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THE EFFECT OF ETOMOXIR ON THE mRNA LEVELS OF ENZYMES INVOLVED IN KETOGENESIS AND CHOLESTEROGENESIS IN RAT LIVER

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Abstract—The effects of acute treatment with 2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxylate (etomoxir), an antiketonaemic and antidiabetic drug, on the mRNA levels of several regulatory enzymes of ketogenesis, cholesterogenesis, and fatty acid synthesis in rats were determined. In rats treated with etomoxir, mRNA levels for mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and carnitine palmitoyl transferase I (CPT I) remained unchanged, while mRNA levels for carnitine palmitoyl transferase II (CPT II) significantly increased 2-fold. Injection of etomoxir produced no effect on the mRNA levels of cytosolic HMG-CoA synthase but increased the mRNA levels of HMG-CoA reductase 2.5-fold. Etomoxir led to a 3-fold increase in the mRNA levels of fatty acid synthase of rats under acute treatment. Rats fed with a fat diet significantly increased the expression of mitochondrial HMG-CoA synthase, CPT I and CPT II 3-fold in all cases, while 2-(diethylhexyl)phthalate (DEHP) produced increases in the expression of these genes (5-, 4- and 12-fold, respectively). The mRNA levels of HMG-CoA reductase were not changed by either DEHP or fat diet, while DEHP increased cytosolic HMG-CoA synthase 2.5-fold. DEHP did not change the mRNA levels for fatty acid synthase. It was concluded that etomoxir does not produce its hypoketonaemic, hypocholesteraemic or hypolipogenic effects through changes in the genetic expression of the regulatory enzymes of these pathways, but probably due to the shortage of their common substrate, acetyl-CoA, because of the inhibitory action on CPT I.

Key words: etomoxir; HMG-CoA reductase; HMG-CoA synthase; D1 2-(diethyl hexyl)phthalate

Etomoxir† is a member of a family of substituted 2-oxirane-carboxylic acids that inhibit fatty-acid oxidation [1]. This family also includes palmoxiratetetradecyl - 2 - oxiranecarboxylate, 2-TDGA), and 2 - [5 - (4 - chlorophenyl)pentyl] - oxirane - 2 - carboxylate (clomoxir, POCA). Their CoA esters, formed in the cytosol from free acids, inhibit long-chain fatty acid oxidation by inhibiting CPT I, a mitochondrial enzyme located in the inner face of the outer mitochondrial membrane [2]. Etomoxir binds to this enzyme at the catalytic site producing a decrease in its activity. Because of this effect a powerful inhibition of mitochondrial β-oxidation of long-chain fatty acids is also produced as a result of the low availability of its substrate [3].

Etomoxir also provokes inhibition of gluconeogenesis and increased utilization of glucose, since the fatty acids, which are the preferred fuel for most tissues during fasting and diabetes, are unsuitable since they cannot enter mitochondria. Therefore, this compound and the other members of the family have been used for the management of diabetes [4]. Etomoxir, in addition, inhibits ketogenesis in normal fed and fasted animals and also in diabetic rats [4–7]. There are two important regulatory sites in the ketogenic process: the CPT I and CPT II system and mitochondrial HMG-CoA synthase (EC 4.1.3.5). A line of evidence, progressively sounder, shows that mitochondrial HMG-CoA synthase is a major control point in ketone body synthesis [8, 9]. Studies carried out in our laboratory showed that mitochondrial HMG-CoA synthase mRNA levels change with the activity of the ketogenic process [10].

Recent reports state that etomoxir also inhibits cholesterol synthesis in isolated rat hepatocytes [11]. A detailed study of this effect shows that the main inhibition takes place before the synthesis of mevalonate, since the conversion of mevalonate to cholesterol was only weakly inhibited by etomoxir [11]. Cholesterol synthesis and CPT activity are closely related metabolically: clomoxir, which inhibits CPT I activity, decreases cholesterol concentrations by about 50% [12]. Because of the correlation between the two systems, the evaluation of the effects of etomoxir on cholesterol homeostasis is important.

Our specific aim was to determine whether the decrease in ketone body concentration produced by etomoxir was related to the genes that control this process. Accordingly, we studied the effect of etomoxir on the expression of the genes for mitochondrial HMG-CoA synthase, CPT I and CPT II by measuring their mRNA levels. In addition, we have also studied the expression of the two control

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† Abbreviations: etomoxir, 2-[6-(4-chlorophenoxy)-hexyl]-oxirane-2-carboxylate, HMG-CoA, 3-hydroxy 3-methylglutaryl coenzyme A; CPT, carnitine palmitoyl transferase (EC 2.3.1.21); DEHP, 2-(diethylhexyl)-phthalate; FAS, fatty acid synthase (EC 2.3.1.85).

genes of cholesterogenesis previous to mevalonate synthesis [13], that is, HMG-CoA reductase (EC 1.1.1.34) and cytosolic HMG-CoA synthase, and the expression of fatty acid synthase, which is the main control point of fatty acid synthesis. We have also measured the effect of other treatments, such as fat diet and DEHP on the expression of these genes. Results show that the inhibition of ketogenesis by etomoxir is not related to the gene expression of either mitochondrial HMG-CoA synthase, CPT I or CPT II, since etomoxir does not change mRNA levels of the first two, but doubles the mRNA levels of the latter. In addition, the genetic expression of HMG-CoA reductase increased whereas that of the cytosolic HMG-CoA synthase was unchanged by the drug. Moreover, fatty acid synthase mRNA levels were tripled. All these findings suggest that the availability of the substrate in these processes, i.e. acetyl-CoA, regulated by the activity of CPT I could be the main factor responsible for decreased cholesterogenesis and ketogenesis under the action of etomoxir.

MATERIALS AND METHODS

Chemicals. Etomoxir sodium salt (B 827–33), was generously provided by H.P.O. Wolf, Byk Gulden Lomberg Chemische Fabrik GmbH (Konstanz, Germany). [α-^{32}P]dATP (3000 Ci/mmol) was from Amersham (Amersham, U.K.). The random-primed DNA labelling kit was from Boehringer Mannheim (Mannheim, Germany). The cDNA first strand synthesis kit was from Stratagene. DEHP was from Merck-Schuchardt. Other biochemical reagents were from either Boehringer Mannheim or the Sigma Chemical Co. (Deisenhofen, Germany). All chemicals were of analytical grade.

Animals. Sprague–Dawley rats (150–200 g) bred in our laboratory and fed *ad libitum* were divided into six groups. Group 1 (control) were fed standard laboratory chow: 3% lipids, 58% carbohydrate, 27% casein and 21% fibre; group 2 were fed standard chow containing DEHP: (2% w/w added as a 10% w/w solution in acetone, followed by air drying), for 14 days; group 3 were fed with a high fat diet 43% lipids (butter fat and corn oil), 10% carbohydrate, 30% casein and 17% fibre; group 4 were control-fed rats to which etomoxir (10 mg/kg) was injected i.p. 3 hr before being killed. Groups 5 and 6 were treated respectively with DEHP or a fat diet, to which etomoxir was injected 3 hr before being killed. After each treatment, rats were decapitated, and their livers were quickly removed and placed in liquid N_2. The livers were then powdered in a porcelain mortar under liquid N_2, distributed in aliquots and kept at −80°.

RNA analyses. Total RNA was prepared from rat liver by extraction with guanidine isothiocyanate and centrifugation through a CsCl cushion [14]. Aliquots of 10 μg (determined by absorbance at 260 nm) were fractionated on a 1% agarose gel containing formaldehyde, and subjected to northern transfer using an NY13 nytran filter (Scheicher & Schuell). Filters were fixed with ultraviolet light at 254 nm. After 6 hr prehybridation, using high stringency conditions (at 42° in 1 M NaCl, 50% formamide, 7.5 × Denhardt's solution [14], 0.1% SDS, 50 mM NaH_2PO_4 pH 6.3, 10% dextran sulphate and heat-denaturated salmon-sperm DNA, 100 mg/mL), the filters were hybridized overnight using the ^{32}P random-primed-labelled cDNA probe. The radioactivity was 2×10^6 cpm/mL. Filters were washed briefly at 42° in 300 mM NaCl, 30 mM sodium citrate pH 7.0 and 0.1% SDS followed by three 20 min washes at 65° in 30 mM NaCl, 3 mM sodium citrate pH 7 and 0.1% SDS. Filters were autoradiographed at −70° in contact with Kodak X-ray film with an intensifying screen.

The levels of mRNA as determined by densitometric scanning of the autoradiograms measured by Bioprofil (Vilber-Lourmat). Densitometry values were corrected by using rat albumin as a constitutive probe, which was used as control. Filters were dehybridized for 30 min at 100° with the same washing system and then rehybridized. Statistical analysis was carried out by the Student's *t*-test with significance levels chosen as $P < 0.05$ and $P < 0.01$.

DNA-hybridization probes. The following cDNA fragments were used as probes: a specific 1.5 kb *Pst*I fragment corresponding to the cDNA for rat mitochondrial HMG-CoA synthase [15], a 1.1 kb specific fragment (*Apa*I-*Sac*I) of the rat liver cytosolic HMG-CoA synthase cDNA [16], a 2.9 kb *Xba*I specific fragment from cDNA clone (pRed-227) of Chinese hamster HMG-CoA reductase [17], a 1.7 kb specific fragment *Pst*I from cDNA clone (FAS-3) of avian fatty acid synthase [18], a 1.4 kb (*Xho*I-*BamH*I) fragment from cDNA clone (pBKS-CPT II.4) for rat CPT II [19], and a 1.1 kb *Pst*I fragment from cDNA clone (pRSA 13) for rat serum albumin [20].

A partial fragment of rat CPT I cDNA, covering the region corresponding to positions 1272–2020 in the rat mRNA CPT I [21] was produced by a reverse transcriptase/polymerase chain reaction procedure using two specific primers [(5′ GCAGGGA-TACAGAGAGGAGG 3′, (CPTa), and 5′GGAAAGGTGAGTCGACTGC 3′, (CPTb)]. First strand cDNA was synthesized from 100 ng of hepatic poly $(A)^+$ RNA rat using 300 ng of random hexamers and 20 U of Moloney murine leukemia virus reverse transcriptase according to the manufacturer's recommendations (Stratagen). Amplification of cDNA was performed by adding a 2 μL aliquot of first strand cDNA reaction mixture to 98 μL containing 20 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM dNTP, 0.1 μM each of the two primers CPTa and CPTb and 1.25 U of *Taq* polymerase. After an initial denaturation step at 94° for 5 min, the following amplification program was used: denaturation 94° for 1 min; annealing at 55° for 2 min; and extension at 72° for 3 min. Thirty cycles were used for amplification. Upon completion of the thirtieth cycle the mixtures were incubated for 10 min at 72°. The amplified fragment was cloned in a *BamH*I-*Sal*I site in pBluescript SK^+ vector (Stratagene) and sequenced by using the dideoxy chain termination method [22] to confirm the identity of the CPT I cDNA. This *BamH*I-*Sal*I 715 pb fragment was used as a probe for detection CPT I mRNAs.

All DNA probes were generated by labelling

with [α-^{32}P]dATP to a specific radioactivity of 1.5×10^9 cpm/μg of DNA by random priming with Klenow polymerase.

RESULTS

Ketogenesis

Since etomoxir decreases blood ketone body levels, we attempted to determine whether the genetic expression of enzymes involved in ketogenesis regulation could be modified by etomoxir. Representative northern blot analyses for hepatic mRNA for two rats from each diet or treated group are shown in Fig. 1 panel A. The combined data from six rats per diet group are presented graphically (mean ± SEM) in Fig. 1, panels B, C and D. Injection of etomoxir to normal-fed rats produced no significant change in the mRNA levels for mitochondrial HMG-CoA synthase with respect to untreated rats, Fig. 1 panel B. In contrast, the addition to diet of either DEHP or fat produced significant increases in mRNA levels: 5- and 3-fold, respectively ($P < 0.01$). The combined action of DEHP and etomoxir increased the mRNA levels 5.5-fold with respect to control. This increase with respect to DEHP alone is not significant ($P = 0.09$). Etomoxir plus the fat diet produced a 1.5-fold decrease with respect to the effect produced by fat alone ($P < 0.01$). Taken together, these results suggest that the decrease in ketone bodies observed after administration of etomoxir cannot be attributed to a change in the mRNA levels of mitochondrial HMG-CoA synthase.

The changes produced by the same effectors on CPT I and CPT II hepatic mRNA levels are presented graphically in Fig. 1, panels C and D. Etomoxir produced no change in expression of CPT I but led to a 2-fold increase in the expression of CPT II versus control ($P < 0.05$), while DEHP increased CPT I and CPT II mRNA levels 4- and 12-fold, respectively ($P < 0.01$). High fat diet increased the mRNA levels of CPT I and CPT II; 3-fold in each case ($P < 0.01$). The combined action of DEHP and etomoxir did not significantly increase the mRNA levels with respect to DEHP alone either in CPT I or CPT II. The combination of etomoxir and fat increased the mRNA levels for CPT I 6.5-fold. This increase is significant versus fat alone ($P < 0.01$).

Cholesterogenesis

It has been described that etomoxir inhibits the conversion of [2-^{14}C]acetate into cholesterol, and that this effect is produced before the synthesis of mevalonate [11]. To test whether the action of etomoxir was bound to the decrease in expression of the enzymes involved in cholesterogenesis regulation, cytosolic HMG-CoA synthase and HMG-CoA reductase mRNA levels were determined after the injection of etomoxir to a group of rats. Results seen in Fig. 2 show that there was no significant change versus control ($P = 0.1$) in cytosolic HMG-CoA synthase and a 2.5-fold increase *versus* control in mRNA levels of HMG-CoA reductase ($P < 0.02$). The action of fat was negligible with respect to control. In contrast, DEHP produced a 2.5-fold increase in cytosolic HMG-CoA synthase ($P < 0.01$) but did not change HMG-CoA reductase mRNA levels. The association of etomoxir and each of these two effectors did not significantly change the RNA levels versus the other effectors alone. These changes in the mRNA levels of the regulatory enzymes of cholesterol synthetic pathway are insufficient to explain the decrease observed in cholesterol synthesis caused by etomoxir.

Fatty acid synthesis

To explore whether etomoxir modifies the expression of fatty acid synthase, the main control point of fatty acid synthesis, the mRNA levels for this enzyme were determined. As shown in Fig. 3 there is a 3-fold increase in the mRNA levels produced by etomoxir compared to control and a 2-fold increase produced by fat ($P < 0.05$). The increase produced by the fat diet on fatty acid synthase mRNA levels is of the same order as that described by Schillabeer *et al.* [23] for a high fat diet. The influence of DEHP alone or in combination with etomoxir did not produced significant changes with respect to control.

DISCUSSION

Etomoxir and other related substances produce notable changes in metabolism, which may be summarized as follows: a decrease in blood glucose, concomitant with a more rapid utilization of glucose, decreased gluconeogenesis, a marked decrease in blood ketone body levels, a decrease in plasma cholesterol together with a large decrease in the carbon flux through the isoprenoid pathway, and a decrease in fatty acid synthesis.

The molecular mechanisms of etomoxir action have not been completely elucidated. It is known that etomoxir covalently and irreversibly binds to the molecule of CPT I leading to its inactivation [24], and, in addition, other carnitine acyltransferases are also inhibited [25]. CPT II is inhibited by etomoxir but at higher concentrations, and the binding is reversible [26]. However, nothing has been published, to our knowledge, on the influence of etomoxir on regulation of gene transcription or translation. Other hypolipidaemic drugs such as clofibrate or DEHP act by binding to a specific receptor modulating DNA transcriptional activity and inducing peroxisomal proliferation [27, 28]; other drugs such as cipofibrate and clofibric acid increase the amount of carnitine palmitoyl transferase protein by 23 and 97%, respectively; changes in protein and RNA synthesis precede the drug-induced increases in carnitine acetyltransferase activity [29]. The possibility that etomoxir may have a similar mechanism of action cannot be ruled out.

Ketogenesis changes dramatically by the action of etomoxir. In less than two hours ketone body levels practically disappear from blood in both normal fed and fasted, and in diabetic rats [4–7]. It has recently been reported that mitochondrial HMG-CoA synthase is a major control point of ketogenesis. Several effectors that increase ketone body synthesis also increase the expression of mitochondrial HMG-CoA synthase: dibutyril cAMP, fasting, fat diet and

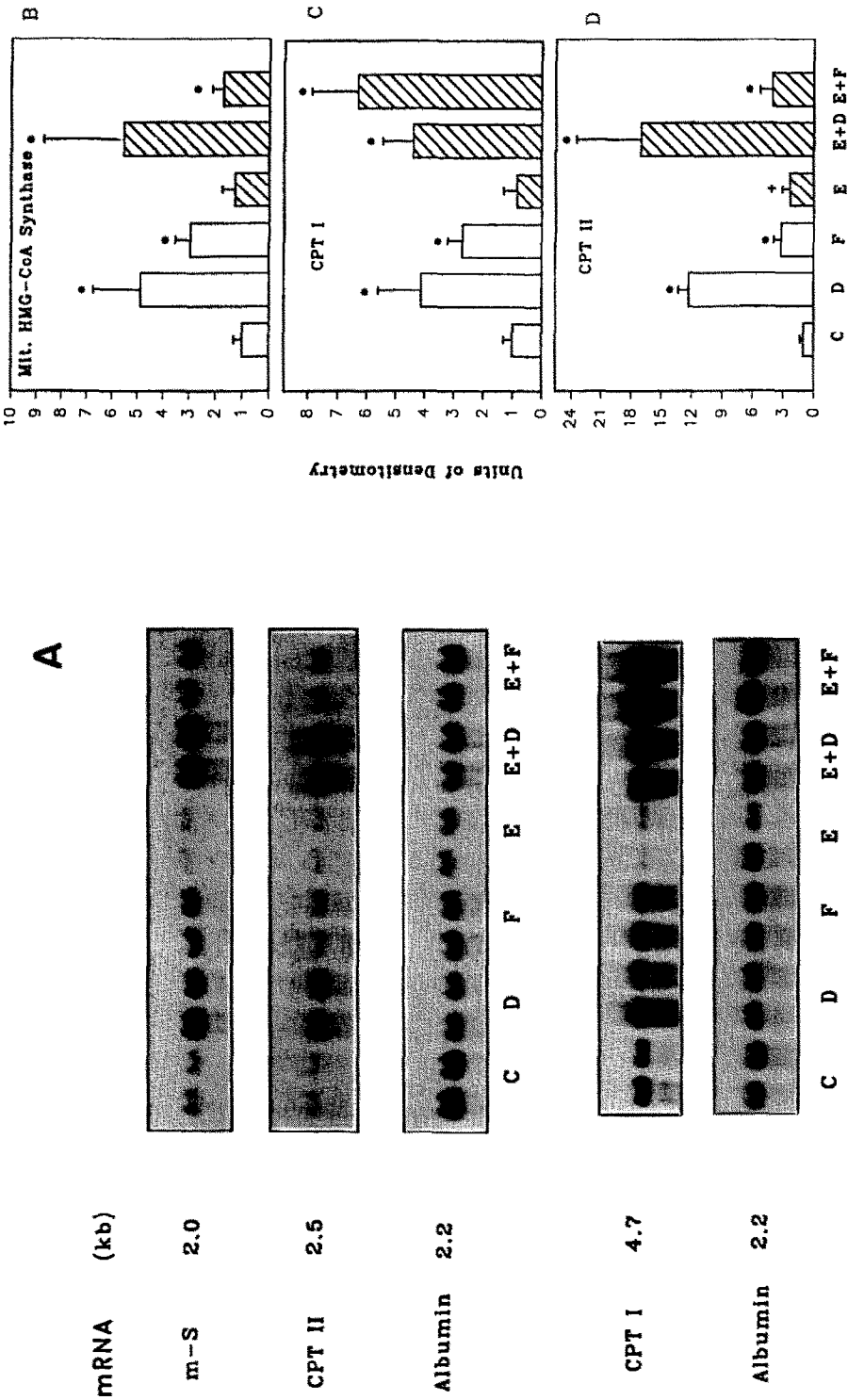

Fig. 1. Effect of etomoxir, DEHP and fat diet on the hepatic mRNA levels of mitochondrial HMG-CoA synthase, carnitine palmitoyl transferase I and carnitine palmitoyl transferase II. To the left (panel A) is shown one representative northern blot analysis for hepatic mRNA from two rats fed with standard diet (C), diet supplemented with DEHP (D) and a high fat diet (F). Administration of etomoxir (10 mg/kg) ip 3 hr before killing to rats feeding on standard diet, DEHP diet and high fat diet is denoted as E, E + D and E + F, respectively. In the northern blot analysis, 10 μg of total RNA were transferred to nylon membranes after electrophoresis in 1% agarose and hybridized using as a probe the cDNA for mitochondrial HMG-CoA synthase (m-S), carnitine palmitoyl transferase II (CPT II) and albumin, respectively. In the lower part of panel A, another representative northern blot was hybridized using as a probe the cDNA for carnitine palmitoil transferase I (CPT I). The mRNA levels of *CPT I* are shown together with those of the corresponding albumin. The mRNA size is indicated. To the right (panels B, C and D) quantitative comparison of mRNA levels of livers of rat measured by densitometeric scanning of the autoradiograms The data were corrected for the amount of albumin mRNA. The values represent means ± SEM of six different animals for each diet or treatment. Significance of differences versus control rats are, * $P < 0.01$ and + $P < 0.05$.

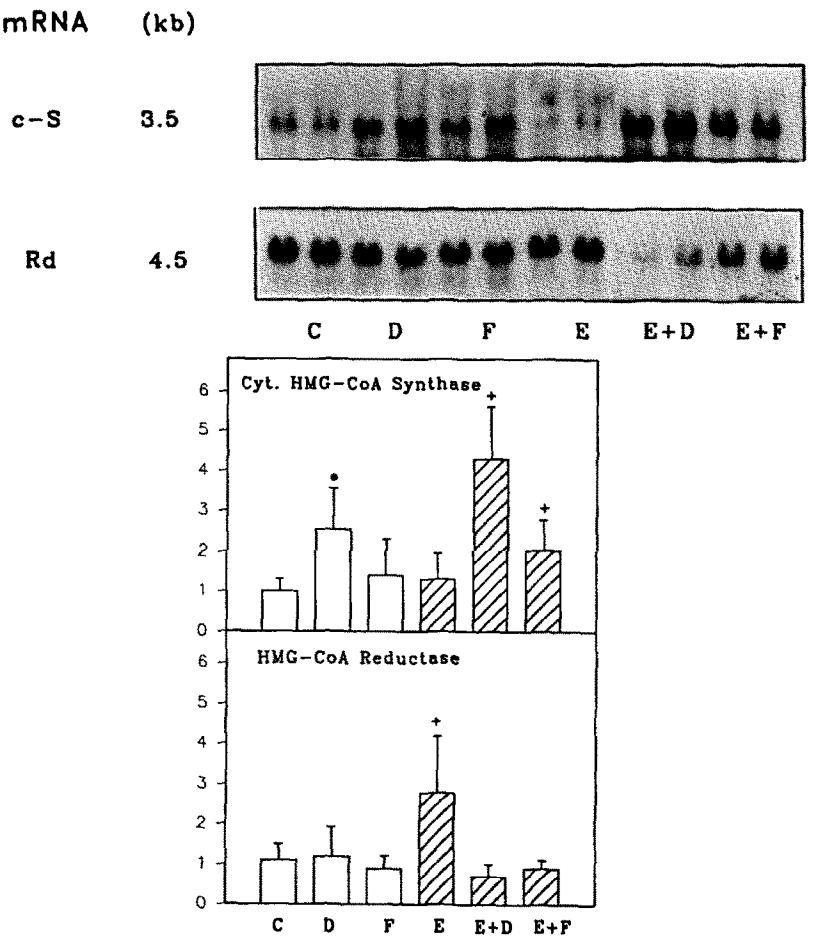

Fig. 2. Effect of etomoxir, DEHP and fat diet on the hepatic mRNA levels of cytosolic HMG-CoA synthase and HMG-CoA reductase. At the top is shown one representative northern blot analysis for two rats from each diet or treatment group. Notations and procedures as in Fig. 1. The filter was hybridized using as a probe the cDNA for cytosolic HMG-CoA synthase (c-S) and HMG-CoA reductase (Rd). At the bottom, quantitative comparison of mRNA levels measured by densitometric scanning of the autoradiograms. The data were corrected for the amount of albumin mRNA present in Fig. 1. The values represent means ± SEM of six different animals for each diet or treatment. Significance of differences versus control rats are, * $P < 0.01$ and + $P < 0.05$.

diabetes increase the expression, while refeeding and insulin, which decrease blood ketone body concentrations, also repress the expression of this gene [10]. In order to determine whether etomoxir could influence the genetic expression of this enzyme, the mRNA levels were measured. There appeared to be no change in the expression of mitochondrial HMG-CoA synthase under this drug treatment; changes caused by a fat diet were as expected: that is, an increase in expression [8]. An increase in the expression of mitochondrial HMG-CoA synthase has also been observed under DEHP treatment. It is known that DEHP not only produces peroxisome proliferation but also indices mitochondrial enzymes such as delta 3, delta 2-3-enoyl-CoA isomerase [30] and enzymes associated with smooth endoplasmic reticulum such as cytochrome P452 (clorfibrate-induced cytochrome P450) [31].

The study of etomoxir action on CPT I and CPT II showed that only CPT II mRNA levels increased. It is difficult to explain how etomoxir may promote the entry of fatty acid to mitochondria through the increase in CPT II mRNA levels. However, the β-oxidation process remains arrested. The mRNA levels of CPT I and CPT II, together with those of mitochondrial HMG-CoA synthase suggest that the decrease in blood ketone body concentration produced by etomoxir is not due to a repression of the genes involved, but probably to the low substrate availability for CPT II and mitochondrial HMG-CoA synthase, produced by the inhibition of CPT I activity.

The inhibition of cholesterogenesis by etomoxir before mevalonate synthesis reported in literature, suggested that cytosolic HMG-CoA synthase and/or HMG-CoA reductase could be involved in this inhibition. As in the case of ketogenesis, the action of etomoxir was the opposite of what was expected;

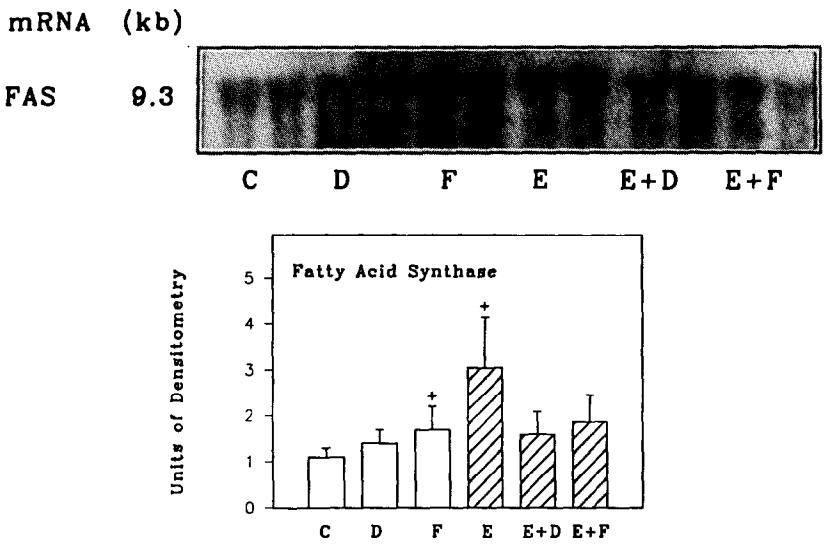

Fig. 3. Effect of etomoxir, DEHP and fat diet on the hepatic mRNA levels of fatty acid synthase. Notations and procedures as in Fig. 1. The northern blot of a representative experiment is shown at the top. The filter was hybridized using as a probe the cDNA for fatty acid synthase (FAS). The data are corrected for the amount of albumin mRNA present in Fig. 1. The values represent means ± SEM of six different animals for each diet or treatment. Significance of differences versus control rats are, * $P < 0.01$ and + $P < 0.05$.

HMG-CoA reductase increased and cytosolic HMG-CoA synthase mRNA levels were unchanged. Again, it seems that the absence of acetyl CoA may be responsible for the decreased cholesterogenic carbon flux, since there was no repression of etomoxir on the genes for either enzyme.

The inhibition of long-chain fatty acid synthesis by etomoxir has also been reported, although FAS activity was not affected [11]. We explored whether the genetic expression of FAS was the main reason for this phenomenon. There was no decrease in the mRNA levels compared with control, but rather an increase.

The increase in the expression of several genes related to lipid metabolism caused by etomoxir (3-fold in FAS mRNA levels, 2.5-fold in HMG-CoA reductase mRNAs and 2-fold in CPT II mRNAs) suggests that etomoxir may either stabilize the mRNA levels or interact with the promoters of these genes, modifying their transcriptional rate. Other factors derepressing the genes involved, such as absence of final product (e.g. cholesterol acting on the promoter of both of the genes for HMG-CoA synthase and HMG-CoA reductase) cannot be ruled out [32].

Our results seem to suggest that a common cause may link the metabolic pathways studied in relation with etomoxir action: CPT I is strongly inhibited by etomoxir. Under this hypothesis, the entry of fatty-acyl CoA and its β-oxidation is slowed by the absence of its substrate, acyl-CoA. Accordingly, the levels of acetyl-CoA inside the mitochondria may be severely reduced, and the HMG-CoA pathway that produces ketone bodies is extremely slow. Levels of cytosolic acetyl CoA are also drastically reduced because that the absence of acetyl CoA in mitochondria prevents the synthesis of citrate, which cannot leave the mitochondria for cytosol. Under these conditions ATP citrate lyase does not produce cytosolic acetyl CoA and then both cytosolic fatty acid synthesis and cholesterol synthesis slow because of the absence of substrate, and levels of both plasma cholesterol and triglycerides fall. The need for ATP synthesis induces the glycolytic pathway, and an important lowering of blood glucose is produced.

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