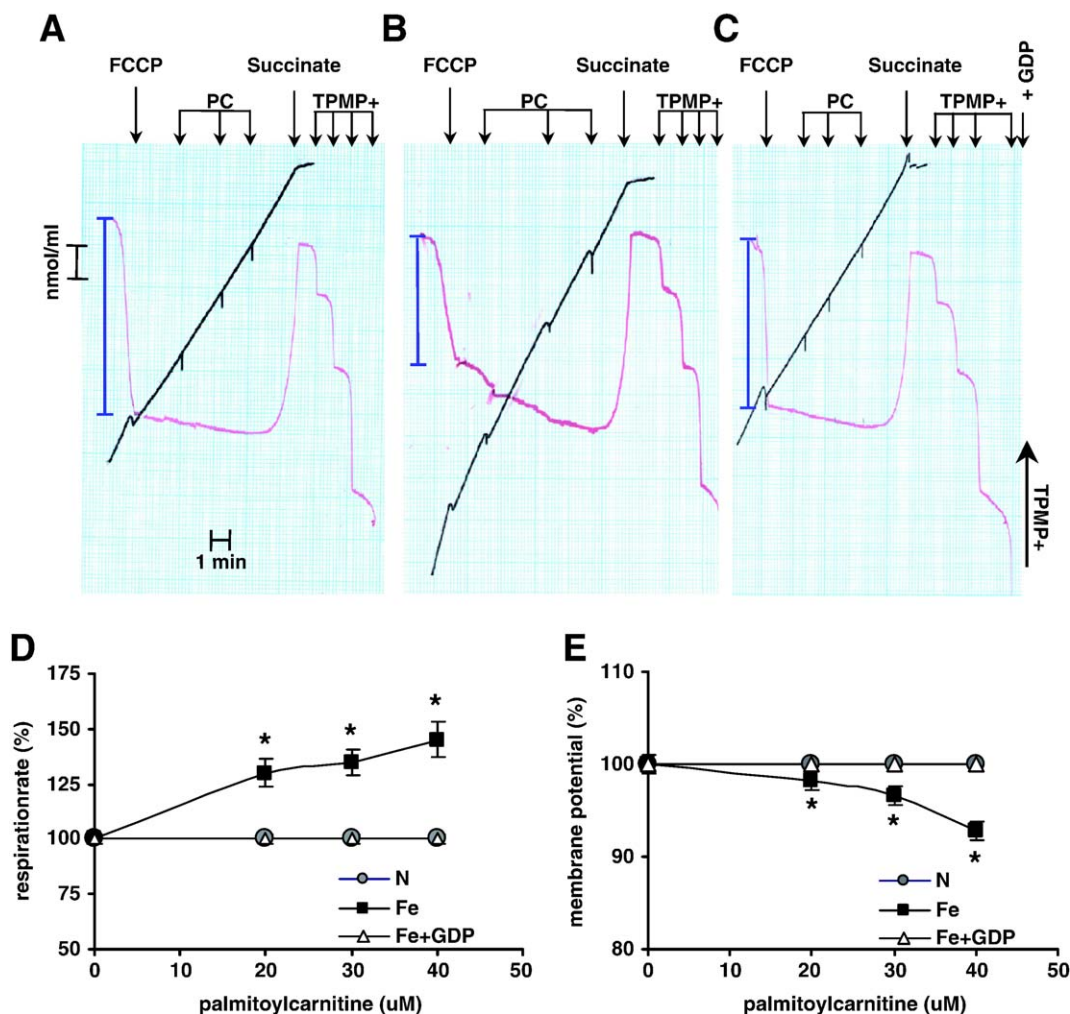


Fig. 5. Effect of palmitoyl-carnitine titration on respiration rate and membrane potential of succinate-energized liver mitochondria from N and Fe rats. Panels A–C, representative recordings showing simultaneous measurements of mitochondrial respiration and membrane potential in liver from N (A) and Fe (B and C) rats. In each panel, the black and red traces show the oxygen and TPMP<sup>+</sup> concentrations, respectively. Liver mitochondria (0.5 mg/ml) were incubated at 37 °C in the oxygen electrode chamber in a total volume of 1 ml of assay medium containing rotenone, nigericin, oligomycin, and CAT. The TPMP<sup>+</sup> and reference electrodes were inserted into the chamber, and calibrated using four sequential 1 μM additions up to 4 μM TPMP<sup>+</sup> before succinate was added to 6 mM. As shown in the recordings, the TPMP<sup>+</sup> electrode indicated uptake of TPMP<sup>+</sup> to equilibrium in response to the generation of a membrane potential following addition of succinate (where indicated). Respiration rate and membrane potential were titrated by sequential additions of palmitoyl-carnitine (PC, where indicated) up to 40 μM. After that, FCCP was added (as indicated) to dissipate the membrane potential completely, allowing evaluation of: i) the amount of TPMP<sup>+</sup> released back into the medium, ii) any small electrode drift, and iii) how much respiration could still be stimulated. GDP (where present) was added to the incubation medium before the mitochondria (C). The vertical blue line in each panel represents the amount of TPMP<sup>+</sup> released from the mitochondria back into the medium after FCCP addition. Panels D and E, the values obtained from each titration were quantified, and respiration rate and membrane potential were plotted against the palmitoyl-carnitine concentration. For each group, data are expressed relative to the value obtained in the absence of palmitoyl-carnitine (taken as 100%). Results are each the mean ± S.E.M. from 4 preparations. \**P* < 0.05 vs. N.

liver. However, it seems evident that since following fenofibrate treatment, the fatty acid supply exceeds their rate of oxidation [the fenofibrate-induced increase in CPT-system activity being almost two times the increase in the oxidation rate), such treatment will lead to an accumulation of fatty acids within the mitochondrial matrix, unless a compensatory mechanism exists. These results are in line with the hypothesis that UCP3 may be involved in the export of fatty acids and/or of their peroxidized derivatives, effects that might represent a protective mechanism for the mitochondrial matrix when the supply of fatty acids exceeds their rate of oxidation [20,24].

In this study, fenofibrate treatment enhanced the UCP2 mRNA level, but did not alter the gene expression of the recently cloned HDMCP or that of AdNT1 plus AdNT2. While the de novo-induced UCP3 mRNA expression was accompanied by the presence of the protein in the mitochondrial fraction, UCP2 protein was not detected, most likely due to the presence of an inhibitory post-transcriptional mechanism. Indeed, our sequence alignment of rat, human, and mouse UCP2 genes indicated that the rat UCP2 gene shares a very high homology with the region of the mouse and human UCP2 genes containing the repressor sequence described by Pecqueur et al. [30] in the mouse that encodes a putative peptide of 36 amino

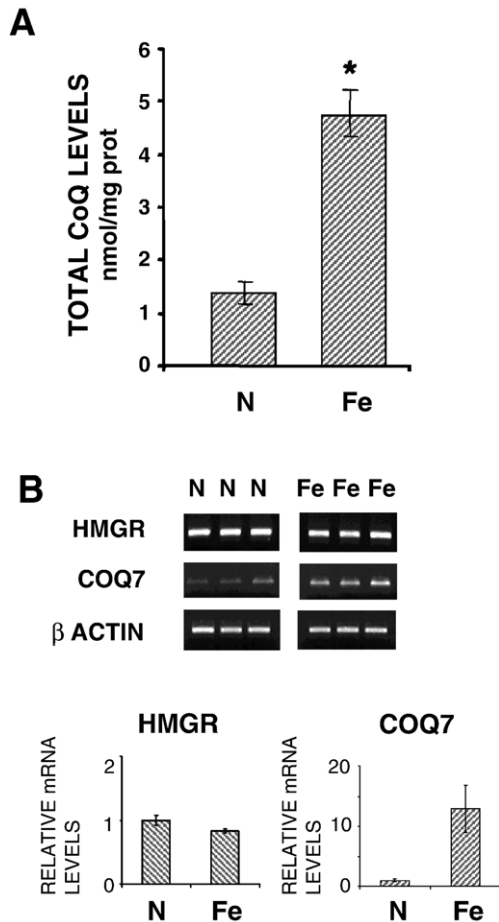


Fig. 6. Total CoQ levels, and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and Ubiquinone biosynthesis protein COQ7 homolog (COQ7) mRNA levels in liver of N and Fe rats. (A) Endogenous mitochondrial content of total CoQ (CoQ9+CoQ10, in both quinol and quinone forms) as determined by HPLC analysis of liver from N and Fe rats. Results are each the mean  $\pm$  S.E.M. from 4 preparations. \* $P < 0.05$  vs. N. (B) RT-PCR-based measurements of HMGR and COQ7 mRNA levels. Lower panels: quantification of the data shown in the upper panels. Data are expressed relative to the value obtained for N liver, which was set as 1, and are presented separately for each treatment (as indicated below the bars). Error bars represent S.D. of the mean ( $n=3$ ). \* $P < 0.05$  vs. N.

acids able to mediate cis-downregulation of the translation of the gene. Our data suggest that a similar mechanism could be operative in rat liver, too.

Fenofibrate also affected MTE-I expression and increased mitochondrial acyl-CoA-thioesterase activity, thus leading to an enhanced availability of: (i) CoA, a lack of which would lead to an inhibition of  $\beta$ -oxidation and of the Krebs-cycle, and (ii) intra-mitochondrial fatty acid anions, which could be involved in a possible UCP3-mediated uncoupling associated with a higher rate of entry into the mitochondria of activated FA, as allowed by the CPT system. Indeed, using our system – in which the addition of palmitoyl-carnitine to succinate+rotenone-energized mitochondria could provide FA inside the mitochondria by means of palmitoyl CoA hydrolysis, potentially catalyzed by MTE-I and an increased NADH level – palmitoyl-carnitine induced mitochondrial uncoupling only in mitochondria from Fe rats,

since it simultaneously induced an increase in the respiration rate and a decrease in the membrane potential. As stated in Introduction, in a previous study, in the presence of the de novo expressed UCP3, we did not observe differences in the kinetic responses of the proton leak to change in membrane potential [16]. However, in the above-cited study, we measured the basal proton leak (in the presence of BSA, which chelates fatty acids) without addition of FA such as palmitoylcarnitine. On the other hand, here we measured inducible proton conductance in the presence of palmitoylcarnitine as a provider of FA inside the mitochondria which are recognised as UCPs activators [27,28].

In the present study, in view of the presence of CAT in the incubation medium and the absence of UCP2 protein, the uncoupling effects are presumably attributable to UCP3. The simultaneous increases in MTE-I expression/activity and UCP3-mediated uncoupling would have effects on: (a) the modulation of the levels of CoA in the matrix, (b) the mitochondrial FA trafficking, and (c) the rate of ROS production, when FA utilization is stimulated. In addition, the UCP3-mediated uncoupling would result in a decrease in redox pressure by reducing ROS production. Indeed, the production of ROS is positively correlated with, and is very sensitive to, changes in mitochondrial membrane potential [51]. The data on aconitase activity reported here support this idea. The effect exerted by fenofibrate on UCP3 activity may also be related to its actions on some cofactors involved in UCP3-mediated uncoupling. Indeed, fenofibrate modulates the mitochondrial level of CoQ (one of the intracellular cofactors for UCP3-mediated uncoupling both in vitro and in vivo [27,48]) by affecting the pathways involved in its biosynthesis. It was noteworthy that while decreasing the expression levels of HMGR, which is known to be involved in systemic CoQ synthesis, fenofibrate treatment significantly upregulated the expression level of COQ7, which encodes a mitochondrial protein necessary for intramitochondrial CoQ synthesis [52,53]. In turn, its inhibition of HMGR and activation of such a mitochondrial biochemical pathway would allow fenofibrate to exert an inhibitory effect on cholesterol synthesis (via HMGR), while at the same time increasing the mitochondrial levels of CoQ, thus demonstrating that the mitochondrial hepatic levels of CoQ can be increased by fenofibrate in a manner that is independent of changes in cholesterol synthesis. As is the case for UCP3 and CPT1b, the COQ7 gene is predominantly expressed in skeletal muscle and heart under normal conditions [52]. Again, the upregulation of the expression of such genes in liver, together with the same effect on the known liver-specific ones, suggest the need for additional mechanisms to support the action of fenofibrate at the mitochondrial level. In conclusion, this study demonstrates that in rat liver, fenofibrate induces a remodelling of the pattern of gene expression that serves to modulate the biochemical pathways related to lipid handling and UCP3 activation. It seems evident that UCP3-mediated uncoupling depends on the concomitant activation of several interrelated biochemical pathways, and that the fenofibrate-treated standard-diet-fed rat may be a useful model for investigations of the biochemical functions of UCP3.

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