Biochemical Pharmacology, Vol. 41, No. 11, pp. 1773–1775, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Enhancement of NAD-linked isocitrate dehydrogenase activity in rat liver by clofibrate feeding

(*Received* 19 *September* 1990; *accepted* 10 *December* 1990)

Clofibrate (ethyl 2-(4-chlorophenoxy)-2-methylpropionate) is used as a hypolipidemic drug in humans [1]. Its chronic administration to rats induces hepatomegaly [2] and proliferation of liver peroxisomes [3]. It induces many enzyme systems concomitantly: peroxisomal fatty acid β-oxidation [4], peroxisomal, microsomal, and mitochondrial carnitine acetyl- and octanoyltransferase [5], (acetyl-CoA): carnitine *O*-acetyltransferase, EC 2.3.1.7 and octanoyl-CoA: carnitine *O*-octanoyltransferase, EC 2.3.1.x), mitochondrial glycerol-3-phosphate dehydrogenase (G3PDH*; *sn*-glycerol-3-phosphate: (acceptor) oxidoreductase, EC 1.1.99.5) [3] and choline dehydrogenase (choline: (acceptor) oxidoreductase, EC 1.1.99.1) [6] have all been reported to be affected. These effects may contribute to the lowering of plasma lipids caused by clofibrate.

Some enzymes of the mitochondrial compartment, namely malate and succinate dehydrogenase [7], (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37 and succinate: (acceptor) oxidoreductase, EC 1.3.99.1), and NADP-linked isocitrate dehydrogenase (NADP-ICDH; threo-D_s-isocitrate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) [8], are essentially unchanged by clofibrate. The remaining enzymes of the citrate cycle have not been studied in this respect. In particular the NAD-linked isocitrate dehydrogenase (NAD-ICDH; threo-D_s-isocitrate: NAD^+ oxidoreductase, EC 1.1.1.41) deserves some attention in connection with clofibrate. The activity of this citrate cycle enzyme, which is increased by high NAD^+/NADH and ADP/ATP ratios [9], influences the intramitochondrial concentration of citrate. Citrate in turn is the key intermediate for conveying acetyl groups for cytosolic lipid syntheses [10]. Therefore we investigated the influence of clofibrate treatment on the activity of NAD-ICDH in rat liver.

Materials and Methods

Male rats (Sprague–Dawley, about 150 g body wt) received a standard diet containing 7.5 g/kg clofibrate (Serva, Heidelberg, F.R.G.) and water *ad lib.* for 3 weeks. A large particle (LP) fraction [11], essentially consisting of mitochondria, lysosomes and peroxisomes, was pelleted from the postnuclear supernatant (PNS) of rat liver homogenates at 20,000 *g* for 20 min [12]. A fraction containing the purified mitochondria (marker: G3PDH) was recovered from a discontinuous sucrose gradient (d = 1.195 kg/L) consisting of 50 mL of 0.25 M, LP-fraction in 50 mL 1 M, 70 mL 1.4 M, 70 mL 1.6 M and 2 M sucrose to a final volume of 365 mL. The gradient was performed on a Beckman L8-55 ultracentrifuge in a Z-60 rotor at 50,000 rpm for 4 hr. All steps were carried out at 4°.

NAD-ICDH activity was measured at 340 nm. The reaction mixture contained 84 mM K-phosphate, pH 7.6, 6.7 mM $MgCl_2$, 0.67 mM KCN, 2 mM ADP, 2 mM NAD^+, 0.84 g/L Triton X-100 and 1.6 mM D,L Na-isocitrate [13]. The reaction mixture for NADP-ICDH consisted of 47 mM triethanolamine-HCL, pH 7.6, 7.5 mM $MgCl_2$, 4.7 mM EDTA, 0.67 mM KCN, 0.13 mM $NADP^+$ and 1.6 mM D,L Na-isocitrate [14]. Mitochondrial G3PDH was assayed with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium-chloride (INT) as electron acceptor and with 14 mM D,L-glycerol-3-phosphate as substrate under test conditions described previously [15]. Protein was determined according to Bradford [16].

Results

Clofibrate treatment increased the specific activity of liver mitochondrial G3PDH sixfold in the LP-fraction (Table 1). This finding is in agreement with previous work [3]. The efficacy of the drug was also reflected by the observed hepatomegaly (Table 1, 1st column) and by the increased yield of protein in the LP-fraction (Table 2).

The activity of NAD-ICDH rose by 100% when expressed as gram of tissue in the LP-fraction. This increase was not solely due to the rise of mitochondrial protein induced by clofibrate, because the specific activity also rose significantly in the LPs (57%) as well as in purified mitochondria (62%) (Tables 1 and 2). On the other hand the NADP-ICDH remained unchanged (Table 1), which supports previous findings [7]. As a consequence the ratio of the specific activities of NAD-ICDH to NADP-ICDH in the LP-fraction increased after clofibrate feeding from 0.38 to 0.56 (Table 1).

Discussion

Among the mitochondrial enzymes, NAD-ICDH, G3PDH and choline dehydrogenase are responsive to the proliferative stimulus of clofibrate. Other mitochondrial enzymes like succinate dehydrogenase and NADP-ICDH are only increased in the same proportion as the mitochondrial protein. As a result the specific activities of these enzymes remain essentially constant. There are also enzymes which do not respond to the clofibrate stimulus at all. This behaviour has been described for some enzymes of the outer mitochondrial membrane [8].

In animal mitochondria the non-equilibrium steps at which the flux through the citrate cycle is regulated are catalysed by citrate synthase (citrate oxaloacetate-lyase (pro-3S-CH_2COO^-->acetyl-CoA, EC 4.1.3.7), 2-oxoglutarate dehydrogenase (2-oxoglutarate: lipoamide oxidoreductase (decarboxylating and acceptor-succinylating, EC 1.2.4.2) and most critically by NAD-ICDH [9]. The activation of this last enzyme by clofibrate might lead to an increased flux through citrate. A higher flux through the citrate pool would lead to an increased utilization of acetyl-CoA and lower the availability of acetyl-groups for lipid synthesis.

The enhancement of the mitochondrial G3PDH could lower the cytosolic glycerol-3-phosphate : dihydroxyacetone phosphate ratio by enhancing the conversion of glycerol-3-phosphate to dihydroxyacetone phosphate. This in turn would diminish the triacylglycerol synthesis at rate limiting cytosolic NADH concentrations.

In summary, after feeding rats for 3 weeks with clofibrate specific activities of the liver mitochondrial enzymes glycerol-3-phosphate dehydrogenase (G3PDH) and NAD-linked isocitrate dehydrogenase (NAD-ICDH) were found

* Abbreviations: G3PDH, glycerol-3-phosphate dehydrogenase; ICDH, isocitrate dehydrogenase; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium-chloride; LP, large particles; PNS, postnuclear supernatant.

Table 1. Effect of clofibrate feeding on G3PDH, NAD-ICDH and NADP-ICDH activities in the LP-fraction and purified mitochondria (G3PDH peak fraction) prepared from rat liver

	Liver wt/body wt (g/100 g)	G3PDH (mUnits/mg)	NADP-ICDH (mUnits/mg)	NAD-ICDH (mUnits/mg)
LP control	4.26 ± 0.57	9.30 ± 1.0	25.0 ± 1.5	9.50 ± 1.2
LP clofibrate	6.53 ± 0.75*	56.8 ± 10.6*	26.4 ± 1.7	14.9 ± 1.6*
Mitochondria				
Control		39.0	114	42.0
Clofibrate		281	121	68.1

LPs from 15 animals per group were assayed individually. One LP-fraction each of the control and of the clofibrate group was purified on a sucrose gradient. Specific activities (mUnits/mg protein) are given in nmol of INT, NAD^+ or $NADP^+$ reduced per min (mUnits). Values represent means ± SD.
* Significantly different ($P < 0.005$) from the control (Student's *t*-test).

Table 2. Activity of NAD-ICDH in PNS and LP-fraction from rat liver after clofibrate treatment

	Protein (mg/g liver)	NAD-ICDH (mUnits/g liver)	NAD-ICDH (mUnits/mg protein)
PNS control	153 ± 8	450 ± 52	3.52 ± 0.40
PNS clofibrate	164 ± 10	940 ± 89*	5.73 ± 0.75*
LP control	40.0 ± 3.0	380 ± 53	9.50 ± 1.2
LP clofibrate	52.2 ± 3.5*	779 ± 98*	14.9 ± 1.6*

For details see Table 1.
* Significantly different ($P < 0.005$) from the control (Student's *t*-test).

to be increased in a large particle fraction 6-fold and 1.6-fold, respectively, whereas the activity of NADP-linked isocitrate dehydrogenase (NADP-ICDH) remained unchanged.

Possibly these effects contribute to the hypolipidemic action of clofibrate.

Acknowledgement—The present study was supported by the Anton Dreher-Gedächtnisschenkung.

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Biochemical Pharmacology, Vol. 41, No. 11, pp. 1775–1777, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Enhanced chiral inversion of *R*-ibuprofen in liver from rats treated with clofibric acid

(*Received* 18 *October* 1990; *accepted* 4 *January* 1991)

The chiral 2-arylpropionate anti-inflammatory drug ibuprofen is administered as a racemate. *In vivo*, the metabolic inversion of *R*-ibuprofen to the pharmacologically active *S*-enantiomer is mediated via a three enzyme pathway [1]. The initial formation of ibuprofen-CoA is stereospecific for the *R*-enantiomer and reportedly catalysed by microsomal long-chain fatty acid CoA ligase (EC 6.2.1.3) [2, 3]. Once formed the *R*-CoA thioester is either hydrolysed releasing *R*-ibuprofen or enzymically racemized. The *S*-CoA thioester thus formed is subsequently hydrolysed releasing the *S*-enantiomer. The identity and subcellular localizations of the racemase and hydrolase have not been established. In addition to a pivotal role in the overall inversion process formation of a CoA intermediate has led to incorporation of *R*-ibuprofen into adipose tissue triglycerides [4]. To date there is no information as to factors which may modulate either of these two metabolic pathways. Clofibric acid treatment induces a number of hepatic enzymes associated with fatty acid metabolism including the microsomal long chain CoA ligase [5] and various acyl-CoA hydrolases [6, 7]. In addition, administration of clofibrate causes a 2–3-fold increase in the total amount hepatic CoA [8]. It was conceivable therefore that clofibric acid treatment could modulate the chiral inversion of *R*-ibuprofen by virtue of either inducing both ligase and hydrolase activities and/or increasing synthesis of cofactor. In this context, the present investigation examined the effect of clofibric acid treatment on the *in vitro* chiral inversion of *R*-ibuprofen in rat liver homogenate.

Materials and Methods

The *R*- and S-enantiomer of ibuprofen (*R*, 97.3% and *S*, 98.4% purity) were obtained from the Boots Company (Australia). [1-^{14}C]Palmitic acid (50 mCi/mmol) was purchased from Amersham (Australia) and all other chemicals from the Sigma Chemical Co. Male hooded Wistar rats (250–300 g) were treated with either clofibric acid orally (Group I, 280 mg/kg/day, N = 5) or vehicle (Group 2, 5% w/v methylethycellulose) for 1, 2 or 5 days. Food and water were allowed *ad lib*. The livers were removed, perfused, homogenized with ice-cold buffer (10 mM phosphate containing 1.15% KCL, pH 7.4) and centrifuged at 700 *g* for 10 min. The supernatant was assayed for protein [9] and diluted to a final concentration of 30 mg/mL. A reaction mixture (2 mL) containing 150 mM Tris (pH 7.4), 6.2 mM $MgCl_2$, 0.05% Triton X-100, 2 mM EDTA, 1 mM dithiothreitol, 2.5 mM ATP, 0.6 mM CoASH, 6 mg homogenate protein and 0.2 mM *R*-ibuprofen was incubated at 37° and aliquots (100 μL) collected at various times (5–120 min) and analysed for *R*- and *S*-ibuprofen using a modified enantiospecific HPLC technique [10]. The microsomal fraction was prepared from the homogenate by differential centrifugation and long chain CoA ligase activity determined using [^{14}C]palmitic acid as previously described [11]. The statistical significance of the difference between groups was determined by either an analysis of variance or Student's *t*-test with Bonferroni correction [12].

Results and Discussion

Preliminary experiments established that the chiral inversion of ibuprofen was stereospecific for the *R*-enantiomer, dependent on the presence of ATP, CoASH, $MgCl_2$ and exhibited a pH optimum of 7.4. Omission of EDTA, dithiothreitol or Triton X-100 resulted in diminished inversion. In addition, inversion of *R*-ibuprofen was not observed in the presence of denatured hepatic protein. In all animals studied there was an initial rapid decrease in the concentration of *R*-ibuprofen which reached a maximum at 20 min. The *in vitro* system used was not constrained by lack of availability of cofactors as further addition of ATP and CoASH at that time produced no further formation of the *R*-CoA conjugate. It is conceivable that the CoA ligase was inhibited by either the reaction products of the initial step i.e. *R*-ibuprofen-CoA and AMP or adenosine [13]. Although a rapid decrease in the concentration of the *R*-enantiomer occurred this was not reflected by a corresponding quantitative increase in the *S*-enantiomer thus suggesting that the initial activation step was not rate limiting in the overall sequence of inversion. These results are in accord with the data of Knihinicki *et al.* [3] however in that study formation of the CoA conjugate was maximal at 10 min. Over the remaining 100 min the concentration of *R*-ibuprofen slowly increased, presumably due to hydrolysis of the *R*-CoA conjugate. In contrast, the concentration of the *S*-enantiomer continually increased such that at 120 min the total amount of *R*- and *S*-ibuprofen equated to the initial concentration of the *R*-enantiomer. Clofibric acid treatment for 1 day did not produce an increase in liver weight and there was no difference between the concentration time profiles for either *R*- or *S*-ibuprofen in liver homogenate from both the control and the treated animals (11.3 ± 0.1, 26.8 ± 1.6 and 11.9 ± 0.5 g, 26.1 ± 1.6%, liver weight and inversion, respectively). This was in marked contrast to the effects observed following administration of clofibric acid for either 2 or 5 days. Liver weight was markedly increased in Group 1 animals 13.48 ± 0.85 and 15.02 ± 0.6 g ($P < 0.001$) compared to Group 2 11.85 ± 0.35 and 11.45 ± 0.2 g for 2 and 5 days, respectively. A significant reduction (57%, $P < 0.01$) in the initial concentration of the *R*-enantiomer was observed after 20 min in the treated group when compared to liver homogenate from untreated animals (34%, Fig. 1A). This was a reflection of the increased catalytic activity ($P = 0.05$) of the microsomal long-chain fatty acid CoA ligase (Table 1). This enzyme has previously been implicated in the formation of both fenoprofen and ibuprofen-CoA and shown to be inducible by clofibric acid [2, 3, 5]. Once