Effects of clofibric acid on the activity and activity state of the hepatic branched-chain 2-oxo acid dehydrogenase complex

Yu ZHAO, Jerzy JASKIEWICZ and Robert A. HARRIS*
Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202-5122, U.S.A.

Feeding clofibric acid to rats caused little or no change in total activity of the liver branched-chain 2-oxo acid dehydrogenase complex (BCODC). No change in mass of liver BCODC was detected by immunoblot analysis in response to dietary clofibric acid. No changes in abundance of mRNAs for the BCODC E1a, E1b and E2 subunits were detected by Northern-blot analysis. Likewise, dietary clofibric acid had no effect on the activity state of liver BCODC (percentage of enzyme in the dephosphorylated, active, form) of rats fed on a chow diet. However, dietary clofibric acid greatly increased the activity state of liver BCODC of rats fed on a diet deficient in protein. No stable change in liver BCODC kinase activity was found in response to clofibric acid in either chow-fed or low-protein-fed rats. Clofibric acid had a biphasic effect on flux through BCODC in hepatocytes prepared from low-protein-fed rats. Stimulation of BCODC flux at low concentrations was due to clofibric acid inhibition of BCODC kinase, which in turn allowed activation of BCODC by BCODC phosphatase. Inhibition of BCODC flux at high concentrations was due to direct inhibition of BCODC by clofibric acid. The results suggest that the effects of clofibric acid in vivo on branched-chain amino acid metabolism can be explained by the inhibitory effects of this drug on BCODC kinase.

INTRODUCTION

Clofibric acid (p-chlorophenoxypropionic acid), a well-known antihyperlipidaemic drug [1], has been observed to lower tissue levels of branched-chain amino acids [2] and both to inhibit [3-5] and to stimulate [2,6] flux through the branched-chain 2-oxo acid dehydrogenase complex (BCODC), the enzyme recognized as rate-limiting in the oxidation of branched-chain amino acids [7]. Clofibric acid therapy also induces a muscular weakness syndrome called myotonia [8-10], and this condition may be linked to altered branched-chain amino acid metabolism [2,4,11]. This work was initiated after the report by Ono et al. [12] of induction of BCODC by clofibric acid in the liver of the rat. Marked induction of hepatic BCODC by clofibric acid was not found under the experimental conditions used in the present study. The drug was found, however, to activate BCODC in the liver of rats fed on a protein-deficient diet via inhibition of the kinase responsible for phosphorylation and inactivation of the complex. It is proposed that inhibition of BCODC kinase in the major tissues of the body may indirectly account for the myotonic effect of clofibric acid in rat and man.

MATERIALS AND METHODS

Materials

13H1-Protein A and radioactive nucleotides were obtained from New England Nuclear Corp., Boston, MA, U.S.A. All chemicals and dihydrolipoamide reductase were from Sigma Chemical Co., St. Louis, MO, U.S.A. Male Wistar rats (175-200 g; Harlan Industries, Indianapolis, IN, U.S.A.) were housed in a temperature- and light-controlled room. Rats were fed on either a chow diet (Purina Rodent Laboratory Chow 5001; minimum 23% protein, by wt.) or a low-protein (8 %, by wt.) diet (ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.) described previously [13]. Clofibric acid was mixed with powdered diet at various levels as indicated. Rats were maintained on the various diets for 10-14 days before being killed.

Assay of enzyme activities

BCODC activity was assayed in extracts of freeze-clamped liver tissue by the procedure described by Goodwin et al. [14]. Protein content was determined by a modified Lowry method [15].

For BCODC kinase assay, homogenates were prepared as described by Goodwin et al. [14] for assay of BCODC activity, except that 2-chloro-4-methylpentanoate and rat serum were omitted to prevent carry-over of inhibitors of the kinase into the final preparation. For samples from rats fed on the low-protein diet, the resuspended extracts were incubated with a broad-specificity phosphatase [14] to activate the complex completely. After complete activation, the BCODC kinase complex was precipitated with 9 % poly(ethylene glycol) (PEG) a second time. The PEG pellets were resuspended in a kinase buffer, which consisted of 50 mM-K,HPO4 (pH 7.3), 0.5 % (v/v) Triton X-100, 2 mM-KF, 0.1 mM-N-α-tosyl-L-lysylchloromethane, 0.1 mg of trypsin inhibitor/ml, 1 µg of apritinin/ml, 0.5 µM-leupeptin and 0.5 µM-pepstatin-A. Kinase reactions were conducted at 15 °C. Reactions were initiated by addition of tissue extracts to an incubation cocktail made in the kinase buffer with final concentrations of 0.4 mM-ATP, 7.5 mM-MgCl2, 10 mM-phosphocreatine and 5 units of creatine kinase/ml. The reactions were terminated by taking samples at the indicated times and diluting them with the assay cocktail for spectrophotometric determination of the remaining dehydrogenase activity. The activity of BCODC kinase is expressed as the first-order rate constant of inactivation of BCODC, which is the absolute value of the slope of a semi-logarithmic plot of BCODC activity remaining versus incubation time with ATP.

The method of Lazarow [16] was used for the extraction and assay of liver peroxisomal palmitoyl-CoA dehydrogenase. One unit of enzyme activity corresponds to the formation of 1 µmol of NADH/min at 30 °C.

Abbreviations used: BCODC, branched-chain 2-oxo acid dehydrogenase complex; PEG, poly(ethylene glycol).

* To whom correspondence should be addressed.
Immunoblot analysis of the mass of BCODC

The pellet obtained by precipitation with PEG for the assay of BCODC activity [14] was dissolved in a solution consisting of 4% (w/v) SDS, 0.125 M-Tris/HCl, pH 6.8, 20% (v/v) glycerol and 1.5 mg of dithiothreitol/ml. The samples were boiled for 90 s and applied to nitrocellulose paper in a dot-blot apparatus in serial dilutions made with a solution of 0.14 M-NaCl, 2.7 mM-KCl, 8.1 mM-Na$_2$HPO$_4$, 2H$_2$O, 15 mM-KH$_2$PO$_4$ and 10 mM-Na$_2$SO$_4$. The dot-blotted paper was blocked with 5% (w/v) BSA, incubated with polyclonal antibodies against the E2 subunit of BCODC [17], washed with 0.1% BSA, incubated with 125I-Protein A and washed again. Autoradiography was carried out with Kodak XAR-5 film for 12 h, and quantification was by densitometry.

RNA isolation and Northern-blot analysis

Total cellular RNA was extracted from freeze-clamped livers by the RNAzol method (Cinna/Biotech Laboratories International, Houston, TX, U.S.A.) by following the instructions of the manufacturer. A 25 μg portion of total RNA from each sample was electrophoresed on a 2.5% agarose gel containing 2.2 M-formaldehyde and transferred to a Nytran membrane (Schleicher & Schuell Corp., Keene, NH, U.S.A.). Additional steps for Northern-blot analysis were carried out as described previously [17].

Isolation and incubation of hepatocytes

Hepatocytes were isolated from chow-fed and low-protein-fed rats by the procedure of Berry & Friend [18] with modifications described previously [19]. Hepatocytes (30–40 mg wet wt.) were incubated in 2 ml of Krebs–Henseleit buffer [20] supplemented with 1.25% BSA (Powder CRG-7; Armour Pharmaceutical Co., Kankakee, IL, U.S.A.: dialysed) and as described previously [21]. Clofibric acid (sodium salt) was added to the flask at the final concentrations indicated. After 15 min of incubation in a shaking water bath at 37 °C, 3-methyl-2-oxo[1-14C]butanoate (0.2 mm) was injected through the serum cap to initiate the reaction. The incubation was continued for an additional 15 min before termination and collection of 14CO$_2$ as described previously [21].

RESULTS

The initial experiment of this study was similar to that described by Ono et al. [12], except that a higher concentration of clofibric acid was used. Rats were fed on a chow diet for 14 days with and without supplementation of the diet with 0.5% clofibric acid. In contrast with the findings of Ono et al. [12], clofibric acid treatment failed to cause a significant increase in liver total BCODC activity (1.3 ± 0.2 units/g wet wt. in chow-fed rats; 1.6 ± 0.3 units/g wet wt. in clofibric acid-treated rats; mean ± s.e.m. for five animals in each group; P > 0.05 by two-tailed Student’s t test). As an independent verification of these findings, hepatocytes were prepared from rats fed for 10–14 days on chow diet or chow diet supplemented with 0.5% clofibric acid. BCODC flux measurements, carried out by determining the rate of decarboxylation of 3-methyl-2-oxo[1-14C]butanoate by intact hepatocytes as described in the Materials and methods section, revealed no significant difference between cells prepared from control and clofibric acid-treated rats (217 ± 18 nmol/ml per g wet wt. for hepatocytes from chow-fed rats; 259 ± 54 nmol/min per g wet wt. for hepatocytes from clofibric acid-treated rats; mean ± s.e.m. for hepatocyte preparations from three animals in each group). Preincubation of the hepatocytes with 2-chloro-4-methylpentanoate, an inhibitor of BCODC kinase established previously to be effective in isolated hepatocytes [21], had no effect of BCODC flux rates (results not shown). This result was obtained regardless of whether hepatocytes were prepared from chow-fed rats or clofibric acid-treated rats, suggesting that latent BCODC activity due to phosphorylation was not present in hepatocytes isolated from clofibric-treated animals.

Since Ono et al. [12] reported very dramatic effects on BCODC activity with only 0.1% dietary clofibrate, another study was conducted with animals fed on chow diet supplemented with 0.1, 0.25 and 0.5% clofibric acid (Fig. 1). A significant but modest increase in liver total BCODC activity, expressed per mg of total liver protein, was observed in this study, but only at a clofibric acid concentration of 0.5% (P < 0.05). The magnitude of this increase (20%) was disappointing relative to the 3-fold increase in liver BCODC activity reported by Ono et al. [12]. The increase was significant also on a liver-g-wet-wt. basis (Table 1), but again the increase was not impressive (27%). Furthermore, no significant increase in mass of BCODC could be detected by immunoquantification with antibodies to the BCODC E2 subunit (Fig. 2), arguing that the lack of a greater difference in activities cannot be explained on the basis of an error in the measurement of total enzyme activities. Furthermore, clofibric acid treatment had no effect on the activity state of BCODC, i.e. the complex was completely active (dephosphorylated) with or without clofibric acid in the chow diet (Table 1).

Clofibric acid treatment caused an increase in liver weight and an increase in activity of the liver peroxisomal marker enzyme palmitoyl-CoA dehydrogenase (Table 1), i.e. effects of clofibric acid expected from the work of others [22,23].

Since feeding rats on a diet deficient in protein has been shown in previous studies to lower liver total BCODC activity [13], an experiment was conducted to examine the effects of clofibric acid on BCODC activity of rats fed on a low-protein (8%) diet. Clofibric acid increased liver weight and palmitoyl-CoA dehydrogenase activity of animals on this diet (Table 1), but again...
Table 1. Effect of clofibric acid on body weight, liver weight, palmitoyl-CoA dehydrogenase activity and BCODC activity and activity state

<table>
<thead>
<tr>
<th>Dietary treatment</th>
<th>Final body wt. (g)</th>
<th>Liver wt. (g/100 g body wt.)</th>
<th>Liver protein (mg/g wet wt.)</th>
<th>Palmitoyl-CoA dehydrogenase activity (units/g wet wt.)</th>
<th>Liver BCODC activity (units/g wet wt.)</th>
<th>BCODC activity state (% active)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>277 ± 7</td>
<td>4.5 ± 0.4</td>
<td>181 ± 4</td>
<td>0.7 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>Chow + clofibric acid</td>
<td>249 ± 6</td>
<td>7.1 ± 0.2*</td>
<td>192 ± 3</td>
<td>8.0 ± 0.8*</td>
<td>1.5 ± 0.2*</td>
<td>107 ± 3</td>
</tr>
<tr>
<td>Low-protein</td>
<td>211 ± 7</td>
<td>4.3 ± 0.7</td>
<td>131 ± 3</td>
<td>0.3 ± 0.2</td>
<td>0.13 ± 0.04</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>Low-protein + clofibric acid</td>
<td>167 ± 4†</td>
<td>5.6 ± 0.2†</td>
<td>167 ± 3†</td>
<td>5.0 ± 0.9†</td>
<td>0.81 ± 0.13†</td>
<td>99 ± 8†</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of clofibric acid on the mass of hepatic BCODC

Rats were fed for 14 days on a chow diet with and without 0.5% clofibric acid. Extracts were prepared from freeze-clamped liver and blotted on nitrocellulose paper with a 2-fold serial dilution based on identical wet tissue weight. Immunooquantification was carried out with polyclonal antibodies against the E2 subunit of BCODC, followed by 125I-labelled Protein A. Lanes 1–5 correspond to extracts prepared from five different control animals; lanes 6–10 correspond to extracts prepared from five different clofibric acid-treated animals.

Fig. 3. Effect of clofibric acid on relative abundance of mRNAs of BCODC subunits

Rats were fed for 14 days on a chow diet with and without 0.5% clofibric acid. Total RNA was prepared from freeze-clamped liver and electrophoresed and blotted on to a Nytran membrane. The membranes were probed with 32P-labelled cDNAs for rat Elx (A), E1β (B), E2 (C) and tubulin (D). The autoradiogram was exposed for 72 h. Lanes 1–3 contained RNA from three different rats fed on chow diet; lanes 4–6 contained RNA from three different rats fed on chow diet plus 0.5% clofibric acid.

Northern-blot analysis failed to reveal any effect of clofibric acid treatment of rats on the relative abundance of the liver mRNAs for BCODC E2, Elx and E1β subunits (Fig. 3). Tubulin mRNA was also quantified to ensure that comparable amounts of RNA were analysed for both groups of animals (Fig. 3).

Feeding rats on a protein-deficient diet has been demonstrated [24] to result in a substantial increase in the activity of liver BCODC kinase towards its complex. This finding is confirmed in the present study (Fig. 4). Since clofibric acid activates BCODC in low-protein-fed rats (Table 1), it was decided to determine whether a stable change in BCODC kinase activity was caused by this compound. However, clofibric acid treatment had no effect on liver BCODC kinase activity in either chow-fed or low-protein-fed rats (Fig. 4). Thus the increase in activity state of BCODC caused by clofibric acid cannot be explained by an induced change in BCODC kinase activity.
concentration of protein-fed reveals hepatocytes incubated with 2-chloro-4-methylpentanoate (Fig. 4) inhibitory of the enzyme.

Hepatocytes prepared from rats fed on a low-protein (8\%) diet for 10–14 days were incubated for 20 min with the indicated additions. Column headed ‘−Pase’ refers to BCODC activities measured before phosphatase treatment. Column headed ‘+Pase’ refers to BCODC activities measured after phosphatase treatment. All values are means ± S.E.M. for three animals: *P < 0.05 by Student’s $t$ test for paired samples versus no additions (None).

<table>
<thead>
<tr>
<th>Addition (mm)</th>
<th>BCODC activity (units/g wet wt.)</th>
<th>BCODC activity state (% active)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.09 ± 0.02</td>
<td>0.70 ± 0.08</td>
</tr>
<tr>
<td>Clofibricacid (0.5)</td>
<td>0.57 ± 0.08*</td>
<td>0.75 ± 0.09</td>
</tr>
<tr>
<td>2-Chloro-4-methylpentanoate (0.1)</td>
<td>0.66 ± 0.07*</td>
<td>0.68 ± 0.06</td>
</tr>
</tbody>
</table>

**DISCUSSION**

It was reported several years ago by Wagenmakers et al. [3] that clofibrac acid treatment of rats results in nearly a 3-fold increase in liver total BCODC activity. The findings of the study are complicated, however, because the BCODC assay used [25] depended on flux measurements with intact mitochondria and the rates reported correspond to only a fraction of the total enzyme activity known to be present in the tissue [26]. As an added complication, Wagenmakers et al. [3] could detect the increase in liver BCODC activity with one substrate (4-methyl-2-oxopentanoate), but not with another well-established substrate (3-methyl-2-oxobutanoate) [27] of the enzyme. Nevertheless, Ono et al. [12] also recently reported a dramatic induction of liver BCODC by clofibrac acid. A 3-fold increase in activity of the enzyme was matched by 3-fold increases in mass of the Elα, Elβ and E2 subunits as well as in the levels of the corresponding mRNAs for these subunits. Since previous work from our laboratory has focused on the mechanisms responsible for the regulation of hepatic BCODC activity [13,21,26,28], the findings of Ono et al. [12] stimulated further work on this action of clofibrac acid. However, evidence was not found for a dramatic induction of BCODC by clofibrac acid under the experimental conditions of the present study. Experiments were repeated to verify the results and also to determine the effects of different concentrations of dietary clofibrac acid. A broad-specificity phosphoprotein phosphatase was used to ensure that BCODC was in the completely activated, dephosphorylated, state before measurement of BCODC activity [13]. Clofibrac acid treatment produced the expected effects on liver weight and the activity of the peroxisomal enzyme palmitoyl-CoA dehydrogenase [22,23], but had only a relatively small effect on liver total BCODC activity that was not observed in every experiment. No effect at all could be shown by immunoblot analysis of the mass of BCODC subunits. Likewise, hepatocytes prepared from clofibrate-treated rats did not oxidize branched-chain 2-oxo
Clofibric acid and branched-chain 2-oxo acid dehydrogenase

acids at rates significantly greater than for hepatocytes prepared from control animals. Although a relatively small increase in liver BCODC activity in response to 0.5% dietary clofibric acid was observed in one experiment, this increase was not greater than the increase in mitochondrial mass that occurs in response to clofibric acid [29].

Since Wagenmakers et al. [3] only found the increase in BCODC activity with 4-methyl-2-oxopentanoate as substrate, and since 3-methyl-2-oxobutan-2-0late is routinely used in our BCODC assay, liver extracts of control and clofibric acid-treated rats were also assayed for BCODC activity with the former substrate of the complex. As found with 3-methyl-2-oxobutan-2-0late, no significant increase in activity occurred with clofibric acid treatment when BCODC was assayed with 4-methyl-2-oxopentanoate (1.2±0.2 units/g wet wt. in chow-fed rats; 1.6±0.3 units/g wet wt. in 10-day 0.5% clofibric-acid-treated rats; means ± S.E.M. for five animals in each group; P > 0.05 by two-tailed Student’s t test).

The study with low-protein-fed rats was conducted because dietary protein deficiency is known to decrease total liver BCODC activity [13], and Ono et al. [12] did not specify the protein content of the diet used in their study. The specific activity reported by Ono et al. [12] for BCODC in isolated mitochondria was low, i.e. approx. 25% of those reported previously by our laboratory [26]. Indeed, the activities induced by clofibric acid in the study by Ono et al. [12] were still not as high as those reported by our laboratory for liver mitochondria isolated from normal animals [26]. The reason for the differences between the laboratories in estimates of BCODC activity remains to be defined. Perhaps liver BCODC is already induced in the rats used by our laboratory because of the presence (or absence) of some component in our rat chow diet that exerts control over BCODC gene expression. This is not likely to be dietary protein, because no effect of clofibric acid was found on total liver BCODC activity of low-protein-fed rats.

That clofibric acid can activate BCODC in liver of low-protein-fed rats is a new finding of the present study. This was shown to occur in vivo in animals given clofibric acid in the low-protein diet and in vitro with hepatocytes prepared from low-protein-fed rats. Clofibric acid was shown in a previous study from this laboratory [6] to stimulate BCODC flux in the perfused rat heart and greatly to increase BCODC activity state as measured in cell-free extracts of perfused rat hearts. Honda et al. [30] likewise reported activation of BCODC by clofibric acid in primary-cultured rat hepatocytes. All these observations can be readily explained by our previous finding that clofibric acid is a potent inhibitor of BCODC kinase activity associated with BCODC [6]. By inhibiting BCODC kinase, clofibric acid indirectly activates BCODC, because BCODC phosphatase acts unopposed to dephosphorylate and activate the complex.

Feeding rats on a diet deficient in protein is known to increase the activity of the kinase responsible for covalent modification of BCODC [24], and it has been proposed that this change in kinase activity is a factor in the low activity state of BCODC in the liver of low-protein-fed rats [24]. Since treatment with clofibric acid reversed or prevented this effect of dietary protein deficiency, i.e. caused activation of the BCODC, it was of interest to determine whether clofibric acid prevented the apparent induction of BCODC kinase activity that occurs in protein deficiency. However, clofibric acid treatment had no effect on BCODC kinase activity of low-protein-fed rats, and it appears therefore that clofibric acid does not suppress the apparent inducing effect of low-protein feeding on BCODC kinase activity, but rather acts simply as a BCODC kinase inhibitor to overcome the effects of the increased activity of BCODC kinase in the liver of the low-protein-fed rats.

Previous studies by Paul & Adibi [2] demonstrated that chronic administration of clofibric acid stimulates leucine oxidation by the intact rat, decreases branched-chain amino acid concentration in muscle tissue, and increases leucine oxidation by homogenates of gastrocnemius muscle. These effects are consistent with the observations reported here for liver and previously for heart [6], and are readily explained by clofibric acid inhibition of BCODC kinase.

Clofibric acid was shown in previous studies to inhibit branched-chain amino acid oxidation by rat skeletal-muscle cells [4,5] and muscle homogenates [3], an effect likely to be due to direct inhibition of BCODC, as demonstrated with the purified enzyme [6,11]. This inhibitory effect was apparent also with isolated hepatocytes in the present study, but occurred at higher clofibric acid concentrations than those required to activate BCODC by inhibition of its kinase. As demonstrated previously [6] with rat heart and in the present study with rat liver, activation of BCODC occurs at lower clofibric acid concentrations because BCODC kinase is more sensitive to clofibric acid inhibition than is BCODC itself.

Chronic treatment of experimental animals and man with clofibric acid can result in a muscular syndrome called myotonia, which is characterized by muscle pain, weakness and wasting [8–10]. Both inhibition [4,11] and activation of branched-chain amino acid oxidation by clofibric acid [2] have been suggested to be responsible for the development of this condition. Clofibric acid treatment of animals lowers tissue branched-chain amino acid concentrations [2], suggesting that activation rather than inhibition of BCODC occurs in vivo. Although the mechanisms involved are poorly understood, leucine is known to promote muscle protein synthesis and inhibit protein degradation [31,32]. Thus the lowering of muscle leucine concentration by clofibric acid, most likely caused by activation of BCODC, may be a factor in muscle wasting characteristic of myotonia.

This work was supported in part by grants from the U.S. Public Health Service (Grant No. DK19259), the Grace M. Shaylor Residency Trust and a March of Dimes Predoctoral Fellowship (Y.Z.).

REFERENCES


Received 19 September 1991/2 January 1992; accepted 29 January 1992