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# EFFECTS OF CLOFIBRATE AND ACETYLSALICYLIC ACID ON HEPATIC CARNITINE PALMITOYLTRANSFERASE SYNTHESIS

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**Abstract**—Clofibrate and acetylsalicylic and have both been reported to increase carnitine palmitoyltransferase (CPT) activity when administered to rats. The purpose of the present study was to determine the mechanism of the increase in CPT activity. Rats (150–200 g) were fed one of the following: chow, chow with 0.5% clofibrate, or chow with 1% acetylsalicylic acid for 2 weeks. At the end of this time, hepatic CPT activity was increased 4-fold over control in the clofibrate group and 3.6-fold over control in the acetylsalicylic acid group. Immunoreactive protein increased 4.0- and 3.6-fold, respectively, over control. Transcription rates of hepatic nuclei were increased 2.8- and 1.9-fold over control in the clofibrate and acetylsalicylic acid groups, and hepatic mRNA levels increased 2.8- and 2.0-fold respectively. These data indicate that increases in CPT activity caused by clofibrate and acetylsalicylic acid administration are due, at least in part, to increased CPT protein, resulting from increased transcription rate and levels of mRNA specific for CPT.

Clofibrate is a widely used hypolipidemic drug that leads to peroxisomal and mitochondrial proliferation in rodent liver. The mitochondrial proliferation has been found to be associated with increases in β-oxidation, ketogenic capacity, and carnitine palmitoyltransferase (CPT) activity [1–4]. However, there is currently no information on how clofibrate acts to increase CPT activity. We found that CPT mRNA and transcription rates were increased 3- to 5-fold by feeding the plasticizer 2-(diethylhexyl)-phthalate (DEHP) to rats (P. S. Brady and L. J. Brady, unpublished observations). The ability of DEHP to proliferate peroxisomes is similar to that of clofibrate and other hypolipidemic drugs and, thus, it has been suggested that these agents may act by similar mechanisms. Therefore, our initial hypothesis was that clofibrate may act to increase CPT mRNA levels and transcription rates. Acetylsalicylic acid is another pharmacological agent which has been reported to increase CPT activity. Ishii and Suga [5] documented that acetylsalicylic acid has clofibrate-like effects and increases CPT activity per g liver. However, their data failed to show whether the increase in CPT activity was due to increased mitochondrial mass per g liver or to increased CPT activity per mitochondrial mass.

Thus, the purpose of the present study was to determine if clofibrate and acetylsalicylic acid act to increase CPT activity by increasing synthesis. We examined the effects of clofibrate and acetylsalicylic acid on CPT activity, immunoreactive protein, mRNA levels, and transcription rates in rat liver.

The CPT which we studied here is the 68 kD protein. There is a general agreement that this is one of the mitochondrial CPTs. Whether it is the only mitochondrial CPT is an area of active dispute [6–12]. In the present paper we do not attempt to assign location of the protein to a specific mitochondrial site, nor do we dispute that there may be a second CPT protein other than the one reported here. However, this protein does respond to physiological, pharmacological and nutritional manipulations with changes in its synthesis. Thus, it must be considered in discussion of the regulation of mitochondrial fatty acid oxidation by carnitine palmitoyltransferase(s).

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## METHODS

*Animals and diets.* Male Sprague–Dawley rats (150–200 g) were adapted to Purina laboratory chow for 4 days and then placed on the following diets for 2 weeks: chow, chow with 0.5% clofibrate added, or chow with 1% acetylsalicylic acid added. The drugs were added to the chow by dissolving in acetone and then spraying onto the chow pellets and allowing the acetone to evaporate. Body weights of rats on each diet at the end of 2 weeks were: 257 ± 3 g (mean ± SE) for chow-fed, 240 ± 5 g for clofibrate-fed, and 247 ± 11 g for acetylsalicylic acid fed rats respectively. Liver weights were: 14.4 ± 5 g, 18.6 ± 0.6 g, and 14.0 ± 0.7 g respectively. Mitochondria were isolated as described, omitting the extensive washing steps [13]. CPT specific activity (nmol/min/mg) was lower in these preparations than in the more extensively washed preparations.

*CPT activity, immunoreactive protein, mRNA determination, and transcription.* CPT activity was determined as described, with a modification to a 25° temperature [14]. The purification of CPT and the documentation of the antibody specificity have been presented previously [14]. The protein does not

cross-react with anti-peroxisomal carnitine octanoyltransferase (provided by Dr. Loran Bieber) or to anti-peroxisomal "soluble carnitine palmitoyltransferase" (provided by Dr. Rona Ramsay). Immunoreactive CPT was determined by solubilizing isolated mitochondria in 0.1% Triton. An equivalent quantity of mitochondrial protein (10 μg) was plated onto 96 well plates and dried *in vacuo*. The plates were blocked with 0.1% bovine serum albumin. The primary antibody (rabbit anti-CPT) was diluted (1:1000) in 10 mM Tris, (pH 7.5), 150 mM NaCl, and 0.5% Tween 20, and allowed to react for 1–2 hr. The plates were washed three times with this buffer and allowed to react with goat anti-rabbit IgG linked to horseradish peroxidase for 1 hr. The plates were washed five times with the same buffer, and then substrate was added [1:1 mixture of hydrogen peroxide and 2,2′-azino-d(3-ethyl-benzthiazoline sulfonate)] and developed for 1 hr. The absorbance was then determined at 414 nm (using a BioRad plate reader model 2550) relative to samples without the primary antibody.

A 17-mer oligonucleotide probe was developed based on the N-terminal amino acid sequence of the protein as described [15] and used to hybridize total cellular RNA. This probe gave identical results to the cDNA clone probe in Northern blots. Total RNA was glyoxylated and separated by submarine electrophoresis in 1% agarose in 0.01 M $NaH_2PO_4$ (pH 7.5) with recirculation of the buffer and subsequently transferred to nitrocellulose in 20× saline sodium citrate (SSC) [16]. An RNA ladder was used as standard. The lanes containing standards were cut from the gel prior to Northern transfer and visualized with ethidium bromide staining. Prehybridization, hybridization, and washing at 42° were as described [17]. The CPT mRNA corresponded to approximately 2500 base pairs. (bp). A representative Northern blot is presented in Fig. 1. After we verified that the probe was effective, a dot blot procedure was used for quantitation of mRNA as described [15]. The dot blot data for this experiment are presented in Fig. 2.

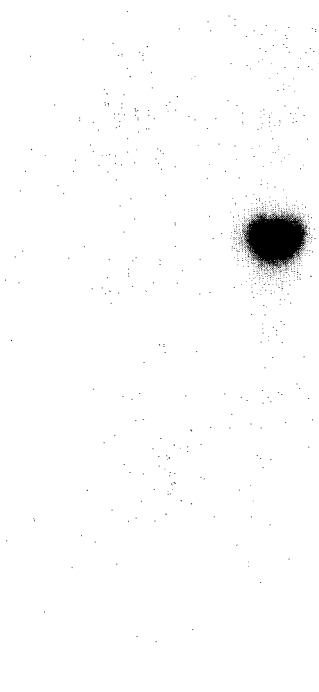

Fig. 1. Representative northern blot of CPT RNA. Total RNA was prepared from rat liver by the guanidium/cesium chloride method and 10 μg glyoxalated RNA and was run on a 1% agarose submarine gel as described (Methods). The RNA was capillary transferred to nitrocellulose using 20 × SSC. The blot was processed as described in Methods and hybridized with the $^{32}$P-labeled 17-mer. The blot was exposed to X-ray film at −70° for 7 days using intensifying screens. The position of the band was determined by comparison to an RNA ladder which was ethidium bromide stained. The RNA corresponded to 2.5 kb.

For the transcription assays, $pBluescript_{3A15.1.1}$ (Clone described previously—Ref. 15) was used after linearization with Bam HI and alkaline denaturation. $pBluescript_{3A15.1.1}$ appears to have an insert size of 2.5 kilobases (kb) based on agarose electrophoresis following Eco R1 digestion. The identity of the clone was confirmed by generating $T_3$ RNA polymerase transcript with 5′7meGppp5′G cap from Hind III restricted $pBluescript_{3A15.1.1}$. The resulting mRNA (2.1 to 2.3 kb) was translated in the standard reticulocyte lysate system with [$^{35}$S]methionine and containing RNasin. CPT activity was detected using the standard activity assay modified to include 20% dimethyl sulfoxide. No CPT activity was found using brome mosaic virus mRNA. The size of the translation product was 70.6 kD as determined from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) autoradiogram [15]. [$^{14}$C]Protein standards were included for this determination.

For the *in vitro* transcription assays (nuclear run-on assays), the $pBluescript_{3A15.1.1}$ clone was restriction digested with BAM HI and denatured as described by Marzluff and Huang [17]. The $pBluescript_{3A15.1.1}$ was spotted onto nitrocellulose filters (5 μg per dot in a Schleicher & Schuell dot blot manifold) in 2× SSC and baked at 80° for 2 hr *in vacuo*. The filters were prehybridized at 52° prior to hybridization with the RNA for 48 hr. Rat liver nuclei were isolated as described by Morris *et al.* [18]. Nuclei were stored at −70° following centrifugation through a 2 M sucrose cushion. The *in vitro* transcription assay reaction mix and RNA isolation were as described by Morris *et al.* [18].

*Materials.* Eco R1, Hind III, and Pst 1 were purchased from Bethesda Research Laboratories (Bethesda, MD). pBluescript and $T_7/T_3$ mRNA capping kit were obtained from Stratagene (San Diego, CA). Rabbit reticulocyte lysate translation and processing kits (endonuclease-treated) and RNasin were obtained from Promega Biotech (Madison, WI). CoA was purchased from Pharmacia-PL Biochemicals (Piscataway, NJ). l-Carnitine was the gift of Sigma-Tau (Rome, Italy). Labeled methionine, ATP, and UTP were obtained from DuPont-New England Nuclear (Boston, MA). Nitrocellulose was obtained from Schliecher & Schuell (Keene, NH). Clofibrate and acetylsalicylic acid were obtained

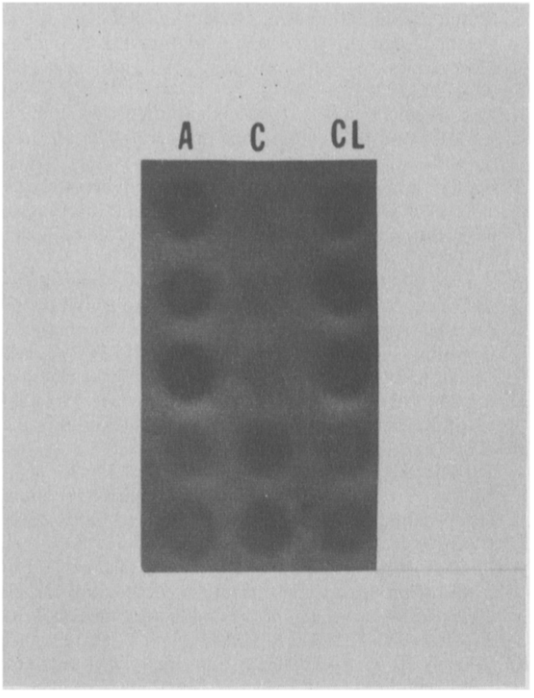


Fig. 2. RNA dot blot of control, acetylsalicylate- and clofibrate-treated rats. Total hepatic RNA was prepared and 10 μg applied to nitrocellulose in 15× SSC. The blot was probed as described in the legend of Fig. 1, and intensity of the dot was determined by densitometry. Each column corresponds to a treatment which contained five samples (five dots represented/column): A, acetylsalicylate; C, control; and CL, clofibrate.

from Sigma Chemical, St. Louis, MO. All other chemicals were molecular biology grade, where available, or highest purity. For most purposes, water was diethylpyrocarbonate-treated.

*Statistics.* Data were analyzed by analysis of variance (ANOVA) for a multifactor design with unequal numbers, using a general linear model with the PC-SAS system. Statistical significance is defined as $P < 0.05$.

## RESULTS AND DISCUSSION

Administration of both clofibrate and acetylsalicylic acid increased CPT activity and immunoreactive protein. Clofibrate increased CPT activity 4-fold (control = 7.1 vs 28.2 nmol/min/mg; SEM = 2.6, $P < 0.01$) and immunoreactive protein 4-fold (SEM = 0.4, $P < 0.01$), whereas acetylsalicylic acid increased CPT activity 3.6-fold (7.1 vs 25.5 nmol/min/mg; SEM = 2.6) and immunoreactive protein 3.6-fold. These relationships are presented in Fig. 3A ($r = 0.84$, $P < 0.01$).

The relationship between CPT mRNA and transcription rates is presented in Fig. 3B ($r = 0.78$, $P < 0.01$). Compared to control, clofibrate increased $mRNA_{CPT}$ 2.8-fold and acetylsalicylic acid, 2.0-fold (SEM = 0.4, $P < 0.05$); transcription of $mRNA_{CPT}$ was increased 2.8-fold with clofibrate treatment and 1.9-fold with acetylsalicylic acid (SEM = 0.2, $P < 0.01$).

These strong correlations between CPT activity, immunoreactive protein, mRNA, and transcription rate indicate that one mechanism for the increase in CPT activity seen with clofibrate or acetylsalicylic acid administration is an increase in CPT protein, caused by increased $mRNA_{CPT}$ and an increased transcription rate of $mRNA_{CPT}$. We have also shown that the increase in CPT activity and translation with DEHP administration is due to increased $mRNA_{CPT}$ levels and transcription (P. S. Brady and L. J. Brady, unpublished observations). Chatterjee and Roy [19] have shown previously that mRNA for carnitine octanoyltransferase (COT) is increased with Wy-14,643, a potent peroxisomal proliferating drug. Reddy *et al.* [20] have found that mRNA and transcription rate for fatty acyl CoA oxidase and enoyl CoA hydratase in peroxisomes are co-ordinately regulated by DEHP, clofibrate, and ciprofibrate. Thus, there is previous evidence that these drugs do influence mRNA levels and transcription rates of enzymes involved in β-oxidation in peroxisomes. The correlation of activity, immunoreactive protein,

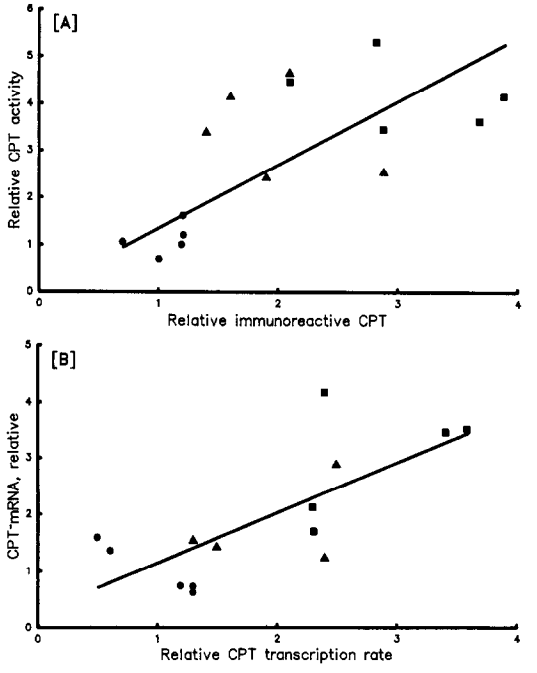


Fig. 3. Relation of CPT activity and immunoreactive CPT, and mRNA and transcription rate in acetylsalicylate- and clofibrate-treated rats. (A) CPT activity and immunoreactive CPT were determined as described in Methods. Each value is expressed relative to the mean control value (7.1 nmol/min/mg). Treatments were as follows: (●) control; (▲) acetylsalicylate; and (■) clofibrate. Correlation was determined by least squares procedures. The correlation coefficient was 0.84 (13 df, $P < 0.01$). (B) CPT RNA levels were determined by dot blot (Fig. 2), and CPT transcription rate was determined by nuclear run on assays (see Methods). Values are expressed relative to mean control values. Correlation was determined by least squares procedures. The correlation coefficient was 0.78 (12 df, $P < 0.01$).

mRNA, and transcription rates for these pharmacological manipulations corresponds well with data obtained for physiological and pathological states, such as starvation, riboflavin deficiency, and diabetes [15, 21]. Thus, a major role of long-term regulation of CPT is via synthesis. Data obtained in our laboratory also indicate that glucagon and cAMP administration increased $mRNA_{CPT}$ and transcription rate, while insulin depressed these variables to control levels (P. S. Brady and L. J. Brady, unpublished observations). Therefore, glucagon may be responsible for the physiological increases in CPT synthesis and activity seen in states with a low insulin/glucagon ratio, such as starvation and diabetes. It is more difficult to envision the mechanism of clofibrate and acetylsalicylic acid that leads to increases in CPT mRNA and transcription. Do they, or a metabolite derived from them, act directly on transcription of RNA from DNA or do they act via a common cellular intermediate? DEHP administration caused increased transcription rate and mRNA for CPT, as did glucagon, but there was no additive effect of DEHP + glucagon. Does this indicate that these agents act via a similar mechanism on a common regulatory unit?

Finally, the data presented in this report suggest that the regulation of CPT is more complex than previously thought. Most work has focused on short-term regulation of the "outer CPT" by malonyl CoA. The "outer CPT" has been assumed to be the only regulatory enzyme based on these data. The question of whether CPT is one or two distinct proteins has not been answered, and the data presented here certainly do not answer the question. We do not attempt to assign a location to the 68,000 dalton CPT at this point, nor do we suggest that it is the only CPT. The major conclusion of the present paper is that the CPT protein that we have purified and cloned appears to be regulated by synthesis in response to various physiological and pharmacological stimuli. This does not rule out a regulatory role for another distinct CPT protein, nor does it diminish the role of malonyl CoA in short-term regulation of the "outer" CPT.

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